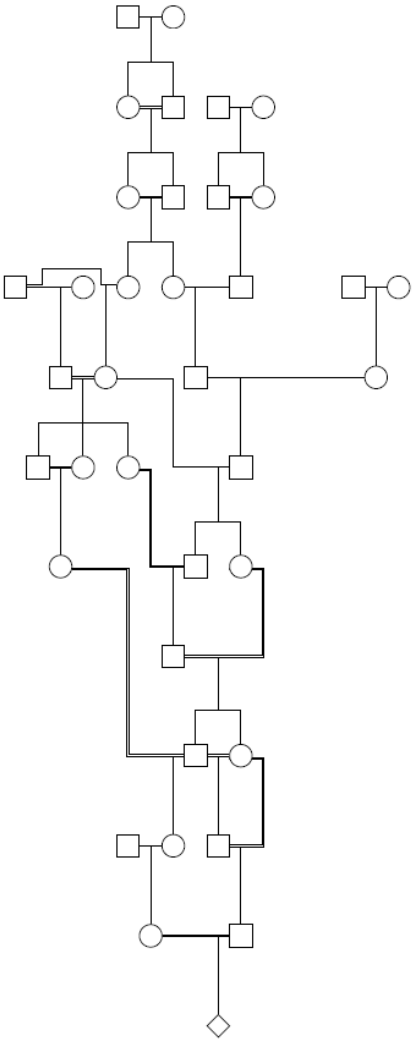
Investigating adaptation to captivity: a data-driven approach



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A thesis submitted to fulfil the requirements for the degree of Doctor of Philosophy

Declaration

This is to certify that the content of this thesis is my own work. Information sourced from published or unpublished works has been acknowledged in the text, with a list of references given at the end of this thesis. This work has not been submitted for any other degree.

Katherine Farquharson

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Table of Contents

List of Tables

List of Figures

Authorship contribution statements

A NOTE ON THE STYLE AND LAYOUT OF THIS THESIS

Each research chapter of this thesis is formatted for submission to academic journals, and as a result there is some duplication of background material between chapters. Three of the chapters of this thesis (Chapters 2, 4, and 5) have been published, one (Chapter 6) is in review, and two are formatted for submission (Chapters 3 & 7). The inclusion of published manuscripts follows the University of Sydney's Thesis and Examination of Higher Degrees by Research Policy 2015 guidelines for a thesis with publications. Supplementary materials are provided in appendices, referred to for example as A1, and a single list of references is given at the end of this thesis. Throughout this thesis, the term F is used to refer to the captive generation of an individual, e.g. F_0 for wild-born animals, F_1 for first-generation captive-born animals, and does not represent a hybrid cross.

Listed below are the published manuscripts that form this thesis and the associated co-authorship confirmation statements. These publications have benefited from several anonymous reviewers. A list of additional publications that I contributed to during my PhD candidature that do not form research chapters follows, with these publications provided in the appendices.

PUBLISHED MANUSCRIPTS INCLUDED IN THIS THESIS

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*contributed equally.

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Confirmation of co-authorship of published work

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Author contributions

Katherine A. Farquharson, the candidate collected the data, analysed the data, prepared the figures and drafted the manuscript. Carolyn J. Hogg provided conceptual guidance on the analysis and critically revised the manuscript. Catherine E. Grueber conceived the project, provided technical guidance on the analysis, and critically revised the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

(A copy of the signed statement by all co-authors is available on request)

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Publication

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*contributed equally.

Author contributions

Belinda Wright performed analyses using the SAMtools and GATK pipelines and genotype ratios and concordances. Katherine A. Farquharson, the candidate, wrote the custom R script and with Elspeth A. McLennan analysed data using the Stacks pipeline and custom R script including all PCoAs. Carolyn J. Hogg provided all information on the devil breeding program and oversaw the project. Katherine Belov provided direction on study design and oversaw the project. Catherine E. Grueber provided direction on study design, statistical analyses, and oversaw the project. All authors contributed to the writing and editing of the final manuscript. All authors read and approved the final manuscript.

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Author contributions

Katherine A. Farquharson, the candidate, performed DNA extractions, processed sequence data, performed pedigree reconstruction and drafted the figures, tables and manuscript. Carolyn J. Hogg provided devil management information, assisted with effective population size comparisons in PMx, oversaw the project and critically revised the manuscript. Catherine E. Grueber provided funding for this project, oversaw the project and critically revised the manuscript.

I, as a co-author, endorse that the level of contribution by myself and the candidate indicated above is appropriate.

(A copy of the signed statement by all co-authors is available on request)

In addition to the above authorship contribution statements, permission to include the published material has been granted by the corresponding author.

Katherine A. Farquharson

20/09/2019

As supervisor for the candidate, I confirm that the authorship attribution statements above are correct.

Catherine E. Grueber

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Presentations

Farquharson, K.A., Wright, B., McLennan, E.A., Belov, K., Hogg, C.J. & Grueber, C.E. (2019) *Do you want reliable reduced representation sequencing SNPs?* Genetics Society of Australasia Annual Conference, Melbourne, 30th June - 3rd July 2019; poster presentation.

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Abstract

Captive breeding programs are an increasingly common tool to prevent extinction and provide a source population for reintroductions to the wild. Breeding programs attempt to 'halt evolution' in captivity by ensuring that captive populations are genetically representative of the original wild population. However, there will always be differences between captive and wild environments. Genetic adaptation to captivity as a result of artificial or unintended selection is therefore likely. While the importance of genetic management in captivity is widely recognised, minimising adaptation to captivity is often given lower priority than strategies to avoid inbreeding and maintain wild genetic diversity. It is unclear how widespread adaptation to captivity is across taxa, yet individual breeding programs may not have enough data to sufficiently examine changes over time. Additionally, changes may be difficult to detect and/or have varied consequences. The increasing need for captive breeding and reintroduction programs necessitates investigation of adaptation to captivity, particularly as guidelines to minimise consequences may conflict with other program goals. The rise of data available, both through improved record-keeping from zoos, and the increased accessibility of thousands of genetic markers for conservation purposes presents new opportunities to address these challenges.

In this thesis, I aimed to examine (1) whether there are differences in reproductive success in captive environments between wild-born and captive-born animals and how widespread any changes are, (2) long-term multi-generational changes in reproductive success in captivity, and (3) how changes may occur between generations of captive breeding, including through variation in reproductive success and undetected selection. As the consequences of adaptation to captivity are of relevance to all captive breeding programs, I used a data-driven approach to examine the response of multiple species to captive breeding in Chapters 2 and 3. In chapters 4 - 7 I then used the Tasmanian devil as a case study to allow a closer examination of genetic change in captivity. The Tasmanian devil insurance population was established in 2006 in response to the spread of a contagious cancer, devil facial tumour disease (DFTD), and is now the largest captive breeding program in Australia.

In Chapter 2, I synthesised published literature to discover differences in reproductive success between captive-born and wild-born animals across 44 diverse species and in a broad range of captive breeding programs, including aquaculture, laboratory studies and conservation. In captivity, captive-born animals had lower reproductive success than wild-born animals across a variety of measures. The differences were most pronounced for offspring effects such as offspring survival. My findings highlight the importance of a meta-analytical approach to expand knowledge that is otherwise limited to individual, often low-powered studies. However, my results showed that the literature was not comprehensive enough to disentangle differences between first-generation captive-born animals and multi-generational effects.

Chapter 3 investigates the factors driving reduced offspring survival in captive-born animals and disentangles generational effects. I examined studbook data from 15 diverse long-running conservation breeding programs, totalling over 38,000 individual records, to determine whether there were generational effects on survival and if this was a consistent trend across taxa. I found that an individual's level of inbreeding was the strongest predictor of its survival. Generational effects were strong in some cases but had varied impacts across species.

I then explored possible genetic mechanisms that may explain the fitness changes I observed in captivity, including individual variation in reproductive success and undetected selection. This first required the development of methods to reliably process reduced representation sequencing data, which I tested in two wildlife species (Chapter 4). I then applied the method to resolve the pedigree of 81 Tasmanian devils held in semi-natural free-range enclosures (Chapter 5), in order to examine variation in reproductive success and the consequences of variation on captive population management. I found high variation in reproductive success of both sexes, which may reduce genetic diversity and accelerate adaptation to captivity if unmanaged. Additionally, I quantified the management benefits of my molecular pedigree reconstruction to justify the utility of applying genetic techniques in conservation.

Incorporating mate choice into conservation breeding programs is recommended to improve reproduction and prevent adaptation to captivity. However, genetic-based mate choice can have varied consequences for the management of genetic diversity. To determine the possible genetic mechanisms of the high variation in reproductive success detected in Chapter

5, I examined three mate choice hypotheses in Tasmanian devil free-range enclosures (Chapter 6) at both 1,948 genome-wide SNPs and 12 MHC-linked microsatellite markers. I found that non-genetic factors such as age were the best predictors of breeding success and found no evidence of genetic mate choice under the tested hypotheses. I demonstrated that integrating mate choice into captive management may be more difficult than previously considered.

In Chapter 7, I investigated another possible driver of adaptation to captivity: undetected early viability selection. I examined known parent-offspring triads at 123 loci across five neutral and functional amplicons to detect deviations from Mendelian inheritance that cannot be explained by pre-copulatory mate choice. Deviations varied across a gradient of captive management, from intensive monogamous pairings to semi-wild environments, suggesting a possible mechanism for genetic changes to occur undetected in captive breeding programs.

My work has provided new information about the consequences and possible mechanisms of adaptation to captivity. The curation and analysis of large datasets has allowed me to identify patterns across taxa in order to provide useful recommendations to conservation managers considering the impact of adaptation to captivity in their species.

Abbreviations

AIC – Akaike information criterion

BAM – binary alignment map

BAQ – base alignment quality

BWA – Burrows-Wheeler aligner

CI – confidence interval

CPU – central processing unit

CV – coefficient of variation

DArTseq – Diversity Arrays Technology sequencing

ddRAD – double digest restriction-site associated DNA

DFTD – devil facial tumour disease

DIC – deviance information criterion

f – inbreeding coefficient

F_0 – wild-born generation

F_1 – first-generation captive-born

FRE – free-range enclosure

F_{ST} – fixation index

GATK – genome analysis toolkit

GBS – genotyping-by-sequencing

GLMM – generalised linear mixed model

H^2 – phylogenetic heritability/signal

H_E – expected heterozygosity

H_{GW} – standardised genome-wide heterozygosity

H_{MHC} – standardised MHC-based heterozygosity

H_O – observed heterozygosity

HPD CI – highest posterior density credible interval

I^2 – heterogeneity

IP – insurance population

IUCN – International Union for the Conservation of Nature

lnOR – log odds ratio

MAF – minor allele frequency

MCMC – Markov chain Monte Carlo

MHC – major histocompatibility complex

MLH – multi-locus observed heterozygosity

MULT – multiple putative parents, apportioned fractions of parentage in the pedigree management software PMx

N – sample size

N_e – effective population size

NGS – next-generation sequencing

PCoA – principal coordinates analysis

PCR – polymerase chain reaction

PRISMA – preferred reporting items for systematic reviews and meta-analyses

RADseq – restriction-site associated DNA sequencing

RI – relative importance

RRS – reduced representation sequencing

SAM – sequence alignment map

SD – standard deviation

SE – standard error

SNP – single nucleotide polymorphism

STDP – Save the Tasmanian Devil Program

VCF – variant call format

WGS – whole genome sequencing

ZIMS – Zoological Information Management Software

Chapter 1: Introduction

Worldwide over one million species are threatened with extinction (IPBES, 2019). The rapid rate at which species are becoming extinct as a direct and indirect consequence of human action has justified claims of a sixth mass extinction (McCallum, 2015). In the most recent International Union for the Conservation of Nature (IUCN) Red List update, not a single animal, plant, or fungi species exhibited a genuine improvement in its threat status between 2018 and 2019 (IUCN, 2019).

The IUCN recognises the role of captive (*ex situ*) management to reverse species declines and prevent extinction (IUCN/SSC, 2014; McGowan *et al.*, 2017). Captive breeding has contributed to the recovery of a number of threatened species, including 19 of 64 species where the IUCN Red List status was down-listed (improved) between 1980 - 2004 (Hoffmann *et al.*, 2010). Captive breeding has prevented extinction for species such as the Arabian oryx (*Oryx leucoryx*, Islam *et al.*, 2011), black-footed ferret (*Mustela nigripes*, Dobson & Lyles, 2000) and California condor (*Gymnogyps californianus*, Meretsky *et al.*, 2000). The proportion of zoo-bred source populations in published North American translocations has increased since 1974 (Brichieri-Colombi *et al.*, 2019), highlighting the ongoing potential of captive methods to contribute to conservation efforts. Captive breeding programs can take various forms, including insurance populations that aim to sustain captive populations in the long-term until threats in the wild have abated (Conde *et al.*, 2011; Conway, 2011); breed-for-release programs that bring wild animals in to captivity, breed them and release their progeny (see for example McCleery *et al.*, 2014); and source populations for the reintroduction/translocation of individuals to restore wild populations (McGowan *et al.*, 2017).

Captive management must balance a number of genetic challenges (Conway, 2011). These breeding programs aim to halt evolution by ensuring captive populations retain wild genetic diversity (Lacy, 2009). However, genetic change can occur in captivity as a result of inbreeding, genetic drift, or adaptation to captivity (Lees & Wilken, 2009; Frankham *et al.*, 2010). Heritable genetic changes in a population differ from plastic changes that occur within individuals' lifetimes; the latter including learned behaviours (e.g. Shier & Owings, 2007) and changes in microbiome composition (e.g. Chong *et al.*, in press). Population-level evolutionary

changes from one generation to the next form the focus of this thesis. Generational change may occur as a result of neutral change, such as genetic drift. With small population sizes, allele frequency changes due to drift can lead to fixation. In conservation breeding programs, quantitative goals, such as the retention of over 90% wild gene diversity over 100 years, are typically used to establish annual demographic and genetic targets to minimise the effects of neutral change for a given population size (Frankham *et al.*, 2010).

Pedigree-based management is widely implemented in captive management to minimise the effects of neutral genetic change and inbreeding (Lacy *et al.*, 1995). By incorporating information from studbooks that record the parents of offspring, and pedigree-based software such as PMx (Ballou *et al.*, 2010b; Lacy *et al.*, 2012), the relationships among individuals can be used to calculate genetic metrics for management. Currently, the method of maintaining genetic diversity in captive breeding programs is to equalise founder representation. This is achieved by mean kinship minimisation, which uses known relationships among individuals to form breeding pairs with low average relatedness to the rest of the breeding population, and similar mean kinship values to each other (Montgomery *et al.*, 1997; Frankham *et al.*, 2010). Combined with avoidance of mating close relatives, simulations have shown that the mean kinship strategy is effective in maximising retained genetic diversity and minimising inbreeding (Ivy & Lacy, 2012), and is more efficient in retaining genetic diversity than random mating (Montgomery *et al.*, 1997; Fernández & Caballero, 2001). Furthermore, by prioritising the least represented lineages for breeding, the mean kinship strategy equalises family sizes, which maximises the effective population size and the retention of wild genetic diversity (Caballero & Toro, 2000). When relationships among wild-caught founders are unknown, the mean kinship strategy is effective in minimising the loss of genetic diversity in the long-term, although unidentified variance in founder relatedness can cause inbreeding to increase in the short-term (Rudnick & Lacy, 2008). Pedigree-based management can therefore be very effective for minimising neutral change due to genetic drift when pedigrees are known and accurate. Nevertheless, differential survival and reproductive success can occur in captivity, despite pedigree-based management efforts to minimise this inter-individual variance, potentially resulting in adaptive population change. Management strategies to mitigate this adaptive change have been given less attention than strategies to retain genetic diversity (Leus *et al.*, 2011), even

though adaptation to captivity is recognised as a top priority for genetic management (Frankham, 2010b).

Adaptation to captivity broadly refers to the changes that can occur at an individual or population level as a result of the captive environment. Such changes may be behavioural, phenotypic, genetic or a combination of these. Genetic adaptation to captivity can occur at a population level if alleles that are deleterious in the wild are favoured and become more frequent in captivity. If the individuals best suited to the captive environment have higher survival and/or reproductive success than those less suited (even despite management efforts to equalise contributions), alleles underpinning the corresponding traits will increase in frequency and reproductive success in captivity can improve. Increases in fitness over generations in captivity have been experimentally demonstrated in the model organisms *Drosophila melanogaster* (Frankham & Loebel, 1992; Gilligan & Frankham, 2003) and white-footed mouse (*Peromyscus leucopus*, Lacy *et al.*, 2013). In conservation breeding populations, reproductive improvements such as increased offspring survival (delta smelt [*Hypomesus transpacificus*, Finger *et al.*, 2018]), and increased gamete production (Houbara bustard [*Chlamydotis undulata*, Chargé *et al.*, 2014a]) have been found over captive generations. Fitness changes do not necessarily take many generations of captive breeding to be of note: Fraser *et al.* (2018) found substantial variation in fitness across brook trout (*Salvelinus fontinalis*) populations after just one generation of captive breeding. Nevertheless, when the ultimate aim of reintroduction/translocation to the wild is considered, captive adaptations are likely to be inappropriate.

Reintroduction attempts using captive-born animals are generally less successful than wild-to-wild translocations (Fischer & Lindenmayer, 2000). A review in carnivores found that wild-caught animals are more likely to survive translocation than captive-born conspecifics (Jule *et al.*, 2008). The loss of predator avoidance behaviours in captive populations may contribute to poor survival upon reintroduction. For example, a generational deterioration in antipredator response has been observed in the Mallorcan midwife toad (*Alytes muletensis*, Kraajieveld-Smit *et al.*, 2006), the red jungle-fowl (*Gallus gallus*, Håkansson & Jensen, 2008), and the oldfield mouse (*Peromyscus polionotus subgriseus*, McPhee, 2004). In the Tasmanian devil (*Sarcophilus harrisii*), captive devils released to the wild had a higher probability of death by roadkill with increasing generations in captivity (Grueber *et al.*, 2017). Even if captive-born

animals survive upon release, they must also reproduce to contribute to successful reintroduction efforts. However, numerous studies of fish species have found lower relative reproductive success of hatchery-origin fish spawning in the wild compared to wild-origin fish (Araki *et al.*, 2007; Milot *et al.*, 2013; Christie *et al.*, 2014; Ford *et al.*, 2016; Janowitz-Koch *et al.*, 2019; Skaala *et al.*, 2019). The effects of captive breeding may also have a detrimental carry-over effect to the wild-born descendants of hatchery-bred fish (Araki *et al.*, 2009). Agent-based models predicted that the release of captive-born animals with even slightly lowered heritable fitness in the wild will lower population sizes and reduce genetic diversity over time, especially in short-lived species (Willoughby & Christie, 2019). In addition to lowered reproductive success, captive-born animals may also demonstrate assortative mating upon release due to changes in mate preference (Slade *et al.*, 2014), or shifts in phenological traits, such as the timing of migration in Atlantic salmon (*Salmo salar*, Horreo et al. 2017). Assortative mating will limit the genetic contribution of captive-born animals to the wild, potentially negating the demographic boost a reintroduction provides. Reduced success of reintroduced captive animals is a concern, particularly given that genetic adaptation to captivity can occur in just one generation of captive breeding (Christie *et al.*, 2012; Christie *et al.*, 2016), and may be difficult to avoid.

Reduced reproductive success may also occur within captivity. In most settings, adaptation to captivity is expected to increase reproductive success in captivity. Yet, in conservation breeding programs that are managed using the mean kinship strategy, a counter-intuitive consequence of differential selection could be a *decrease* in the population mean reproductive success over time. The mean kinship strategy targets the least represented lineages for breeding. If these groups have low representation due to heritably poor reproduction in captivity, then over time these underrepresented individuals will become increasingly targeted, while family lines better-suited to breeding in captivity, and which therefore easily become overrepresented, are given lower priority to breed. For example, in the Tasmanian devil, I previously found that the probability of breeding at the first attempt declined from 56% in wild-born females to 2.8% in generation five captive-born females (Farquharson *et al.*, 2017, Appendix 10). It is therefore critical to the long-term sustainability of conservation breeding programs to consider the effect of adaptation to captivity on reproductive success in captivity, and not just after reintroduction. Captive management

must balance the demand for successful reproduction in captivity with the need to avoid adaptation to captivity.

1.1 MINIMISING ADAPTATION TO CAPTIVITY

The rate of genetic change as a result of adaptation to captivity is expected to increase with increasing generations in captivity, selection intensity, genetic diversity and effective population size based on Frankham's equation:

$$GA_t \sim Sh^2 \sum (1 - \frac{1}{2N_e})^{t-1}$$

where S is the coefficient of selection, h^2 is heritability, t is the number of generations in captivity and N_e is the effective size of the captive population (Frankham *et al.*, 2002).

Based on this equation, a number of management strategies have been proposed by Williams and Hoffman (2009) to minimise genetic adaptation to captivity, including:

a) Minimising the number of generations in captivity
b) Population fragmentation
c) Minimising selection

A more detailed background to each of these strategies, along with the challenges of applying them is outlined below.

Minimising the number of generations in captivity

Minimising the number of generations in captivity reduces the potential for genetic adaptation to captivity either absolutely (e.g. breed-for-release) or relative to time (e.g. extend generation length to reduce number of generations occurring over a specified time-frame) (Williams & Hoffman, 2009). Breed-for-release programs, also referred to as supportive breeding, do not accumulate captive generations because captive-born animals are immediately released. This resource-intensive approach is commonly applied for taxa producing many offspring, such as fish, amphibians and insects (Fiumera *et al.*, 2004; Fisch *et al.*, 2015). The breed-for-release strategy is not feasible for species without an appropriate wild source population (such as the extinct-in-the-wild scimitar-horned oryx, *Oryx dammah*), suitable habitat for reintroduction (Hardman *et al.*, 2016) or if there is a risk of disease transmission between captive and wild populations (e.g. Tasmanian devil, threatened in the wild by the contagious devil facial tumour disease [Hawkins *et al.*, 2006; Pearse & Swift,

2006]). Instead, populations held as part of insurance populations are unlikely to be returned to the wild until multiple generations of captive breeding have accumulated. Gene flow from immigration of wild-born founders will mitigate shifts away from the wild population's mean phenotype (Ford, 2002), but obtaining wild-born animals is not always possible.

Strategies to extend the generation length so that a population experiences fewer generations in captivity over time include delaying the age at breeding and assisted reproductive technologies. Age at breeding can be delayed by isolating males and females (Asa, 2016), or through contraception (Cope *et al.*, 2018b). However, delaying the age at breeding can risk the female never producing offspring ("use it or lose it"; Penfold *et al.*, 2014), and lead to reproductive and health problems (acyclicity in captive elephants and rhinoceroses [Hermes *et al.*, 2004]; endometrial hyperplasia in canids [Asa *et al.*, 2014]). Preventing natural behaviours at the appropriate age can also lead to social development issues that may result in inexperience and mismothering at later reproductive opportunities (Asa, 2016). Allowing animals to breed but culling surplus offspring extends generation intervals without fitness consequences (Penfold *et al.*, 2014; Asa, 2016), though this comes with numerous ethical considerations. Assisted reproductive technologies such as artificial insemination are a more acceptable strategy to extend generation length (Ballou, 1984). In the black-footed ferret, artificial insemination using cryopreserved semen from wild-born founders 20 generations removed from the captive population successfully enhanced gene diversity (Wildt *et al.*, 2016), demonstrating the utility of this strategy. However, developing these technologies is technically difficult for use in endangered species, particularly for those without a close domestic relative or with under-researched reproductive biology (Herrick, 2019). In the early stages of captive breeding programs, where population growth is prioritised, delaying the age at reproduction or otherwise minimising the number of generations in captivity is unlikely to be a focus.

Population fragmentation

Larger populations retain higher adaptive potential (Willi *et al.*, 2006; Hoffmann *et al.*, 2017). Small effective populations will minimise adaptive change (Williams & Hoffman, 2009), however, this overtly conflicts with targets of large effective population sizes in order to minimise neutral genetic changes (drift), and inbreeding depression. To balance goals of minimising adaptive and neutral change, the fragmentation of large captive populations into

small separately managed subpopulations has been proposed (Frankham, 2008). Although small populations are at greater risk of genetic drift, because drift is stochastic each subpopulation will likely retain different genetic profiles so that the overall population is more genetically diverse than one large population. Pooled subpopulations will have less genetic adaptation to captivity than one large population of the same size (Margan *et al.*, 1998). Global-scale captive breeding programs with an international studbook are essentially already managed under this strategy if the different regions are considered as subpopulations (Leus *et al.*, 2011).

Minimising selection in captivity

Genetic adaptation to captivity will be reduced if both intentional and unintentional selective pressures in captivity are minimised. Species are expected to have different responses to the captive environment due to their inherent shyness/boldness and behavioural flexibility (Mason, 2010), with highly variable heritability of different behavioural traits (Courtney Jones & Byrne, 2017). Intentional selection against individuals with perceived negative traits (such as stereotypic behaviours as a result of stress [Mason, 2010]), in favour of tame individuals (Wielebnowski, 1999; McDougall *et al.*, 2006), should be avoided. Otherwise, detrimental adaptation to captivity may be accelerated if negatively perceived traits are beneficial in the wild, and captive productivity could decline if such traits are linked to reproduction (Lacy *et al.*, 2013). Pedigree-based management using mean kinship successfully reduces intentional selection by equalising family sizes and removing bias to choose “good breeders” (Tetley & O'Hara, 2012). The mean kinship strategy can in fact halve the rate of genetic adaptation to captivity (Allendorf, 1993; Frankham *et al.*, 2000), though equal founder representation is rarely achieved in practice (Schulte-Hostedde & Mastromonaco, 2015). However, even under best-practice mean kinship pedigree-based management, mechanisms such as selective sweeps have been shown to reduce genetic diversity in captive *Drosophila* (Montgomery *et al.*, 2010). Captive management therefore aims to reduce variation among individuals in order to prevent selection and maximise the retention of genetic diversity.

1.2 MECHANISMS OF CHANGE

Captivity may drive unintentional "domestication" as a result of differences between the captive and wild environments, such as the provision of veterinary care and often high-quality resources. Altered selective environments, such as the change to higher-density housing, are hypothesised to have resulted in hundreds of differentially expressed genes involved in wound-healing, immune and metabolic responses, in one generation of captive breeding of the steelhead trout (*Oncorhynchus mykiss*, Christie *et al.*, 2016). To reduce unintentional selection, many zoos/wildlife parks aim for wild-like captive environments that, where possible, reflect natural social and environmental conditions. For example, large free-range enclosures where animals are housed in groups reflective of wild social structures allow for the expression of normal behaviours including mate choice (Swaisgood & Schulte, 2010). Providing opportunity for mate choice can also overcome issues of mate incompatibility or lack of mating experience that can otherwise hinder captive breeding programs (Asa *et al.*, 2011; Schulte-Hostedde & Mastromonaco, 2015). Mate choice has been demonstrated to improve fitness of various species, including the eastern barred bandicoot (*Perameles gunnii*, Hartnett *et al.*, 2018), stripe-faced dunnart (*Sminthopsis macroura*, Parrott *et al.*, 2019b), koala (*Phascolarctos cinereus*, Brandies *et al.*, 2018), giant panda (*Ailuropoda melanoleuca*, Martin-Wintle *et al.*, 2015), and others (see Martin-Wintle *et al.*, 2019 for review).

Nonetheless, it is important to consider that if mate choice is underpinned by genetic variation among individuals (which it typically is [Andersson & Simmons, 2006]), it could in fact accelerate adaptation to captivity (Chargé *et al.*, 2014b). For example, under the 'good genes' hypothesis, mate choice that favours individuals with certain characteristics over others will drive variation in individual reproductive success, increasing reproductive skew (proportion of individuals that fail to breed) and decreasing population-level genetic diversity and effective population size (Andersson, 1994; Chargé *et al.*, 2014b). Furthermore, it is plausible that mate choice preferences vary to suit the expected environmental conditions of the offspring (Tregenza & Wedell, 2000): if mate choice preferences differ in captivity from the wild, mate choice could drive adaptation to captivity. This mechanism of adaptive change relies on variation in reproductive success and/or mate choice being heritable — fitness traits typically have low heritability, so would require strong selection to respond (Hansen *et al.*, 2011; Hoffmann *et al.*, 2016).

In addition to pre-copulatory mate choice, post-copulatory selection may occur. Neither pre-copulatory nor post-copulatory selection can be detected using traditional pedigrees alone, necessitating molecular genetic approaches. Genetic management in captivity is underpinned by neutral theory and Mendelian inheritance: the offspring in a pedigree are assumed to be representative of the Mendelian proportions expected given the combination of parental genotypes. However, early offspring losses can occur on a non-random genetic basis if certain genotypes are incompatible with survival, receive differential parental investment, are immunogenetically incompatible with maternal genotypes, or are outcompeted by siblings with different genotypes (Grueber *et al.*, 2015a). The stage at which selection occurs may be difficult to pinpoint. Mammalian species with reproductive delays such as delayed implantation may exhibit post-copulatory sexual selection that could bias offspring genotypes (Orr & Zuk, 2014). Selection could alternatively, or additionally, operate at later stages. For example, the Tasmanian devil gives birth to over 20 embryos, yet only a maximum of four can attach to teats to survive (Guiler, 1970), generating competition between siblings that may result in offspring being a non-genetically representative sample of the original litter (Grueber *et al.*, 2015a). If genotypes favoured in captivity differ from those favoured in the wild, early viability selection may provide a mechanism for adaptive genetic change.

Strategies to minimise selection in captivity are theoretically sound but may be difficult to implement in practice, and the trade-offs of allowing mate choice in group-housing demands further exploration. As unintentional evolutionary change cannot be detected from pedigrees, molecular genetic tools are needed.

1.3 DETECTING ADAPTIVE CHANGE

Decreased costs of sequencing and the development of new techniques has enabled a variety of genetic approaches to be more widely used in conservation settings (Puckett, 2017; Norman *et al.*, 2019). Microsatellites, the most popular application in the 2000s, typically provide low marker density (Witzenberger & Hochkirch, 2011), limiting the ability to detect pre- or post-copulatory selection. Single nucleotide polymorphism (SNP) arrays generate many more markers, yet often require genomic resources for development. SNP arrays are therefore usually restricted to species with close domestic relative such as the addax (*Addax nasomasculatus*, Ivy *et al.*, 2016) or species with a reference genome (e.g. Tasmanian devil; Wright *et al.*, 2015). With the dawn of the genomics era, new approaches that do not require

a reference genome, such as reduced-representation sequencing (RRS) (Peterson *et al.*, 2012) have enabled conservation researchers to cost-effectively obtain the thousands of SNP markers needed to investigate adaptive change. However, analysing RRS data presents new challenges for conservation managers accustomed to working with tens of loci. Analytical choices can influence results (Shafer *et al.*, 2017), so methods to assess markers for their reliability are needed, particularly in applications such as pedigree reconstruction. Molecular resolution of pedigrees not only assists in genetic management of captive populations but can also be used as the basis of investigating mechanisms of adaptive change, including individual variation in reproductive success and early-viability selection.

1.4 DATA-DRIVEN APPROACHES

Adaptation to captivity is likely to occur in diverse taxa, yet is rarely investigated in conservation settings: most experimental hypothesis testing has used model organisms and fish. Generational changes in captivity may occur as a result of shifts in population genetic parameters from one generation to the next, or via transgenerational mechanisms (e.g. maternal effects, epigenetics, behavioural transmission), or an interaction of the two. Disentangling genetic and non-genetic effects can be difficult and requires large multi-generational datasets. A major challenge to previous investigation in conservation contexts has been the limited data available. Data from captive contexts is restricted by small population sizes and low number of generations in captivity for recent programs or long-lived species. In addition, species may vary in their response to captive breeding, so an investigation in one species might not apply more broadly. To overcome these challenges in a conservation setting, this thesis uses two main approaches: combining datasets from multiple species and applying recently developed molecular genetic approaches to a large, diverse study system, the Tasmanian devil.

The first approach overcomes sample size limitations by combining data from multiple species. This can be achieved by synthesising available primary literature (i.e. systematic review and meta-analysis) or by analysing large studbooks from diverse species. The collation of huge databases such as studbooks and husbandry records by zoos and regional zoo associations provides an opportunity to retrospectively investigate generational fitness changes without the need for interventional experiments that could disrupt best-practice management. By incorporating phylogenetic comparative methods, new inferences can be

gained on patterns across taxa in order to make broad conclusions that will inform future research directions and conservation management.

The second approach uses RRS markers to explore pre- and post-copulatory selection that would otherwise go undetected by pedigree-based management, in the largest managed captive breeding program in Australasia. The Tasmanian devil is endangered due to the emergence of devil facial tumour disease (DFTD) in 1996, a transmissible cancer that is nearly always fatal (Hawkins *et al.*, 2006). In response to large-scale population declines, an insurance population was established in 2006 and has since grown to over 700 individuals (Hogg *et al.*, 2017b). Although this is a relatively recent captive breeding program, devils have a short lifespan of 5 to 7 years (in captivity and disease-free populations) and a rapid generation time, reaching sexual maturity at 1 to 2 years (Keeley *et al.*, 2012). As a result, the number of captive generations has accumulated quickly, enabling an investigation of generational change. The captive population is housed across a gradient of management intensity, including small zoo enclosures with one-on-one pairings and large free-range enclosures with approximately 20 adults (Hogg *et al.*, 2017b). The range of housing types makes the devil program ideal for investigating pre- and post-copulatory selection as selective pressures may vary across contexts (Grueber *et al.*, 2018b). In addition to the large captive breeding program, the devil's reproductive biology allows for an investigation of mechanisms of adaptive change. As introduced above, female devils produce an excess of embryos (Guiler, 1970), providing an ideal opportunity to examine early-viability selection. Furthermore, mate competition that may drive variation in reproductive success is likely, as devils are polygamous, polyoestrous seasonal breeders and can have mixed-paternity litters (Russell *et al.*, 2019). Due to the success of the insurance population, captive devils are now being released to support wild populations (Grueber *et al.*, 2018b), so it is timely to investigate adaptive change in captivity.

1.5 RESEARCH AIMS

This thesis aims to investigate adaptation to captivity and possible mechanisms of generational fitness changes using a data-driven approach. This is achieved as follows:

- In **Chapter 2**, I used a systematic review and meta-analytic approach to quantify differences in reproductive success between wild-born and captive-born animals in captivity, and to establish whether specific or diverse taxa are affected. I observed lower reproductive success of captive-born animals across taxa, especially when offspring survival was measured. These findings informed the questions and approaches for Chapter 3.
- **Chapter 3** uses global and regional studbooks of 15 taxonomically diverse species, to examine long-term multi-generational changes in offspring survival in captivity. The results showed that generational fitness changes are occurring, yet are highly variable, in managed captive populations. These findings inform recommendations around adaptation to captivity in diverse, global conservation contexts.
- To be able to test the general patterns seen in Chapters 2 and 3 in more detail using my model species, the Tasmanian devil, **Chapter 4** presents a method to process high-throughput reduced-representation sequencing data. This method was then applied in Chapters 5, 6 and 7.
- Group housing is often suggested as a strategy to reduce unintentional selection in captivity and promote mate choice by housing individuals in 'wild-like' settings. In **Chapter 5**, I applied the method developed in Chapter 4 to a real-world setting to resolve the pedigree of group-housed Tasmanian devils and compare the molecular pedigree reconstruction to traditional pedigree methods. I uncovered high variation in reproductive success between individuals and identified non-breeding wild-born founders.
- Chapter 5 uncovered high inter-individual variation in devil reproductive success, so in **Chapter 6**, I investigated a variety of possible genetic and non-genetic drivers of reproductive success that may explain this variation. I examined various mate choice hypotheses in a non-experimental captive setting as a possible mechanism of genetic change in captivity. None of the mate choice hypotheses that I tested predicted

reproductive success, although non-genetic factors such as age and weight did contribute to variation.

- **Chapter 7** investigates a further mechanism for adaptive change in the devil breeding program: undetected early viability selection. I performed a triads (sire-dam-offspring) analysis by comparing offspring genotypes to the ratio of genotypes expected under Mendelian inheritance given known parents, the basis of traditional pedigree management. I identified deviations from expected ratios across a gradient of management intensity in captivity.

Together, the results of this thesis provide new insights into adaptation to captivity in conservation settings and possible mechanisms of adaptive genetic change.

Chapter 2: A meta-analysis of birth origin effects on reproduction in diverse captive environments

2.1 BACKGROUND

Chapter 2 comprises the published manuscript:

Farquharson, K.A., Hogg, C.J., & Grueber, C.E. (2018) A meta-analysis of birth origin effects on reproduction in diverse captive environments. *Nature Communications*, **9**, 1055.

This chapter investigates differences in reproductive success in captivity between captive-born and wild-born animals. In order to understand broad patterns in birth origin effects, a systematic review and meta-analysis covering diverse literature including aquaculture, laboratory research and conservation contexts was performed (up to June 2016). The meta-analysis revealed substantially lower reproductive success in captive-born animals in captivity compared to their wild-born counterparts. Supplementary Material is available in Appendix 1. The Supplementary Dataset is available in Appendix 2 and the R code written to perform the meta-analysis is provided in Appendix 3.

I undertook the research in this chapter and drafted the manuscript. Carolyn Hogg and Catherine Grueber oversaw the project, provided technical and conceptual guidance, and critically revised the manuscript. Note that while this chapter has been formatted for the thesis for consistency, it still follows the format of *Nature Communications* which is Introduction, Results, Discussion and then Methods in line with the published version.

2.2 MAIN ARTICLE

A meta-analysis of birth origin effects on reproduction in diverse captive environments

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Abstract

Successfully establishing captive breeding programs is a priority across diverse industries to address food security, demand for ethical laboratory research animals, and prevent extinction. Differences in reproductive success due to birth origin may threaten the long-term sustainability of captive breeding. Our meta-analysis examining 115 effect sizes from 44 species of invertebrates, fish, birds, and mammals shows that, overall, captive-born animals have a 42% decreased odds of reproductive success in captivity compared to their wild-born counterparts. The largest effects are seen in commercial aquaculture, relative to conservation or laboratory settings, and offspring survival and offspring quality were the most sensitive traits. Although a somewhat weaker trend, reproductive success in conservation and laboratory research breeding programs is also in a negative direction for captive-born animals. Our study provides the foundation for future investigation of non-genetic and genetic drivers of change in captivity, and reveals areas for the urgent improvement of captive breeding.

Introduction

Animals have been kept by humans since the change from a hunter-gatherer lifestyle to farming approximately 8,500 years ago (Diamond, 2002). Successful reproduction is the most fundamental requirement of captive breeding programs across a range of industries including commercial production, conservation, and research. The domestication of wild animals involves increasingly diverse species to address global food security (Subasinghe *et al.*, 2009). In particular, the growth of the aquaculture industry from less than one million tonnes of aquatic food (including fish, crustaceans, molluscs, echinoderms, and amphibians) in the 1950s, to an expected 85 million tonnes by 2030, has driven the diversification of species used (Subasinghe *et al.*, 2009). The establishment of closed-cycle breeding programs is essential for the growth and sustainability of aquaculture as wild fishstocks continue to be depleted (FAO, 2016). In conservation, captive breeding has been recommended by International Union for the Conservation of Nature (IUCN) Red List assessors for 2,199 species as a tool to reduce the threat of extinction (CBSG, 2017). For research populations, some countries have banned the use of wild-caught non-human primates in modern laboratory research and insufficient captive-born animals are produced to meet demand (Carlsson *et al.*, 2004). Successful captive breeding, as opposed to continual supplementation of captive populations with wild animals, can also help avoid additional welfare concerns arising from wild-born animals adjusting to a captive environment (Mason *et al.*, 2013). Thus, identifying limitations and opportunities for captive breeding across all industries is an urgent priority.

Considerable research has explored differences between captive and wild populations in terms of their health, genetics, nutrition, behaviour, physiology, and reproduction (for examples see Clauss *et al.*, 2008; Knibb *et al.*, 2014; Bailey *et al.*, 2015; Edwards *et al.*, 2015; Work *et al.*, 2015; Scheun *et al.*, 2016; Van der Weyde *et al.*, 2016). However, far less attention has been given to differences that may exist between wild-born and captive-born animals when both are considered in a captive environment. Although many breeding programs aim to replicate some wild conditions in the captive environment in order to promote successful reproduction, it is inevitable that differences in nutrition, social structures, and breeding strategies will occur. Genetic change in captive populations is likely, and potentially unavoidable, as a result of founder effects, inbreeding, drift and adaptation to captivity, among other processes (Frankham, 2008). If these processes combine to result in captive-

born animals that are less successful than their wild-born counterparts (Farquharson *et al.*, 2017, Appendix 10), closed-cycle aquaculture may not be economically viable and the long-term sustainability of conservation breeding and laboratory research is threatened. Conversely, genetic adaptation to captivity may increase the reproductive success of captive-born animals, however this comes at the cost of a potential reduction in fitness if animals are released to the wild (Araki *et al.*, 2007; Frankham, 2008).

Genetic change in captivity may be beneficial or deleterious depending on a program's goals. Aquaculture systems aim to domesticate species through selecting highly productive individuals over generations of captive breeding (Mignon-Grasteau *et al.*, 2005), while conservation breeding programs aim to avoid selection (Williams & Hoffman, 2009) in order to retain wild traits and genetic diversity in the eventual prospect of reintroduction to the wild (Frankham *et al.*, 2010). The role of selection in research breeding programs is less clear and depends on the species involved and the purpose of the research. Nevertheless, all three of these captive breeding industries share a reliance on successful reproduction among captive-born animals. Differences in reproductive success as a result of birth origin may arise as a result of genetic effects such as inbreeding depression (Boakes *et al.*, 2007) and adaptation to captivity (Christie *et al.*, 2012); non-genetic effects, such as inappropriate social development, stress (Kiik *et al.*, 2013), and nutrition (Levallois & de Marigny, 2015); and complex interactions, such as the early rearing environment and maternal effects (Matos, 2012). Due to this complexity, assessing the success of captive breeding programs by examining only one metric, such as breeding success (i.e., producing an offspring), fails to account for life-history trade-offs that may occur, and/or differential impacts of captivity throughout a species' life history. For example, if captive-born animals produce more offspring per breeding event than their wild-born counterparts but have higher juvenile mortality, life-time reproductive success (i.e., total genetic contribution to the next generation), may be similar to, or perhaps even lower, than wild-born individuals.

Birth-origin effects have been examined in a number of species with mixed results (Schwitzer & Kaumanns, 2009; Kiik *et al.*, 2013). As the majority of studies in this area focus on single species, it has not previously been possible to quantitatively ascertain whether differences in reproductive success follow general trends across taxa and captive environments or whether they are specific to the study species, the captive environment of interest, or the type of

reproductive trait examined. Here we provide a systematic review and meta-analysis to examine the influence of birth origin on reproductive success across multiple species, a variety of life-history traits and in various captive environments. We take a broad definition of 'reproductive success' to refer to diverse measures of reproductive traits, encompassing production of gametes/offspring at multiple stages throughout the life history of breeders. Specifically, our objective was to quantify differences in reproductive success between captive-born and wild-born animals, in captivity, across diverse animal species to determine whether birth-origin effects are specific to taxa or follow a general trend regardless of phylogeny. As all captive breeding programs (aquaculture, conservation, and laboratory research) require successful reproduction for their management objectives, all are included in this review. Diverse literature (115 effect sizes from 44 species) shows that, overall, captive-born animals have a 42% decreased odds of reproductive success in captivity compared to their wild-born counterparts. The strongest trends are seen in commercial aquaculture settings, with weaker effects (but in the same direction), in conservation and laboratory settings. The choice of traits measured also impacts the reported effect of birth origin on reproductive success, with offspring survival and quality being the most sensitive traits. Examining varied measures of reproductive success in this study gives insight into the possible drivers of birth-origin effects that have important implications for the establishment, efficacy and long-term viability of captive breeding programs.

Results

Wild-born animals are more productive in captivity

A total of 39 papers published between 1967 and 2015 contributed 115 comparisons of reproductive traits between captive-born and wild-born animals in captive environments for analysis (some papers compared more than one reproductive measure, or more than one species) (Supplementary Dataset A2). The final dataset included 44 species from phylogenetically diverse taxa including invertebrates, fish, birds, marsupials, and eutherian mammals (Figure 2.1).

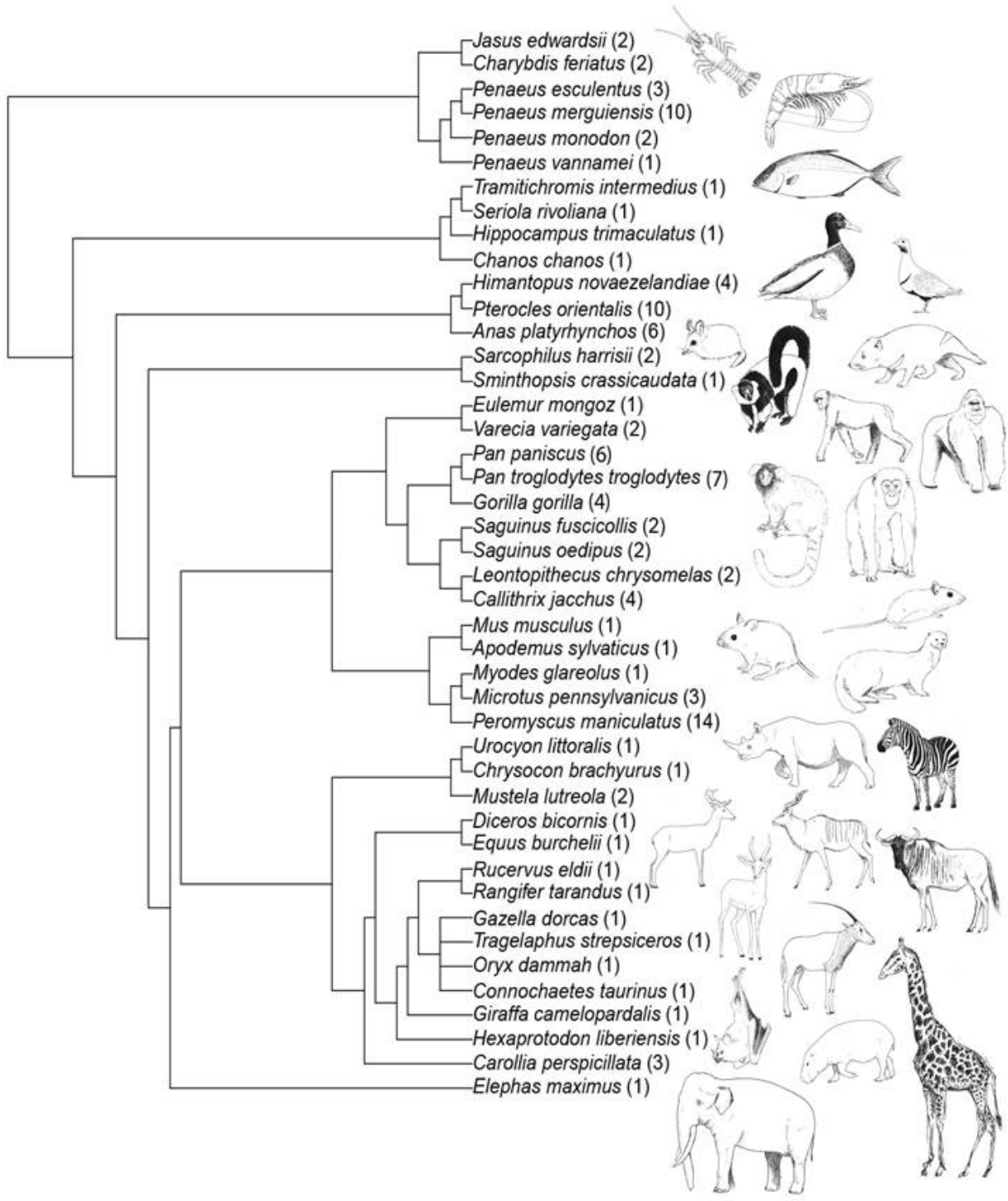

Figure 2.1: Phylogenetic tree of 44 species included in the meta-analyses.
The tree was created using the 'rotl' package (Michonneau *et al.*, 2016) in R. The total number of comparisons between captive-born and wild-born animals included for each species is given as (N).

We used the log odds ratio (lnOR) to quantify the standardised effect size of differences in reproductive success, where negative log odds ratios represent higher reproductive success of wild-born animals compared to their captive-born counterparts. Overall, wild-born animals have a 74.2% (lnOR = −0.56, 95% HPD CI:[−1.01, −0.10], Table 2.1) increased odds of reproductive success in captive environments compared to captive-born animals, equivalent to a small-medium effect size. Accounting for phylogenetic non-independence occurring as a result of shared evolutionary history did not greatly alter the point estimate but broadened the CI resulting in it crossing zero (Figure 2.2). Phylogeny accounted for only 0.29% of heterogeneity (see Table A1.4.1 for full extended heterogeneity statistics for both models), and phylogenetic heritability was low (H^2 = 0.0026), therefore our result is generalisable across species. As the non-phylogenetic model had a lower DIC than the phylogenetic model (DIC = 317.4 vs. 317.5) all subsequent analysis (meta-regression) proceeded without phylogeny. The high heterogeneity (I^2 total = 94%) observed within our dataset is not surprising given the diverse species, captive environments and reproductive traits included, and is consistent with other ecological and evolutionary meta-analyses (Senior *et al.*, 2016). We next examined the source of this heterogeneity by fitting moderator variables to our analysis, and determined the contribution of environment and trait type on the effect of birth origin.

Birth-origin effects vary with captive environment

Our dataset included data collected from four study environments: aquaculture (*N* = 9 publications, 23 comparisons), conservation (*N* = 14 publications, 51 comparisons), research (*N* = 15 publications, 40 comparisons) and other (*N* = 1 publication, 1 comparison). Effect sizes varied according to the captive environment of the study (Figure 2.2). In aquaculture systems, wild-born animals had a 328.7% increased odds of reproductive success relative to captive-born animals (lnOR = −1.45, 95% HPD CI: [−2.46, −0.56]; a large, statistically significant effect). In conservation and research environments, the estimated effect was in the same direction (negative, i.e., wild-born animals more reproductively successful), but not statistically significant at α = 0.05 (Table 2.1). The one study that we categorized as 'other' examined studbook data from Burmese timber elephants (Mar, 2002), which are bred as working elephants and do not fit any of our other categories. The estimated effect was positive (captive-born animals were more successful), though this had poor precision (Figure 2.2).

Table 2.1: Meta-analytic effect size estimates of differences in reproductive success between wild-born and captive-born animals in captive environments.

Posterior mode gives the meta-analytic log odds ratio (lnOR) estimate from the MCMCglmm models, with lower and upper 95% higher posterior density credible intervals given. Estimates with the 95% HPD CI excluding zero are marked with *. Percentage odds refers to the % increase (+) or decrease (-) in the odds of reproductive success of captive-born or wild-born animals, relative to the other group.

	Posterior mode (lnOR) [95% HPD CI]	% odds of captive-born reproductive success	% odds of wild-born reproductive success	*N*
Overall model*	-0.56 [-1.01, -0.10]	-42.3%	+74.2%	115
Overall model + phylogeny	-0.65 [-1.45, 0.04]	-47.7%	+91.3%	115
Captive environment				
Aquaculture*	-1.45 [-2.46, -0.56]	-76.7%	+328.7%	23
Conservation	-0.38 [-1.06, 0.30]	-31.8%	+46.6%	51
Research	-0.34 [-1.08, 0.35]	-29.0%	+40.8%	40
Other	1.84 [-0.98, 4.49]	+527.6%	-84.1%	1
Trait type				
Fertility & hatchability	-0.38 [-0.94, 0.15]	-31.5%	+45.9%	30
Reproductive yield	-0.52 [-1.06, 0.05]	-40.6%	+68.4%	28
Offspring quality*	-1.22 [-2.01, -0.46]	-70.5%	+238.8%	8
Offspring survival*	-1.26 [-1.85, -0.65]	-71.5%	+250.9%	33
Reproductive phenology	-0.04 [-0.69, 0.57]	-3.5%	+3.6%	16

Captive-born animals are less productive across life stages

Our dataset included comparisons from five, broad, trait type categories: fertility/hatchability (e.g., probability of breeding; *N* = 30 comparisons), reproductive yield (e.g. litter size; *N* = 28), offspring quality (e.g., birth weight; *N* = 8), offspring survival (*N* = 33) and reproductive phenology (e.g., interbirth interval; *N* = 16). For a full list of the specific reproductive traits included in each category see Table A1.4.2. Birth-origin effects were negative across all trait type categories (Figure 2.2). Wild-born animals had a statistically significant 238.8% greater odds of reproductive success relative to captive-born breeders when measured as offspring quality traits, and a 250.9% greater odds of offspring survival (both considered large effects, Table 2.1). No statistically significant effects of birth origin were observed when reproductive success was measured as fertility/hatchability, reproductive yield or reproductive phenology (Table 2.1).

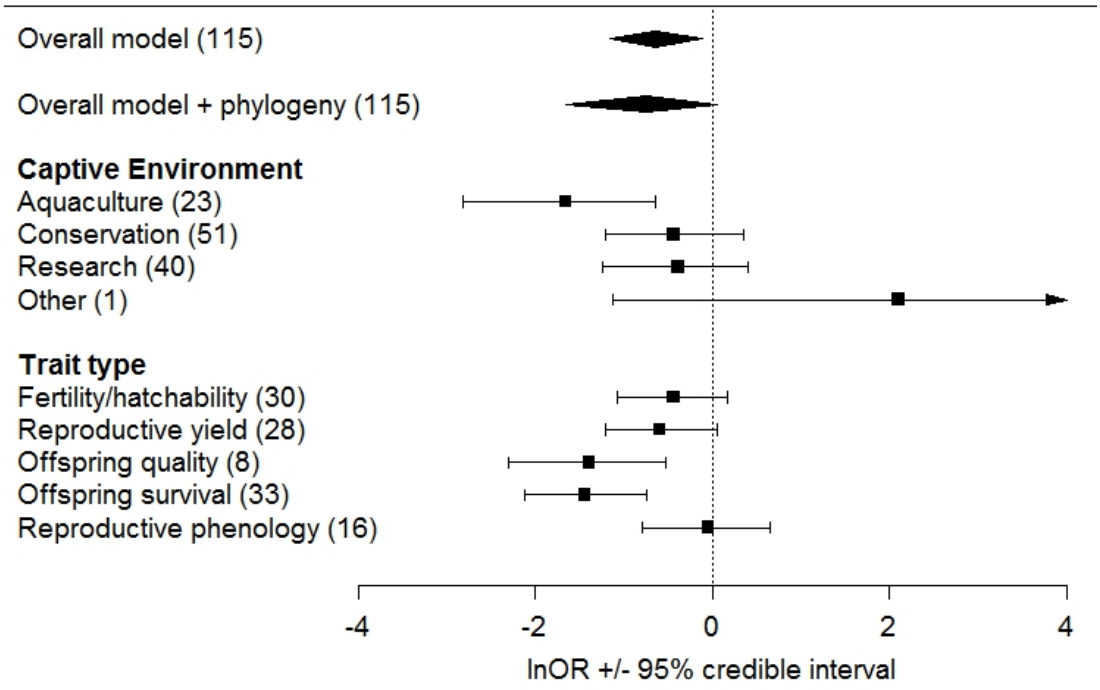

Figure 2.2: Forest plot of overall meta-analytic results (diamonds), and meta-regression models of captive environment and trait type (squares).
A negative log odds ratio (lnOR) indicates wild-born animals have higher reproductive success than their captive-born counterparts, with a positive log odds ratio referring to increased reproductive success of captive-born animals compared to wild-born. Squares represent the posterior mode (or parameter estimate) with error bars showing the 95% highest posterior density credible intervals (95% HPD CIs). (N) refers to the number of effect sizes. See Methods section for definition of study environments, and Table A1.4.2 for the comparisons included in each trait type category.

Drivers of birth-origin effects

As the difference between wild-born and captive-born reproductive success was detectable with some trait types and not with others, we examined whether the dataset was evenly distributed across study environments and trait types (Table 2.2). The two trait type categories showing a strong, significant influence of birth origin, offspring quality and offspring survival, were dominated by conservation and research comparisons (*N* = 7/8, *N* = 29/33, respectively). Data from aquaculture studies contributed to all trait type categories; but data from conservation environments largely contributed to fertility/hatchability (60%), offspring quality (62.5%) and offspring survival (51.5%) (Table 2.2). Comparisons made in research environments comprised the largest proportion of the reproductive yield trait category (57.1%), with correspondingly high contributions to data in the fertility/hatchability and offspring survival categories.

Table 2.2: Number of effect sizes in analysis, grouped by captive environment and trait type.

	Fertility & hatchability	Reproductive yield	Offspring quality	Offspring survival	Reproductive phenology	Total
Aquaculture	4	7	1	4	7	23
Conservation	18	5	5	17	6	51
Research	8	16	2	12	2	40
Other	0	0	0	0	1	1
Total	30	28	8	33	16	115

While we were primarily interested in comparing the effect of birth origin on reproductive success, we also recorded whether each study specified the number of generations of captive breeding of the captive-born population. The majority of the studies included did not specify the generation, nor range of generations, of the captive-born population (26/39 studies, 66.7%; 80/115 comparisons, 69.6%, Table A1.4.3), so we could not statistically analyse the effect of generations in captivity on reproductive success. Of the studies that did report generation, the most common comparison was to an F_1 (first generation) captive-born population (5/13 studies, 38.5%; 17/35 comparisons, 48.6%), and this comparison was found exclusively in aquaculture and research study environments.

We found no strong evidence that our results are influenced by publication bias (Supplementary Note A1.1, Figure A1.3.1). A total of 74 comparisons of interest were

excluded from our analysis because they did not report all the data required for inclusion; values for 17–41 of these comparisons could be recovered using multiple imputation (Supplementary Note A1.2). Although multiple imputation increased uncertainty in our results, the main effects were in the same direction and of similar magnitude to those obtained by our main analysis. We therefore do not believe that our overall conclusions are biased by missing data (Supplementary Note A1.2, Table A1.4.4).

Discussion

We synthesised the results of studies across different species, captive environments, and measures of reproductive success to provide an overall estimate of the effect of birth origin on reproductive success in captivity. Our analysis included 44 species across diverse animal taxa, including vertebrates and invertebrates. Surprisingly, across all species and captive environments, it was wild-born animals that had higher odds of productivity in captivity, relative to their captive-born counterparts (74.2%). As phylogenetic signal was low (<1%), it is likely that our overall result is generalisable across species, indicating a general trend toward declines in the reproductive success of captive-born animals relative to wild-born animals, in captive environments. Our meta-analysis enables us to examine the available data on this topic in more detail, and draw inferences about the possible causes of this unexpected pattern.

When our data were stratified by captive environment, aquaculture was the only environment to show a large, statistically significant mean difference between wild- and captive-born animals (Figure 2.2). Again, it was the wild-born animals that showed higher odds of reproductive success (328.7%), relative to their captive-born counterparts. This result is unexpected given that aquaculture aims to improve reproduction among captive-born stock in the process of domestication. This strong, significant effect has important implications for the sustainability of closed-cycle commercial production systems, suggesting that wild-stock supplementation, or other solutions, may be required. Effects in conservation and laboratory breeding contexts were in the same direction as seen for aquaculture, but weaker (Figure 2.2). There are several possible explanations for this variation among contexts, which is consistent with the differing goals of various captive breeding programs. For example, conservation breeding programs aim to minimise adaptation to captivity (Williams & Hoffman, 2009), while phenotypic and/or marker-assisted selection for favoured traits is

often an important goal of agricultural breeding programs (Dekkers & Hospital, 2002). These different goals predict decreased genetic change in conservation programs, compared to agricultural programs.

A further possible source of the variation among environments is differences in the length of the captive breeding programs included in publications. Captive-born animals in different environments varied in the number of generations of captive breeding, data that were often not reported (Table A1.4.3). For aquaculture data, 12 of the 23 comparisons originated from studies comparing the reproductive success of wild-born (F_0) to first generation (F_1) captive-born animals (Table A1.4.3). It is therefore likely that much of the difference in reproductive success we observe in aquaculture is related to changes occurring within the first generation of captive breeding, rather than across multiple generations. In contrast, none of the 51 comparisons made in conservation studies specified a wild versus F_1 comparison. In our conservation dataset, four comparisons were from studies comparing wild-born to a captive-born population ranging from F_1–F_3 or to F_4, while the other 47 comparisons did not report the captive generation of comparison used in the study (Table A1.4.3). Given the long-running nature of many conservation breeding programs, it is probable that many of the captive-born populations in those studies that did not specify the generation depth comprised a range of generations. Similarly, for research studies, captive-born animals were F_1 for only 5/40 of the comparisons made, 25 of the remainder were from studies that did not report generation depth (Table A1.4.3). As a result, we are unable to conclude whether the differences in birth-origin effects on reproductive success are influenced by general factors associated with captivity (such as purely environmental factors), or by characteristics of the captive-born population (such as genetic factors correlating with generation depth, including neutral or adaptive change). Conservation and research breeding programs could still be experiencing a reduction in reproductive success in the first generation of captive breeding. It is imperative that potential declines in the early stages of conservation breeding programs are reported and prevented, otherwise founder genetic diversity and the evolutionary potential of the captive population may be lost (Lacy, 1989).

Understanding possible causes of differences in reproductive success in the first generation of captive breeding is useful for the successful establishment of breeding programs. We suggest comparing changes within the first generation of captive breeding to long-term

changes over multiple generations in order to disentangle possible causative factors, such as environmental effects versus long-term genetic change. For example, one possible explanation of increased fitness of wild-born animals, relative to F_1 captive-born animals, is that animals caught in the wild have survived early and ongoing natural selection pressures, and are therefore 'fit'. Relaxed selective pressures in captivity mean that even F_1 animals that would be 'unfit' in the wild may survive to reproductive age. If these unfit animals are also unproductive, the captive-bred population would exhibit a reduction in reproductive success in the first generation, relative to wild-born animals. In an aquaculture setting this is not necessarily a cause for concern—artificial selection can act to increase population productivity where there is variation in heritable reproductive success over generations of captive breeding. This is most simply demonstrated by the breeder's equation: $R = h^2S$, where R is the response to selection, h^2 is the narrow-sense heritability and S is the selection differential (Falconer, 1960; Hill & Caballero, 1992). However, as traits linked with broad evolutionary fitness (such as reproductive success) tend to have low heritability (Merila & Sheldon, 1999), selection cannot be relied upon to improve them. Furthermore, processes such as antagonistic pleiotropy can complicate the response to selection on life-history traits in captivity (Bryant & Reed, 1999). In the establishment phase of an agricultural program, the potential benefits of long-term selection and domestication must be weighed against short-term productivity losses. In a conservation setting, unintentional selection may be disastrous. For example, offspring survival in utero that differs from Mendelian expectations provides an opportunity for early viability selection that is difficult to prevent and may impact on the effectiveness of pedigree management (Grueber *et al.*, 2015a; Chapter 7). Instead, efforts to address variation among breeders during the first generation of captive breeding, such as improved nutrition, should be prioritised (Izquierdo *et al.*, 2001).

The magnitude of birth-origin effects on reproductive success was influenced by the type of reproductive trait measured. Offspring quality and offspring survival showed the most pronounced decrease of captive-born relative to wild-born reproductive success (Figure 2.2). This result indicates the crucial importance of measuring fitness outcomes at multiple life-history stages. Our observation is consistent with a recent meta-analysis (Ronget *et al.*, 2017) that found a close link between offspring quality traits and offspring survival, estimating that a one standard deviation increase of offspring body weight increased survival odds by 71% in

mammals and 44% in birds. We observed that fertility/hatchability, reproductive yield and reproductive phenology trait types did not significantly differ between captive-born and wild-born animals, suggesting no evidence that captive-born animals compensate for reduced offspring survival (all lnOR estimates were negative) in a life-history trade-off framework (Table 2.1). Unnatural social environments or disrupted maternal contact during the early life-stages of captive-born animals may lead to maladaptive development and changes in behaviour (Mason *et al.*, 2013) such as mismothering and offspring abandonment. The mechanisms leading to maladaptive development may explain why we observed a significant decrease in offspring survival without significant differences in other traits that may be less influenced by behavioural changes (e.g., reproductive phenology). Taken together, our results indicate that, if the overall reproductive success of captive breeding programs is to be improved, population managers would be best placed to focus efforts on improving offspring quality and survival outcomes, as effects on other traits are likely to be weaker. For example, the effect of offspring body weight on juvenile survival in mammals is stronger in captive environments than in wild environments, and offspring mass is positively correlated with maternal mass (Ronget *et al.*, 2017). Thus, improving maternal nutrition in captive environments may increase offspring quality and survival through increased offspring birth weight.

Reduced offspring survival among captive-bred animals may also result from inbreeding depression—the reduction in fitness as a result of increased homozygosity of inbred animals, and accumulation of deleterious recessive mutations that may be lethal in early life (Charlesworth & Willis, 2009). Captive populations managed for conservation breeding purposes already implement strategies to avoid inbreeding, such as the use of pedigree-based management, and the incorporation of molecular techniques to assist in determining parentage (Ivy *et al.*, 2009; Chapter 5). Likewise, for laboratory research, genomic information is required for long-term management of non-human primate populations (Kanthaswamy *et al.*, 2009), and is particularly important not only for preventing inbreeding, but can also reveal genetic variance as a result of mixed ancestry that may influence treatment effects in biomedical research (Kanthaswamy *et al.*, 2013). The avoidance of inbreeding may not be as carefully managed in aquaculture settings, with short-term inbreeding even encouraged to some extent to develop homogenous stock that have uniform body sizes for easier

management, and to protect the intellectual property and commercial interests of breeders that supply stock to other fisheries by decreasing genetic variance available for further improvement by selection (Janhunen *et al.*, 2013; Doyle, 2016). Aquaculture species are not immune to the effects of inbreeding depression (Gallardo *et al.*, 2004), so applying management strategies at a population-level to prevent the effects of inbreeding depression remains a priority for captive breeding programs across all industries.

In this analysis, various measures of reproductive success were included within the offspring survival category, such as juvenile mortality rate, stillbirth/abortion rate and cannibalism, or abandonment of young (Table A1.4.2). As we have discussed, both genetic (e.g., inbreeding) and non-genetic (e.g., management practices, stress) factors could be responsible for decreased offspring survival in captive-born animals (Wielebnowski, 1996; Terio *et al.*, 2004; Yordy & Mossotti, 2016). Our dataset precludes determining the cause of this effect; without experimental data it is difficult to disentangle genetic and non-genetic effects. In aquaculture and research environments, we recommend designing experiments to separate these effects, as experimental crosses are more feasible than in conservation programs. For example, Christie *et al.* (2016) identified changes in gene expression between offspring of first-generation hatchery stock of steelhead trout (*Onchorhynchus mykiss*) and offspring of wild stock in captivity, and through a series of crosses were able to rule out maternal effects or chance events. Identifying causative factors will allow captive managers to address these changes, and may inform conservation breeding management. Conservation breeding programs can also benefit from the retrospective analysis of their large detailed datasets in the form of studbooks that are available for many species (see Mason (2010) for sources of data; Chapter 3). The incorporation of husbandry and behavioural data in regression analyses, possible through the release of the Zoological Information Management System (ZIMS) (Species 360, 2018), will assist in determining the factors affecting reproductive success and juvenile mortality.

Our systematic review has identified key areas where the reporting of additional data for captive-breeding studies could be improved, to increase the suitability of these observations for analysis of the effects of captivity in future meta-analysis. In total, 74 comparisons we identified in our systematic review were excluded from the main analysis solely on the criterion of missing data that precluded calculation of effect sizes. Most commonly, reports

of variance (such as the standard deviation or the standard error of the mean), and sample size were missing and unable to be inferred from the text. Together the 12 excluded publications made comparisons involving all four study environments (aquaculture, research, conservation and other), and all five trait type categories. The excluded data covered 10 additional species not otherwise included in our meta-analysis (Table A1.4.5), which were not taxonomically distinct from other species included in our analysis. Our results did not change greatly with the inclusion of 17 of these comparisons using multiple imputation (Table A1.4.4). The call for careful reporting of all relevant statistics required for meta-analysis in primary studies has been made often; recently Gerstner *et al.* (2017) provided a useful guide to authors as to what to include.

In conclusion, our meta-analysis shows that wild-born animals generally have higher reproductive success than their captive-born counterparts in captive environments, across multiple industries and irrespective of taxonomy. The increased reproductive success of wild-born relative to captive-born animals was particularly evident in aquaculture environments, which were more likely to report wild versus first-generation comparisons than studies from other environments. We urge greater reporting of the general characteristics of captive population studies, in particular generations of captive breeding, to enable a greater understanding of effects at the first and subsequent generations. Our literature search uncovered a large body of literature on other types of captive to wild comparisons that were not the target of our search criteria (Figure A1.3.2) and which therefore cannot be considered a systematic survey. Nevertheless, future systematic searches into these areas, especially captive-born to wild-born animals in the wild (e.g., reintroductions) may reveal long-term effects of captive breeding. Now that we have found strong evidence of birth-origin effects on reproductive success within captive environments, future research should experimentally investigate the factors driving these changes, to inform management decisions, such as preventing adaptation to captivity, avoiding inbreeding, reducing juvenile mortality, and establishing successful closed-cycle breeding programs.

Methods

Data collection

Following the PRISMA guidelines for systematic reviews and meta-analyses (Liberati *et al.*, 2009; Nakagawa & Poulin, 2012), we searched the 'Web of Science' database on 26 April 2016 and the 'Scopus' database on 7 June 2016, with no language or time restrictions, using the following terms related to reproductive traits and birth origin: (reproduct* OR product* OR hatch* OR fecund* OR "breeding success" OR "litter size" OR "juvenile mortality" OR "infant mortality") AND (captiv* OR "zoo-born") AND ("wild-born" OR "wild-caught" OR "wild-laid" OR "wild-bred" OR "free-ranging"). We also screened reference lists in relevant papers to obtain the broadest possible coverage. We obtained 1,065 results from our search of the 'Web of Science', and 600 results from our 'Scopus' search.

See Figure A1.3.2 for the overview and outcomes of our search strategy. We first removed duplicates between and within the two databases, leaving 1,160 unique works. We next examined the abstract and title of all works to identify potentially relevant primary sources, and downloaded full texts of sources that appeared to meet our inclusion criteria (see below). We considered only published papers that were the primary source of data (i.e., excluded reviews, books, conference proceedings and syntheses) to avoid the duplication of reporting. In order to isolate papers on our research topic of interest (the effects of birth origin in captivity), we classified all papers by the study populations they compared:

a) wild-born vs. captive-born in captivity (comparison of interest),
b) wild-born vs. captive-born in the wild (such as in reintroductions),
c) wild-born in captivity compared to the wild,
d) wild populations compared to captive populations,
e) other comparisons (such as those made at the level of the ancestors), and
f) two or more of the above.

Of 1,160 papers examined, 126 (10.9% of unique results) were screened by two people to ensure agreement and minimise the risk of researcher bias. After grouping papers by study population (i.e., a—f, above), papers that compared wild-born to captive-born animals in captivity (category a, and 12 papers from category f, total $N = 125$) were further screened to identify studies that reported data for at least one reproductive trait ('comparison', used for

calculating the effect sizes used in our meta-analysis). A total of 56 papers comparing reproductive traits in wild-born and captive-born animals in captivity were identified, encompassing 242 wild-born/captive-born comparisons considered for inclusion in the study.

Data extraction

We developed a data coding strategy to classify the comparisons by recording the first author, year of publication, journal of publication, species of study (common name and scientific name), study environment (see below), whether the captive generation of comparison was specified (e.g., F_1, F_2), comparison (reproductive trait) for each study, trait type (see below), measurement/statistic, error, and sample size.

Study environment was determined from the reported purpose for keeping the captive population, as described by the authors of each publication, and categorised as either:

a) Aquaculture—may occur in the laboratory, but the primary purpose is for commercial production/domestication of animals for consumption or trade.
b) Conservation—a captive breeding program with the purpose of propagating the species to reinforce the wild population, to provide an insurance population against extinction in the wild, or to educate members of the public.
c) Research—the purpose of the captive program is to provide animals for research under controlled conditions, for reasons other than developing a closed life-cycle production system, unless this is for a laboratory research species. The results of the study may inform conservation outcomes, but the animals are not propagated for conservation purposes.
d) Other—does not fall into any of the above categories.

Studies were included in the review and meta-analysis if they fulfilled the following inclusion criteria: (i) studies must have made at least one comparison of a reproductive trait between captive-born and wild-born animals of any species in a captive environment. Some studies hold animals in 'semi-natural' enclosures—we considered a study to take place in a captive environment if there were human barriers to movement for the purpose of holding animals and if some form of provisioning of resources (such as shelter, food and/or water) occurred. We did not require that the animals in a study were housed in the same physical location to be included in the meta-analysis, as long as the enclosure types were similar. For example,

captive-born and wild-born animals of the same species across multiple zoos were included. We considered animals to be 'wild-born' if they were brought into captivity from the wild either as eggs, young or mature individuals. (ii) Studies did not duplicate other included studies. In cases where duplicates were identified (by species studied, population reported, years of analysis and sample size), we selected the study that was most recent, or which had the greatest sample size (*N* = 1 study comprising 1 comparison was excluded for this reason). (iii) Papers (including any supplementary material) must contain extractable data (for example means, standard deviations and sample sizes, or other statistics or raw data that could be used to calculate effect sizes), this criterion resulted in the exclusion of *N* = 74 comparisons. (iv) The study must not have experimentally manipulated reproductive success, for example through the restriction of diet (*N* = 4 comparisons excluded). (v) Reproductive success was not systematically influenced by bias in opportunity to breed (*N* = 22 comparisons excluded). For example, many comparisons such as lifetime reproductive output can be influenced by captive management if wild-born animals are prioritised for breeding over captive-born animals as is the case in conservation breeding programs that aim to maximise the genetic contribution of founder animals (Ballou, 1984). (vi) Data were not duplicated within the study (*N* = 5 comparisons). For example, if male reproductive success, female reproductive success, and overall reproductive success were reported, only overall reproductive success was included. The excluded studies and the reasons for their exclusion are given in Table A1.4.5, with a flowchart of data filtering provided in Figure A1.3.3.

As our studies included diverse species and breeding strategies, we obtained many different comparisons related to reproductive success or failure. These were broadly categorized into 'trait types' as comparisons relating to the following: fertility/hatchability, reproductive yield, offspring quality, offspring survival, and reproductive phenology (Table A1.4.2). For each comparison, we determined whether it had a positive or negative relationship with overall reproductive success (Table A1.4.2). An increase in a comparison with a positive relationship would result in increased reproductive success. For example, an increase in probability of breeding, fertility rate, hatching rate, juvenile survival rate, and litter/clutch/spawn size are expected to be typically positively correlated with reproductive success. Increased interbirth intervals, juvenile mortality and age at first parturition are expected to be typically negatively correlated with productivity. For other comparisons, the directionality of a relationship with

reproductive success was unclear, for example date of parturition, gestation length and offspring sex ratio. As such, comparisons for which the direction of the effect on overall reproductive success could not be characterised were excluded from the meta-analysis (*N* = 20 comparisons).

After filtering on our inclusion criteria, 39 papers contributed 115 reproductive comparisons between captive-born and wild-born animals in captive environments for analysis. All 39 papers were coded by the same person, with 18 of these (46%) coded by an additional person to ensure agreement with the coding strategy.

Effect size extraction and calculation

For each comparison that satisfied our inclusion criteria, we extracted raw data for both the captive-born and the wild-born population reported in the text or in tables/figures (including supplementary material) to calculate an effect size, a measure of the magnitude and direction of the difference between the two populations (detailed below). Data that were reported only graphically were extracted using GetData Graph Digitizer 2.26 (Fedorov, 2002). For continuous comparisons (such as number of offspring), we obtained the mean, standard deviation and sample size for each group. Where the standard error was the only variance measure reported, we calculated the standard deviation as $SD = SE \times \sqrt{N}$.

If only 95% confidence intervals were presented, we calculated the standard deviation as $SD = \sqrt{N} \times \frac{(upper\ 95\%\ CI - lower\ 95\%\ CI)}{3.92}$. For proportional comparisons (such as hatching rate), we recorded the number of events out of the total sample size (*n*/*N*). Some studies reported the frequency of singletons, twins, triplets, and quadruplets between wild-born and captive-born animals in captivity. Where possible, overall litter size was calculated from this data and used as the comparison instead.

Stochastic dependency can occur when multiple comparisons are made of the same data, resulting in biased wild-born to captive-born comparisons (Gleser & Olkin, 2009; Noble *et al.*, 2017). In our dataset, this non-independence occurs in studies that reported productivity for the population of wild-born individuals, compared multiply to each generation of captive breeding. In such cases, we obtained the overall mean for the captive-born animals for effect size calculation, where possible. If overall values were not calculable, we used only the data from the first generation of captive breeding (F_1) to compare to the wild-born generation (F_0).

Likewise, for studies that compared more than two populations (e.g., wild-born animals compared to two groups of captive-born animals), we included the effect size associated with only one comparison, chosen as the pair of populations most comparable to one-another in all other respects (e.g., housed at the same location under the same conditions, comparison reported for the same year), or by pooling data from the multiple captive-born populations if they were identical treatments (e.g., tanks of fish).

We chose the log odds ratio (lnOR) as our measure of effect size, as it could be calculated for both the continuous and proportional data present in our analysis. Log odds ratios between the wild-born population and captive-born population and their unbiased estimates of sampling variances were computed for each comparison using the 'metafor' package in R (Viechtbauer, 2010; R Core Team, 2016). The log odds ratio is a symmetric measure centered around zero; data were input such that a positive log odds ratio refers to increased reproductive success of captive-born animals relative to wild-born counterparts and a negative log odds ratio refers to the converse. A small constant (0.5) was added to zero values in proportional data to allow for estimation of the effect size; this applied to 3/48 (6%) of effect sizes calculated from proportional data.

Meta-analytic procedures

To account for the non-independence of effect sizes as a result of the shared evolutionary history of closely related species, we obtained the phylogenetic correlation between the species in our meta-analysis using the 'rotl' package (Michonneau *et al.*, 2016) in R, based on published phylogenies available through the Open Tree of Life (Hinchliff *et al.*, 2015). Taxon names were matched to records in the Open Tree Taxonomy, to obtain relationships between species. *Chironex fleckeri* was used as the outgroup to obtain the full variance-covariance matrix of phylogenetic relationships. Due to the diverse species in our meta-analysis, accurately estimating branch lengths was not plausible, so we computed branch lengths based on topology (Figure 2.1) using the 'ape' package (Paradis *et al.*, 2004) in R.

We fitted multi-level hierarchical models in the 'MCMCglmm' package (Hadfield, 2010) in R. Each model was run for 5×10^6 iterations, with a burn-in of 1.5×10^5 and a thinning interval of 3,000, with an inverse-gamma prior (V = 1, nu = 0.002). We report the posterior mode and the 95% highest posterior density credible intervals (95% HPD CIs) for each model set. Model

diagnostics were checked so that autocorrelation <0.1. Chain convergence was confirmed visually by passing the Heidelberg stationarity test and by a Gelman-Rubin statistic <1.1 based on three runs of each model.

We performed sensitivity analyses by comparing the overall model (with study ID as a random effect) to one with both study ID and phylogeny as random effects. We considered the model with the lowest Deviance Information Criterion (DIC) value the best model. Cohen's established recommendations for the interpretation of small (Pearson's correlation coefficient φ = 0.1), medium (φ = 0.3) and large (φ = 0.5) effects are equivalent to odds ratios of 1.22, 1.86, and 3.00 with equal treatment-control sample sizes (Cohen, 1992; Olivier & Bell, 2013). These correspond to estimates of log odds ratios from our models of ± 0.20, 0.62, and 1.10 as small, medium, and large, respectively. Estimates with a 95% HPD CI excluding zero were taken as statistically significant at α = 0.05. We note that these benchmarks do not establish biological importance (Nakagawa & Cuthill, 2007), so we discuss our results in terms of their practical implications for captive breeding programs.

Traditional calculations of heterogeneity such as I^2 assume that effect sizes are independent, however this is not the case for multi-level models so the extended heterogeneity statistic was instead calculated following Nakagawa and Santos (2012). Doing so enabled us to partition total heterogeneity (I^2_{total}) into phylogenetic variance ($I^2_{phylogeny}$), study ID variance (I^2_{study}) and residual variance ($I^2_{residual}$). Heterogeneity well above the I^2_{total} >75% benchmark for high heterogeneity (Higgins *et al.*, 2003) is common across ecological and evolutionary meta-analyses (Senior *et al.*, 2016). For the phylogenetic model, we obtained lambda, a measure of phylogenetic signal or phylogenetic heritability (H^2), where H^2 = 0 indicates no phylogenetic relatedness among effect sizes (Lynch, 1991). As both our models had high heterogeneity, but phylogenetic signal was low, we proceeded with non-phylogenetic meta-regression models to fit moderators including 'captive environment' and 'trait type'.

Publication bias

We assessed publication bias in our meta-analysis using three methods. First, we fitted a non-phylogenetic meta-regression model with year of publication as a moderator. Evidence of time-lag bias is indicated if the 95% HPD CI of the slope estimate excludes zero. Second, we used funnel plots to visualize possible publication bias (evident by funnel plot asymmetry), by

plotting the effect sizes and the meta-analytic residuals against their precision ($\sqrt{\frac{1}{variance}}$). Funnel plot asymmetry can also result from high heterogeneity, so applying these publication bias tests to the meta-analytic residuals instead of the raw effect sizes minimises the effect of heterogeneity on funnel plot asymmetry (Nakagawa & Santos, 2012). Third, we performed Egger's regression (Egger *et al.*, 1997) on the meta-analytic residuals obtained from the overall model to formally test for evidence of funnel plot asymmetry, by fitting a linear model of the meta-analytic residuals against their precision. If the intercept of the Egger's regression is significantly different from 0 (at $\alpha = 0.05$), publication bias may be present. We then performed a trim-and-fill analysis using the 'trimfill' function in 'metafor' to estimate the number of effect sizes potentially missing from our dataset. Finally, all models were rerun on a subset of the dataset after outliers identified in the funnel plot were removed, to examine whether any model results changed substantially. Publication bias results are presented in Supplementary Note A1.1 and Figure A1.3.1a–c.

Multiple imputation

We performed multiple imputation to recover missing data. Of the 74 comparisons excluded for missing data, 17 of these were continuous traits with the mean reported but not the standard deviation, and which could be estimated. We performed 20 imputations using the 'mice' function in the R package 'mice' (van Buuren & Groothuis-Oudshoorn, 2011) and re-ran all models with each imputation. The posterior mode of the pooled posterior distributions from each imputed meta-analysis was used for inference and qualitatively compared to the main dataset results to check support for our conclusions. We also considered the effect of imputing missing sample sizes for a further 24 comparisons. Full multiple imputation details and results are presented in Supplementary Note A1.2 and Figure A1.3.4. R code for all analyses reported herein is included in Supplementary Code A3.

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Chapter 3: Changes in fitness over generations in captivity in conservation breeding programs

3.1 BACKGROUND

In Chapter 2, I identified reduced reproductive success of captive-born animals in captivity compared to their wild-born counterparts. The largest birth-origin effects occurred in offspring traits, including offspring survival. Due to under-reporting of statistics, such as the number of captive generations, in the literature reviewed it was not possible to disentangle first-generation effects from multi-generational trends using existing literature. In this Chapter, I obtained studbook data from long-running conservation breeding programs to examine multi-generational changes in offspring survival. This manuscript has been prepared for submission to a broad-interest journal. Supplementary Material is provided in Appendix 4.

For this study, I obtained studbook data, performed data cleaning and analysis, prepared figures and tables and drafted the manuscript. Carolyn Hogg assisted in obtaining and converting studbook data, provided conceptual guidance on the analysis, critically revised the manuscript and oversaw the project. Catherine Grueber provided technical advice on data analysis, critically revised the manuscript and oversaw the project.

3.2 MANUSCRIPT

Changes in fitness over generations in captivity in conservation breeding programs

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Abstract

Saving global biodiversity relies on many strategies, and successfully breeding animals in controlled conditions (such as zoos) has been credited with preventing extinction of many species. However, the long-term sustainability of conservation breeding programs is challenged by genetic changes that occur in the captive population, such as might occur via neutral (e.g. genetic drift) or selective (e.g. adaptation to captivity) processes. We analysed studbook (pedigree) data from 15 animal conservation breeding programs – comprising over 30,000 individuals – to identify changes in offspring survival over generations of captive breeding. Generational effects were highly variable, with some species demonstrating substantial increases or decreases in offspring survival over generations in captivity, independent of time. Disentangling first-generation from multi-generational effects revealed further complexity, as dam and sire effects differed at this scale. We also investigated effects of inbreeding and parental age. Offspring inbreeding (equivalent to kinship of the parents) strongly and consistently decreased offspring survival across taxa. Parental age effects, such as decreased reproduction in older animals, were generally consistent with species' reproductive biology and mating systems. Across our 15 taxa, species' responses to captivity could not be predicted by their evolutionary relationships, demonstrating the importance of quantifying generational changes within individual breeding programs. Our data shows that, even in captive populations under best-practice management, generational fitness changes that cannot be explained by known processes such as inbreeding depression, are occurring.

Introduction

Captive breeding is increasingly relied upon to prevent extinction (McGowan *et al.*, 2017). Conservation programs aim to halt evolution over time so that captive populations remain representative of wild sources (Lacy, 2009). However, even the best efforts cannot fully replicate the wild in captivity. Different selective pressures experienced in captivity than in the wild can drive captive adaptations. Adaptation to captivity may improve population-level fitness in the captive environment if the individuals best suited to captivity are more successful (Frankham, 2008). Yet, when animals are returned to the wild, captive adaptations may be maladaptive (e.g. selection for tameness), and contribute to the low success of reintroduction programs (Jule *et al.*, 2008). Adaptation to captivity has been investigated in multiple fish species and non-model organisms (see for examples Gilligan & Frankham, 2003; Araki *et al.*, 2007; Lacy *et al.*, 2013; Milot *et al.*, 2013; Janowitz-Koch *et al.*, 2019), but is underexplored in conservation settings (Frankham, 2010b). Although conservation breeding programs employ strategies to minimise the effects of adaptation to captivity (such as avoiding intentional selection, attempting to replicate natural environments, and fragmenting populations) (Williams & Hoffman, 2009), the extent and consequences of adaptation to captivity are largely unknown. Genetic changes as a result of captive breeding have been demonstrated to occur in as little as a single generation in steelhead trout (Christie *et al.*, 2016), so conservation breeding programs are unlikely to be immune to the effects of adaptation to captivity.

A recent systematic review and meta-analysis investigating birth origin effects on reproductive success revealed captive-born animals have substantially lower reproductive success in captivity than their wild-born counterparts, particularly for offspring survival traits (Farquharson *et al.*, 2018b, Chapter 2). This result seems to conflict with the expectation of improved fitness in captivity (e.g. Christie *et al.*, 2012), however many of the studies reviewed considered only the first generation (F_1) of captive breeding. The response of species to captive breeding may differ in the first generation relative to later generations, as different pressures may apply. For example, first generation changes may occur as a consequence of non-genetic effects such as husbandry or maternal effects (Matos, 2012). Longer-term, multi-generational changes may instead reflect heritable genetic changes. Therefore, it is essential

to disentangle first generation and multi-generational changes when investigating adaptation to captivity.

Other genetic factors such as inbreeding can also contribute to fitness changes over time. Inbreeding depression can act upon various life-history traits, such as fertilisation, embryo survival, offspring survival and total lifetime reproductive success (Van Oosterhout *et al.*, 2007; Xu *et al.*, 2007; Grueber *et al.*, 2010; Harrisson *et al.*, 2019). In conservation contexts, inbreeding has been demonstrated to reduce offspring survival with no detectable purging to reverse negative fitness effects (Boakes *et al.*, 2007; Kennedy *et al.*, 2014). As a result, strategies to minimise inbreeding are widely applied in conservation contexts (Frankham *et al.*, 2010). However, few studies of inbreeding consider generations in captivity: as inbreeding is often positively correlated with time in captivity, any effects of inbreeding and other mechanisms of genetic change over time may be confounded. Typically small sample sizes in conservation settings have limited the power available to detect inbreeding depression or other population genetic processes (Hedrick & Kalinowski, 2000).

As the biodiversity crisis continues to threaten species worldwide, the diversity of species bred in captivity will need to increase if extinctions are to be prevented (Fa *et al.*, 2014; Martin *et al.*, 2014). The species that are currently managed in captivity are not just phylogenetically diverse, but also differ in their life-histories, reproductive biology and social structures. Phylogenetic comparative methods can be useful to observe trends and patterns across multiple species, so that the results can be extended to related taxa (Fisher & Owens, 2004), overcoming small sample size limitations. Captive breeding programs routinely use pedigrees to measure and manage genetic diversity and inbreeding (Frankham *et al.*, 2010). Studbooks recording births, deaths and parentage information can be analysed to retrospectively investigate aspects of reproduction without the need for experimental manipulation of often threatened species. With the increasing accessibility of standardised studbook data through large curated online databases, such as the Zoological Information Management Software (ZIMS) (Species 360, 2018), there are now opportunities to examine important traits such as offspring survival at a much larger scale.

In this study, we used data from 15 diverse and long-running conservation breeding programs to investigate the drivers of change in offspring survival to reproductive maturity. We specifically aimed to examine generational trends, by comparing main effects estimated from

all 15 species to species-level responses. We also aimed to disentangle first-generation and multi-generational changes in offspring survival. In addition to generational effects, we examined offspring inbreeding (kinship of parents), dam and sire inbreeding, and the effects of dam and sire age at breeding on offspring survival.

Methods

Studbook data

We obtained the international or regional studbooks of 15 species from the relevant regional zoo associations and studbook keepers, totalling 58,611 individuals, including 11 eutherian mammals, 1 marsupial and 3 reptiles (Table 3.1). These studbooks were selected on the basis of availability, size, taxonomic diversity, generations of captive breeding, and limited unknown ancestry. The pedigree management software PMx (Ballou *et al.*, 2010b; Lacy *et al.*, 2012) was used to generate a dataset with one datapoint per offspring, containing information on the sire, dam, birth date, birth location, death date (if dead) and pedigree inbreeding coefficient (*f*). Pedigree-based inbreeding is calculated using known relationships and by assuming that wild-born founders are unrelated, so founders are assigned an inbreeding coefficient of 0, even though this assumption is not always met (Ballou, 1983; Hogg *et al.*, 2019b). Therefore, individuals with unknown parents could not be included in the analysis. However, individuals with unknown ancestry further back in the pedigree could be included (using the PMx option "set unknown parents to wild"). We conducted further data cleaning in R (version 3.5.1, R Core Team, 2018), whereby for each offspring we used the known parents to calculate age at birth, generations in captivity (F_X), and inbreeding coefficient (*f*) of the sire and dam. Wild-born animals are assigned generation F_0. For captive-born animals, generation is calculated as the average generation of the parents plus 1, meaning that it can be a non-integer e.g. $(F_0 + F_1)/2 + 1 = F_{1.5}$. We also calculated the age at death of the individual, or current age if still alive. We truncated the last 364 days of data from the studbook to minimise the possibility that recent deaths had not yet been updated in the studbook.

Table 3.1: Summary statistics for the fifteen studbooks included in the main analysis (*N* = 37,484 individuals).

Species (Scope of studbook)[1]	N^2	N_e^3	First record (year)	Age (days) at maturity female; male	Pedigree *f* (mean, sd, max) Dam (D); sire (S); offspring (O)	Generation (mean, sd, max) Dam (D); sire (S)	Age at breeding (days) (min, mean, max, sd) Dam (D); sire (S)
Prehensile-tailed skink *Corucia zebrata* (AZA)	534	40.1	1969	4502[4]; 5702[4]	D: 0, 0, 0; S: 0, 0, 0; O: 0.012, 0.049, 0.250	D: 0.208, 0.431, 1.750; S: 0.301, 0.527, 1.750	D: 636, 3696, 9114, 1808; S: 576, 3897, 13000, 1979
Western swamp tortoise *Pseudemydura umbrina* (ZAA)	581	28.9	1949	2910[4]; 2100[4]	D: 0, 0, 0; S: 0, 0, 0; O: 0, 0, 0	D: 0.184, 0.388, 1; S: 0.201, 0.401, 1	D: 3261, 11686, 22388, 5062; S: 2156, 11652, 25243, 5999
Radiated tortoise *Astrochelys radiata* (AZA)	757	58.0	1900	3720[4]; 3000[4]	D: 0, 0, 0; S: 0, 0, 0; O: 0, 0, 0	D: 0.178, 0.404, 1.5; S: 0.135, 0.342, 1	D: 3907, 12951, 22795, 5018; S: 3043, 12716, 23835, 5474
Tasmanian devil *Sarcophilus harrisii* (ZAA)	1111	163.4	1982	730; 730	D: 0.012, 0.052, 0.375; S: 0.007, 0.033, 0.250; O: 0.016, 0.049, 0.375	D: 1.348, 1.204, 5.408; S: 1.084, 1.152, 4.500	D: 231, 944, 2470, 295; S: 231, 1177, 2466, 414
Cheetah *Acinonyx jubatus* (INTL)	4932	222.5	1850	456; 456	D: 0.010, 0.034, 0.250; S: 0.011, 0.042, 0.250; O: 0.022, 0.055, 0.268	D: 1.567, 1.387, 5.353; S: 1.339, 1.290, 5.156	D: 651, 2313, 6084, 770; S: 590, 2535, 6329, 981
Meerkat *Suricata suricatta* (AZA)	1659	17.0	1908	365; 365	D: 0.078, 0.132, 0.406; S: 0.073, 0.146, 0.434; O: 0.115, 0.156, 0.516	D: 1.828, 1.244, 5.391; S: 1.594, 1.348, 5.250	D: 217, 1793, 4918, 925; S: 89, 1836, 4798, 863
Red wolf *Canis rufus* (AZA)	958	108.0	1966	330[4]; 330[4]	D: 0.036, 0.035, 0.125; S: 0.032, 0.034, 0.250; O: 0.055, 0.039, 0.250	D: 2.985, 1.624, 6.059; S: 2.935, 1.601, 6.133	D: 345, 2056, 4033, 818; S: 722, 2288, 5499, 1037

African wild dog *Lycaon pictus* (INTL)	5391	81.1	1887	639; 639	D: 0.058, 0.117, 0.503; S: 0.065, 0.122, 0.503; O: 0.107, 0.137, 0.594	D: 2.211, 1.462, 6.594; S: 2.037, 1.477, 6.594	D: 361, 1698, 6880, 710; S: 361, 2070, 6721, 982
Red panda *Ailurus fulgens* (INTL)	2926	382.2	1868	550; 550	D: 0.020, 0.035, 0.274; S: 0.020, 0.035, 0.298; O: 0.036, 0.048, 0.375	D: 2.828, 1.723, 6.969; S: 2.714, 1.767, 7.098	D: 357, 2005, 5157, 905; S: 361, 2329, 6945, 1120
European mink *Mustela lutreola* (EAZA)	1480	74.5	1932	323; 323	D: 0.084, 0.111, 0.504; S: 0.083, 0.108, 0.481; O: 0.117, 0.119, 0.504	D: 4.218, 2.758, 10.397; S: 4.195, 2.955, 10.514	D: 282, 919, 3309, 567; S: 295, 998, 3645, 647
Scimitar-horned oryx *Oryx dammah* (INTL)	6435	216.0	1872	639; 210[4]	D: 0.086, 0.124, 0.646; S: 0.087, 0.126, 0.601; O: 0.112, 0.137, 0.675	D: 2.938, 1.848, 7.870; S: 2.962, 1.879, 8.027	D: 564, 2552, 10429, 1408; S: 518, 2478, 8359, 1334
Eastern bongo *Tragelaphus eurycerus isaaci* (INTL)	2443	174.9	1931	806; 914	D: 0.066, 0.100, 0.614; S: 0.066, 0.106, 0.614; O: 0.086, 0.110, 0.614;	D: 2.966, 1.538, 6.812; S: 2.933, 1.512, 6.166	D: 396, 2471, 7004, 1250; S: 582, 2607, 7054, 1153
Red-ruffed lemur *Varecia rubra* (INTL)	1737	171.4	1959	609; 650	D: 0.085, 0.084, 0.375; S: 0.097, 0.086, 0.445; O: 0.138, 0.102, 0.445	D: 2.576, 1.217, 5.484; S: 2.651, 1.261, 5.672	D: 707, 3105, 10925, 1487; S: 719, 3414, 10990, 1761
Black-and-white ruffed lemur *Varecia variegata* (INTL)	3516	275.6	1959	605; 649	D: 0.046, 0.077, 0.375; S: 0.053, 0.081, 0.344; O: 0.085, 0.101, 0.438	D: 2.353, 1.295, 5.969; S: 2.175, 1.358, 5.805	D: 292, 3017, 9911, 1598; S: 568, 3270, 14335, 1777
Goeldi's monkey *Callimico goeldii* (INTL)	3024	187.5	1913	365; 395	D: 0.019, 0.049, 0.375; S: 0.019, 0.049, 0.375; O: 0.034, 0.069, 0.500	D: 2.648, 1.475, 6.326; S: 2.621, 1.496, 6.336	D: 441, 2304, 7763, 1144; S: 184, 2471, 7518, 1221

[1]INTL = international (WAZA studbook), AZA = Association of Zoos & Aquariums, EAZA = European Association of Zoos and Aquaria, ZAA = Zoo and Aquarium Association.

[2]With complete data (both parents known, no missing values), 9 outliers removed as per methods.

[3]N_e obtained from PMx using whole studbook prior to data filtering.

[4]No AnAge record available, so age obtained from PMx.

For each species, we defined the age at reproductive maturity for each sex in days using the AnAge (Animal Ageing and Longevity) database (Tacutu *et al.*, 2017), or PMx for the species without data in AnAge (all three reptile species, red wolf, and male scimitar-horned oryx). We excluded all individuals born within the timeframe of the reproductive maturity age from the 364 days before the current date of the studbook, as these animals would not yet have had the opportunity to reach reproductive maturity. We removed individuals that had been identified as hybrids in the red wolf studbook, and those that were born in the wild or released to the wild before the age of reproductive maturity (affected the red wolf and Tasmanian devil studbooks). A further 13,089 individuals did not have a known sire or dam or both, meaning that predictors of interest were unknown, and these individuals were excluded from the above analysis. Multiple imputation could not be attempted as for 8,530 individuals all values of interest were missing. Of the remaining 37,493 individuals with complete data, we established whether they had survived to the defined age of reproductive maturity (1) or not (0). For individuals with unknown sex, we defined age at reproductive maturity as the shortest of the two sexes. Data appeared to be missing at random with respect to time.

There was high variation in our predictors of interest between species (Table 3.1), due to historic captive management and variation in species biology. We therefore standardised numeric variables of interest (dam/sire *f*, dam/sire generation, dam/sire age at breeding and offspring *f*) within each species by centring on the mean and dividing by 1 standard deviation with the 'standardize' package (Eager, 2017) to avoid species with extreme values unduly influencing the results and to assist interpretation of model parameter estimates. This method of standardising does not affect the relative variances around predictors.

Phylogenetic correlations

We assessed phylogenetic correlations in our dataset by creating a tree in the 'rotl' package (Michonneau *et al.*, 2016) based on phylogenies available through the Open Tree of Life (Hinchliff *et al.*, 2015). The topology of the tree was used to calculate lambda, an estimate of phylogenetic signal ranging from 0 (no signal) to 1. Phylogenetic signal would indicate that closely related species are more similar in their offspring survival rates than distantly related species. As species varied in their mean offspring survival but phylogenetic signal was very weak, we proceeded to model offspring survival controlling for variation among species, but not phylogenetic relationships among species.

Random factors and model fitting

Generalised linear mixed models (GLMMs) were fit in 'lme4' (Bates *et al.*, 2015) with a binomial response and a nested random factor design. The random factors we controlled for were Species, Birth Program and Year. Birth Program refers to the region where an individual was born as defined by ZIMS (Africa, Australasia, East Asia, Europe, Latin America, Middle East, North America, South East Asia, South Asia, Unknown and Other). Birth Program was nested within Species to account for regional specialisation. For example, while a region may have particularly high offspring survival of one species, it may have below-average offspring survival of a different species. This can be due to a range of factors including taxonomic expertise, population management practices and varied husbandry. The year of birth controls for improvements in offspring survival made over time with improved husbandry, and was also nested within Species as the studbooks covered very different time-frames (the year of first captive-born offspring ranged from 1881 [scimitar-horned oryx] to 1991 [Western swamp tortoise]). Our global model therefore consisted of:

Survival ~ Dam generation + Sire generation + Dam age at breeding + Sire age at breeding + Dam *f* + Sire *f* + Offspring *f* + (1|Species/Birth Program) + (1|Species:Year)

We examined model fit using the 'DHARMa' package (Hartig, 2019). Nine data points with high leverage were identified in residual plots, removed and models refitted (*N* = 37,484 individuals). We calculated Variance Inflation Factors (VIFs) to ensure multi-collinearity of predictors was < 2 (Harrison *et al.*, 2018). Our model satisfied the Kolmogorov-Smirnov test of uniformity, outlier test, non-parametric dispersion test and zero-inflation test.

Model selection

We proceeded with model inference under an information theoretic approach following Grueber *et al.* (2011). All possible sub-models were fitted using the 'dredge' function from the 'MuMIn' package (Barton, 2018), and models within the top 2 AIC_C of the top model were retained and model averaged (conditional average method). We interpreted predictors based on the size, direction and precision of the model estimate and its relative importance (sum of Akaike weights for top models containing the predictor).

First-generation vs. multi-generational changes

We may expect differences between the survival rates of offspring of wild-born parents and the offspring of captive-born animals. Therefore, we ran a second analysis as above but excluding all offspring with either one or both wild-born parents (i.e. F_{2+} offspring, $N = 27,734$).

Non-independent litter-mates

Animals born as part of the same litter or clutch share the same dam (and often the same sire). We identified litters as animals born to the same dam on the same day for mammalian species, and as animals born to the same dam in the same year for the tortoises and skink. One offspring from each litter was randomly selected and models were re-run as above (N = 21,282 independent individuals). Random selection was repeated a total of five times and analyses pooled to obtain model estimates. We repeated the F_{2+} analysis using these five subsets (N = 16,514 - 16,516 offspring, varies because litter-mates identified by the same dam may have a different sire, and the generations in captivity of the sire may vary).

Random slope models for between-species differences

We selected one of the independent litter-mate subsets that was representative of the five sets of results to further investigate trends across species (Figure A4.2.1). We fitted separate random-slope models for each predictor to estimate species-level effects, where the main effect is interpreted as the mean across all species, and the random component quantifies the amount of variation in that slope among species. For example, while the main effect may suggest a negative relationship between dam age at breeding and offspring survival, the effect of dam age may vary between species. Random-slope models were fitted from the global model, as model averaging cannot provide random slope estimates. No parameters were dropped from the top model set with model averaging (Table A4.1.2), so we do not expect the random slopes models to differ substantially if estimation after model selection was possible. We also fitted random slopes model from the F_{2+} random litter-mate subset for the dam and sire generation parameters, as we may expect species responses to differ after removing the offspring of wild-born animals from the analysis.

Results

Phylogenetic correlations

While the species varied in their mean offspring survival (due to life-history traits e.g. R- vs. K-selected species, single-offspring vs. multiple offspring, differential maternal investment; and management, e.g. date of accession to studbook), there was no evidence of phylogenetic signal (lambda = 7×10^{-5}) (Figure 3.1A). We therefore excluded phylogeny from our subsequent modelling (although we did include "Species" random effects, see Methods).

Offspring survival

Table 3.2 presents the pooled model estimates of the five models run with one offspring randomly selected per litter/clutch to overcome non-independence of litter-mates to offspring survival (N = 21,282). Offspring f had the strongest negative relationship with offspring survival, while sire and dam f effects were estimated close to zero (Figure 3.2). Dam age at breeding had a negative effect on offspring survival, while sire age at breeding had a similar-sized positive effect. Dam and sire generation showed no clear overall effect on offspring survival (Table 3.2).

When examining random slope models, generation effects were highly variable among species, with some showing steep positive slopes (e.g. red wolf, African wild dog, western swamp tortoise), and others having steep negative slopes (black-and-white ruffed lemur, Tasmanian devil) (Figure 3.1B). Sire f also showed variation among species, with the Eastern bongo, red panda and red wolf displaying a positive slope and the Tasmanian devil, red-ruffed lemur and black-and-white ruffed lemur having a negative slope (Figure 3.1B). Dam f had less variation among species, with none showing particularly strong effects. Offspring survival was negatively associated with dam age for most species, although the prehensile-tailed skink had a steep positive slope (Figure 3.1B). A random slope could not be fitted for sire age at breeding due to model convergence issues, possibly due to a lack of variation in between-species responses. All species had a negative slope for offspring f, with the three primate species and the European mink experiencing the strongest effects of inbreeding (Figure 3.1B).

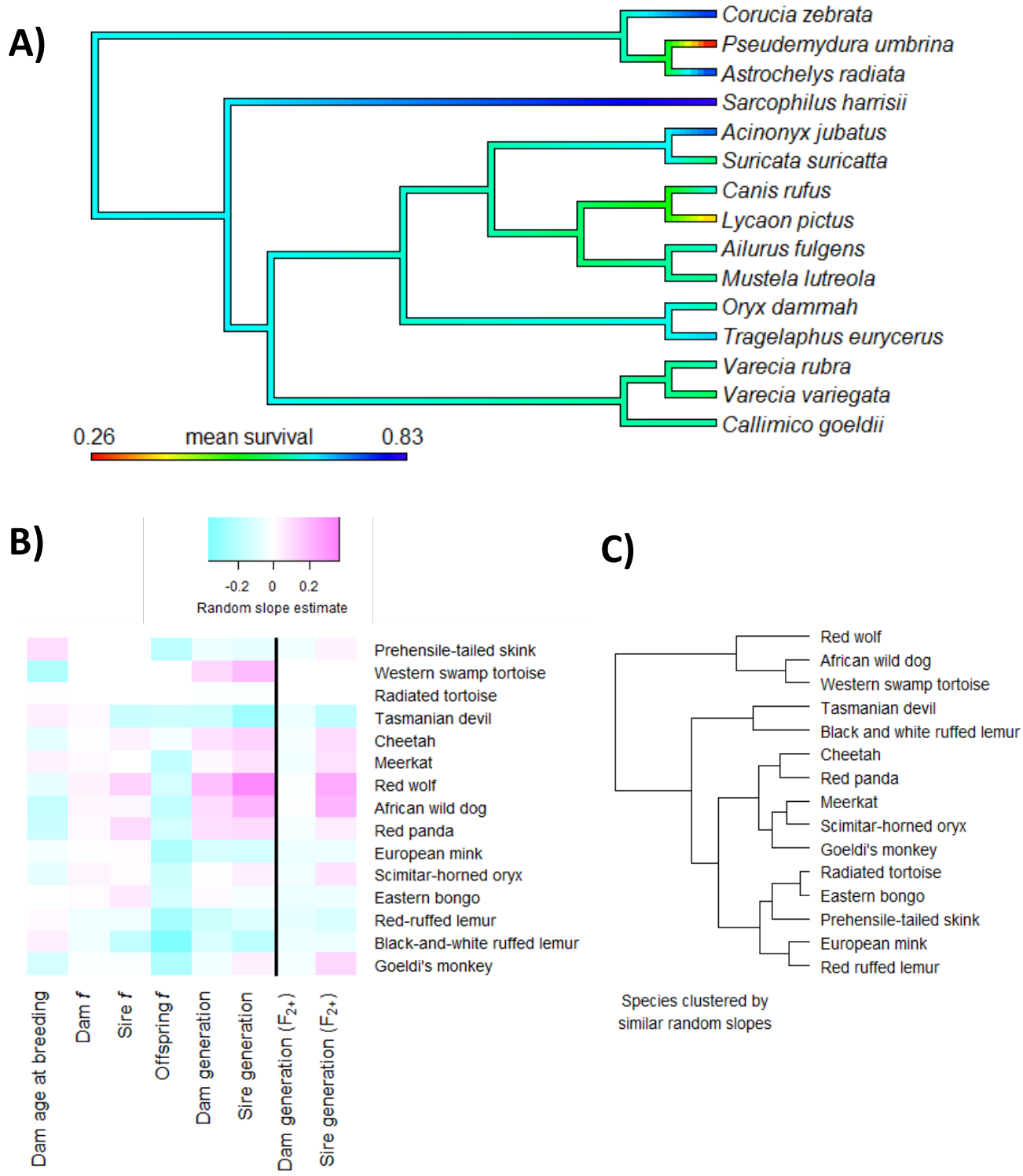

Figure 3.1: Phylogeny and random slope results.
A) Phylogenetic relationships of the 15 species included in this study, shaded by the mean offspring survival. No evidence of phylogenetic signal was detected using the topology of this tree. B) Heatmap of random slope estimates for each parameter across the 15 species. Note that no random slopes could be estimated for sire age at breeding. The vertical black line separates the model with all offspring from the model with F_{2+} offspring (no wild-born parents) only. C) Dendrogram of species clustered based on similar random slope values, not phylogenetic relationships.

Table 3.2: Model estimates when one offspring is selected from each litter/clutch.
A) Standardised parameter estimates of pooled (*N* = 5) analyses after randomly selecting one offspring from each litter and model averaging (*N* = 21,282 individuals). B) Standardised parameter estimates of pooled (*N* = 5) random litter-mate F_{2+} analyses (*N* = 16,514 - 16,516 individuals, see Methods).

Predictor	Mean estimate	Mean SE	95% CI
A) All offspring			
Intercept	0.4610	0.1853	0.0977, 0.8242
Dam generation	0.0048	0.0203	-0.0350, 0.0446
Sire generation	0.0216	0.0188	-0.0152, 0.0584
Dam age at breeding	-0.0624	0.0156	-0.0931, -0.0318
Sire age at breeding	0.0583	0.0159	0.0271, 0.0895
Dam *f*	0.0020	0.0164	-0.0300, 0.0341
Sire *f*	0.0086	0.0165	-0.0237, 0.0409
Offspring *f*	-0.1632	0.0157	-0.1940, -0.1323
B) F_{2+}			
Intercept	0.4226	0.1554	0.1181, 0.7272
Dam generation	-0.0322	0.0281	-0.0872, 0.0228
Sire generation	0.0441	0.0278	-0.0103, 0.0985
Dam age at breeding	-0.0893	0.0191	-0.1267, -0.0518
Sire age at breeding	0.0787	0.0188	0.0418, 0.1156
Dam *f*	-0.0064	0.0189	-0.0435, 0.0307
Sire *f*	0.0177	0.0189	-0.0194, 0.0548
Offspring *f*	-0.1831	0.0186	-0.2196, -0.1465

F_{2+} effects on offspring survival

After removing offspring of wild-born parents from the dataset (F_{2+} offspring remaining) model estimates for most parameters were similar to those obtained from the model containing all offspring, but with slightly less precision as expected given the smaller sample size (Table 3.2B, Figure 3.2). However, the overall estimate for dam generation became negative. When we fitted the random slopes for the generation effects to the F_{2+} data subset, sire generation reflected a similar pattern to the model with all offspring (Figure 3.1B), with high variation between species. However, the random slopes for dam generation all became slightly negative, including the red wolf and African wild dog that had positive slopes in the model with all offspring (Figure 3.1B). Detailed illustrations of species-level random slopes are presented in Figures A4.2.3 - A4.2.17, with raw data points included to demonstrate the range of statistical power across species and parameters.

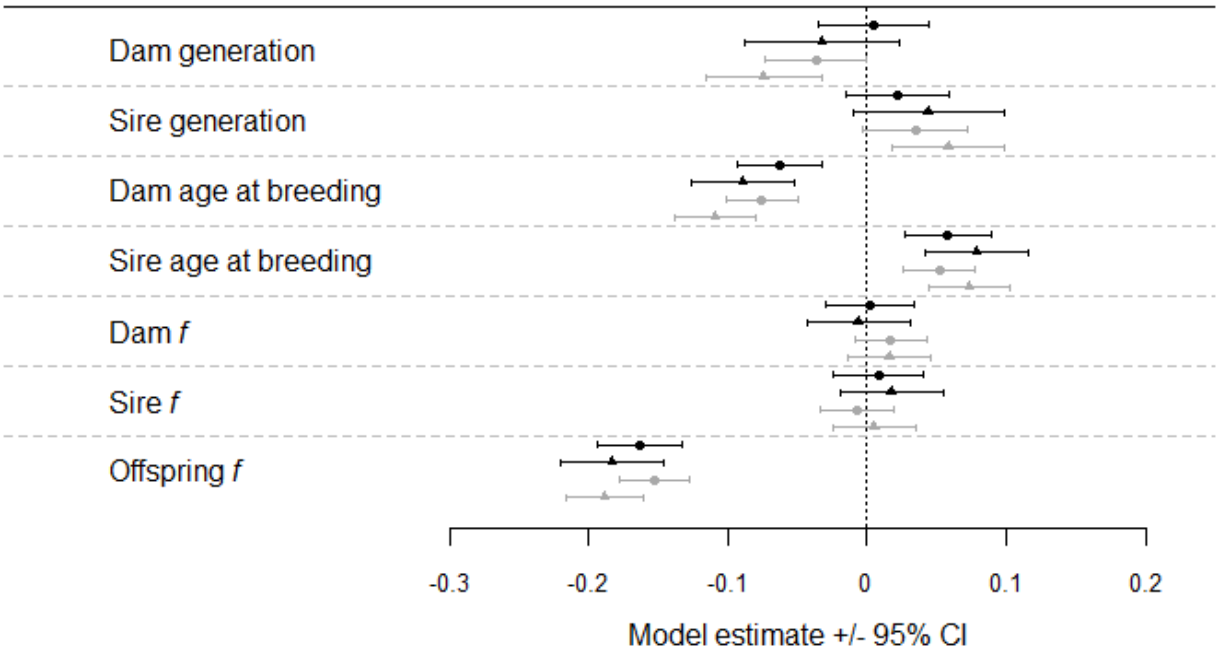

Figure 3.2: Model estimates for offspring survival analyses (+/- 95% CI).
Black circle is the pooled results (*N* = 5) from random selection of one offspring per litter/clutch (*N* = 21,282 individuals), black triangle is pooled results (*N* = 5) from F_{2+} subset of this model (*N* = 16,514 - 16,516 individuals), grey circle is the complete-cases model (*N* = 37,484) after model averaging, grey triangle is the F_{2+} subset of this model (*N* = 27,734) after model averaging.

Extended dataset

Our main analysis included random litter-mate selections, so we compared these results to an extended dataset model that incorporates all data points regardless of their shared litters. It was not possible to fit litter/clutch-level random effects. Some of the species in our dataset usually give birth to only one offspring and would be unlikely to drive any differences between the extended dataset relative to our main analysis. The amount of data contributed to each model by each species is shown in Figure A4.2.2.

In the extended dataset model, most estimates were similar in their direction and magnitude, relative to our main analysis, with offspring *f,* and dam and sire age at breeding remaining the most important effects on offspring survival (Table A4.1.1A, Figure 3.2). Estimated effects were also similar for the extended dataset F_{2+} model, with the estimate of dam generation negative and sire generation positive (Table A4.1.1B, Figure 3.2).

Discussion

Here, we used multi-species mixed-effects models to investigate the effect of generations in captivity, inbreeding, and parental age, on offspring survival to reproductive maturity in 15 vertebrate species encompassing over 30,000 datapoints whilst controlling for mean differences between years, regions and species. Overall, we found effects of inbreeding and age that were largely consistent with conventional predictions. However, changes in survival over generations in captivity were more complicated, as trends varied among species, between sexes within species, and between first and subsequent generations in captivity. Alongside a lack of phylogenetic signal, our analysis implies that intensive human management can drive complex patterns of ongoing change in conservation breeding programs and that these changes will be difficult to predict for any given taxon.

Generational changes

We found a high degree of inter-species variation in the effect of dam and sire generations in captivity on offspring survival from our random-slopes models, and no consistent overall effect from our multi-species model (Figure 3.1B, Figure 3.2). Generational changes in survival were observed despite controlling for other processes that may result in changes over time such as changes in husbandry/population management or the accumulation of inbreeding.

It is plausible that generational effects of captivity primarily occur in the first generation, when wild animals are brought into captivity, with little subsequent change. This first generation change has been observed in some fish species (Christie *et al.*, 2016). Differentiating this process from ongoing, accumulating change is important for management planning. We addressed this issue by comparing our models fitted across all generations, with subset models that included only generation F_{2+} animals (i.e. animals with two captive-born parents). Our results showed that despite the potential for major changes in the first generation, continual captive breeding still impacted many species, although there was substantial among-species variation in the effects, as well as important differences between sire and dam effects. For breeding males (sires), the pattern of inter-species variation in the effect of generation on offspring survival was consistent regardless of whether all offspring, or only F_{2+} offspring, were examined (compare sire generation to sire generation F_{2+}, Figure 3.1B). This result indicates that for a given species, whatever effect captivity has on the

survival of a male's offspring, this effect is maintained from the moment animals are brought in from the wild and continues through subsequent generations. For example, red wolves and African wild dogs showed strong positive effects of sire generation on offspring survival in both the full model and the F_{2+} only model. Conversely, Tasmanian devils, European minks, black-and-white ruffed lemurs and red-ruffed lemurs showed negative effects of sire generation on offspring survival in the full model, which were also maintained in the F_{2+} only model. For breeding females (dams), the results were more complicated. In the full model (incorporating all generations) the patterns of inter-species variation in the effect of generation were remarkably similar to the effects seen for sires (compare dam generation to sire generation, Figure 3.1B). However, the effects of dam generation on F_{2+} offspring were uniform and slightly negative across all the taxa we studied, even for those species that had strong positive responses when offspring of wild-born parents were included (Figure 3.1B). This result suggests that regardless of whether captivity negatively impacts female breeders in the first generation or not, generations F_{2+} are likely to see slight declines in offspring survival.

Fitness changes over captive generations may occur as a result of genetic processes (both neutral e.g. drift, and non-neutral e.g. adaptive changes in allele frequencies), non-genetic processes (e.g. behavioural changes), and/or epigenetic effects (e.g. maternal effects, transgenerational changes). These processes may act on the first generation, and/or across multiple generations and could have different effects on males and females. In aquaculture settings, other processes influencing survival such as maternal effects can be ruled out by performing experimental crosses of wild and captive parents (Matos, 2012; Christie *et al.*, 2016; Finger *et al.*, 2018). In a conservation context involving highly threatened species, it is not practical to implement experimental breeding pairs. As described above for many of the species we attempted to separate the first generational effects from the subsequent generation effects. Our use of a long-term measure of offspring survival, age at reproductive maturity (ranging from < 1 year to > 15 years across our dataset, Table 3.1), provides ample opportunity for parental effects to influence survival beyond the influence of inherent factors such as genetic disorders. The differences we observed between the sexes when removing first-generation effects may be a result of the strength of maternal effects relative to paternal effects. Generally in mammals (12 of the 15 species in our dataset), reproduction is a greater

investment for females than males (Wells, 2014), so maternal care may provide a greater opportunity to influence offspring survival than paternal effects. The birth origin of the dam (wild or captive) appears to have a greater influence on offspring survival than that of the sire, where generational effects are consistent between the inclusion and exclusion of offspring of wild-born parents (Figure 3.1B). Although different species had different effects and different magnitude of the effect, we conclude that time in captivity impacts species' biology.

Inbreeding depression

The strongest driver of an offspring's survival to reproductive maturity was that individual's inbreeding coefficient (equivalent to the kinship of the parents), a trend that was consistent across all species in our study. Inbreeding depression is widely recognised in the literature, including in captive settings where inbreeding has been shown to negatively affect offspring survival in multiple species (Boakes *et al.*, 2007). Our study is the first, that we are aware of, in a conservation setting to disentangle inbreeding effects from generations in captivity and time. Notably, our dataset includes genetically depauperate species that have undergone substantial historic and/or recent bottlenecks such as the cheetah (Menotti-Raymond & O'Brien, 1993), the Tasmanian devil (Brüniche-Olsen *et al.*, 2014), and the red wolf (captive population founded with only 14 wild-caught animals; Hedrick & Fredrickson, 2008). Even these historically inbred species experienced inbreeding depression in captivity (Figure 3.1B), suggesting that any purging to reduce the frequency of deleterious alleles is weak or ongoing. We reiterate the recommendations of Leberg and Firmin (2008) to avoid inbreeding in captive breeding programs, as purging is unpredictable and any benefits are unlikely to outweigh the costs of inbreeding depression (Boakes *et al.*, 2007).

The strongest inbreeding effects were observed at the offspring level (i.e. vital-stage inbreeding depression), rather than parental inbreeding (i.e. reproductive-stage inbreeding depression). Captive management should therefore continue to prioritise the avoidance of breeding related individuals (i.e. minimising kinship). Previous studies examining pedigree-based inbreeding have suggested that inbred females should not be bred from, even to unrelated individuals (i.e. pairings that are not inbred) (Boakes *et al.*, 2007). However, in our study (also using pedigree-based inbreeding estimates) there was no general effect of dam *f* and our species-level random slopes models suggest that dam *f* is far less likely to substantially impact offspring survival to reproductive maturity than offspring *f*. Similar results were

obtained for sire inbreeding. Nonetheless, parental inbreeding should still be minimised, with some species demonstrating negative effects for both dam and sire *f*, including the two lemur species (Figure 3.1B). Avoidance of inbreeding is standard practice for captive management programs (Frankham *et al.*, 2010) including the ones analysed here. A growing interest in group housing of animals to promote more natural social settings and minimise adaptation to captivity (Williams & Hoffman, 2009) means that it may become harder to avoid inbreeding. Additional considerations such as the use of contraceptives (Cope *et al.*, 2018b) or molecular pedigree reconstruction (Farquharson *et al.*, 2019; Chapter 5) or relatedness estimation (Ivy *et al.*, 2016) will be required for managing inbreeding in these settings.

Parental age

Parental age at breeding had large effects on offspring survival: negative for dam age and positive for sire age. These results likely reflect the biology of the species chosen in this analysis, with 12 mammal and three reptile species modelled. Older males may be expected to improve offspring survival through social factors and male experience. For example, in herd species, older males are more likely to be dominant and to time mating better than younger males to maximise reproductive success (L'Italien *et al.*, 2012; Tennenhouse *et al.*, 2012). Overall, we found that younger mothers had higher offspring survival across the taxa studied, noting that some species showed exceptions when random slopes were fitted (e.g. black-and-white ruffed lemur, red-ruffed lemur, meerkat, prehensile-tailed skink, Tasmanian devil). This may seem unexpected given that younger mothers may experience lower offspring survival due to inexperience or subordinate status (Henry *et al.*, 2013; Lukas & Huchard, 2019). However, in line with our findings, older females may have reduced offspring survival as senescence reduces viability (Descamps *et al.*, 2008). Our study did not investigate the causes of offspring mortality, nor did we investigate the effect of parity on offspring survival, so testing of such biological hypotheses and interactions will require a more detailed species-level investigation.

Conclusions

This study makes use of studbook records collected as part of routine management, such as to record parentage, avoid inbreeding and retain wild genetic diversity. With the availability of pedigree-based management software such as PMx, we have been able to investigate

generational change in conservation breeding programs without any additional imposition on studbook keepers to collect data, nor without conducting experimental studies. The phylogenetic relationships of the species in our dataset (Figure 3.1A) were not reflected in the species' responses to captivity (clustered dendrogram of Figure 3.1C shows no apparent trends). Although phylogenetic comparative methods may be useful for other fields of enquiry, such as assessing extinction risk (Ripple *et al.*, 2017), responses to climate change (Kellermann *et al.*, 2018), and investigating captive welfare and stereotypic behaviours (Kroshko *et al.*, 2016; Mellor *et al.*, 2018); our data show that they are unlikely to be useful for predicting species' responses to generations of captive breeding. Instead, species-level investigations are necessary.

We have identified generational changes in fitness within captivity in diverse conservation breeding programs that are managed to avoid such change. Further research is needed to investigate the possible underlying mechanisms, such as genetic processes, of this change at a species level. Understanding the effect, and potential drivers, will go a long way to assisting conservation breeding programs to minimise this generational effect into the future as captive breeding programs remain a vital tool to prevent extinction. We acknowledge that population-level genetic change in captivity is not intrinsically detrimental for individual animals being held in zoos, but note that other studies have identified potential negative consequences upon reintroduction programs (Araki *et al.*, 2009; Christie *et al.*, 2014). Will fitness changes in captivity reduce survival and reproduction in the wild? Taken together, the results of this study demonstrate that generational changes in fitness are difficult to predict, but are occurring in some long-running conservation breeding programs even with best-practice management under the minimising mean kinship strategy. Here we advocate that conservation managers investigate their particular species to understand the generational effects occurring in their managed program and adjust management practice accordingly to account for changes in fitness over captive generations.

Acknowledgements

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Chapter 4: From reference genomes to population genomics: comparing three reference-aligned reduced representation sequencing pipelines in two wildlife species

4.1 BACKGROUND

Chapter 4 comprises the published manuscript:

Wright, B*, Farquharson, K.A.*, McLennan, E.A., Belov, K., Hogg, C.J. & Grueber C.E. (2019) From reference genomes to population genomics: comparing three reference-aligned reduced-representation sequencing pipelines in two wildlife species, *BMC Genomics*, **20,** 453.

*contributed equally

In Chapters 2 & 3 I identified a diverse range of species exhibiting fitness changes in captivity. Investigating mechanisms of adaptive change requires molecular genetic approaches. Reduced costs of sequencing and improved methodologies for molecular sequencing of non-model organisms has meant that conservation genetic approaches now generate hundreds to thousands more SNP markers than the tens of microsatellites historically used in conservation genetics. High-density molecular markers present new opportunities to investigate mechanisms of change but can be challenging to analyse. The bioinformatic choices made in processing SNPs can influence results, so a method is needed to ensure SNPs are reliable across several different SNP calling software. This chapter compares three pipelines used for SNP calling, SAMtools, GATK and Stacks. In this chapter, the ability of the three pipelines to detect population structure in the Tasmanian devil is compared to a non-threatened species, the pink-footed goose. The goose data was publicly available and sourced from another peer-reviewed publication which is appropriately cited in-text. Of particular relevance to this thesis, this chapter reports a new method to improve the reliability of SNP calls and process data in a format for downstream applications such as pedigree analysis. The pipeline was applied to Chapters 5, 6 & 7 to perform accurate parentage assignment in order to investigate mechanisms of genetic change in captivity. Supplementary Material is provided in Appendix 5, and Supplementary Code in Appendix 6.

I joint first-authored this publication with Belinda Wright. Belinda performed SNP calling with the GATK and SAMtools pipelines and drafted sections of the manuscript. I performed SNP calling with the Stacks pipeline and generated PCoAs and F_{ST} analyses, with contributions from Elspeth McLennan. I wrote the custom R script and applied this to all three pipelines, and drafted sections of the manuscript. Katherine Belov, Carolyn Hogg and Catherine Grueber oversaw the project, provided conceptual and technical guidance, contributed funding, and critically revised the manuscript. Note that this chapter is presented in the order Background, Results, Discussion, Methods, in line with the *BMC Genomics* format and published version.

4.2 MAIN ARTICLE

From reference genomes to population genomics: comparing three reference-aligned reduced representation sequencing pipelines in two wildlife species

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Abstract

Background: Recent advances in genomics have greatly increased research opportunities for non-model species. For wildlife, a growing availability of reference genomes means that population genetics is no longer restricted to a small set of anonymous loci. When used in conjunction with a reference genome, reduced-representation sequencing (RRS) provides a cost-effective method for obtaining reliable diversity information for population genetics. Many software tools have been developed to process RRS data, though few studies of non-model species incorporate genome alignment in calling loci. A commonly-used RRS analysis pipeline, Stacks, has this capacity and so it is timely to compare its utility with existing software originally designed for alignment and analysis of whole genome sequencing data. Here we examine population genetic inferences from two species for which reference-aligned reduced-representation data have been collected. Our two study species are a threatened Australian marsupial (Tasmanian devil *Sarcophilus harrisii*; declining population) and an Arctic-circle migrant bird (pink-footed goose *Anser brachyrhynchus*; expanding population). Analyses of these data are compared using Stacks versus two widely-used genomics packages, SAMtools and GATK. We also introduce a custom R script to improve the reliability of single nucleotide polymorphism (SNP) calls in all pipelines and conduct population genetic inferences for non-model species with reference genomes.

Results: Although we identified orders of magnitude fewer SNPs in our devil dataset than for goose, we found remarkable symmetry between the two species in our assessment of

software performance. For both datasets, all three methods were able to delineate population structure, even with varying numbers of loci. For both species, population structure inferences were influenced by the percent of missing data.

Conclusions: For studies of non-model species with a reference genome, we recommend combining Stacks output with further filtering (as included in our R pipeline) for population genetic studies, paying particular attention to potential impact of missing data thresholds. We recognise SAMtools as a viable alternative for researchers more familiar with this software. We caution against the use of GATK in studies with limited computational resources or time.

Background

Decreasing sequencing costs and increasing availability of genomic resources mean that population genetic studies are more often utilising genomic data. Whereas in the past tens of microsatellites may have been used to infer population structure and answer fundamental and applied questions, now thousands of single nucleotide polymorphisms (SNPs) can be generated and aligned to reference genomes (Andrews *et al.*, 2016; Maroso *et al.*, 2018). Reduced-representation sequencing (RRS), also referred to as genotyping-by-sequencing (GBS), or restriction-site associated DNA sequencing (RADseq, also ddRAD), is an approach to generate genome-wide high-throughput sequencing data (Baird *et al.*, 2008; Peterson *et al.*, 2012). This is achieved by reducing the genomic data to be sequenced using restriction enzyme digestion and next-generation sequencing (NGS) of the resultant fragments (Peterson *et al.*, 2012). While RRS provides a cost-effective method of sequencing a large number of genome-wide loci across many individuals, coupling this approach with an assembled reference genome improves the reliability of genotype calls (Torkamaneh *et al.*, 2016) and subsequently improves any downstream inferences (Shafer *et al.*, 2017).

One of the initial benefits of RRS approaches was the lack of a need for a reference genome (Peterson *et al.*, 2012). However, now that the costs of generating reference genomes are declining, genetics researchers may take a top-down approach, whereby the genome sequencing project is undertaken first to provide the scaffold for later population genetic studies using RRS (e.g. Pujolar *et al.*, 2017b; Ekblom *et al.*, 2018; Johnson *et al.*, 2018). In this context, biologists who start with a reference assembly may develop familiarity with, and in-

house pipelines for, bioinformatic software designed for whole genome sequencing (WGS), such as SAMtools (Li *et al.*, 2009) and the Genome Analysis Toolkit (GATK) (McKenna *et al.*, 2010). While these software can be used for analysing RRS data, specialist tools such as Stacks (Catchen *et al.*, 2013) are purpose-built for RRS, and designed for use with or without a reference genome (Catchen *et al.*, 2011; Catchen *et al.*, 2013; Rochette & Catchen, 2017). In practice, the algorithms underlying software tools for analysing WGS versus RRS data can differ considerably, which in turn may influence conclusions drawn. For example, calibration of GATK SNP calling parameters is highly dependent on known variant datasets (Van der Auwera *et al.*, 2013), making parameterisation problematic for non-model species.

Many studies have found major differences in resultant datasets produced using various WGS (O'Rawe *et al.*, 2013; Yu & Sun, 2013) or RRS (Torkamaneh *et al.*, 2016) software tools, but none have specifically compared the analysis of reference-aligned RRS data in Stacks versus two widely used genome software packages, SAMtools and GATK. This knowledge gap has been noted by the software developers themselves (Rochette & Catchen, 2017) and so our study serves to fill this gap. Furthermore, comparisons between analysis tools have focused largely on computational efficiency and the total number of SNPs obtained (Torkamaneh *et al.*, 2016; Wickland *et al.*, 2017) and few have examined the critical problem of whether biological interpretations of real data are affected by alternate pipelines (Shafer *et al.*, 2017). This application is important, because fundamental genomic differences between threatened and non-threatened species (such as variation in levels of diversity, inbreeding and linkage disequilibrium) have the potential to impact our analytical choices, inference, and the transferability of population genetic findings (Grueber *et al.*, 2008). As concerns over the current biodiversity crisis deepen, there has been a call for the greater use of genetic and genomic data in the management of species both in captivity and the wild (Shafer *et al.*, 2015; Taylor *et al.*, 2017).

In this study, we employed three widely-used programs, Stacks, SAMtools and GATK, to call variants from reference-aligned RRS data collected from two species with very different demographic histories, and determine how differences between these analysis pipelines impact population interpretations across contexts. Our first study species is a threatened Australian marsupial, the Tasmanian devil (*Sarcophilus harrisii*, hereafter “devil”). The devil has exhibited a severe population crash due to the emergence of a contagious cancer, devil

facial tumour disease (DFTD) in the 1990s (Grueber *et al.*, 2015b; Lazenby *et al.*, 2018). To aid conservation of the species, the devil genome was sequenced in 2012 (Murchison *et al.*, 2012). We generated RRS data from devil samples and anticipated moderate population structure between wild devils of western and eastern Tasmania origin, based on previous analyses using microsatellites (Jones *et al.*, 2004; Grueber *et al.*, 2019) and genomics (Miller *et al.*, 2011; Hendricks *et al.*, 2017). Our second study species is the pink-footed goose *Anser brachyrhynchus* (hereafter "goose"), which breeds in the Arctic and overwinters in Northern Europe and has a reference genome available (Pujolar *et al.*, 2018). For the goose, we re-analysed a subset of the data made available by Pujolar *et al.* (2017b). Their study used population genetic analyses to examine connectivity between two putatively separate populations and infer the effects of climate and human activities on demography of this migrant species. The purpose of our analysis here was not to specifically recapitulate the population genetic investigations for these two species. Rather, we aimed to discover how inferences in two very different species, both with known population structure, are impacted by variation in analysis tools.

Results

Within-population diversity

We applied our three analysis pipelines (Stacks, SAMtools, GATK; all further processed with the custom R script [Appendix 6]; Figure 4.1) to a total of 131 devil samples and 40 goose samples. Our main results focus on two major study populations of each species, which were expected to show genetic differentiation (devil [Jones *et al.*, 2004; Miller *et al.*, 2011; Hendricks *et al.*, 2017; Grueber *et al.*, 2019]; goose [Pujolar *et al.*, 2017b]). The devil dataset also contains a third population of captive individuals which are mixed provenance between east and west (Hogg *et al.*, 2015). We used this latter population to test how well each analysis pipeline discriminates among populations with mixed lineages.

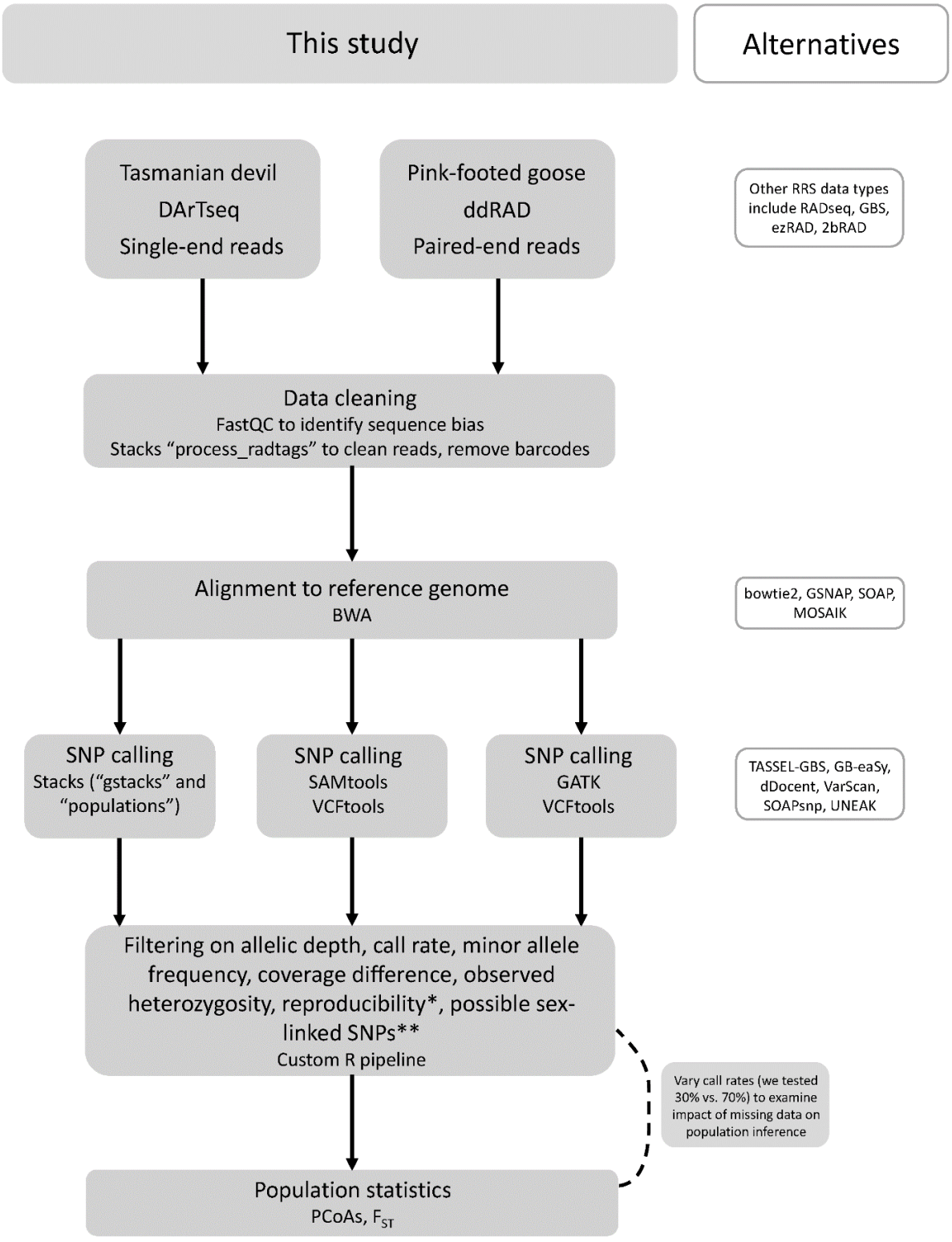

Figure 4.1: Methodology flowchart.
Overview of methods used in this study to process reduced representation sequencing data with reference genomes, with some alternatives to software used indicated where appropriate. *Reproducibility filtering only possible if replicates or technical replicates are performed. **Possible sex-linked SNP filter requires knowledge of sex of samples and is based on XX/XY system, but could be reversed for ZZ/ZW systems.

Table 4.1: Summary statistics for the resultant SNP loci datasets of three pipelines.
Data filtered at a 70% call rate (see Table A5.2.1 for data filtered on 30% call rate), for Tasmanian devil (*N* = 131) and pink-footed goose (*N* = 40), including total number of loci (total loci), average number of loci sequenced across individuals (mean loci), amount of missing data (%), calculated error rates (%), mean observed heterozygosity across loci (H_O), mean expected heterozygosity across loci (H_E), and average multilocus heterozygosity of individuals (MLH).

Dataset	Pipeline	CPU hours[1]	Total loci	Mean loci (min; max)	% missing	Error rate (%)[2]	H_O (± SD)	H_E (± SD)	MLH (± SD)
Devil	Stacks	16	1,359	1,177.3 (500; 1,326)	13.4	2.9	0.207 (0.149)	0.248 (0.163)	0.205 (0.043)
	SAMtools	55	251	205.8 (96; 236)	18.0	6.6	0.308 (0.160)	0.327 (0.115)	0.298 (0.092)
	GATK	325	1,464	1297.2 (604; 1442)	11.4	5.3	0.185 (0.139)	0.256 (0.161)	0.184 (0.040)
Goose	Stacks	11	52,053	44,914.4 (954; 50517)	13.7	NA	0.132 (0.127)	0.156 (0.136)	0.127 (0.026)
	SAMtools	14	26,437	22,035.0 (732; 23,732)	16.7	NA	0.256 (0.160)	0.307 (0.142)	0.563 (0.158)
	GATK	65	277,362	245,412.2 (6787; 270,008.4)	11.5	NA	0.137 (0.121)	0.187 (0.149)	0.132 (0.034)

[a] CPU hours represent total computational time for each pipeline excluding alignment and the further filtering in R. Note that while some steps can be parallelised for quicker computation, not all steps allow for this.
[b] Error rates could not be calculated for the pink-footed goose dataset as no replicates were included in the current analysis. Error rate is calculated after filtering on SNPs with > 85% reproducibility, so is lower than initial error rates.

Mean sequencing coverage was similar for both species, although more variable for geese. Mean coverage for devils was 12.8× (S.D. = 3.5, range = 7.8–32.0). For geese mean cover was 13.3× (S.D = 6.7, range = 1.4–26.4). Unsurprisingly, considering the demographic histories of the two species, the number of SNPs returned for each differed substantially (Table 4.1), although we acknowledge that the laboratory methods for the two datasets were also different (see Pujolar *et al.*, 2017b; Supplementary Methods A5.1). After all filtering steps (including a 70% call rate), the GATK pipeline obtained the highest number of SNPs for both species: 1,464 for devil and 277,362 for goose. Stacks returned a similar number of SNPs as GATK for devils, while Stacks and SAMtools approaches returned a substantially smaller number of SNPs than GATK for goose (Table 4.1); we note we used the same stringency cut-offs for all three data processing pipelines, as far as the user-definable parameters of each software permitted (Figure 4.1).

For both species, mean multilocus heterozygosity estimates obtained using Stacks and GATK were noticeably lower than for SAMtools (Table 4.1). Genotype ratios (ratios of genotypes called as either of the two homozygotes or as heterozygotes) were similar between species but varied across pipelines (Figure A5.3.1). SAMtools was more likely to call heterozygous genotypes than either Stacks or GATK, explaining the higher heterozygosity estimates for SAMtools (Table 4.1). Stacks was more likely to call the most common homozygote (Figure A5.3.1).

By aligning our datasets to reference genomes we were able to unambiguously identify each SNP based on its genomic position and determine the degree of consistency among the three analysis methods. For devil, across all three pipelines, a total of 2,060 unique SNPs were identified; 155 (7.5%) of these were identified by all three methods (Figure A5.3.2a). For goose, this pattern was similar: 78,235 unique SNPs were identified, of which 3,283 (4.2%) were common to all three methods (Figure A5.3.2b). Concordance rates between genotype calls across pipelines, calculated according to shared loci, were high (Table 4.2). Concordance rates were slightly higher for devils (for which SNPs were filtered on their reproducibility; see below) than goose (where no replicates were performed so the error rate could not be reduced). Concordance was also higher between both Stacks and GATK and Stacks and SAMtools than for GATK and SAMtools for both species (Table 4.2). Comparing the genotypes that differed between samples across the different pipelines, Stacks was more likely to call a

genotype heterozygous that was called homozygous in either SAMtools or GATK. There were very few homozygous to alternate homozygous discordant genotype calls between all pipelines (Table 4.2).

Table 4.2: Genotypic differences between loci common to the three pipelines for devils (155 loci) and geese (3,283 loci).
Concordance rates (identical genotype calls between samples) between pipelines are in parentheses. Discordant genotype calls are presented as the percent of total genotypes. Homozygous → Homozygous refers to those loci where an AA is called a TT in the other pipeline for example. Homozygous → Heterozygous are any genotype calls that are homozygous in the first pipeline but called heterozygous in the other for that sample at the same locus. Heterozygous → Homozygous are those calls that are heterozygous in one pipeline but called homozygous in the other for that sample at the same locus.

	Stacks:SAMtools	Stacks:GATK	GATK:SAMtools
Devil	(97.77)	(98.15)	(98.92)
Homozygous → Homozygous	0.00005	0.00010	0.00005
Homozygous → Heterozygous	0.00039	0.00227	0.00197
Heterozygous → Homozygous	0.01719	0.01369	0.00670
Goose	(97.06)	(97.64)	(97.85)
Homozygous → Homozygous	0.00019	0.00018	0.00043
Homozygous → Heterozygous	0.00395	0.00628	0.00394
Heterozygous → Homozygous	0.01920	0.01355	0.01327

For devil only, a subset of 35 individuals were sequenced twice, allowing us to compare the reproducibility of genotype calls from our three pipelines. The Stacks pipeline had the highest reproducibility, with an error rate prior to filtering on reproducibility of 5.9%, which reduced to 2.9% after filtering out loci with poorest reproducibility. The error rate between technical repeats was 12.3% for both SAMtools and GATK. Error rates improved to 6.6 and 5.3% respectively after filtering on reproducibility.

Between-population divergence

All three pipelines recovered the expected population structuring of both study species, with some variation among analysis methods. For both species, differentiation visualised using a principal coordinates analysis (PCoA) was clearest with the GATK pipeline, relative to the Stacks and SAMtools pipelines (Figure 4.2). For devils, we also reanalysed our dataset with the addition of *N* = 66 captive animals (with a mixture of genetic heritage) and found that these fell intermediate to the two major populations, as expected (Figure A5.3.3).

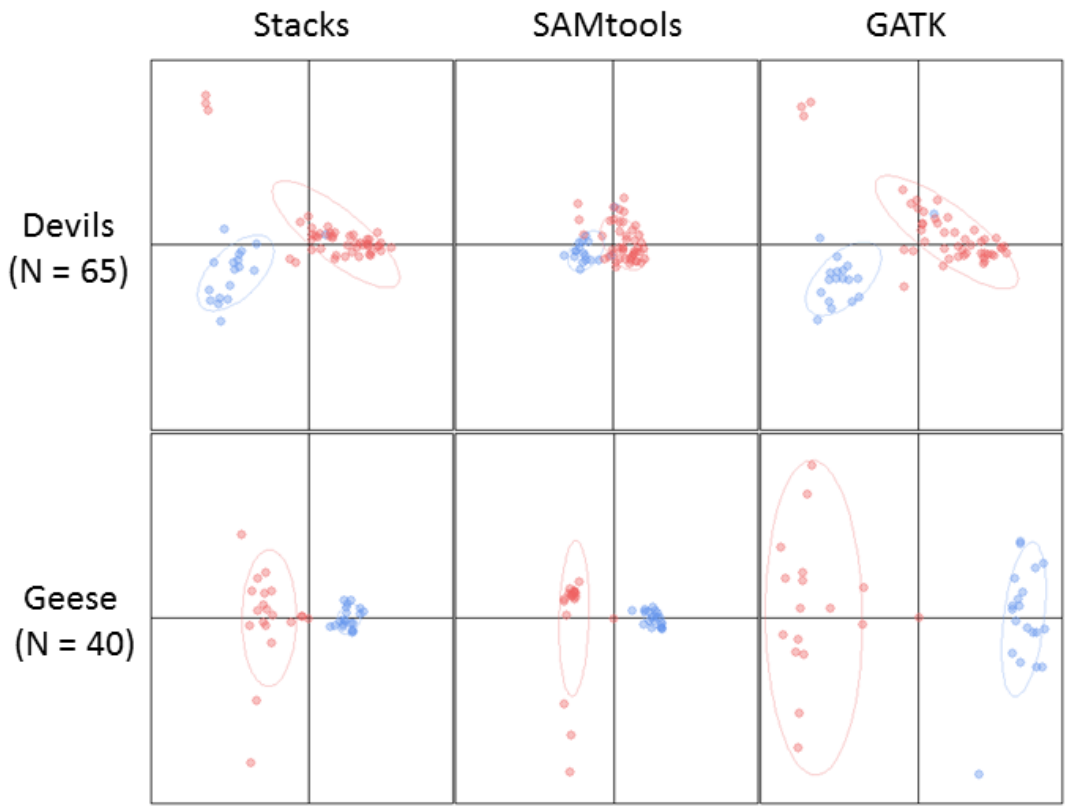

Figure 4.2: PCoAs of the two datasets after processing through three pipelines.
Data processed with a call rate of 70% and the custom R script (Appendix 6) as outlined in Figure 4.1. For devils, red is the "west" (*N* = 47) and blue is the "east" (*N* = 18) population. For goose, red is the "Iceland" (*N* = 20) and blue is the "Denmark" (*N* = 20) population. Inertia ellipses illustrate groupings and do not necessarily indicate confidence.

When analysed utilising pairwise F_{ST}, we saw higher differentiation between our two major populations for devil than for goose (Table 4.3). Nevertheless, patterns across the three analysis methods were similar for both species: data processed by all three pipelines provided F_{ST} values that were similar (Table 4.3). These findings are consistent with our PCoA results, described above. Both species showed evidence of statistically significant population differentiation (Table 4.3).

Each analysis method produced a varying amount of missing data (Table 4.1), but filtering less stringently (30% vs 70% call rate) to allow more missing data (and thus a greater number of loci) did not generally change the qualitative interpretation of our results by PCoA nor F_{ST} for either species. The exception is the Stacks analysis for goose, where the inclusion of many thousands more SNPs with low call rate obscured population inference (Figure A5.3.4).

Table 4.3: Population pairwise F_{ST} values for each analysis with 95% confidence intervals generated over 2,000 bootstraps.
In devils, Pop1 refers to the Western population (*N* = 47), Pop2 refers to the Eastern population (*N* = 18), and Pop3 refers to the insurance population (*N* = 66). In geese, Pop1 refers to the Iceland population (*N* = 20) and Pop2 refers to the Denmark population (*N* = 20).

Dataset	Pipeline	Pop 1:Pop 2	Pop 1:Pop 3	Pop 2:Pop 3
Devil (70% call rate)	Stacks	0.100 (0.090, 0.110)	0.030 (0.027, 0.034)	0.025 (0.021, 0.029)
	SAMtools	0.071 (0.056, 0.088)	0.019 (0.014, 0.025)	0.025 (0.017, 0.033)
	GATK	0.094 (0.084, 0.103)	0.029 (0.026, 0.033)	0.025 (0.021, 0.029)
Devil (30% call rate)	Stacks	0.091 (0.084, 0.100)	0.026 (0.023, 0.029)	0.025 (0.021, 0.029)
	SAMtools	0.067 (0.057, 0.078)	0.026 (0.022, 0.030)	0.015 (0.011, 0.021)
	GATK	0.091 (0.083, 0.099)	0.028 (0.025, 0.031)	0.026 (0.022, 0.030)
Goose (70% call rate)	Stacks	0.034 (0.032, 0.035)		
	SAMtools	0.038 (0.036, 0.039)		
	GATK	0.033 (0.032, 0.033)		
Goose (30% call rate)	Stacks	0.017 (0.016, 0.019)		
	SAMtools	0.092 (0.091, 0.093)		
	GATK	0.046 (0.045, 0.047)		

Discussion

We examined population genetic inferences drawn from RRS data for two very different species with reference genomes using three analytical pipelines. Reference-aligned RRS analyses are poised to become much more common as a greater number of reference genomes become available. Genomes are no longer restricted to model species, and global initiatives such as the Earth BioGenome Project (Lewin *et al.*, 2018) aim to sequence all eukaryotic life, whilst targeted initiatives focus either on regionally important species (such as the Oz Mammals Genome Project [Oz Mammals Genomics Framework Data Initiative, 2018]) or on particular taxa (such as the Birds 10K Project [Zhang, 2015]). With an increasing proliferation of reference genomes, researchers skilled in the use of WGS alignment and assembly software (such as SAMtools [Li *et al.*, 2009] and GATK [McKenna *et al.*, 2010]) may prefer to use these tools when expanding their studies to include population-level RRS data. However, our results demonstrate the utility of purpose-built RRS pipelines with reasonable computational demands (such as combining Stacks with our custom R script [Appendix 6]) intended for use in non-model organisms.

Although all of the analytical pipelines we examined were able to detect genetic structure between the two populations of both species, there were differences in the resultant datasets. Due to the greater number of SNPs obtained, GATK may perform better for conducting analyses such as genome-wide associations that require a high marker density, however we note that computational resources required may be a limiting factor for use of GATK when studying non-model organisms (Table 4.1). Both SAMtools and GATK had higher initial error rates than Stacks, which could impact reliability for individual-level analyses, although our custom R script allows SNPs to be filtered out based on reproducibility to improve error rates, if replicates are performed. Stacks produced a comparable number of SNPs to GATK for devils, but far fewer for geese, and yet performed similarly well in detection of population structure in both species, with far less computational investment (Table 4.1, Figure 4.2).

For both species, we observed a low percentage of shared loci across pipelines, which may introduce a source of ascertainment bias if extending a study to include more samples and using a prior set of defined loci. This observation may raise a potential red flag for many types of analyses (such as estimating allele frequencies or calculating linkage disequilibrium [Lachance & Tishkoff, 2013]), although it did not impact the population structure analyses we conducted here. Nevertheless, we note that genotype concordance across the shared loci was high. The tighter clustering of the two devil populations demonstrated by the SAMtools PCoA and the lower estimations of pairwise F_{ST} relative to Stacks and GATK, is likely influenced by the greater proportion of heterozygous genotype calls in that dataset. The apparent over-representation of heterozygous genotype calls in SAMtools can of course be addressed with additional data filtering which would be specific to each study so should be parameterised at the outset in future population genetic studies. Considering compute time and downstream population inferences, Stacks combined with the custom R script was the best performer of the three software packages we tested, and provided results that were independent of number of loci or percentage missing data for devils, but was influenced by missing data for geese.

In this study, we compared analysis pipelines using real datasets for two very different study species. Tasmanian devils are known to have low genetic diversity (Miller *et al.*, 2011) and their numbers are declining due to DFTD (Lazenby *et al.*, 2018). The pink-footed goose, on the

other hand, has higher genetic diversity and an expanding population (Pujolar *et al.*, 2017b). As shown here, there are differences between the three pipelines observed in the PCoAs and pairwise F_{ST} comparisons. These may result in different recommendations, which may impact the genetic outcomes of the populations in question. We used the same parameters for each species for the purpose of comparison and note that our MAF thresholds may not be suitable for both populations given expected levels of diversity and sample sizes. The sample sizes were quite different and may have resulted in more alleles being sampled in the devil dataset, which has likely influenced population-level results (Linck & Battey, 2019). We recommend MAF thresholds are parameterised at the outset of studies using RRS approaches.

Here we provide researchers with a customisable R pipeline (Appendix 6) that can be used for downstream analysis with data outputs in VCF format from any of these, or similar, software packages. The R pipeline works with VCF outputs from either initial alignment to a reference genome or *de novo* assembly and SNP calling. Our script allows for flexibility in choosing filtering thresholds by visual assessment of SNP data, as appropriate thresholds will differ between species, genotyping methods and downstream applications (Paris *et al.*, 2017). Filtering options include minimum read depth of both alleles (a feature that can be controlled in *de novo* alignment in Stacks with the -m parameter, but which is not implemented within Stacks for reference alignment), coverage difference, call rate, minor allele frequency (MAF), heterozygosity and potentially sex-linked SNPs (based on XX/XY sex determination, though this could easily be reversed for ZZ/ZW organisms). An additional feature designed specifically to make use of the technical replicates performed by DArT PL is the reproducibility filter and error rate calculation, which can be extended to any RRS project where replicates have been used. The dartR package (Gruber *et al.*, 2018) contains functions for many of these filtering steps, however requires the proprietary DArT PL results spreadsheet as input for full functionality. Our custom R script can reproduce metrics provided by DArT PL from user-processed data, including SNP data from other RRS methods, allowing researchers to fully customise their analytical pipelines. The R script can be run on a standard personal computer in most scenarios, or on high performance computers, as is required with the thousands of SNPs output from GATK. We have specifically designed this pipeline so that researchers who work closely with conservation managers (Hogg *et al.*, 2017a) can use genomic data to assist in making informed management decisions for species of conservation concern.

Conclusion

While all pipelines performed well, they each have pros and cons which differ depending on the diversity present in the population and the amount of missing data. Stacks was less than optimal when missing data levels were high for goose as the populations could no longer be discriminated. SAMtools did not perform as well when the number of SNPs were low for devils so the diversity present was not great enough to discriminate between the populations as well as Stacks and GATK. GATK performed well but computational burden may exclude its use in some species of conservation concern where access to high performing compute resources may be limited and management decisions need to be made quickly following data collection. For our datasets, the Stacks pipeline combined with our custom R script is a robust and computationally efficient method for analysis of RRS data for both conservation-dependent and widespread species.

Methods

Datasets

Devil RRS data were obtained using DArTseq following McLennan *et al.* (2019), with full details provided at Supplementary Methods A5.1 (see Figure A5.3.5 for sample quality). The restriction enzyme combination used was PstI-SphI, with fragments sequenced on an Illumina HiSeq 2500 as 77-bp single-end reads. Our devil dataset included animals originating from Western Tasmania ("Population 1", *N* = 47) and Eastern Tasmania ("Population 2", *N* = 18). In a further analysis we also considered data from *N* = 66 captive animals, which collectively comprised a mix of these two source populations and offspring thereof ("Population 3"). Methods for the goose RRS are reported at Pujolar *et al.* (2017b). In brief, a ddRAD protocol was used with restriction enzymes Pst-HF and MSp1, and libraries sequenced on an Illumina HiSeq 2500 as 79-bp paired-end reads. We used data (Pujolar *et al.*, 2017a) for the Iceland ("Population 1", *N* = 20) and Denmark ("Population 2", *N* = 20) sites, as reported in Pujolar *et al.* (2017b).

Data cleaning

Stacks 'process_radtags' was used on both devil and goose datasets to clean reads, removing those with any uncalled bases or low quality scores prior to aligning, and remove barcodes if

necessary (devil data only, goose data already de-barcoded; see Supplementary Methods A5.1).

Alignment to reference genomes

Stacks pipeline

For both species, we used the Burrows-Wheeler aligner (BWA) v0.7.15 'aln' function (Li & Durbin, 2009) to align single-end reads (devil) or paired-end reads (goose) following Rochette and Catchen (2017) to the respective reference genome (Murchison *et al.*, 2012; Pujolar *et al.*, 2018). For our devil data, bias in per base sequence content was detected in the first 5 bases of reads (adaptor region) with FastQC so these were trimmed during the genome alignment step (−B 5) to remove the restriction enzyme cut site (PstI-HpaII). The BWA 'samse' function (devil, single-end reads) or 'sampe' function (goose, paired-end reads) was used to generate alignments in SAM format, which were converted to BAM format and ordered and indexed using SAMtools v1.6. Cleaned, trimmed, aligned data were then used as input for further analyses.

SAMtools and GATK pipelines

For our devil data, the first 5 bases were trimmed prior to alignment using bbDUK (Bushnell, 2014). The 'mem' function in BWA was used to align reads following best practise guidelines (Van der Auwera *et al.*, 2013), to the devil reference genome (Murchison *et al.*, 2012) followed by the SAMtools 'sort' function (Li *et al.*, 2009) to sort by genomic coordinate. Local realignment around indels was conducted using GATK IndelRealigner (McKenna *et al.*, 2010). For our goose data, cleaned reads were aligned to the pink-footed goose genome (Pujolar *et al.*, 2018) with BWA 'mem' followed by SAMtools sort and local realignment with GATK as per the devil data.

Calling loci

Our three bioinformatic pipelines use slightly different methods to identify SNPs. To summarise, Stacks builds a catalogue of loci grouped across individuals (Catchen *et al.*, 2013), and applies a Bayesian maximum-likelihood approach developed by Maruki and Lynch (2017) that incorporates population genotype frequency information. GATK and SAMtools implement Bayesian approaches to call genotypes. GATK considers all reads covering a locus,

as well as expected heterozygosity, to compute the posterior probability of a genotype (McKenna *et al.*, 2010). SAMtools additionally includes Hidden Markov models to calibrate SNP calls using base alignment quality (BAQ) scores (Li, 2011a; Li, 2011b).

Stacks pipeline

We used the Stacks v2.0b pipeline to process the sorted BAM files. The 'gstacks' module was run with default parameters (--model marukilow and --var-alpha 0.05) to create a catalogue of SNPs across our sample set as a single population. We ran the 'populations' module with the following parameters: a minimum call rate of 70% (−r 0.70), a maximum observed heterozygosity of 70% for devils (--max_obs_het 0.70) or 80% for goose (a higher threshold was chosen due to the much lower sample size), a minimum minor allele frequency (MAF) of 0.01 (--min_maf 0.01), and the --write_random_snp flag to randomly select only one SNP per locus.

SAMtools pipeline

SNPs were called from the realigned, sorted BAM files using SAMtools mpileup (Li *et al.*, 2009) with minimum base and mapping quality scores of 30. The coefficient for down-grading mapping quality of reads with excessive mismatches was set to 50 and bcftools call -m 6 was used to set a minimum depth of six reads to call a locus. This value was chosen to most closely simulate the Stacks parameter m = 3, which is minimum depth to call an allele, hence this was doubled to equate to minimum depth to call a locus. BCFtools merge (Li *et al.*, 2009) was used to merge single sample VCFs into a multi-sample VCF and filter on genotyping rate (min 70%, similar to Stacks -r) and MAF of 1% with VCFtools (Danecek *et al.*, 2011), to reflect the values used in the Stacks pipeline.

GATK pipeline

The realigned, sorted BAM files were used as input into GATK's HaplotypeCaller (McKenna *et al.*, 2010) to produce individual gvcf files that were input into GATK's GenotypeGVCFs to create a multi-sample gvcf file. VCFtools was again used to conduct preliminary data filtering using the same parameters as the SAMtools pipeline.

Custom R script

Within our custom R script (Appendix 6), we converted the VCF files from each of the three pipelines for the two species using the vcfR package (Knaus & Grünwald, 2017) in order to extract the genotypes and associated metadata such as read depth. We further filtered the SNP set on average allelic depth, coverage difference, reproducibility and sex-linked SNPs. For SAMtools and GATK datasets, we also filtered on maximum observed heterozygosity as per the parameters used in Stacks. We set a minimum average read depth for both the reference and SNP allele as 2.5×. We calculated coverage difference as the percentage difference at each SNP between the read depth of the reference allele and SNP allele, and used a coverage difference of ≤ 80% as our cut-off. DArT PL performs technical replicates during the sequencing process, so for our devil dataset we calculated a measure of reproducibility as the genotype call error rate at each SNP between technical replicates once missing data is removed and filtered at > 85% reproducibility. We then recalculated error rate post-filtering. The goose dataset did not have replicates available for calculation of error rates or filtering on reproducibility.

In mammals, females are the homogametic sex with two X chromosomes, and males are heterogametic XY, whilst in birds females are heterogametic ZW and males are homogametic ZZ. We had accurate sex data for all devil samples and could therefore identify and filter out SNPs that may be sex-linked if no heterozygotes were present in the heterogametic sex but at least one heterozygote was present in the homogametic sex. We note however that this is a stringent filter and could be adjusted for sequencing errors. We did not have this information for goose and so did not apply any further filtering in this respect.

Within-population diversity

The three resulting SNP datasets (Stacks, SAMtools and GATK) for each species were assessed for their ability to examine our study populations using a set of markers mapped to the genome. Data filtering and transformations were conducted using the custom R script for all datasets. For each of the datasets, summary statistics of observed (H_O) and expected heterozygosity (H_E) across loci were calculated using the 'adegenet' package for R (Jombart, 2008; Jombart & Ahmed, 2011). The multilocus observed heterozygosity of individual devils (MLH) was calculated as a proportion of heterozygous loci across each individual. We

extracted the shared loci between each pipeline for both species and used the 'merge' function in PLINK (Purcell *et al.*, 2007) to identify concordance rates between genotype calls across pipelines and output differing genotype calls for comparison.

Between-population divergence

We performed principal coordinates analyses (PCoA) to discriminate population structuring and genetic clustering in the 'adegenet' and 'ade4' (Dray & Dufour, 2007) packages. This method calculates squared pairwise Euclidean distances between individuals allowing visualisation of population differentiation. PCoAs were run using population information to examine the structuring between "west", "east", or "IP" (insurance population, captive-born) samples for devil, and Iceland and Denmark for goose. For devils, two different analyses were performed for each of the three pipelines, the first including all individuals sequenced (*N* = 131), and the second only the founding wild-born individuals (*N* = 65). For devil samples with a technical replicate (*N* = 35), the sample with the least missing data from the SAMtools pipeline was selected (same sample selected across all pipelines). Pairwise fixation indices (F_{ST}) were calculated using the 'StAMPP' package for R (Pembleton *et al.*, 2013), with 95% confidence intervals calculated via 2,000 bootstraps across loci.

Impact of missing data

For both species, we refiltered all three pipelines less stringently (genotyping rate of 30% rather than 70%) to examine the impacts of missing data on population inference. Calculation of summary statistics and F_{ST}, and visualisation with PCoA were performed as above on the less stringently filtered SNP datasets.

Acknowledgements

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Data availability

Sequence data for devils has been deposited on the NCBI SRA (BioProject no. PRJNA540395; sample SRA accessions: SRR9001456-SRR9001623). Goose data is available on the NCBI SRA (BioProject Accession no. PRJNA400851/SRA accession SRP116633) and Dryad (https://doi.org/10.5061/dryad.c4r81).

Animal Ethics Note

All devil samples were collected under Save the Tasmanian Devil Program standard operating procedures for routine management of the species. Samples were shared with us for analysis.

Chapter 5: A case for genetic parentage assignment in captive group housing

5.1 BACKGROUND

Chapter 5 comprises the published manuscript:

Farquharson, K.A., Hogg, C.J.* & Grueber, C.E.* (2019) A case for genetic parentage assignment in captive group housing, *Conservation Genetics*, **20,** 1187-1193.

*contributed equally to this work.

Group housing is often suggested as a strategy to minimise adaptation to captivity by providing more natural social structures. One of the challenges of group housing for pedigree-based management is that parentage is difficult to assign. Molecular methods can be used to assign parentage in group-housed populations. In this chapter, I apply the genotyping method I developed in Chapter 4 to obtain reliable high-density marker data in order to perform parentage assignment and examine between-individual variation in reproductive success in group-housed Tasmanian devils. Accurate pedigrees are needed to investigate possible mechanisms of adaptive change, so the pedigree reconstruction performed in this chapter also informs the methods applied in Chapters 6 and 7.

I undertook the research in this chapter and drafted the manuscript (including table and figures). I also extended the custom R script of Chapter 4 to convert data to the required format and perform parentage analysis. The extended R script is provided in Appendix 7. Carolyn Hogg and Catherine Grueber oversaw the project, provided technical and conceptual assistance and provided funding. Note that this chapter is presented in the order Introduction, Materials & Methods, Results and Discussion, in line with the *Conservation Genetics* format and published version.

5.2 MAIN ARTICLE

A case for genetic parentage assignment in captive group housing

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Abstract

Captive animals are commonly housed in groups to make efficient use of limited resources and allow for natural social behaviour. Captive management relies on accurate pedigrees to estimate various population genetic parameters, such as genetic contributions of breeders, but pedigrees of group-housed offspring can be uncertain. Pedigree analysis software incorporates genetic information from multiple putative parents ("MULT"). Molecular pedigree reconstruction to resolve pedigree uncertainties can be costly. We quantify the need for molecular parentage assignment by comparing predicted offspring contributions (based on uncertain "MULT" pedigrees) to contributions obtained from a molecular genetic pedigree reconstruction. Parentage of 81 insurance population Tasmanian devils (*Sarcophilus harrisii*) born in free-range enclosures from 2011 to 2017 was resolved using 891 single nucleotide polymorphisms. We observed large discrepancies between the MULT pedigree and molecular pedigree data, revealing both overestimates and underestimates of genetic contributions of individuals, and different pedigree-based effective population sizes (102 vs. 158 respectively). The molecular data revealed that reproductive skew (proportion of adults that failed to breed) was high for both sexes. Over half of the wild-born individuals in our dataset were found to have not bred. If undetected, variation in breeding success undermines the utility of pedigree management and may threaten the success of captive breeding. Molecular techniques are increasingly cost-effective, and our data demonstrate that they are critical to devil management. Where feasible, we recommend molecular management of group-housed species in captivity to avoid inaccurate estimates of genetic diversity and to identify non-breeding individuals, in particular founders, for targeted breeding.

Introduction

Conservation breeding programs often house captive animals in groups rather than pairs to promote natural behaviours, lower management costs, and enable mate choice (Wedekind, 2002; Wang, 2004). To maximise genetic diversity retention, breeding in managed populations follows a mean kinship strategy, whereby individuals with the lowest relatedness to the rest of the captive population are prioritised for breeding (Frankham *et al.*, 2010; Chapter 1). This approach requires accurate pedigrees to calculate relatedness. Group housing, however, can complicate parentage assignment, particularly for species with polygamous mating structures, low parental care, or secretive, or cooperative, breeding behaviour.

From a conservation management perspective, uncertainty in a pedigree differs from wholly unknown parentage. Group housing may result in uncertain pedigrees, where true parents of offspring are amongst a limited set of known males and females. In contrast, ancestry of wild-born (founder) animals is unknown. This is an important distinction, because "unknown" founder animals are considered unrelated to all other animals in the population, while individuals with uncertain pedigree are known to be related to some (uncertain) degree. Current population management software, such as PMx (Lacy *et al.*, 2012), a widely used captive population management program, allows for this distinction. In PMx, offspring with uncertain parents are attributed multiple sires and/or dams, selected using knowledge of the adults in the enclosure, termed "MULT". MULT animals are apportioned fractions of parentage (Lacy, 2012). For example, a diploid offspring with two equally likely possible sires and a known dam will have half its genome allocated to the dam, and a quarter attributed to each sire. Incorporating probabilistic parents improves upon other methods, such as excluding unknown parents or using behavioural data alone, e.g. assuming the dominant male is the sire (Lacy, 2012). In reality, only one sire and one dam can truly contribute to an offspring's genome. In theory, if animals breed at random, incorporating MULT parentage will provide population-level parameters that closely match true values. In practice, many factors influence which animals will breed in a group (e.g. Gooley *et al.*, 2018; Martin-Wintle *et al.*, 2019), which may lead to deviations between expected and observed values that affect individual-level management, such as identifying individuals to translocate or breed. Here, we quantify this challenge in Tasmanian devils (*Sarcophilus harrisii*) by measuring deviations

between MULT pedigree-based expectations and true breeding success as determined by molecular pedigree reconstruction.

Devils are an endangered marsupial with a large (> 700 living individuals) captive breeding program established in 2006, housed across a number of institutions including 10–22 ha free-range enclosures (FREs) with ~ 20 adults per enclosure (Hogg *et al.*, 2017b). Females are polyovular, polyoestrus seasonal breeders, producing up to four offspring per litter each year (Keeley *et al.*, 2012). Although the frequency of mixed-paternity litters is unknown, they have been observed in intensive captive housing (Russell, 2017), group housing (Gooley *et al.*, 2017; this study), an island population (McLennan *et al.*, 2018) and in the wild (McLennan, unpublished data). Altricial offspring remain in the female's pouch for ~ 105 days (Guiler, 1970) before being denned. As offspring are not trapped until they are partially independent (~ 6 months, Guiler, 1970), determining parentage in FREs is not possible by observation alone, so population management relies on field observations of pouch young and MULT assumptions. We used molecular genetics to resolve parentage of 85 devil offspring born in FREs from 2011 to 2017 and provide recommendations for group-housed species management.

Materials and Methods

Records provided by the Save the Tasmanian Devil Program and the studbook (Srb, 2018) were used to establish males, females and resulting offspring housed in two FREs (Table 5.1). Extracted DNA from ear biopsies, or whole blood, of 155 devils (79 males, 76 females), was sent to Diversity Arrays Technology Pty Ltd (DArT) for reduced representation sequencing (RRS) with the PstI-SphI enzyme combination and HiSeq 2500 sequencing. Raw reads were processed in Stacks v2.0b (Catchen *et al.*, 2013) and aligned to the reference genome (Murchison *et al.*, 2012) with BWA aln (Li & Durbin, 2009) and SAMtools (Li *et al.*, 2009). The 'gstacks' module was used to build a catalogue of SNPs across these samples alongside an additional 430 unique devil samples sequenced for other purposes. The 'populations' module was run to obtain an initial set of 9,894 SNPs in linkage equilibrium (using the --write_random_snp flag) with a minimum call rate (proportion of samples genotyped) of 20%, maximum observed heterozygosity of 70% and minimum minor allele frequency (MAF) of 1%. We used our custom R script (R Core Team, 2018, version 3.4.4; Appendix 7) based on Chapter 4 (Wright *et al.*, 2019; Appendix 6) for further downstream filtering on average allelic

depth (average number of reads for an allele) > 2.5, MAF ≥ 5%, reproducibility (proportion of mismatching calls between technical replicates) ≥ 90%, coverage difference (% difference in average read depth of the reference and alternate allele) ≤ 60%, and potentially sex-linked SNPs (heterozygous in at least one female and homozygous in all males). Our script also combined data from technical replicates by adding genotype information from the sample with the lower call rate to the sample with the higher call rate only at loci with missing data.

Table 5.1: Summary statistics for the free-range enclosures.
Number of adults in each enclosure from 2011 - 2017, and number of offspring produced with the associated reproductive skew (% that failed to reproduce in that enclosure year), calculated using the number of sequenced offspring with parentage resolved. May be overestimated in males in years where some offspring are unsequenced or unassigned.

FRE	Year	No. in enclosure (plus no. not sequenced)			Reproductive skew (%)	
		Adult males	Adult females	Offspring	Males	Females
Bridport						
	2011	9	8 (1)	17	44.4	44.4
	2012	8	10 (1)	2	75.0	81.8
	2013	7	11 (1)	20	28.6	41.7
	2014	10	11	14 (2)	60.0	54.5
	2015	6 (1)	3[a]	4	85.7	33.3
	2016	9	7 (2)	2	88.9	88.9
	2017	2 (1)	4 (3)	12 (1)	0	28.6
Freycinet						
	2011	7 (1)	6 (4)	5 (1)	75.0	70.0
	2012	8 (1)	7 (1)	3	77.8	75.0
	2013	11 (1)	7 (1)	6 (1)	75.0	62.5
Total/Average				85 (5)	61.0	58.1

[a] An additional 4 adult females were housed in the enclosure but were contracepted as part of another study (Cope *et al.*, 2018a).

Parentage assignment with SNP data is optimised with fewer SNPs in low linkage disequilibrium, with low missing data and a high MAF, so SNPs are more informative and more computationally efficient (Huisman, 2017). We selected the samples relevant to our analysis and further filtered on call rate > 80% and MAF > 1%, resulting in 891 SNPs. Genotype data, sex and birth year were used to determine parentage with the 'sequoia' package for R (Huisman, 2017), with the error term set to either 0.05 or 0.01. 'sequoia' is ideal for zoo populations, because it is capable of dealing with multi-generational, overlapping, inbred pedigrees and assigning half-siblings and grandparental relationships with unsampled parents and has a low error rate < 0.1% (Huisman, 2017). Parentage could not be determined with the 891 SNPs for six individuals; a more stringently filtered set of 661 SNPs (MAF > 10%) enabled resolution for two of these.

We calculated expected pedigree contributions (i.e. without molecular data) for each adult in the FREs separately for each sex as $\sum_{e,y} \frac{no.\ offspring_{e,y}}{no.\ males/females\ in\ enclosure_{e,y}}$, where *e* was FRE and *y* was year. This is equivalent to the use of the "MULT" option in PMx given that all individuals in an enclosure have equal probability of being the parent of an offspring. MULT pedigree contributions may be non-integers and vary with the years the devil was in an enclosure, how many other same-sex adults were in the enclosure, and how many offspring were produced in that enclosure and year. For each adult, we compared the MULT pedigree contributions to the total number of offspring allocated from the molecular pedigree across all enclosure years. Additionally, for females, we compared these to the number of offspring estimated by pouch observations, as a check for our molecular pedigree reconstruction. We calculated reproductive skew as the percentage of males/females that failed to reproduce in a given enclosure and year, and also averaged across the dataset. High reproductive skew indicates that only a small number of individuals successfully bred; low reproductive skew would be more consistent with MULT pedigree assumptions of equal contributions. We calculated the N_e (effective population size) to *N* (census size) ratio of the entire captive breeding program (*N* = 446 living devils; excluding Maria Island), current to 31/12/2017 in PMx as a comparison of the MULT pedigree and molecular pedigree and its cumulative impact on the captive population as a whole.

Results

We successfully assigned both sire and dam to 81 of 85 offspring using a combination of genetic and biological data (pouch status). Dam only was assigned to a further three offspring. The relationship between expected MULT pedigree contribution and number of offspring genetically assigned from the molecular pedigree for each adult was highly variable (Figures 5.1 and 5.2). For some, expected MULT pedigree contributions very closely matched the observed number of offspring, while for others the MULT pedigree contribution greatly overestimated, or underestimated, the number of offspring actually produced. For example, 28/53 (52.8%) males did not sire any offspring across up to 3 years, even though some had very high MULT pedigree-based expected contributions (up to 3.81 across all years for this group). Reproductive skew was high for both males and females across enclosures and years (Table 5.1), averaging 61.0% and 58.1% respectively. Of the 36 wild-born devils in our dataset, 9/17 males (Figure 5.1) and 10/19 females (Figure 5.2) did not produce any offspring. The N_e/N ratio was 0.2719 for the MULT pedigree (current N_e = 102.19) and increased to 0.3978 for the molecular pedigree (current N_e = 158.41), a 46% increase over the MULT pedigree N_e/N.

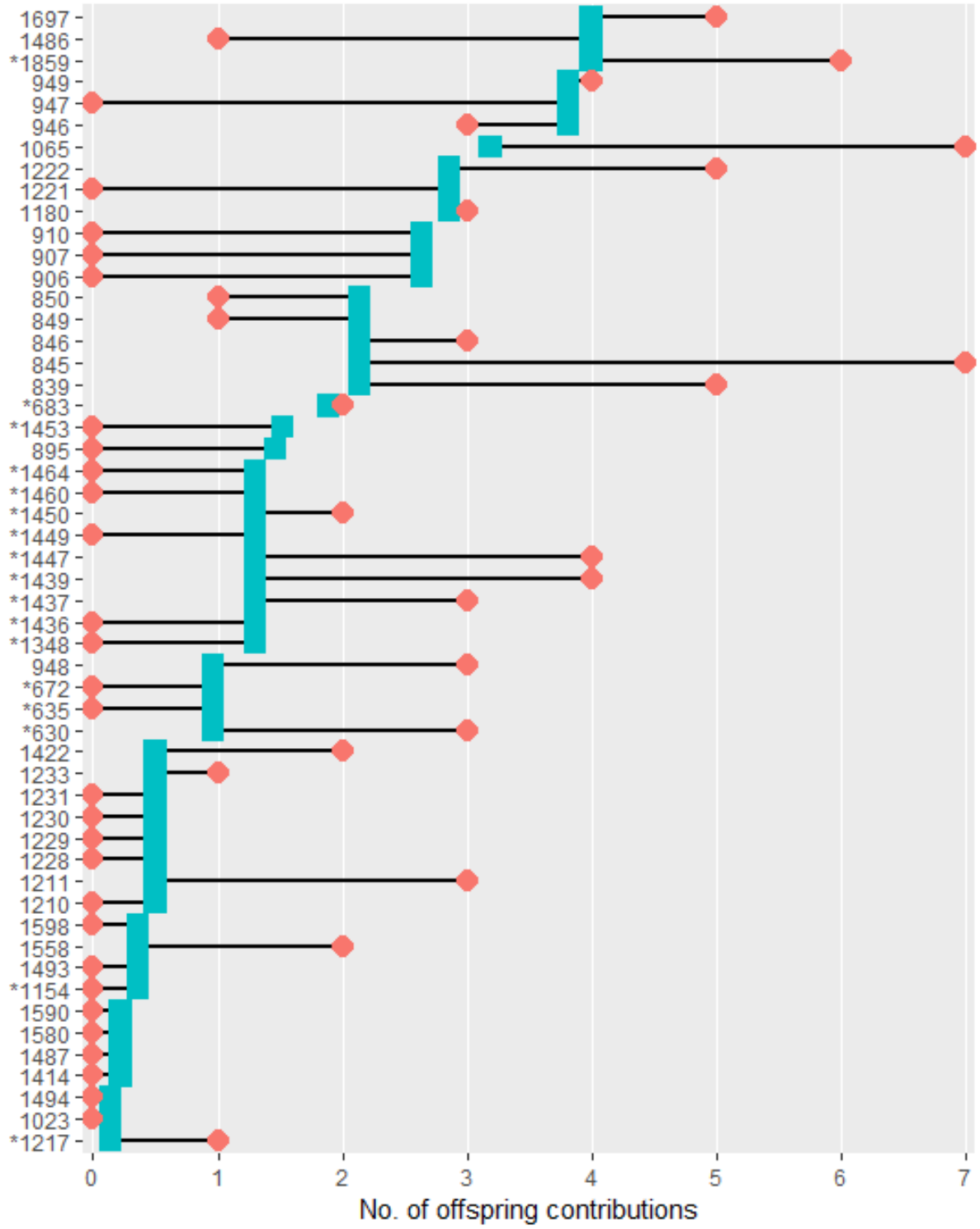

Figure 5.1: Traditional and molecular pedigree discrepancies for males.
Differences between MULT pedigree offspring contributions (squares) and true number of offspring (circles) as determined by molecular pedigree reconstruction for males (represented with studbook number). Wild-born (*).

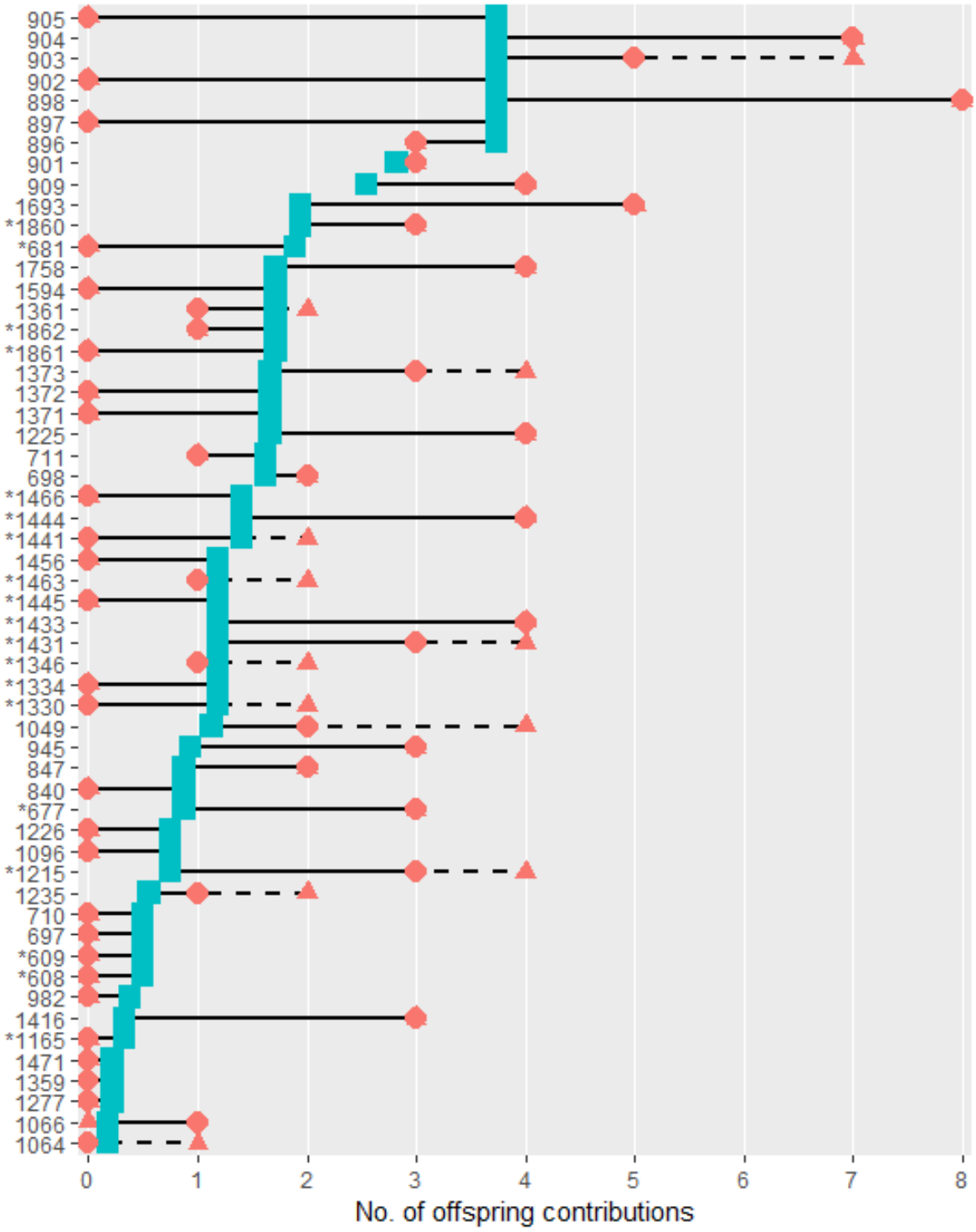

Figure 5.2: Traditional, molecular and observational pedigree discrepancies for females. Differences between MULT pedigree offspring contributions (squares), true number of offspring determined by molecular pedigree reconstruction (circles) and number of offspring allocated from pouch observations (triangles, dashed line) for females (represented with studbook number). Wild-born (*).

Discussion

The high levels of reproductive skew (failure to breed) we observed are slightly higher than those reported by Gooley *et al.* (2018), where male and female devils housed in smaller groups (maximum ten individuals, versus maximum 21 herein) had an average reproductive skew of 58.1% and 53.2% respectively. Reproductive skews were also higher than devils housed in pairs (43.9% and 36.3% respectively) (Gooley *et al.*, 2018). Reproductive skew is concerning if non-molecular pedigree analysis fails to identify the lowered founder genome equivalents and unequal family sizes that lead to genetic diversity losses (Frankham, 2010b). If individuals that consistently fail to breed are identified, they can be targeted for intensive management, and overrepresented individuals can be temporarily (e.g. contracepted [Cope *et al.*, 2018b]), or permanently prevented from breeding.

Although methods exist for genetic management of animal groups (such as the use of MULT), individual-level management is more effective at retaining genetic diversity and avoiding inbreeding (Wang, 2004; Jiménez-Mena *et al.*, 2016). Group-based pedigree methods (such as MULT) will not be as informative if assumptions such as random mating are not met, however are still preferable to no management, or using behavioural data alone (Wang, 2004). For devils, further research is needed to determine whether non-random mating (e.g. mate choice) explains the apparent increase in reproductive skew as group size increases. For other group-housed species, managers should consider the mating system, number of animals group-housed, natural reproductive skews (e.g. dominance structures), cost of acquisition or translocation of animals, costs of analysis versus cost of management, and the impact of accumulative effects of MULTs across multiple generations when deciding whether to apply molecular genetic management to a group-housed species, and if so how many animals should be sampled. Molecular genetic management of groups may even be useful for presumed monogamous species where behavioural data alone is used, as extra-pair paternity may be overlooked (Lee *et al.*, 2018). At face value, group housing may appear to be a cheaper management option than housing in pairs ($2,100 AUD per devil [Parrott *et al.*, 2019a] versus $10,000 per devil housed in pairs), however group housing comes at a genetic diversity cost. That is, group housing using MULT parentage provided an inferred effective population size of 102 whilst the molecular pedigree showed that the true effective population size was closer to 158 (we note that the pedigree was not fully resolved). We calculate that the cost of our

analysis is $125 per devil (including reagents, sequencing, labour costs), an investment that reduces the need to hold 206 more devils to achieve the same N_e (158.41) under the MULT pedigree N_e/N ratio of 0.2719.

Group housing is an important component of many conservation breeding programs. Genetic management is important when key animals, such as potential founders, are group-housed and so are included in calculations of genetic diversity. If these wild-born animals do not breed, and this goes undetected, conservation breeding programs will lose important wild genetic diversity. Molecular genetic approaches continue to decrease in cost (Puckett, 2017), so are becoming more accessible for use in conservation (Norman *et al.*, 2019; Chapter 4). Our results show that molecular genetic information, alongside biological knowledge, should be routinely used to refine pedigree information in group housing contexts.

Acknowledgements

All the Save the Tasmanian Devil Program keeping staff who have worked with the devils in FREs, in particular Karen Fagg and Olivia Barnard. Thanks also to Carla Srb for her ongoing management of the Tasmanian devil studbook, and the Zoo and Aquarium Association Australasia, and its member zoos, who contribute to the insurance population. We thank two anonymous reviewers for comments that improved the manuscript. This work was funded by ARC LP140100508 and DP170101253.

Data availability

The custom R script used to process SNP data and perform pedigree analysis is available in Appendix 7.

Animal Ethics Note

All DNA samples were collected under the STDP Standard Operating Procedures for handling Tasmanian devils as part of their management of the FREs and shared with us.

Chapter 6: Deciphering genetic mate choice: not so simple in group-housed conservation breeding programs

6.1 BACKGROUND

Chapter 6 comprises the following manuscript, which has been reviewed and invited for resubmission:

Farquharson, K.A., Hogg, C.J., Belov, K. & Grueber, C.E. (to be resubmitted) Deciphering genetic mate choice: not so simple in group-housed conservation breeding programs, *Evolutionary Applications.*

In Chapter 5, I identified high variation in reproductive success in a captive population of Tasmanian devils housed in free-range enclosures by reconstructing the pedigree using SNPs processed from the method developed in Chapter 4. In this chapter, I investigated non-genetic and genetic factors that may contribute to this inter-individual reproductive variation. Non-genetic factors including age and weight were investigated and found to be most important to reproductive success. Genetic factors, including SNP-based genome-wide heterozygosity and heterozygosity at MHC-linked microsatellites did not predict reproductive success. I found no clear evidence for the three genetic mate choice hypotheses that were tested: advantage of heterozygous individuals; advantage of dissimilar mates; and optimum genetic distance.

In this Chapter, I performed polymerase chain reactions, analysed the data, prepared figures and tables and drafted the manuscript. Carolyn Hogg provided guidance on the analysis, critically revised the manuscript and provided funding. Katherine Belov critically revised the manuscript and provided funding. Catherine Grueber provided guidance on the analysis, critically revised the manuscript and provided funding. Funding for this work was provided by the Australian Research Council LP140100508 and DP170101253.

6.2 MANUSCRIPT

Deciphering genetic mate choice: not so simple in group-housed conservation breeding programs

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Abstract

Incorporating mate choice into conservation breeding programs can improve reproduction and the retention of natural behaviours. However, different types of genetic-based mate choice can have varied consequences for genetic diversity management. As a result, it is important to examine mechanisms of mate choice in captivity to assess its costs and benefits. Most research in this area has focused on experimental pairing trials, however this resource-intensive approach is not always feasible in captive settings and can interfere with other management constraints. We investigated overall breeding success and three non-mutually exclusive mate choice hypotheses (advantage of heterozygous individuals, advantage of dissimilar mates, and optimum genetic distance) using both genome-wide SNPs and MHC-linked microsatellites in group-housed Tasmanian devils. The managed devil insurance population is the largest such breeding program in Australia and is known to have high variance in reproductive success. We found that non-genetic factors such as age were the best predictors of breeding success in a competitive breeding scenario, with younger females and older males being more successful. We found no evidence of mate choice under the hypotheses tested. Mate choice varies among species and across environments, so there is a need to investigate hypotheses on a case-by-case basis. Our study shows that examining and integrating mate choice into the captive management of species housed in realistic, semi-natural group-based contexts may be more difficult than previously considered.

Introduction

Allowing for mate choice has long been suggested to improve the success of conservation breeding programs (Wedekind, 2002; Quader, 2005; Asa *et al.*, 2011; Schulte-Hostedde & Mastromonaco, 2015; Martin-Wintle *et al.*, 2019). Any examination of the costs and benefits of allowing mate choice in a captive environment must reflect the long-term demographic and genetic sustainability of the captive population (Chargé *et al.*, 2014b). For example, allowing mate choice may result in greater reproductive success overall and confer fitness benefits such as improved offspring health (see Martin-Wintle *et al.* (2019) for a review in *ex situ* populations). Yet populations may also experience high reproductive skew if preferred individuals breed well, while the non-preferred fail to breed. Reproductive skew can then result in loss of genetic diversity and a lower effective population size (Frankham *et al.*, 2010). In their review of mate choice in captive management, Chargé *et al.* (2014b) recognised that "there is currently too little theoretical and empirical evidence to provide any clear guidelines that would guarantee positive fitness outcomes and avoid conflicts with other genetic goals" (p1120). Therefore, an understanding of the mechanisms of mate choice in captivity is needed to ensure that overall genetic goals are not impeded. These goals often include benchmarks such as the maintenance of 95% genetic diversity over 100 years (Ballou *et al.*, 2010a) by equalising genetic representation of wild-born animals (founders) through preferentially breeding individuals with the lowest mean kinship (a measure of relatedness) in the population.

Much of the current literature on mate choice in conservation contexts focuses on experimental pairing trials. In pairing trials, an animal is housed with a test individual of the opposite sex, with behavioural indicators and/or reproductive outcomes used to determine whether the pairing is preferred or non-preferred (see Martin-Wintle *et al.*, 2015; Hartnett *et al.*, 2018; Parrott *et al.*, 2019b for examples). Other studies compare the breeding success of pairings with varying genetic characteristics (Parrott *et al.*, 2006; Parrott *et al.*, 2015; Brandies *et al.*, 2018; Russell *et al.*, 2018). While experimental trials are useful, they are labour-intensive and require resources that may not be available in many conservation breeding programs, for example the space required to house animals in pairs. Conservation breeding programs of threatened species may not be able to risk drops in productivity that could occur during experimental trials and forced monogamous pairings. Furthermore, for social species,

housing individuals in pairs may not be conducive to normal behavioural expression (Lutz & Novak, 2005). As a result, there is a need to investigate mate choice hypotheses in observational studies using populations housed as they would be realistically managed in captivity, such as in group-housed species.

The use of molecular markers in conservation breeding programs is increasingly common for a variety of management purposes, including resolving pedigrees (Chapter 5), inferring population structure (Chapter 4) and investigating hereditary diseases (Norman *et al.*, 2019). Molecular data gathered for these purposes can be extended to investigate mate choice. Evidence of mate choice may be found at the genomic level, which can be investigated using genome-wide single nucleotide polymorphisms (SNPs) such as those generated with reduced representation sequencing (RRS) at a low-cost. Mate choice may also occur in association with specific gene regions. For example, the involvement of the major-histocompatibility complex (MHC) in disease resistance means that mate choice with relation to variation at this region may confer direct fitness benefits to offspring (Consuegra & Garcia de Leaniz, 2008). The MHC region has been widely linked to mate choice in a number of species, for a review of evidence see Kamiya *et al.* (2014).

A number of non-mutually exclusive genetic-based mate choice hypotheses, with varying consequences for genetic goals of captive populations, have been proposed. These include:

1. Advantage of heterozygous individuals, where individuals with the greatest heterozygosity show the greatest fitness (e.g. through disease resistance, also known as quantity of alleles hypothesis; Doherty & Zinkernagel, 1975). If this occurs in a small population in captivity, individuals with lower heterozygosity will be less successful leading to reproductive skew, lower effective population size and poorer founder representation at the population-level over time.
2. Advantage of dissimilar mates, where individuals that breed with mates most dissimilar to themselves will maximise heterozygosity and therefore fitness of their offspring (Landry *et al.*, 2001), for example by reducing inbreeding load (Kempenaers, 2007). As individuals will vary in their choice of mates, no individual should be disadvantaged assuming there is enough genetic diversity in the population to allow dissimilar pairings (Tregenza & Wedell, 2000).

3. Optimum genetic distance, where individuals that breed with partners of an optimum level of genetic dissimilarity experience the greatest fitness, balancing the potential effects of outbreeding depression due to breeding with too-dissimilar mates, with inbreeding depression due to breeding with too-similar mates (also related to the compatible genes hypothesis) (Penn & Potts, 1999; Tregenza & Wedell, 2000). In a small population, genetic diversity will be depleted if the majority of successful pairings have high pairwise similarities (leading to inbreeding), or will increase with dissimilar pairings as per the advantage of dissimilar mates hypothesis (although outbreeding depression may be a potential risk [Chargé *et al.*, 2014b]).

Here we use both genome-wide SNPs and MHC-linked microsatellites to investigate the above three mate choice hypotheses in Tasmanian devils (*Sarcophilus harrisii*) housed in large free-range enclosures. We use seven years of data from the largest managed captive breeding program in Australia (Hogg *et al.*, 2017b), representing the best opportunity to detect mate choice without management intervention. As with many captive programs, the population is managed to meet conservation goals and so was not experimentally manipulated. Free-range enclosures are 22 ha in size and hold up to 21 adult devils in a roughly even sex ratio. Trapping within the free-range enclosures occurs four times per year to monitor the health of devils and record breeding. Relative to one-to-one pairings on one extreme, and free-roaming wild populations on the other extreme, the devil free-range enclosures represent an intermediate level of management: offering a high potential for mate choice, while still under conservation management (health checks and supplementary feeding) (Grueber *et al.*, 2018b). Group-housed devils exhibit high reproductive skew (approximately 60% of individuals fail to breed each season, Farquharson *et al.*, 2019; Chapter 5), so an investigation of potential mate choice mechanisms driving this skew will inform ongoing management. By investigating mate choice hypotheses in a non-manipulated captive setting, we aim to inform management options for other conservation breeding programs that house or plan to house species in groups with the opportunity for mate choice.

Materials and Methods

Sampling

This study included 93 unique adult devils housed in two free-range enclosures, Bridport and Freycinet, between 2011 and 2017. Some adults were present in more than one enclosure across the years, though none appeared in more than three enclosure-years. An additional 15 devils that were housed at the sites during this time could not be included, as no DNA sample was obtained, or the sample was of too poor quality to sequence. A further five females were contracepted during some of the enclosure years for a separate study (Cope *et al.*, 2018a), none of which produced offspring. Contracepted devils were excluded from all analyses. A total of 123 offspring were observed in pouch checks, 34 of which did not survive to weaning (sampling) age so could not be included, and another 4 of which survived but were not sampled. Ear biopsies were collected by the Save the Tasmanian Devil Program under Standard Operating Procedures for handling Tasmanian devils for management purposes, and DNA extracted using a phenol/chloroform protocol (Sambrook *et al.*, 1989). We considered a successful breeder as producing at least one offspring that survived until weaning, using the results of pedigree reconstruction to determine breeding status (Farquharson *et al.*, 2019; Chapter 5).

Non-genetic factors

We used data recorded in the Tasmanian devil studbook (Srb, 2018) and the ZIMS database (Species 360, 2018) to obtain the age and weight for every adult in each enclosure and year. Not all devils were trapped on each occasion, so we took the average weight of any records between 1st January and 30th April where the devil was held in that enclosure, as this time period covers the breeding season (Keeley *et al.*, 2017). Average weight was reasonably consistent throughout the breeding season (female mean = 6.68 kg, SD = 0.90 kg, coefficient of variation [CV] = 13.5%; male mean = 8.88 kg, SD = 1.05 kg, CV = 11.8%). For one male that had no weight measurement, the closest measurement to this time (December of the previous year) was used.

Genome-wide diversity

A reduced representation sequencing (RRS) approach was used to genotype genome-wide SNPs by Diversity Arrays Technology Pty Ltd (DArTseq; Wenzl *et al.*, 2004). We used a modified version of the Stacks (Catchen *et al.*, 2013) and custom R (R Core Team, 2018) pipeline presented in Wright *et al.* (2019) (Chapter 4; Appendix 6) to call and filter SNPs. We built a catalogue of 588 Tasmanian devil samples including those sequenced for this study and for other purposes, and filtered in Stacks on minimum genotyping rate (-r 0.20), heterozygosity (--max_obs_het 0.70), minor allele frequency (--min_maf 0.01) and linkage equilibrium (--write_random_snp). Within the custom R script, we further filtered on minimum average allelic depth (> 2.5; to exclude loci with low allelic depth across the sample set at either the reference or alternate allele), coverage difference (< 80%), reproducibility between technical replicates (> 90%) and minor allele frequency (> 5%) to obtain 1,948 SNPs across the samples relevant to this analysis to calculate diversity metrics.

We chose standardised genome-wide heterozygosity (H_{GW}) as our measure of genome-wide diversity, calculated as the total number of heterozygous loci in a sample divided by the sum of the average observed heterozygosities for all samples at the same genotyped loci, using the 'inbreedR' package in R (Stoffel *et al.*, 2016). A standardised metric reduces the influence of missing data.

MHC diversity

We typed the adults (48 males, 43 females) for which we had sufficient DNA at 12 MHC-linked microsatellite loci (Table A8.1.1) developed by Cheng and Belov (2014) and Day *et al.* (2019). Polymerase chain reactions (PCRs) with Qiagen Type-It Microsatellite PCR Kit were performed in a 10 µl reaction with 1 µl of ~12 ng/µl template DNA, and 0.2 µM of the forward and reverse primer for each locus. Amplification of PCR products was performed on a T100 Thermal Cycler (Bio-Rad) with a 5 minute 95°C enzyme activation step, followed by 30 cycles of 30 seconds at 95°C denaturation, 90 seconds annealing at 65°C and 30 seconds extension at 72°C, before a final 30 minute extension at 60°C. Capillary electrophoresis on an Abi 3130XL Genetic Analyser (Applied Biosystems, USA) separated fragments for allele scoring using GeneMarker 1.95 (Soft Genetics LLC, USA) against the McLab DMSO 100 size standard (Molecular Cloning Laboratories, USA).

Similar to H_{GW}, we standardised MHC-based heterozygosity (H_{MHC}) for each individual. Our two measures of genetic diversity, H_{MHC} and H_{GW}, were weakly correlated (r = -0.25 in females, r = -0.14 in males), as were all other input variables (age and weight correlations < 0.3).

Overall breeding success

We first investigated the factors affecting breeding success using our entire dataset. Modelling both sexes together would require multiple interaction terms to be fitted to account for age and weight differences between the sexes, which was not feasible given our sample sizes. Males and females were therefore analysed in separate models containing age, average weight and the two standardised genetic diversity metrics, H_{GW} and H_{MHC}, as fixed predictors. Ideally, we would include the random effects of enclosure, year and individual ID to account for variation in breeding success amongst the two free-range enclosures, multiple years and repeated breeding attempts of some individuals. However, some of these had very low variance, so could not be fitted due to convergence issues. Therefore, we only fitted random intercepts with adequate variation for each model, being the individual ID for males:

$$\text{Breeding Success} \sim \text{Age} + \text{Average weight} + H_{GW} + H_{MHC} + (1|\text{ID}),$$

and the enclosure year for females:

$$\text{Breeding Success} \sim \text{Age} + \text{Average weight} + H_{GW} + H_{MHC} + (1|\text{EnclosureYear}).$$

Generalised linear mixed models, with a binomial response for successful (1) or unsuccessful (0) breeders were estimated with the 'glmer' function from the 'lme4' package in R. Model averaging and model selection using an information theoretic approach following Grueber *et al.* (2011) was used to obtain the final model. Briefly, effect sizes were standardised by dividing by 2 SD following Gelman (2008). Sub-models of the global model (containing all parameters of interest) were obtained with the 'MuMIn' package (Barton, 2018), and models within the top 2 AICc of the best model were averaged using the full average method. Details of the top model sets are provided in Table A8.1.2. Estimates with a relative importance (RI) of 1 (parameter was included in all top model sets) were back-transformed for interpretation.

Hypothesis 1: advantage of heterozygous individuals

A benefit of our dataset, as opposed to wild studies, is that housing animals in known groups creates a discrete competitive mating environment, allowing us to test specific mate choice

hypotheses such as the fitness benefits of heterozygosity. As distinct from the overall breeding success models, we would expect the individuals with highest heterozygosity, relative to others of the same sex in the enclosure, to be more successful regardless of their heterozygosity ranking relative to the larger population. We tested relative effects by centring all four predictors, age, weight, H_{GW} and H_{MHC} within each enclosure and sex (calculating the difference from the group mean and dividing by 2 SD of the group values) and rerunning the male and female models as above.

Hypothesis 2: advantage of dissimilar mates

To test the hypothesis that breeders are most successful when they pair with dissimilar mates that maximise heterozygosity of their offspring (relative to a random mate selection), we calculated pairwise genetic similarity as $D_{AB} = 2 \times F_{AB}/(F_A + F_B)$, where F_A is the total alleles of female A, F_B is the total alleles of male B and F_{AB} is the total number of unique alleles shared by female A and male B (Wetton *et al.*, 1987). Similarity was calculated between every possible opposite-sex pairing for each enclosure, and separately at the genome-wide SNPs and the MHC loci for which both individuals of the pair were sequenced. For each enclosure, we then compared the average pairwise similarity of the observed successful breeding pairs to an expected average. The expected average was calculated from a structured simulation that selected the same number of pairings as were observed to breed from the set of possible pairings for that enclosure (with equal sex ratios as some males bred with multiple females and vice versa). The simulation was repeated 100,000 times to ensure adequate parameter space exploration. We interpret evidence of advantage of dissimilar mates as an observed mean below the 95% confidence interval of the expected mean similarity under random mating. As some years had small numbers of observed successful pairings, we also pooled all enclosure years to obtain an overall estimate of observed versus expected mean pairwise similarities using the structured simulation, modified to account for the additional structure due to enclosure year group.

Hypothesis 3: optimum genetic distance

Observed pairwise genetic similarities below the expected range (i.e. successful pairs were more different from one-another than expected under random mating) may indicate increased fitness of dissimilar mates. In contrast, observing a high proportion of pairwise

similarities falling within the expected range may be predicted under the optimum genetic distance hypothesis, if the optimum heterozygosity is close to the mean heterozygosity. If animals that mate with individuals at an optimal genetic distance to themselves are more likely to successfully breed than other pairings, we would expect the standard deviation of the observed pairwise dissimilarities to be lower than that of the expected standard deviation of successful pairings that would occur under random mating. We therefore compared the observed standard deviation in pairwise similarities of the observed successful breeders to the standard deviation under simulated random mating.

Results

Overall breeding success

Overall breeding success of females across our dataset had a negative relationship with age (61% probability of breeding success at age 2 vs. 47% at age 3, 34% at age 4 and 22% at age 5, fitted values from model presented in Table 6.1). Average weight was excluded from the final model for females but had a positive relationship with overall breeding success for males (RI = 1, Table 6.1). The two genetic predictors, H_{GW} and H_{MHC}, had low model selection certainty (RI < 1) as predictors of overall breeding success for both females and males.

Table 6.1: Overall breeding success results for females and males after model averaging. Breeding success (1 = success, 0 = failure) was the binomial response variable.

	Predictor	Estimate[1] (unconditional SE)	RI[2]
Females (*N* = 74)	Intercept	-0.1529 (0.3064)	
	Age	-1.0503 (0.5526)	1
	H_{GW}[3]	0.1097 (0.3427)	0.25
	H_{MHC}[3]	0.0567 (0.2699)	0.20
Males (*N* = 69)	Intercept	-0.6325 (0.3783)	
	Age	0.1886 (0.5079)	0.23
	Average weight	1.6426 (1.0120)	1
	H_{GW}[3]	1.1710 (1.0991)	0.78
	H_{MHC}[3]	-0.1011 (0.3748)	0.17

[1] Estimates have been standardised on 2 SD following Gelman (2008).

[2] RI is the relative importance of the predictor in the final model, calculated as the proportion of top models the predictor was included in.

[3] Genome-wide heterozygosity (H_{GW}) and MHC heterozygosity (H_{MHC}) were standardised across all loci for which an individual was genotyped to reduce the influence of missing data on the analysis.

Advantage of heterozygous individuals

We found no evidence that individuals with high heterozygosity, relative to others in the enclosure, had greater breeding success in competitive breeding environments based on genome-wide heterozygosity nor heterozygosity at MHC loci, for either females or males (Table 6.2). H_{MHC} was not included in any of our final models, while H_{GW} had a low relative importance (low model selection certainty). Age showed a negative relationship with breeding success for females in competitive environments as per the overall breeding success model. The youngest female relative to the average age of the other females in the enclosure had a 73% fitted probability of breeding success compared to 17% for the oldest female in the enclosure. Conversely, age had a positive relationship with breeding success for males in competitive environments (the youngest male relative to the average age of the other males in the enclosure had a 17% fitted probability of breeding success compared to 65% in the oldest male), but absolute age was not an important predictor for overall male breeding success (Table 6.1).

Table 6.2: Results of "advantage of heterozygous individuals" hypothesis tested in a competitive breeding scenario.
Breeding success (1 = success, 0 = failure) was the binomial response variable.

	Predictor[1]	Estimate[2] (unconditional SE)	RI[3]
Females (*N* = 74)	Intercept	-0.1576 (0.3088)	
	z.Age	-1.0744 (0.5194)	1
	z.Average weight	-0.1101 (0.3215)	0.25
	z.H_{GW}[4]	0.0606 (0.2615)	0.21
Males (*N* = 69)	Intercept	-0.5914 (0.3293)	
	z.Age	1.3078 (0.7160)	1
	z.H_{GW}[4]	0.5462 (0.7263)	0.54

[1] All predictors were converted to z-scores within each enclosure year and sex before input to models to reflect competition amongst individuals.
[2] Estimates have been standardised on 2 SD following Gelman (2008).
[3] RI is the relative importance of the predictor in the final model, calculated as the proportion of top models the predictor was included in.
[4] Genome-wide heterozygosity (H_{GW}) was standardised across all loci for which an individual was genotyped to reduce the influence of missing data on the analysis.

Advantage of dissimilar mates

For both genome-wide SNPs and MHC-linked microsatellites, observed mean pairwise similarities for each enclosure and year fell within the 95% CI for the expected mean under random mating (Figure 6.1A and 6.1B). We observed no patterns across years or enclosures (i.e. observed values were not consistently below or above the expected mean), providing no evidence to support the advantage of dissimilar mates hypothesis (Figure 6.1A and 6.1B).

Optimum genetic distance

The standard deviation of pairwise similarities among successful breeders was close to the expected value under random mating (within the 95% confidence interval) for both genome-wide SNPs and MHC-linked microsatellites (Figure 6.2A and 6.2B), providing no evidence to support the optimum genetic distance hypothesis.

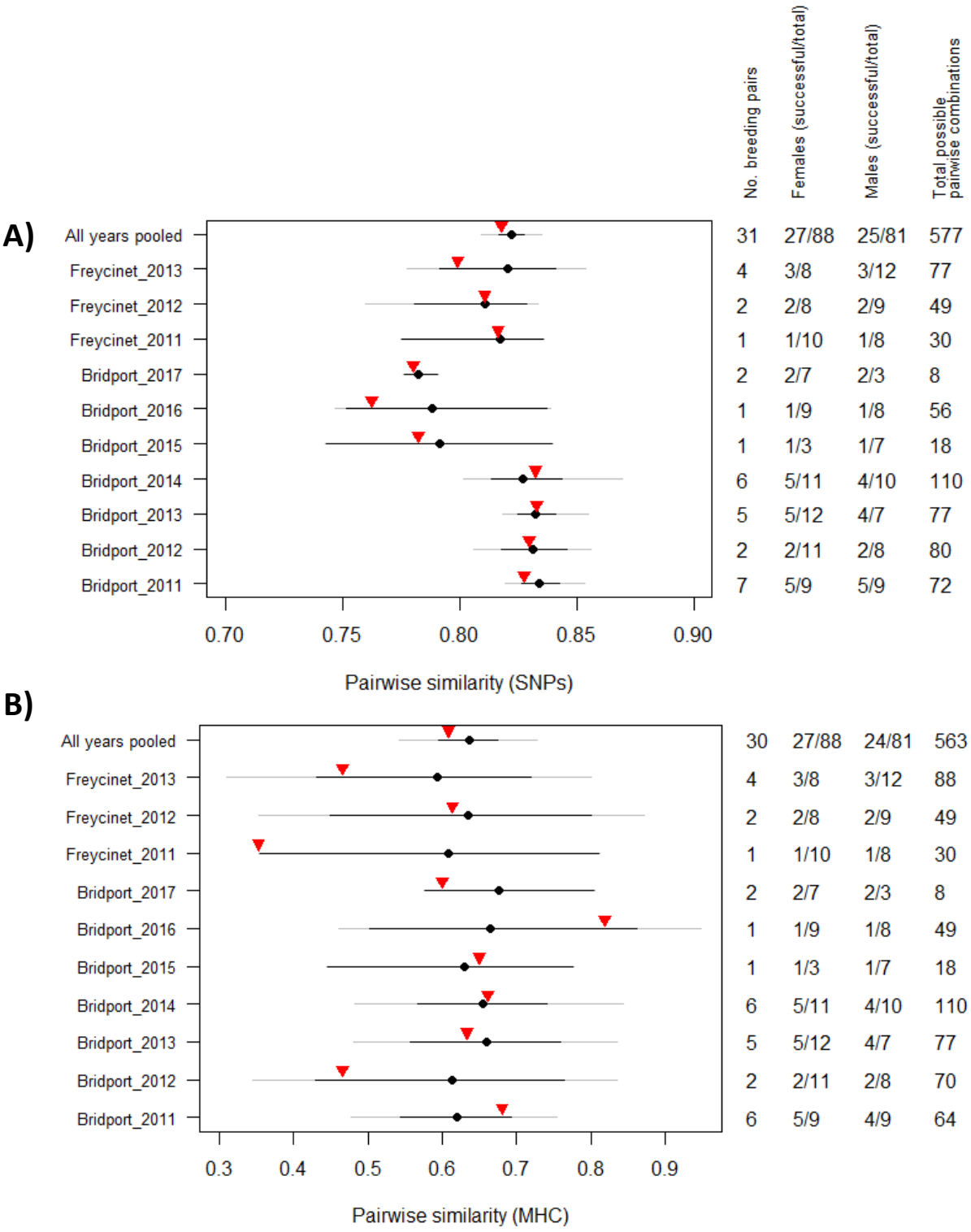

Figure 6.1: Expected vs. observed similarity for advantage of dissimilar mates hypothesis. Observed mean pairwise similarity of successful breeding pairs (red triangle) versus expected mean pairwise similarity (black circle) of 100,000 simulated pairings under the same conditions with 95% CIs, calculated from A) genome-wide SNP data and B) MHC-linked microsatellite loci. Minimum and maximum simulated values outside of 95% CI shown by grey line. Note that the x-axes differ in scale.

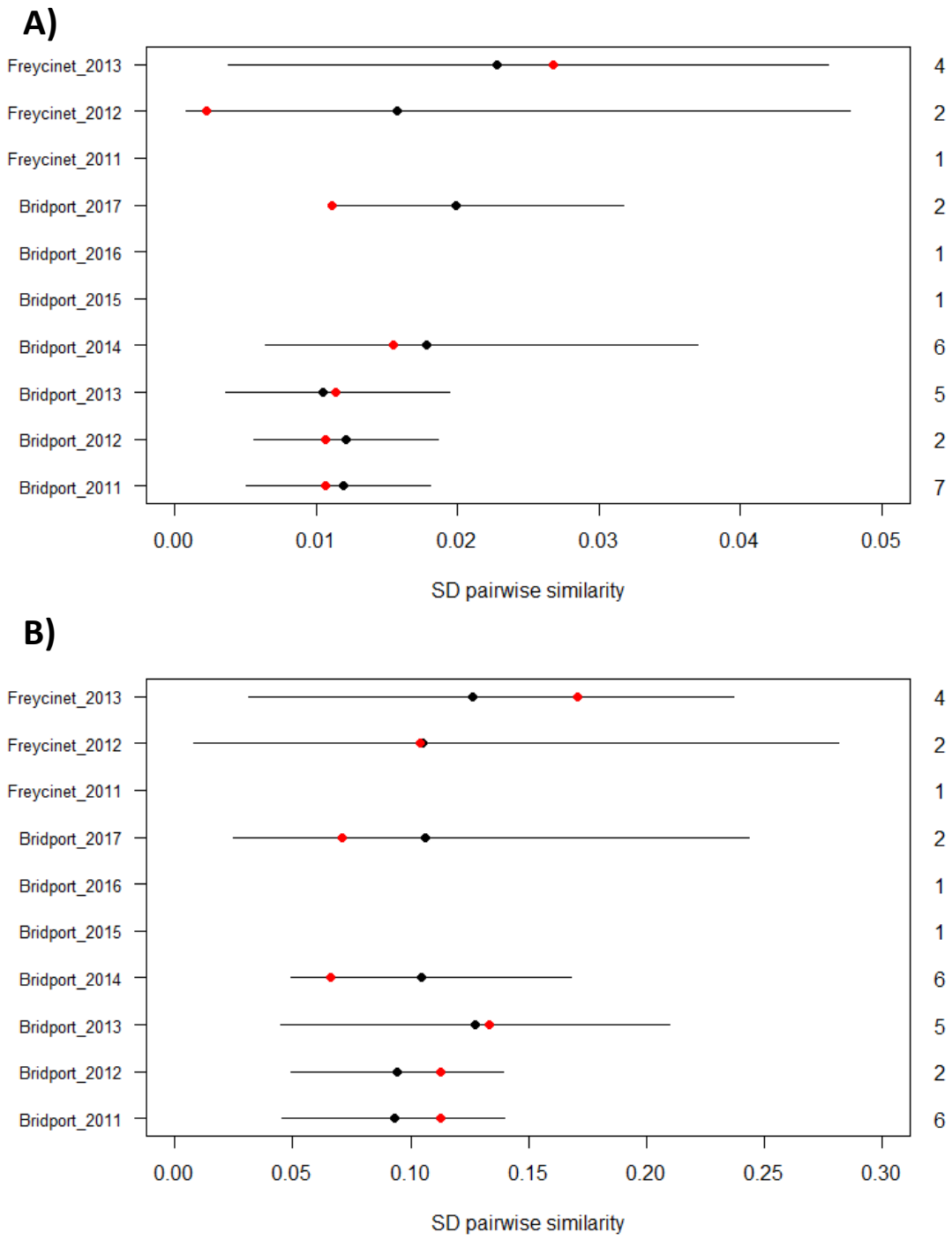

Figure 6.2: Optimum genetic distance hypothesis.
Observed standard deviation (SD) in pairwise similarity of successful breeding pairs (red circle) versus mean expected SD in pairwise similarity (black circle) of 100,000 simulated pairings under the same conditions with 95% CIs (black lines), calculated from A) genome-wide SNP data and B) MHC-linked microsatellite loci. *N* is the number of successful breeding pairs for which sequence data was available in each enclosure year. Note that enclosure years with a sample size of 1 successful pair were excluded as no standard deviation could be calculated. Numbers of total possible pairwise comparisons and numbers of successful males/females are identical to those presented in Figure 6.1.

Discussion

Mate choice is often touted as a reason to group-house individuals in captivity (Wedekind, 2002), yet is rarely tested in realistic group-housing scenarios. As a result, the impact of mate choice on conservation breeding programs that utilise group-housing is not clearly understood. Here, we used a large observational dataset of captive group-housed Tasmanian devils to test three mate choice hypotheses. We found no evidence to support any of these genetic mate choice processes using either MHC-linked microsatellite loci or genome-wide SNP loci. It is therefore likely that none of these account for the high reproductive skew observed in this population (Farquharson *et al.*, 2019; Chapter 5).

Selection coefficients for diversity- and dissimilarity-based mate choice processes are likely to be weak (Kamiya *et al.*, 2014), meaning that a large amount of data would be needed to detect any trend. The range of possible expected values exhibited under our random mating simulations was great enough to potentially observe values outside of the 95% CI in approximately 7 of the 10 enclosure years that we examined (grey bars Figure 6.1), also demonstrating that our captive study population has enough genetic diversity to generate dissimilar pairings. Nevertheless, we did not detect conclusive deviations from random mating, even when data from all years were pooled together. As the observed effects did not follow a pattern (i.e. did not all trend below or above the expected mean), we consider it unlikely that we would detect any pattern even with increased sample sizes.

The MHC is widely used in mate choice studies, yet it is likely that other genomic regions are also involved in mate choice and/or reproductive success. For example, secondary sexual characteristics may be reliable indicators of general mate quality (Møller & Alatalo, 1999), under the "good genes" hypothesis. These secondary sexual characteristics may also be associated with MHC diversity, such as in white-tailed deer where the development of antlers is associated with allelic diversity at the MHC-*DRB* gene (Ditchkoff *et al.*, 2001). Tasmanian devils do not display any known secondary sexual characteristics, and although males are slightly larger than females, the species is not clearly sexually dimorphic. We did note however that the older males and younger females tend to have a higher reproductive success (Table 6.2). While no genetic factors influenced breeding success in our population, age was important for both males and females in competitive breeding environments. Age was also important for the overall breeding success of females, but not for the overall

breeding success of males where average weight was instead strongly positively correlated with breeding success. This suggests that in enclosures with a range of ages, relatively older males may be able to dominate breeding events, though the absolute oldest males may not be the most successful overall.

We can compare our findings here to results of devil studies under other housing conditions, to determine how the influence of non-genetic factors may vary between environments, even within a species. In smaller captive enclosures (up to four males, as opposed to up to 11 males herein), Gooley *et al.* (2018) found that weight influenced male breeding success, similar to our findings for overall male breeding success (Table 6.1). However, weight was excluded from our final competitive breeding models, so may be less important in competition. An explanation for this difference may be that in smaller enclosures (as those used in the Gooley study), heavier (i.e. larger) males are able to dominate breeding by mate guarding, a known behaviour in devils (Guiler, 1970), while in the larger enclosures studied herein the increased male competition may reduce the advantage of weight. Large free-range enclosures with greater number of adults will limit the ability of dominant males to guard all reproductive females. Compared to studies of devils housed in one-on-one pairs without opportunity for mate choice, we found similar effects of female age on reproductive success (Appendix 10; Farquharson *et al.*, 2017; Russell *et al.*, 2018).

A genetic investigation by Russell *et al.* (2018) found that devil pairs with different numbers of heterozygous loci had a higher probability of breeding success than pairs with similar heterozygosities, using 6 of the MHC-linked microsatellites that were also included in our study. On the other hand, Day *et al.* (2019) did not detect mate choice using MHC-linked microsatellites in smaller group enclosures when examining overall MHC heterozygosity. Taken together, these observations indicate that mechanisms of mate choice vary across captive environment types, and specifically that group size may be an important driver of competition between mates. Whilst genetic-based mate choice may influence the reproductive success of forced monogamous pairings that do not experience competition, non-genetic factors contributing to behavioural dominance such as age and weight may be more important in mating competition and could mask any influence of MHC-associated reproductive success.

Several authors have called for empirical studies of mate choice in conservation breeding programs (Asa *et al.*, 2011; Chargé *et al.*, 2014b). By examining the largest managed captive breeding program in Australia, we had a unique opportunity to detect mate choice in a management context without experimental intervention. We did not find any evidence that devil breeding success was driven by any of the mate choice hypotheses we tested. It is possible that mate choice is occurring, either via an untested mechanism, or via the mechanism we tested but with an effect size that is too weak for us to detect in this population. In general, the effect of heterozygosity on fitness is typically weak (Chapman *et al.*, 2009; Szulkin *et al.*, 2010). However, the influence of heterozygosity at specific gene regions such as the MHC can be stronger than the genome-wide average (Hedrick, 2012). If it is true that underlying effect sizes are weak in our study system, it is difficult to conceive of management strategies that could be informed by this process to improve progress toward conservation genetic goals. It is also possible that breeding success in devils is influenced by unmeasured traits, as our study population exhibits a high reproductive skew, with almost two-thirds of individuals failing to breed given an opportunity in free-range enclosures (Farquharson *et al.*, 2019; Chapter 5). Importantly, although reproductive skew decreases effective population size overall (Frankham *et al.*, 2010), our current study shows that allowing mate choice by housing devils in groups does not appear to further exacerbate genetic diversity losses.

Although experimental studies promote the use of group-housing to provide mate choice, the potential costs in respect of genetic diversity may be high (Chargé *et al.*, 2014b). The strength and type of mate choice are not necessarily fixed within species, and can vary based on environmental (e.g. Robinson *et al.*, 2012) or social conditions (such as population density e.g. Sharp & Agrawal, 2008; Martinossi-Allibert *et al.*, 2019). This is likely true for devils, as inferences vary across contexts (e.g. Farquharson *et al.*, 2017; Gooley *et al.*, 2018; Russell *et al.*, 2018; Day *et al.*, 2019; see above). Managers are already aware of the need to collect and genotype samples for all individuals in realistic contexts to accurately assign breeding outcomes. A remaining challenge for conservation managers will be balancing the time taken to obtain sufficient sample sizes to detect any (possibly weak) effect, with the risk that mate choice may influence the genetic structure of the population during that time. For conservation management to be informed by mate choice theory, we advocate for more

studies in realistic captive management contexts, as opposed to solely experimental or wild studies, which may not apply.

Acknowledgements

We are grateful to the Save the Tasmanian Devil Program staff and keepers involved in the management and care of group-housed devils, in particular Olivia Barnard and Karen Fagg, for providing enclosure information and samples for this study. Thank you to Riley Ferguson for technical assistance with MHC PCRs. This study was funded by the Australian Research Council (LP140100508, DP170101253).

Chapter 7: First empirical evidence for selection in captivity in an endangered vertebrate

7.1 BACKGROUND

In this Chapter I analysed Tasmanian devil triads (sire-dam-offspring combinations) sequenced at SNP amplicons to investigate undetected early-viability selection as a possible mechanism of adaptive change in captive breeding programs. Unlike Chapter 6 that examined pre-copulatory mate choice, this chapter specifically excludes mate choice by using known triads with parentage confirmed by various molecular methods including those I developed in Chapter 4. Triad analysis revealed deviations from Mendelian expectations, with meiotic drive being a possible mechanism at certain amplicons. This manuscript has been prepared for submission to a broad-impact journal. Supplementary Material is provided in Appendix 9.

I jointly first-authored this manuscript with Catherine Grueber. Catherine conceived the project, genotyped samples, analysed fitness data, drafted parts of the manuscript and provided funding. I performed data filtering, analysed the expected vs. observed ratios of heterozygotes and contributed fitness data. I also prepared figures, drafted the methods and results and critically revised the manuscript. Belinda Wright genotyped samples and critically revised the manuscript. Graham Wallis contributed conceptual guidance, critically revised the manuscript and provided funding. Carolyn Hogg provided guidance on the Tasmanian devil management and the analysis, contributed to literature review, critically revised the manuscript and provided funding. Katherine Belov contributed to study design, critically revised the manuscript and provided funding. This project was supported by funding from the Australian Research Council (LP140100508 [KB, CEG, CJH], DP170101253 [CEG, KB, GPW]) and an Eric Guiler Grant.

7.2 MANUSCRIPT

First empirical evidence for selection in captivity in an endangered vertebrate

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Abstract

Adaptation to captivity remains a critical concern for the management of threatened species around the world, but little is known about which conservation breeding programs are likely to be compromised by selective processes. Current pedigree-based management assumes classical Mendelian inheritance. In this study, we directly test for deviations from Mendelian proportions in the Tasmanian devil insurance population using known sire-dam-offspring triads (i.e. excluding population-level mechanisms, such as mate choice or inbreeding). The study includes a gradient of captive facilities, from high-intensity zoo enclosures, to wilder, free-range facilities. Overall, heterozygosity of observed offspring was slightly lower than expected given parental genotypes, possibly due to negative heterosis. We found differential effects across environments, but no effect of genotype on female annual productivity. Our molecular data, SNPs obtained by amplicon resequencing, do not allow for direct mechanistic inference. Nevertheless, our findings demonstrate that deviations from Mendelian inheritance can occur in a conservation management program.

Introduction

Many evolutionary processes are important in conservation, including 'unnatural' selection such as biased harvesting (Allendorf & Hard, 2009), response to environmental contaminants (Nacci *et al.*, 1999), adaptation to captivity (Frankham, 2008), and other responses to human activities (Massaro *et al.*, 2013). Understanding the life-history stages at which selection acts helps to reveal the role that adaptation might play in conservation programs. This information can be used to help design management strategies that avoid unintended genetic change in the populations that we are aiming to restore.

In this context, it is important not to ignore *ex situ* management, especially captive breeding, as an essential tool for conservation. IUCN Red List assessors have identified over 2,000 species for which captive breeding is recommended to help prevent extinction (CPSG, 2018). Bringing animals into a controlled environment has great potential to cause unwanted genetic change through artificial selection. Empirical studies have reported potential adaptation to captivity in just a few generations (Frankham & Loebel, 1992; Latter & Mulley, 1995; Christie *et al.*, 2012), with negative consequences for release of animals to the wild (Araki *et al.*, 2007). Almost a decade ago, understanding and mitigating adaptation to captivity, and its effects on reintroduction success, was identified as a "top priority scientific research challenge in conservation genetics" (Frankham, 2010a; p1925). A snapshot of current conservation genetics research activity, taken by examining all 215 papers that cite Frankham's 2010a review (Scopus search conducted 4 December 2018; more details at Table A9.2.1), uncovered very little recent empirical research that directly addresses this challenge. Over 26% (57/215) of the citing works were review or policy documents, and none of the recent works compared the association between genetic diversity and fitness in captive versus wild environments (Table A9.2.1). Although not exhaustive, this survey represents a broad sampling of recent conservation genetic focus highlighting the fact that the urgent question of adaptation to captivity is understudied.

In conservation management, and in particular captive breeding, conservation geneticists typically use neutral models of genetic change for managing small populations because selection intensity is often unknown or thought to be low enough to be overcome by drift in very small populations. However, several studies have suggested that strong selection can occur in the small populations typified by threatened species (Hoffmann & Sgrò, 2011;

Ramírez-Valiente & Robledo-Arnuncio, 2015; Brüniche-Olsen *et al.*, 2016; Wood *et al.*, 2016; Hoffmann *et al.*, 2017). Captive breeding programs are usually managed using pedigrees, an approach that, in addition to neutrality, assumes Mendelian inheritance. The contribution of a breeder's genome to the gene pool of the next generation is estimated by summing its proportional contribution across offspring produced (Lacy, 1989). In pedigree-based management, these calculations are performed for known pairs (i.e. after pre-copulatory mate choice has occurred). Even when parents are known (i.e. extra-pair copulations can be ruled out), offspring genotypes of multiparous or iteroparous species may be biased by embryo losses as a result of cryptic female choice, litter size reduction, or early viability selection (Grueber *et al.*, 2015a). For example, if offspring homozygous for alleles that are identical by descent (i.e. as a result of inbreeding) are disproportionately lost, genomic inbreeding of individuals may differ from the mean predicted values obtained by pedigrees. Prezygotic effects (e.g. meiotic drive) or gametic selection may also be confounding factors but are rarely quantified.

The Tasmanian devil insurance population (IP) is an ideal study system for examining the possibility that artificial selection may impact a conservation breeding program. In response to the threat of devil facial tumour disease (DFTD), the devil IP was established in 2006 to prevent extinction of the species and is now the largest intensively managed conservation breeding program in Australasia. The IP houses over 700 devils across more than 30 institutions representing a wide diversity of environments (Hogg *et al.*, 2017b; see also Methods). Devils are polyoestrous seasonal breeders (Keeley *et al.*, 2012), with females releasing large numbers of eggs per ovulation (on average 30 to 60 per ovulation) with a high percentage of failed embryos (Hughes, 1982). However, female devils are biologically limited to nursing four offspring, as they have only four teats in their pouch, providing an opportunity for early viability selection between the birth and pouch young stages (Grueber *et al.*, 2015a).

Here, we use molecular data to examine offspring and their known parents (i.e. sire-dam-offspring triads), to test for deviations from Mendelian expectations in the devil IP. Large sample sizes, diverse environments, and opportunity for selection via the overproduction of offspring provide ideal conditions for examining selection in captivity. Previous studies of this population have found variation in inbreeding that is not reflected by pedigrees (Gooley *et al.*, 2017), a change in mean population productivity over time (Farquharson *et al.*, 2017;

Appendix 10), and variation in survival outcomes upon release to the wild, dependent on captive heritage (Grueber *et al.*, 2017). The possible impacts of mate choice have also been examined (Chapter 6). In this study, we specifically exclude any effects of extra-pair mating by only examining the offspring of male-female pairs that were known to mate together, to uncover the role of viability selection in a closely managed conservation program.

Materials and Methods

Study populations

Insurance population devils are housed at a range of breeding facilities that represent a gradient of management intensity (defined as the level of human intervention), and which vary across several axes that may influence adaptation. We have partitioned our dataset into three major categories, based on general characteristics of rearing habitat and opportunity for selection, as follows (see also Table 7.1):

1. *High-intensity sites* are zoo-based facilities, housing animals in pairs during the breeding season and providing very controlled environments with respect to food availability, veterinary intervention, and high degree of exposure to human activities.
2. *Medium-intensity sites* are larger enclosures (0.5–22 ha) that house groups of males and females (8–22 individuals) all year round and provide for more natural behavioural and social interactions (including mate choice). These include sites within the IP referred to as "managed environmental enclosures" and "free-range enclosures". Devils in this type of housing are provided with most of their diet by keepers but may also consume small animals within their enclosures. Veterinary intervention is less frequent, occurring during regular "catch-ups".
3. *Low-intensity site* is the population inhabiting a 115 km^2 island reserve (Maria Island) off the east coast of Tasmania. Devils were introduced to Maria Island in 2012 and 2013 (Wise *et al.*, 2016). Biannual trapping trips are conducted to monitor population growth and reconstruct the pedigree of trapped offspring (McLennan *et al.*, 2018), although not all animals are necessarily trapped each year. Although the devils on Maria are monitored and managed at the population level (by selectively harvesting animals for translocation to other sites), individual

devils largely experience conditions very similar to the wild. Animals roam, choose mates, feed and interact with their environment largely in the absence of human intervention.

Together, all study sites contribute to the larger IP. Collectively, both the structure of the IP, and the day-to-day management of individual devils within it, are based on pedigree analysis to inform maximal retention of founder diversity, minimisation of inbreeding, and sustainable reproduction (Hogg *et al.*, 2017b; Grueber *et al.*, 2018b). Annual breeding recommendations are provided to high-intensity sites to specify which pairs of devils should be housed together to breed. At medium-intensity sites, groups of devils are housed together such that breeding combinations satisfy the goals of the program (Hogg *et al.*, 2017b), employing molecular markers to check parentage and construct pedigrees (Gooley *et al.*, 2017; Gooley *et al.*, 2018; Farquharson *et al.*, 2019; Chapter 5). All triads in our dataset were verified by data independent from our main analysis, as described in Supplementary Methods A9.1 ("Parentage confirmation").

DNA was extracted using a standard phenol/chloroform protocol with ethanol precipitation (Sambrook *et al.*, 1989) from ear biopsies collected by IP institutions as part of the ongoing management of the population, from joeys at 7–12 months of age (Table 7.1). We were, therefore, unable to standardise timing of sampling across the animals included in our dataset.

Molecular genotyping

For this analysis, we sequenced devils at a selection of genomic regions using a custom re-sequencing assay developed and described by Wright *et al.* (2015). In short, regions of interest targeting both neutral and functional (immune and behavioural) loci were identified with reference to the devil genome (Murchison *et al.*, 2012). A panel of seven re-sequenced devil genomes were aligned to the genome with BWA (Li & Durbin, 2009) and SNPs called using SAMtools mpileup (Li *et al.*, 2009). Candidate regions were searched for polymorphic loci and primers designed to amplify target loci. Long-range PCRs were conducted using the Sequalprep Long PCR Kit (Invitrogen) and normalised using the Sequalprep Normalisation Kit (Invitrogen) prior to library preparation using Nextera XT Sample Preparation Kit (Illumina), and sequencing on an Illumina MiSeq. Resulting amplicons were then realigned to the devil genome to call bi-allelic variants as per the re-sequenced genomes (Wright *et al.*, 2015).

Table 7.1: Demographic and biological information about Tasmanian devils in each of our data subsets.

Intensity	Sampling age	Breeding conditions				Environmental conditions			
		Reproductive output[1]	Mating system	Opportunity for mate choice	Genetic management	Exposure to environmental stochasticity	Exposure to human activity	Health/ veterinary monitoring	Diet
High	~6 months	38.77 ± 0.09%; 2.63 ± 0.20	Forced monogamy	Largely absent	Individual level	Low	Very high	Daily/weekly	Wholly controlled
Medium	~9 months	54.98 ± 0.19%; 2.51 ± 0.50	Semi-natural	High	Group level	Intermediate	Moderate	Regular catch-up of all animals	Mainly controlled
Low	~9-12 months	59.37 ± 0.21%; 3.09 ± 0.41	Natural	Natural conditions	Population level	Natural conditions	Low	Annual survey of population	Natural conditions

[1] Proportion of females breeding; mean litter size (± standard deviation; [Hogg *et al.*, 2019a]).

A total of 474 IP devils have been sequenced using this general approach since 2014, selected on the basis of a variety of conservation management and scientific values (Morris *et al.*, 2015; Wright *et al.*, 2015; Hogg *et al.*, 2019b). Consequently, and because samples are sequenced in batches, our dataset is highly patchy. For inclusion in our analysis, we identified triads of devils (sire, dam and offspring) that had genotyping data in common. We retained only samples that had genotyping calls at ≥ 75% of the SNPs within a given amplicon. We also removed amplicons with fewer than 10 triads sequenced, or fewer than 2 SNPs.

Although our SNPs were obtained via the sequencing of PCR amplicons, we opted not to phase SNPs into haplotypes, because our dataset does not meet the assumption of a random sample of animals, intrinsic in computational phasing methods (Stephens *et al.*, 2001; Stephens & Donnelly, 2003). Similarly, pedigree-based methods of haplotype reconstruction are premised on Mendelian inheritance and neutrality and could therefore mask any deviations that we are trying to detect. Instead, we investigated each SNP locus separately, with our inference acknowledging that SNPs within an amplicon are likely to show high linkage disequilibrium. Statistical significance of findings are therefore cautiously interpreted, considering non-independence of SNPs within amplicons.

Additional data filtering procedures are provided at Supplementary Methods A9.1 ("SNP mismatch handling"). The final dataset provided data on 123 SNP loci, across five amplicons: IL17B, UNC13B, NF2, DIG12 and AGA (Table A9.2.2). The dataset included 140 offspring of 50 unique sires and 54 unique dams, for a total of 214 genotyped devils. Note that 12 female and 18 male offspring were also represented as parents within the dataset. Although female devils can have litter sizes up to four, our dataset included an average of only 1.94 offspring per unique male-female pairing (range 1 to 6; note that some pairs bred together across multiple years). Due to the non-systematic means by which the molecular data were generated, triads were variously genotyped at different amplicons (Figure A9.3.1).

Viability selection analysis

For a biallelic SNP, differential selection within a litter can operate only when at least one parent is heterozygous (Figure 7.1). We can distinguish three basic types of selection: heterozygote advantage (heterosis, overdominance), heterozygote disadvantage (negative heterosis, underdominance, negative overdominance), and directional. All of these are

potentially detectable in the case of Figure 7.1c–d. However, if there is complete dominance for an allele A_1, differential directional selection will only be detectable in Figure 7.1d. Additionally, meiotic drive (differential representation of an allele in gametes), or gametic level selection (probably unlikely in mammals), can mimic zygotic viability selection. Our dataset was limited to include only data from sire-dam genotype combinations that would be informative of any of these main types of selection.

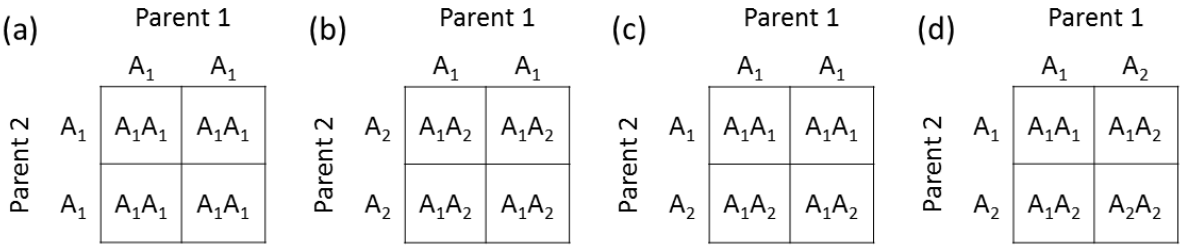

Figure 7.1: Punnett squares showing the expected offspring frequencies from the four possible parental genotype combinations at a diallelic SNP.
(a) homozygous-homozygous pairing for the same allele, (b) homozygous-homozygous pairing for two different alleles, (c) heterozygous-homozygous pairing and (d) heterozygous-heterozygous pairing. There is no genotypic variation on which selection can act differentially in (a-b). In (c-d) however, underdominance or overdominance can cause deviations from the expected 1:1 (heterozygote-homozygote) ratio. Additionally, in (d), differential directional selection acting upon the two homozygotes can cause deviations from the expected 1:2:1 ratio. For pairs of the type shown in (c), directional selection in respect of the "A_1" allele is indistinguishable from selection in respect of zygosity. For pairs of the type shown in (d), examining the relative frequencies of A_1A_1 versus A_2A_2 homozygote offspring allows the detection of directional selection, in addition to selection for or against heterozygotes.

We tested for deviations from Mendelian proportions amongst the offspring represented in our dataset. We interpret any deviations as "early viability selection", because mortality may have occurred at any point between fertilisation and first sampling (Table 7.1). Due to variation in the timing of follow-up sampling regimes amongst our sites, as well as variation in veterinary intervention (Table 7.1), we did not examine causes of mortality later in life.

To test for deviations from Mendelian segregation with respect to zygosity, we calculated the expected heterozygosity of each SNP as the proportion of expected heterozygotes amongst offspring included in triads for that locus. Because only triads capable of producing variable offspring were included (types shown in Figures 7.1c and 7.1d), and all SNPs were bi-allelic, the expected heterozygosity under all conditions is 0.5. We tested whether the observed heterozygosity deviated from expectations by calculating the exact binomial 95% confidence

intervals with the 'binom.confint' function from the 'binom' package (Dorai-Raj, 2014) in R (R Core Team, 2018), using the sample size (number of offspring) at each SNP. Observed heterozygosity values that fell outside of the 95% CIs for the expected proportion are considered statistically significant at $\alpha = 0.05$. Because the 95% CI is dependent on sample size, we also interpret the degree of deviation from the expected 0.5 ratio.

To test for deviations from Mendelian segregation with respect to particular alleles (directional selection, meiotic drive), we examined double-heterozygote parental crosses (Figure 7.1d). Only two amplicons, UNC13B and AGA had at least 10 triads of this type. The 95% CIs were calculated for these loci using an expected genotype frequency (A_1A_1 or A_2A_2, Figure 7.1d) of 0.25, and compared to the observed values, as above. We used the more-common allele (highest frequency in our dataset) as the reference for each SNP.

For the zygosity analysis, we had sufficient data to determine whether selection operates differently under different environments. We binned our data based on offspring birth location into our three environments as described above (high, medium and low intensity), retaining only SNPs genotyped for at least ten triads.

Fitness consequences

We tested whether breeding female devils experience fitness consequences as a result of their genotype. For this analysis, we extracted data on litter sizes from the studbook (Srb, 2018). Because this analysis was not reliant on common genotyping data across triads, we were able to expand the dataset to a larger set of females that were genotyped at the same SNPs as used in our main analysis (N = 119 females with breeding data).

The fitness consequences of genotype at each amplicon was assessed separately. Because we could not use conventional phasing methods, the genotypes of SNPs within amplicons were summarised into "genotype scores". A single numeric value per amplicon per individual was calculated by assigning the genotype of each SNP as homozygous reference (common allele) = 1, homozygous alternative (rare) allele = -1 and heterozygous = 0, and taking the mean across the amplicon. Thus, individuals that scored close to 1 had more common variants across the amplicon, close to -1 more rare variants, and individuals with means close to 0 were more heterozygous (or had roughly equal numbers of rare and common SNPs), within each amplicon. These genotype scores were then included as fixed predictors in linear models

to determine their effect on reproductive fitness in two ways: 1) to test for an effect of particular alleles (directional selection, meiotic drive) genotype scores were fitted directly; 2) to test for the effect of zygosity the scores were converted to a 0/1 predictor. In the latter case, values > 0.5 or < -0.5 (both more similar to homozygotes) were converted to 1, while values $-0.5 \leq x \leq 0.5$ (more heterozygous) were converted to 0. Models of the effect of genotype on female litter size were fitted using logistic regression, as described in Supplementary Methods A9.1 ("Fitness modelling").

Results

Viability selection

Across all triads in our dataset, observed heterozygosities of offspring were generally lower than expected under our null model of Mendelian inheritance (Figure 7.2). At the level of our entire dataset, we expected 50% of offspring genotypes to be heterozygous. In reality, only 2,684 of 6,413 genotypes (41.9%) were heterozygous across the dataset, although this statistic does not account for structuring of SNPs within amplicons. Considering the non-independent SNPs within amplicons, heterozygote deficit verged on statistically significant at $p = 0.05$ for UNC13B, although the magnitude of deviation was similar across UNC13B, NF2, DIG12 and AGA. A greater number of triads were genotyped at UNC13B than the other four loci listed, contributing to the narrower 95% CI for SNPs included in this amplicon (Figure 7.2a). Heterozygote frequencies were, on average, close to expectations for IL17B. Multiple SNPs within an amplicon tended to show consistent patterns, suggestive of a small number of segregating haplotypes and high linkage disequilibrium among SNPs in the study population.

When considering the three different management intensities independently, subtle and interesting patterns emerge (Figure 7.2b–d). High intensity regimes showed slight heterozygote deficits for NF2, DIG12 and AGA, and more substantive for IL17B. Medium intensity sites show slight deficits for UNC13, NF2, DIG12 and AGA, but not for IL17B. Low intensity sites (those with greatest environmental stochasticity; Table 7.1) are close to expectations for NF2 and IL17B, with slight deficits for UNC13B and AGA (see also Figures A9.3.2, A9.3.3). The high-intensity environment differs from the other two in its lack of mate choice (Table 7.1).

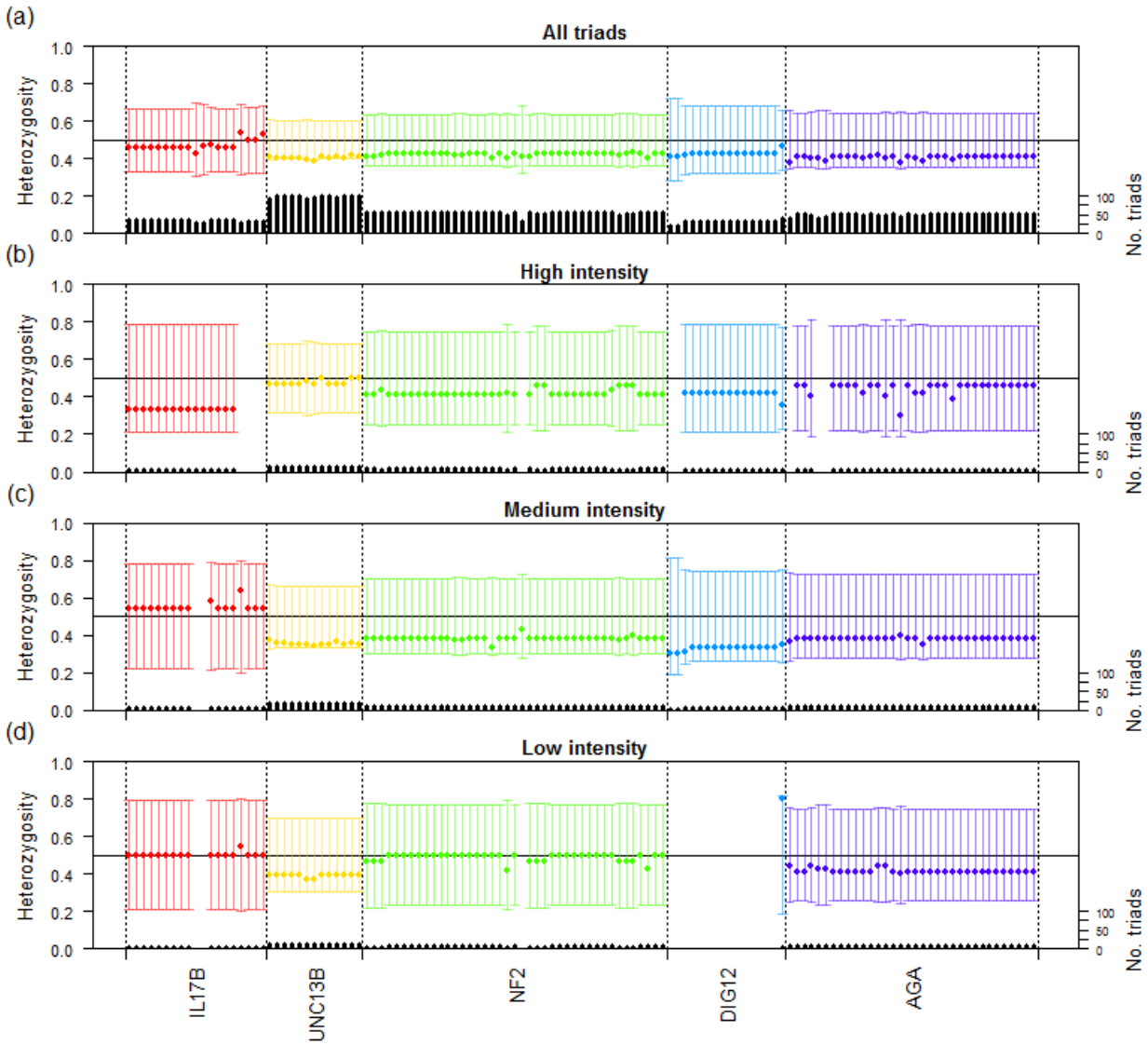

Figure 7.2: Observed vs. expected heterozygosity in offspring.
Ratios of heterozygotes for a total of 123 individual SNPs across 5 amplicons genotyped in > 10 triads with the potential to show deviations from Mendelian expectations. Coloured points indicate observed values, with error bars indicating the 95% CI. Sample size (number of offspring) is shown by small black bars, axis to the right. Results are shown for all triads included in the dataset (a), and triads from high (b), medium (c) and low (d) intensity sites, respectively.

Our dataset was sufficient for testing for evidence of selection on particular variants (directional selection, meiotic drive) within two amplicons: UNC13B and AGA. Overall, we found statistically significant evidence that one allele was over-represented for all SNPs in AGA (note that the observed frequency of homozygotes with the rarer allele was higher than the 95% CI for the expected proportion of 0.25; Figure 7.3). For AGA, the frequencies of common-allele homozygotes were close to the expected 0.25 proportion, suggesting that this is selection that favours the rare allele, rather than selection that disfavours the common

allele. Again, because all SNPs at this locus showed similar patterns (Figure 7.3), it is likely that there is a high degree of linkage amongst them, and that selection is either operating on a common haplotype as a whole, or on a neighbouring linked variant. UNC13B did not show statistically significant deviation from expected proportions (Figure 7.3).

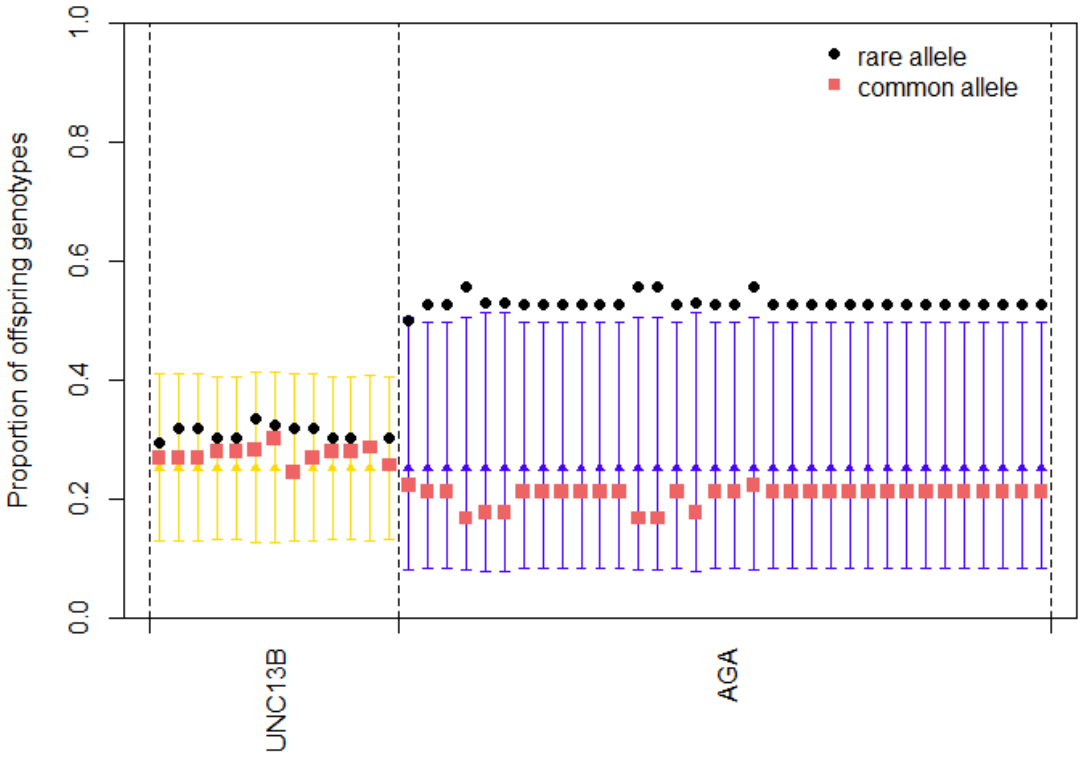

Figure 7.3: Observed vs. expected ratios of alternative homozygotes.
Ratios calculated at SNPs within amplicons genotyped in at least ten triads. Coloured points indicate the observed frequencies of each homozygote (red square = common allele homozygote; black circle = rare allele homozygote). Triangles indicate the expected proportion of 0.25 (see Figure 7.1d), with error bars indicating the binomial exact 95% confidence interval based on the sample size (number of genotyped triads at each SNP).

Fitness analysis

Considering a larger dataset of genotyped female devils, we found little evidence that litter sizes were influenced by the SNP genotypes we report here (Figure 7.4). For those amplicons where there was strong evidence that genotype data should be retained in our final model (i.e. models in which the relative importance of the genotype parameter was greater than 0.9), only IL17B and DIG12 showed effects (Tables A9.2.3, A9.2.4). There was a positive association between mean genotype score and litter size for IL17B, whereby having more common variants led to slightly higher litter sizes (Figure 7.4b). DIG12 showed the opposite trend, whereby having more common variants was associated with a decrease in mean litter size (Figure 7.4b). The latter result may have been largely driven by an effect of decreased litter size in homozygotes generally (Figure 7.4c), as the number of DIG12 rare-type homozygotes observed in our dataset was very low (Figure 7.4a), indicating weak predictive power to distinguish between decreased fitness of homozygotes generally, versus the decreased fitness of the common homozygote specifically.

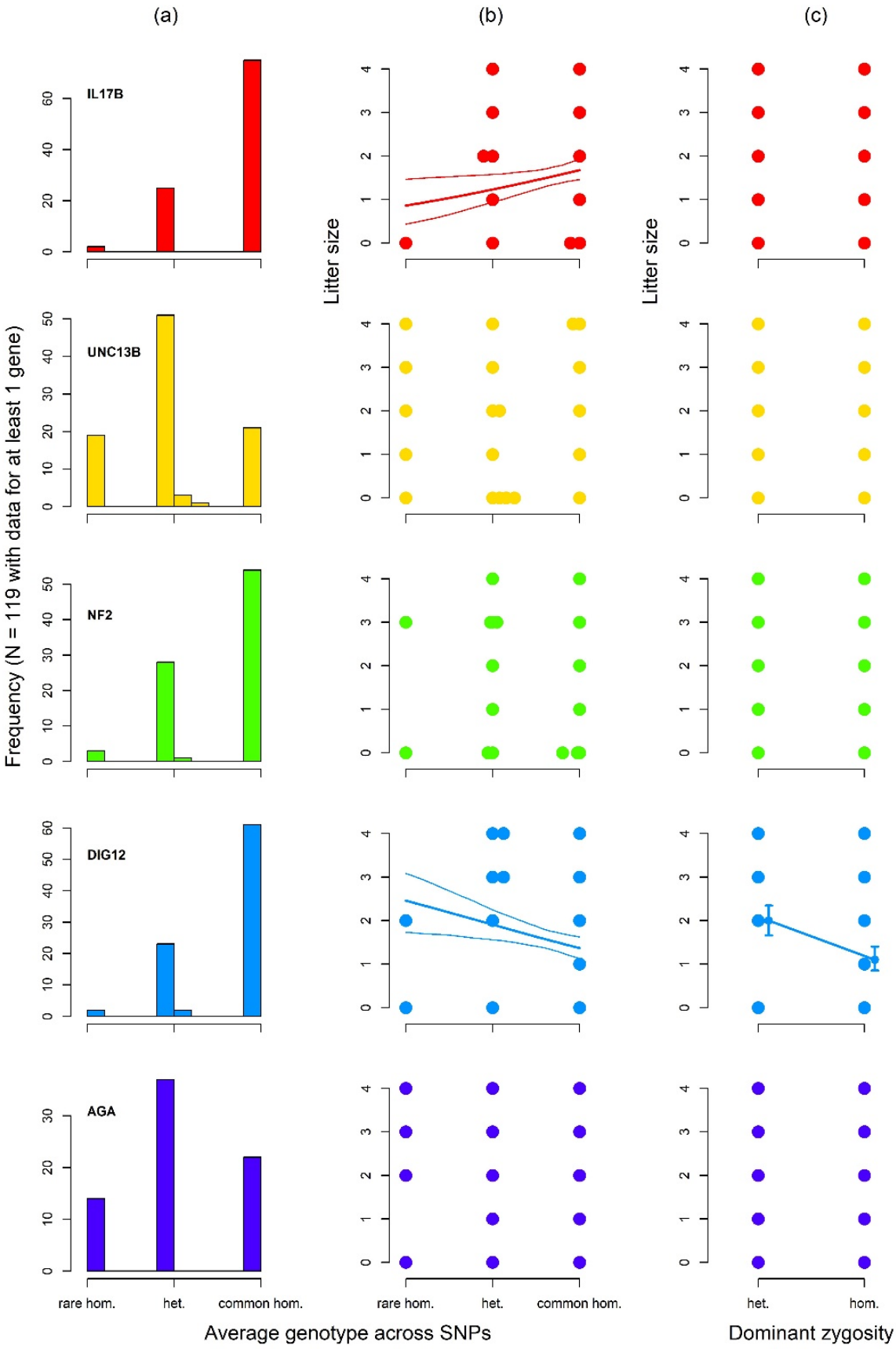

Figure 7.4: Effects of SNP genotypes on female fitness (litter size).
(Figure caption overleaf)

Figure 7.4 continued: For (a), frequency histograms for each amplicon indicate the prevalence of individuals that are more homozygous or heterozygous, on average across the gene (i.e. the "genotype scores", see Methods). Panel (b) shows the effect of genotype score on litter sizes (per Figure 7.1d); thick line is trend from linear regression, with narrow lines the 95% CI. Panel (c) shows the effect of zygosity score on litter sizes (per Figure 7.1c and 7.1d); small filled points are fitted values from linear regression, with error bars indicating 95% CI. In (b) and (c), large points are observed data; plotted semi-transparent to facilitate visualisation of multiple overlaid points. In (b) and (c), fitted trends are only shown where there was strong evidence that genotype influenced litter size (see Results; full model output at Table A9.2.3 and A9.2.4).

Discussion

The special case of superfluous oocyte and embryo production in Tasmanian devils, and varying captive management regimes of the insurance population, have allowed us to test for neonatal selection in a marsupial of great conservation significance. The results we have identified are surprising: devil offspring may be less heterozygous than expected under Mendelian segregation, perhaps due to selection against heterozygotes or meiotic drive. Importantly, this pattern varies with management regime, implicating selection as the more likely scenario. This negative heterosis could impact upon the ongoing genetic composition of the insurance population in coming generations.

Selection against heterozygotes is well-established in the context of hybrids between two species or populations, where it can result from chromosomal differentiation or Bateson-Dobzhansky-Muller effects (Arntzen *et al.*, 2009). Within populations, however, underdominance is rare, because multiallelic under-dominant states are unstable and eventually lead to fixation of one allele (Fisher, 1923; Wright, 1931). If selection is effectively or intrinsically weak, however, polymorphism could persist. Patterns resembling underdominant selection can also occur in subdivided populations where polymorphism is maintained via migration-selection equilibrium (Altrock *et al.*, 2011). Wild devil populations show population structure across the landscape (Jones *et al.*, 2004; Grueber *et al.*, 2019). If the variants we observed are subject to underdominance, and if this diversity is maintained via structure and migration, then we might predict that once populations become isolated (such as in captivity, or via fragmentation due to DFTD) that diversity at such loci may be rapidly lost.

An alternative interpretation of our findings is meiotic drive. We can differentiate between underdominance and meiotic drive by considering the outcome of double-heterozygote

crosses (i.e. crosses of type shown in Figure 7.1d). We were able to separate out crosses of this type for SNPs at two of our genetic regions (UNC13B and AGA; Figure 7.3). If meiotic drive is the cause of decreased heterozygosity, we would expect to see a deviation from expectations at one variant that is greater than the overall heterozygosity deviation, and this is indeed what is seen for AGA (compare Figure 7.2a to Figure 7.3). Although meiotic drive is a plausible explanation for at least part of our findings, we do not favour it as an overall explanation, since this kind of selection would be unlikely to vary across management regimes and therefore cannot explain the differences we see across contexts.

Our molecular data included a range of genomic regions, including loci associated with immunity (IL17B, DIG12) and cancer (NF2), as well as non-coding regions likely to show only neutral variation (AGA, UNC13B) (Table A9.2.2). As most of the SNPs genotyped in our analysis are non-coding (Table A9.2.2), it is unlikely that variation at the SNPs we have genotyped is directly associated with variation in fitness responses. However, it is plausible that our results may be driven by selection at neighbouring or otherwise linked loci. The great consistency among SNPs within loci (e.g. Figures 7.2 and 7.3) is suggestive of near complete linkage (or identity) disequilibrium within genes, and probably over much larger chromosomal regions. This observation is consistent with significant historical bottlenecking inferred from population genetic data (Miller *et al.*, 2011; Morris *et al.*, 2013; Brüniche-Olsen *et al.*, 2014), and cautions against extrapolating a mechanistic association between our results and the inferred function of specific gene regions.

The genomic regions we sequenced were widely distributed across the devil genome (Table A9.2.2), and we found that those regions that showed the strongest effects on offspring genotype (i.e. UNC13B [Figure 7.2a], and AGA [Figure 7.3]) were not the same as those that showed effects for litter size (i.e. IL17B, DIG12; Figure 7.4). This comparison suggests that processes that affect which embryos survive are not closely linked with those that affect litter sizes, an inference that is not necessarily inconsistent. One possible explanation is that, once neonates are produced, other biological processes have a much greater bearing on the number of offspring a female devil can successfully raise (i.e. regardless of their genotype). Our results showed a strong effect of maternal age, a trend that supports several other devil studies undertaken in various contexts (Gooley *et al.*, 2017; Gooley *et al.*, 2018; Grueber *et al.*, 2018a; Farquharson *et al.*, 2017 [Appendix 10]; Farquharson *et al.*, 2018a [Appendix 11]).

We observed subtly different patterns across management levels. The result is consistent with a hypothesis that selection processes in captivity differ from those in the wild. The next question, therefore, is would we predict the patterns we see to negatively affect the diversity of the devil IP in coming generations? If our results are indeed a signal of viability selection, it may not necessarily mean that population genotypes as a whole are driven by that process long term. For example, in a long-term study of Stewart Island robins (*Petroica australis rakiura*), Grueber *et al.* (2013) showed that chance events, such as which individuals successfully breed early after population establishment, can play a major role in the ultimate genetic composition of a population several generations later, even if selection is detected. A similar pattern was observed for devils introduced to Maria Island, whereby variation in breeding success among population founders was a major contributor to the genetic diversity of the population several years on (McLennan *et al.*, 2018). Because the propensity to breed may constitute a separate evolutionary process from that tested here, it is not easy to extrapolate intuitively what the long-term impact of our findings will be on devil IP diversity.

We cannot rule out the possibility that sequencing bias may have influenced our results. The amplicon resequencing dataset that we used contained a lot of missing data (partly as a result of the way the data were collected for various projects over several years, and partly a result of strict filtering to call variants; see Methods; Wright *et al.*, 2015). However, missing data is unlikely to drive our results, as it did not occur systematically across our dataset (Figure A9.3.1). Although our filtering strategy was stringent (Wright *et al.*, 2015), there were a small number of sequencing mismatches observed between known parents and offspring (these were masked from the dataset, see Methods); we cannot determine whether these are mutations, or sequencing/genotyping error. Yet, as noted above, we saw a high degree of consistency amongst the SNPs within each amplicon (e.g. Figures 7.3 and 7.4), probably due to high linkage disequilibrium in the sequenced regions. Given this redundancy, it is unlikely that a small number of sequencing errors, even if these systematically affect particular SNPs, are driving the overall patterns that we have seen. As newer genotyping technologies become commonplace (e.g. reduced-representation sequencing), and the IP progresses, it will be interesting to test whether the patterns observed here are upheld when examining different types of genomic diversity, such as genome-wide SNPs. In any case, considering the reliance of contemporary captive breeding programs on pedigree management and neutral theory,

we believe that our results are sufficient to open a conversation about the potential for deviations from neutral expectations to influence the management of genetic diversity of captive breeding programs (see also Grueber *et al.*, 2015a).

As shown in our literature survey (Table A9.2.1), empirical studies of genetic change in captive breeding programs in relation to selection are few, but a good number of review and opinion papers on this topic indicate that it is a timely issue. Our results suggest that pedigree management alone may not be enough to prevent genetic change in captive breeding programs (see also Chapter 3). Captive breeding is a valuable strategy in the conservation toolkit, and recommended for the preservation of more than 2,000 species worldwide (CPSG, 2018); some species, such as the California condor (*Gymnogyps californianus*; Snyder & Snyder, 2000) and Kihansi spray toad (*Nectophrynoides aspergini*; Harding *et al.*, 2016) would have been permanently lost were it not for their captive breeding programs. It is therefore crucial to identify protocols that will ensure the effective application of this strategy for conservation. Our study contributes to a growing belief that the prolonged maintenance of species in captivity, i.e. over multiple generations, may result in population change that has potential negative consequences during translocations or reintroductions (Christie *et al.*, 2012; Lacy *et al.*, 2013; Grueber *et al.*, 2017; Willoughby *et al.*, 2017).

We recommend that researchers working on threatened populations consider whether the approach taken here could also be used in their study species to empirically test for selection in diverse contexts, using innovative molecular methods. For conservation managers, we recommend that potential genetic change be considered during breeding-program implementation planning, as our results show that pedigree-based management alone might not be enough to prevent all kinds of selection from occurring. If conservation managers have reason to believe that selection in captivity could influence a given population, one solution would be to bring individuals in for breeding and breed over 1–2 generations before releasing them. As the current biodiversity crisis continues to unfold, understanding the drivers of selection in human-managed environments will be essential to those species whose survival depends on captive breeding programs.

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Data availability

All genotype data will be uploaded to the Dryad Digital Repository upon publication.

Animal Ethics Note

All samples were collected as part of routine population management by Zoo and Aquarium Association member zoo staff, and shared with us by the Save the Tasmanian Devil Program for analysis.

Chapter 8: General Discussion

8.1 SUMMARY OF RESULTS

In this thesis, I used a data-driven approach to collate information from multiple species, and generated high-density molecular data from the Tasmanian devil captive breeding program, to make significant discoveries about adaptation to captivity. The findings of my research inform captive population management strategies for conservation and reveal key areas of future research.

My main findings were:

a) Captive-born animals have 42% decreased odds of reproductive success in captivity compared to wild-born counterparts, with consistent effects across diverse captive environments including aquaculture, conservation and laboratory research settings (Chapter 2).
b) The traits that show greatest sensitivity to captivity are offspring traits, such as offspring survival (Chapter 2).
c) Diverse, long-running conservation breeding programs are experiencing generational changes in offspring survival, even under best-practice mean kinship management (Chapter 3).
d) Generational fitness changes in captivity cannot be predicted by taxonomic relationships and vary between the sexes (Chapter 3).
e) Inbreeding depression affects all conservation breeding programs investigated, including historically inbred species (Chapter 3).
f) I have produced a new pipeline for processing high-density SNP markers to obtain reliable genotypes for diverse applications in non-model organisms with or without a reference genome (Chapter 4).
g) There is substantial variation in reproductive success among group-housed Tasmanian devils, which revealed discrepancies between MULT pedigree management and the genetically reconstructed pedigree. The impacts of this deviation on genetic management justifies the use of molecular pedigree reconstruction in group-housed species (Chapter 5).

h) Investigating mate choice in realistic captive settings is complex, and results may differ from experimental pairings. In the Tasmanian devil, reproductive success could not be predicted by any of the genetic mate-choice hypotheses tested. Instead, non-genetic factors, such as age, were the best predictors of breeding success (Chapter 6).

i) Offspring of captive Tasmanian devils showed deviations from expected Mendelian ratios given parental genotypes. Early viability selection may vary across a gradient of captive management styles, reflecting a possible mechanism of adaptation to captivity. Unaccounted early viability selection therefore biases offspring genotypes in ways that are not accounted for by traditional pedigree-based management (Chapter 7).

These key discoveries have contributed to the knowledge of adaptation to captivity in conservation breeding programs and the applications of molecular data for conservation management going forward.

8.2 ADAPTATION TO CAPTIVITY IN CONSERVATION BREEDING PROGRAMS

Prior to undertaking the work reported in this thesis, adaptation to captivity had been identified as a management priority for conservation breeding programs (Frankham, 2010a). Pedigree-based management is targeted towards avoiding neutral change, such as genetic drift and inbreeding. Current strategies to minimise adaptive genetic change are based on pedigree management theory. Adaptive change was acknowledged as likely to occur, but empirical evidence of genetic adaptation was largely limited to model organisms and fish species. The extent of change in captivity was largely unknown: both in the diversity of species likely to be affected, and the magnitude of any change.

Identifying patterns in the diversity of species experiencing fitness changes in captivity, and quantifying such change, required large multi-species approaches. In Chapter 2, I systematically reviewed the literature to collate studies on the effect of birth-origin on reproductive success in captivity from diverse taxa, including invertebrates, fish, birds, and eutherian and marsupial mammals. The studies identified in Chapter 2 usually had small sample sizes and focused on a single species. Considered individually, these articles are valuable case studies, but could not provide general trends. By undertaking a quantitative synthesis, I estimated the magnitude of fitness changes in captivity and provided the first

review of birth-origin differences in captive breeding programs. I found that wild-born animals across diverse taxa had consistently higher reproductive success than their captive-born counterparts in captivity. Quantifying birth-origin differences across various reproductive measures revealed that offspring quality and offspring survival traits had the largest decrease in captive-born success. Rather than identifying taxa particularly prone to birth-origin effects, the data displayed weak phylogenetic signal, indicating that the effects of captivity on reproduction were consistent across taxa.

Captive breeding programs exist for diverse purposes, including food production, research and conservation, but their sustainability is threatened if fitness of captive animals is reduced. It is important to identify the stage at which fitness changes occur. For example, first generation changes may be driven by non-genetic effects such as maternal effects, husbandry and nutrition. Fitness changes that continue over multiple generations are more likely to represent heritable genetic change. Whether the fitness changes I observed in Chapter 2 were due to first-generation changes or multi-generational change could not be ascertained from the literature. Basic summary statistics such as the number of captive generations were rarely reported in the published works I assessed, meaning that captive-born animals could not be broken down by their generation to assess long-term trends in captive breeding.

To answer the question of first versus subsequent generational change, I obtained large studbooks from 15 long-running conservation breeding programs to investigate changes in offspring survival across multiple generations of captive breeding (Chapter 3). As in Chapter 2, phylogenetic signal was low, so the response of different species to captivity was not predicted by their evolutionary relationships. Across the dataset, species varied greatly in their response to captive breeding, with some species showing increases in offspring survival over generations, some no change, and others showing decreases. I disentangled first-generation from multi-generational effects by modelling a subset of the data, excluding offspring with one or both parents wild-born. Sire generation effects remained consistent when offspring of wild-born parents were included or excluded. However, dam generation effects became slightly negative for F_{2+} offspring, even for species that had positive slopes in the larger dataset. This result suggests that, for some species, dam effects are stronger in the first generation. Of the 15 studbooks I analysed, 12 were mammalian species, many of which have greater maternal than paternal care. Life-history variation such as differential parental

investment may contribute to the strength of maternal or paternal effects (Kokko & Jennions, 2012). Life-history traits may explain the variable responses of species to captivity, although more species would need to be examined to uncover general patterns. For example, life-history traits such as size, longevity, dispersal and fecundity are better predictors of nucleotide diversity than phylogenetic relationships (Romiguier *et al.*, 2014), and so similar associations may be predicted for the effects of such traits on species' genetic responses to anthropogenic selection (i.e. captivity). Given the different dam and sire effects observed, my results demonstrate that strategies to manage adaptive change should consider sex differences. Further, species management teams should investigate factors influencing offspring survival as my research has highlighted that there is no consistent pattern across species.

Strategies to minimise adaptation to captivity

In the Introduction (Chapter 1), I described three strategies recommended to minimise adaptation to captivity: population fragmentation; minimising the number of generations in captivity; and minimising selection (Frankham, 2008; Williams & Hoffman, 2009). The results of my research reveal additional considerations for conservation managers when applying these strategies. My findings also reveal conflicts with other strategies to achieve sustainable population growth and maintain genetic diversity.

Population fragmentation

Fragmenting a large population into smaller subpopulations to retain adaptive potential is commonly used in conservation breeding programs, where continental regions are managed as separate subpopulations. This strategy risks inbreeding depression if small populations remain isolated from one another. In Chapter 3, the pedigree-based inbreeding coefficient of the offspring (equivalent to the kinship of the parents) was the strongest predictor of offspring survival across the dataset. All species investigated demonstrated inbreeding depression for offspring survival, including species that suffer from historical inbreeding such as the cheetah, red wolf and Tasmanian devil (Menotti-Raymond & O'Brien, 1993; Hedrick & Fredrickson, 2008; Brüniche-Olsen *et al.*, 2014), suggesting no evidence of purging. The effect of parental inbreeding was less predictable than that of offspring inbreeding. Chapter 3 investigated the effects of parental inbreeding on offspring survival specifically; it is plausible

that parental inbreeding effects could be more substantial for other traits, as the impact of inbreeding can vary across life-history stages (Grueber *et al.*, 2010; Harrisson *et al.*, 2019). My findings add to the literature on inbreeding depression in captive breeding programs and reiterate the importance of pedigree-based management to minimise kinship and avoid inbreeding (Boakes *et al.*, 2007).

The fragmentation strategy requires movement between subpopulations to prevent inbreeding from accumulating. A pedigree-based inbreeding coefficient of approximately f = 0.2 has been suggested as a threshold for when zoos should exchange animals between subpopulations (Frankham *et al.*, 2010). However, this level of inbreeding may be far too high to avoid negative consequences. For the species that were most affected by inbreeding in Chapter 3 (black-and-white ruffed lemur, red ruffed lemur, Goeldi's monkey and European mink), the guideline of f = 0.2 would see offspring survival severely compromised. On the other hand, the slight decrease in offspring survival at f = 0.2 for other species such as the cheetah may be an acceptable balance between the needs to minimise inbreeding versus adaptation to captivity. Managers should therefore make informed decisions around the level of inbreeding accumulation in their species that would necessitate transfers. The empirical evidence for the magnitude of inbreeding depression I provided in Chapter 3 will assist managers in setting these thresholds.

Minimising the number of generations in captivity

Extending the generation length by delaying the age of breeding will minimise the number of generations experienced in captivity over time. My findings in Chapter 3 and Chapter 6 inform the utility of this strategy. In Chapter 3, older females had reduced offspring survival, compared to younger females, across the majority of species in the dataset. Similarly, in the Tasmanian devil free-range enclosures, older females had a lower chance of producing offspring: the youngest female in an enclosure had the greatest probability of success (Chapter 6). Clearly, at least for many mammalian species, the "use it or lose it" risk applies for captive-breeding females (Penfold *et al.*, 2014). On the other hand, for males, age was positively correlated with reproductive outcomes for multiple species (Chapter 3), including the devil (Chapter 6). Strategies to delay the age at breeding should therefore not be discounted entirely. For females, delaying the age at breeding presents a high risk, relative to the benefit gained from extending the generation length. Extending generation lengths via

attempting to breed older females is unlikely to be beneficial on balance. However, for males, delaying the age at breeding could both minimise adaptation to captivity and potentially improve reproduction. Further investigation, such as by simulating reproduction and retention of genetic diversity in populations over time under different strategies, is required to quantify the costs and benefits of this approach.

Minimising selection in captivity

Both intentional and unintentional selection should be avoided to prevent adaptive change in captivity. The minimising mean kinship strategy is the current best-practice method to avoid selection, reducing variation between individuals by equalising family sizes. Nevertheless, I found evidence of multi-generational fitness changes in many conservation breeding programs managed under this strategy in Chapter 3. A limitation of the mean kinship strategy is the need for accurate pedigree information (Hammerly *et al.*, 2016). The pedigree reconstruction performed for group-housed Tasmanian devils in Chapter 5 demonstrates the application of molecular genetic tools to improve pedigree management. Although pedigree methods that to attribute fractions of parentage (MULT) in group-housed species are an improvement on unknown or guessed parentage (Wang, 2004), the molecular pedigree nevertheless revealed large discrepancies. Some individuals, including wild-born animals, never bred, while others were prolific breeders. Without molecular parentage assignment, this high variability in breeding success would go undetected and limit the efficiency of mean kinship management to equalise family sizes and minimise adaptation to captivity. I therefore recommend that molecular parentage tools are applied to captive management of group-housed species, particularly for those species with polygamous mating structures and in groups containing wild-born animals. Molecular genetic approaches are often perceived as high-cost, but were found to be cost-effective for management of genetic diversity in Tasmanian devils held in free-range enclosures compared to the alternative, pedigree-only approach (Chapter 5).

Unintentional selection can be minimised by providing wild-like captive environments, such as group-housing animals to promote natural behaviours, e.g. mate choice. Conversely, group housing could accelerate adaptation to captivity if there is high reproductive skew, as family equalisation is much more difficult to achieve. In Chapter 6, I explored genetic and non-genetic factors contributing to reproductive skew (identified in Chapter 5) in group-housed

Tasmanian devils. Non-genetic factors such as age and weight had the strongest effects, with no clear evidence of mate choice for heterozygous mates, genetically dissimilar mates, or mates of an optimum dissimilarity. Reproductive success was not predicted by either genome-wide diversity, nor diversity at MHC-linked microsatellites. This may seem surprising given the large body of evidence of a genetic basis for mate choice across diverse species (Kamiya *et al.*, 2014). However, it is important to note that most mate choice studies are experimental and are performed as pairing trials. My study instead reflects a realistic captive management setting, in which multiple males and females are housed together. Given competition between potential mates, any genetic influences may be weak in comparison to non-genetic effects, at least for devils. While it is clear that there is great variation in breeding success among group-housed devils, a genetic basis for this skew could not be identified with RRS nor MHC-linked data. These results suggest that high reproductive skew could promote adaptation to captivity, but realistic settings may limit the ability to identify the mechanism of any change. Molecular pedigree reconstruction will assist managers to identify reproductive skew so that underrepresented individuals can be targeted for breeding in order to avoid this unintentional selection. Traditional pedigree-based methods are also unable to detect early viability selection or differences in genomic relatedness (as two siblings will not necessarily share all alleles as identical by descent, whereas pedigree methods only provide a point estimate of the relationship). In Chapter 7, I identified deviations from Mendelian inheritance across a gradient of captive management in devils. The results of this latter study underscore the importance of monitoring genetic change in captivity: even when pedigrees are entirely known and accurate, they fail to account for selection for or against specific genotypes in the offspring.

8.3 AREAS FOR FURTHER RESEARCH

In this thesis, I have advanced knowledge of adaptation to captivity. Importantly, my work has addressed fitness changes in conservation settings rather than in model systems. However, there is still more work needed to understand the mechanisms and consequences of adaptive change. Building on the findings of this thesis, and with knowledge of recent advances in data management and genomics, I have identified three areas of further research that are now testable in conservation contexts:

1. Are phenotypic changes heritable?

2. What genes are involved in heritable or epigenetic changes?
3. What are the consequences of fitness changes in captivity for reintroductions?

Heritability analysis

Management strategies to minimise genetic adaptation to captivity are underpinned by the assumption of heritable adaptive change, but the heritability of fitness traits is rarely examined in captive settings. Heritability analysis combines pedigree information and phenotypic observations to estimate the genetic and non-genetic components of a trait of interest. A number of applications of heritability analysis have been identified as relevant to the investigation of adaptation to captivity (Pelletier *et al.*, 2009; Courtney Jones & Byrne, 2017). For example, quantifying additive genetic variance and phenotypic variance could allow comparisons of heritability across a gradient of captive management intensity. Heritability estimation could also be applied to investigate temporal trends in breeding values, and to estimate the relative strength of genetic drift and selection on divergent captive and wild populations (Pelletier *et al.*, 2009). Although the concept of heritability analysis in conservation is not new (Storfer, 1996), it has been poorly applied due to the limitations of pedigrees. Using studbook information alone, heritability analysis could be performed for offspring survival, but few other traits could be examined without additional phenotypic information. For example, in the captive Cuvier's gazelle (*Gazella cuvieri*), studbook data was used to estimate a moderate heritability of juvenile survival and demonstrate that survival has been selected for over time (Ibáñez *et al.*, 2014). Pedigree-free heritability estimations are also possible using molecular estimates of relatedness (Frentiu *et al.*, 2008), so could be applied to semi-natural or group-housed captive populations where pedigree records may be lacking.

In the time spent undertaking my research, the Zoological Information Management System (ZIMS) was released (Species 360, 2018). ZIMS presents new opportunities for research into threatened species in captive settings by collating studbook data with detailed husbandry observations. It is now possible to apply heritability estimation methods to many important traits including health parameters, behavioural traits, and other aspects of reproductive success such as mating success that are not captured by studbook data alone. Heritability estimation is an achievable goal in a conservation context, as analysis can be performed retrospectively given accurate pedigrees and standardised recording of phenotypic traits, so

does not require experimental manipulation of the study population (Princée, 2016). The results of such research would allow managers to identify the types of traits most likely to be under selection in captivity and ensure that these traits are not being artificially selected for or against over generations.

Molecular methods to detect adaptive change

Only molecular methods can reveal potential drivers of adaptive change that are otherwise overlooked by pedigrees. Deviations from expected offspring genotype ratios in devils (Chapter 7) suggest that undetected early viability selection is a promising area of further research. Of particular importance is understanding which genes show deviations, i.e. which genes are involved in short-term adaptive change? Reduced representation sequencing approaches have proven useful for examining population structure (Chapter 4), reconstructing pedigrees (Chapter 5), and estimating genome-wide diversity metrics (Chapter 6). However, RRS approaches are unlikely to provide the marker density required for functional genomics studies as SNPs do not necessarily fall in coding regions (Wright *et al.*, in prep; Appendix 12). In Chapter 7, I used an amplicon-based SNP typing method that targeted putatively neutral and immune regions of interest (Wright *et al.*, 2015). Going forward, investigating adaptive change will benefit from the use of genomic data. A reference genome provides the basis of functional genomics studies by providing the tool to identify candidate genes of interest (as for devil; e.g. Grueber *et al.*, 2015b). Large genome consortia that aim to sequence representative species across the tree of life, such as the Earth BioGenome Project (Lewin *et al.*, 2018), are driving the development of genomic resources for wildlife species. If a species of interest does not have a reference genome, genomes of closely related species may be used (Galla *et al.*, 2018). For example, regions of genetic divergence in captive endangered Leon Springs pupfish (*Cyprinodon bovinus*) have been identified by aligning to the sheepshead pupfish (*C. variegatus*) genome and inferring functions by annotated orthologues in model species (Black *et al.*, 2017).

Gene ontology enrichment tests can also be used to examine patterns of adaptive differentiation when combined with observations (e.g. reproductive success) by identifying associated candidate genes. Physiological pathways that may be of interest to investigate in a captive breeding context include those implicated in reproduction, behaviour and stress responses. Whole genome resequencing of individuals across numerous captive generations

can then be used to identify regions of adaptive change. Another method that has been applied in captive fish is transcriptomics (Christie *et al.*, 2016). Differential gene expression identified by transcriptome analysis may indicate physiological pathways under selection. Together with experimental work in non-model organisms, these approaches can be used to disentangle epigenetic transgenerational change from heritable adaptive change (Hales *et al.*, 2017). By identifying pathways of adaptive change, management programs can be adjusted to minimise unnatural selective pressures.

Do fitness changes persist in the wild?

The ultimate goal of halting evolution in captivity (Lacy, 2009) is unlikely to be achievable in its entirety. I have shown that fitness changes in captivity do occur in diverse species (Chapter 2), and that generational fitness changes are affecting many captive breeding programs managed even under best-practice mean-kinship management (Chapter 3). Some individuals have much greater reproductive success than others (Chapter 5), and particular genotypes may be favoured by selection in captivity (Chapter 7). Captive managers already accept that neutral change will occur in small captive populations and manage populations accordingly to minimise such change but should also acknowledge that some level of adaptive change is likely to occur. However, it is important to note that genetic changes in captivity are only a concern to the extent that they decrease the long-term sustainability of captive breeding programs, reintroduction success, or the viability of wild populations. This thesis has focused on changes occurring within captive settings - an important next step is to therefore assess the impact of such change on fitness upon reintroduction to the wild. My systematic review in Chapter 2 was targeted towards birth-origin effects in captivity, but also identified a number of studies investigating birth-origin effects in the wild. A systematic review examining wild contexts would be a useful starting point to quantify fitness costs of captive breeding. For example, are reintroduced captive-born populations plastic in their ability to recover wild fitness? Adaptive changes that become fixed and are deleterious in the wild are detrimental not just to the captive-born individual, but could place wild populations at risk (Araki *et al.*, 2009). Long-term monitoring of reintroduction efforts, over multiple generations, will allow researchers to determine the long-term impacts of captive breeding. Molecular genetic approaches may also assist in reintroduction monitoring by identifying parentage and determining whether captive-born animals are successfully reproducing (Attard *et al.*, 2016).

Captive managers should continue to employ strategies such as maximising effective population size to retain adaptive potential.

8.4 CONSERVATION MANAGEMENT RECOMMENDATIONS

One of the rewarding aspects of this thesis has been the involvement of the international zoo community in providing studbook data for my research, for which I am very grateful. The provision of this data has been vital to address knowledge gaps in generational fitness changes in conservation settings. The willingness of the zoo community to address challenging issues such as adaptation to captivity reflects the genuine commitment of conservation managers to improve outcomes for threatened species and ensure the long-term sustainability of captive breeding. Some recommendations for conservation managers include:

a) Continue pedigree-based management strategies to avoid inbreeding depression, specifically by minimising the kinship of pairings.
b) Revise current population-level strategies for minimising adaptation to captivity to consider sex differences in both the responses to captive breeding over generations and reproductive biology limitations such as age effects.
c) Report basic characteristics of captive study populations, such as generations in captivity, in publications to ensure that data can be used for synthesis approaches to investigate broad patterns and inform future management.
d) Apply molecular genetic techniques to group-housed populations to detect variation in reproductive success and ensure that expected contributions of wild-born animals are realised. The pipeline I developed in Chapter 4 can be used to undertake parentage analysis in a wide variety of species, as it can be used on various types of SNP data including RADseq, ddRAD, DArTseq and GBS data and for species with or without a reference genome. By reducing the error rate and improving the reliability of SNPs, this pipeline is of great use for the field of conservation genetics.
e) Managers implementing mate choice strategies should consider fitness benefits in conjunction with possible trade-offs for genetic diversity and adaptive change if high reproductive skew occurs.

f) Population management software should account for generational changes in fitness (see below).

The pedigree-based software PMx calculates the number of breeding pairs required to meet a target population size given historical average breeding success and mortality rate (Lacy *et al.*, 2012). Chapter 3 showed that these reproductive metrics are not necessarily constant across the length of a captive breeding program. PMx will overestimate the number of breeding pairs required if, for example, offspring survival increases over generations. Overestimates may lead to excess production of offspring in zoos that are limited by finite capacity and resources (Asa, 2016). Alternatively, if offspring survival decreases over generations, PMx will underestimate the number of pairs required. Underestimates threaten the long-term sustainability of the captive population. Methods to adjust for biological parameters such as age have been developed for reproductive viability analysis (RVA) (Bauman *et al.*, 2019), which could be expanded to include generation. Given the fitness changes I observed over generations, independent of year or inbreeding, I recommend that an algorithm to adjust probabilities of breeding success and offspring survival is implemented into PMx. Accounting for the captive generation of each parent by simple linear regression, or a non-linear model that separates changes in the first generation of captive breeding from F_{2+} generations, will give more precise predicted probabilities. Given that my results revealed differences between sire and dam effects, separate models should be implemented for each sex. Accounting for generational fitness changes will assist captive managers in meeting target demographic goals but is of course a short-term solution that does not address underlying causes of such fitness changes.

My findings from the Tasmanian devil free-range enclosures have already been shared with the Save the Tasmanian Devil Program (STDP), who have used the results to improve breeding success and better manage genetic diversity. For example, the reconstructed pedigree in Chapter 5 was used to identify under-represented individuals to prioritise them for future breeding events. Some of the devils born in free-range enclosures were subsequently released to Maria Island, in an assisted colonisation of an island where devils had never previously existed. Identifying relationships down to half-siblings has informed management of the genetic diversity of the Maria Island by establishing the relationships between these ‘founders’ of the Maria population. Since undertaking the analysis in Chapter 5, I have

continued to reconstruct the pedigree for the 2018 breeding season. These results were used by the STDP to select devils to release to wild mainland Tasmanian sites in order to provide a demographic and genetic boost to small populations. My findings in Chapter 6 of non-genetic factors affecting breeding success have also helped the STDP to improve predictions of which individuals are likely to breed in free-range enclosures.

8.5 CONCLUSION

The work presented in this thesis has expanded our understanding of adaptation to captivity in threatened, non-model species. Although the concept of adaptation to captivity is not new, I believe it is an emerging area of research for conservation as the molecular tools needed to identify adaptive change continue to advance. In this thesis, I have shown how various data types, including large multi-species datasets and high-density molecular markers, can be used to investigate adaptation to captivity in populations managed for conservation purposes. I have discovered that, while the effects of inbreeding, sex and age are remarkably consistent across vertebrate species, taxonomy cannot necessarily predict the pattern of generational change in conservation breeding programs. I have revealed two important mechanisms of adaptive change: between-individual variation in reproductive success, and undetected early viability selection.

The development of a pipeline to improve the reliability of SNPs will advance the use of molecular genetic applications in threatened species. This pipeline is already being applied to captive and wild Tasmanian devil populations as well as other threatened and non-threatened species by not only members of our lab group, but other research groups and government geneticists. The current extinction crisis has driven the dependence of increasing numbers and diversity of species on *ex situ* management, so the importance of considering adaptive change in captivity cannot be understated.

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Appendix 1: Supplementary Material to Chapter 2

This appendix relates to Chapter 2: A meta-analysis of birth origin effects on reproduction in diverse captive environments.

A1.1 SUPPLEMENTARY NOTE 1: PUBLICATION BIAS

In testing for possible sources of publication bias in our dataset, we observed that the relationship between year of publication and effect size was not statistically significant (posterior mode slope estimate = -0.015, 95% HPD CI: [-0.05, 0.03], Figure A1.3.1a). This suggests no evidence of time-lag bias, such as may occur if studies with larger, significant effect sizes are published and dominate the literature before insignificant results, which may take longer to publish, appear (Higgins & Green, 2011). Egger's regression testing for the symmetry of the funnel plot of the meta-analytic residuals from the overall model against their precision indicated statistically significant asymmetry (t_{115} = -0.825, P = 0.0077, Figure A1.3.1b). While funnel plot asymmetry is used to identify publication bias, it can also be the result of true heterogeneity or chance (Higgins & Green, 2011). Our dataset was characterised by high total heterogeneity (I^2_{total} = 94%), and high heterogeneity between studies (I^2_{study} = 70%), so it is plausible that the funnel plot asymmetry is not reflective of publication bias. Any difference in reproductive success between wild-born and captive-born animals is likely to be of interest to captive managers. Trim-and-fill analysis estimated two effect sizes missing from the right-hand side of the right side of the distribution, however this was not statistically significant (P = 0.125), and the estimated adjustment was small (lnOR = 0.038) and does not qualitatively influence our results.

We observed an outlier in our dataset (Murugan *et al.*, 2013), with an effect size of lnOR = -9.8 (Figure A1.3.1c). As such, we re-ran all of the above models excluding this data point. All model results were qualitatively similar, and the Egger's regression still identified funnel plot asymmetry (t_{114} = -1.06, P = 0.002). However, the trim-and-fill analysis estimated no missing effect sizes. Overall therefore, it is unlikely that publication bias is driving our main results.

A1.2 SUPPLEMENTARY NOTE 2: MULTIPLE IMPUTATION

We observed a strong linear correlation between the absolute mean of an estimate and its standard deviation on the natural log scale, as expected under Taylor's Law (Taylor, 1961) (Figure A1.3.4). To recover missing standard deviations for 17 comparisons, we performed 20 imputations of missing log standard deviations using the 'mice' package in R (van Buuren & Groothuis-Oudshoorn, 2011) and exponentiated the resulting values to calculate effect sizes. These additional 17 comparisons were added to the 115 comparisons in the main dataset, resulting in a total of 132 comparisons, and all meta-analyses re-run. We pooled the posterior estimates from each of the 20 imputations to obtain the posterior mode, and 95% HPD CIs were calculated on the pooled data using the 'hdi' function in the 'HDInterval' package (Meredith & Kruschke, 2016). An additional four species and four papers that were not included in the main analysis were included by imputation, resulting in 48 species and 43 papers in total. The four additional species (cynomolgus macaque *Macaca fascicularis*, cheetah *Acinonyx jubatus*, American lobster *Homarus americanus*, and oval squid *Sepioteuthis lessoniana*) included using multiple imputation were distributed across the taxonomic tree represented by the main analysis. The imputed comparisons covered aquaculture, conservation and research environments, and four of the five reproductive trait categories (all except offspring survival) (Table A1.4.4).

All estimated effects were of similar magnitude to the main analysis for all models (Table A1.4.4). Statistical significance of the overall result, the effects from the model fitted with the 'captive environment' moderator, and the offspring quality and offspring survival traits remained the same as our main analysis. The estimated effects were in the same direction as the original analysis, with the exception of reproductive phenology, which became positive but remained close to zero and not statistically significant (lnOR = 0.14 [imputed] vs. -0.04 [main analysis]). However, the effects based on imputation were estimated with poorer precision than in the main analysis, as evident from the widened 95% HPD CIs for each result (Table A1.4.4).

We also considered whether it was possible to impute missing sample sizes, although the relationship between means and samples sizes was less clear than the relationship between mean and standard deviation (Figure A1.3.4). Imputing sample size provided a further 24 comparisons to the 132 noted above (total $N = 156$). The posterior mode estimates were again

similar and in the same direction as the original analysis, and uncertainty in the estimates did not improve with the inclusion of these additional values (data not shown). Nevertheless, because the estimated effects were similar across our datasets, we do not believe that our overall conclusions are biased by missing data.

A1.3 SUPPLEMENTARY FIGURES

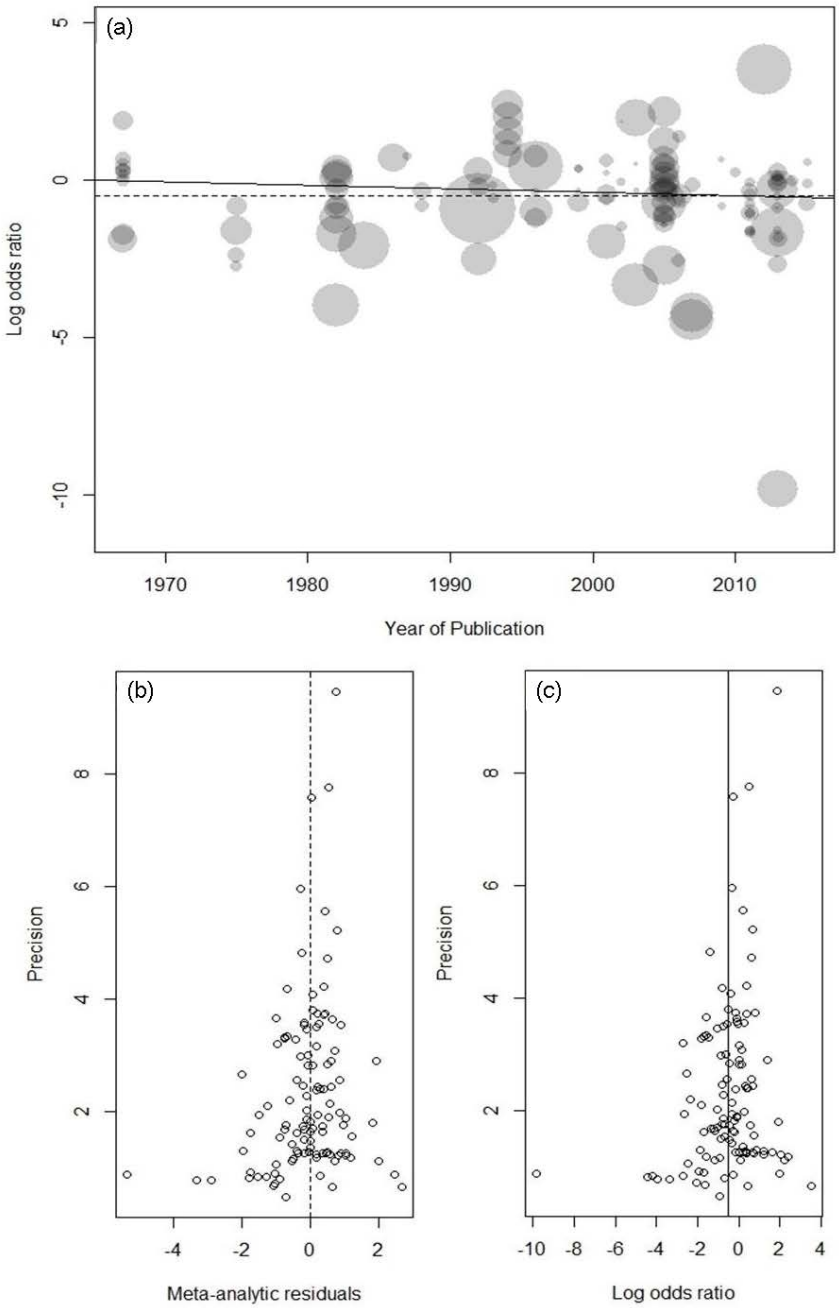

Figure A1.3.1: Tests for publication bias.
(a) Relationship between effect size (log odds ratio) and year of publication (indicated by solid line), to examine evidence of time-lag bias. Dashed line shows meta-analytic mean from the overall non-phylogenetic model. Point size is proportional to the variance of the effect size. (b) Funnel plot of meta-analytic residuals extracted from the overall non-phylogenetic model plotted against their precision $(1/\text{variance})^{1/2}$, with the dashed line at 0. (c) Funnel plot of effect sizes (log odds ratios) plotted against their precision, with solid line showing meta-analytic mean.

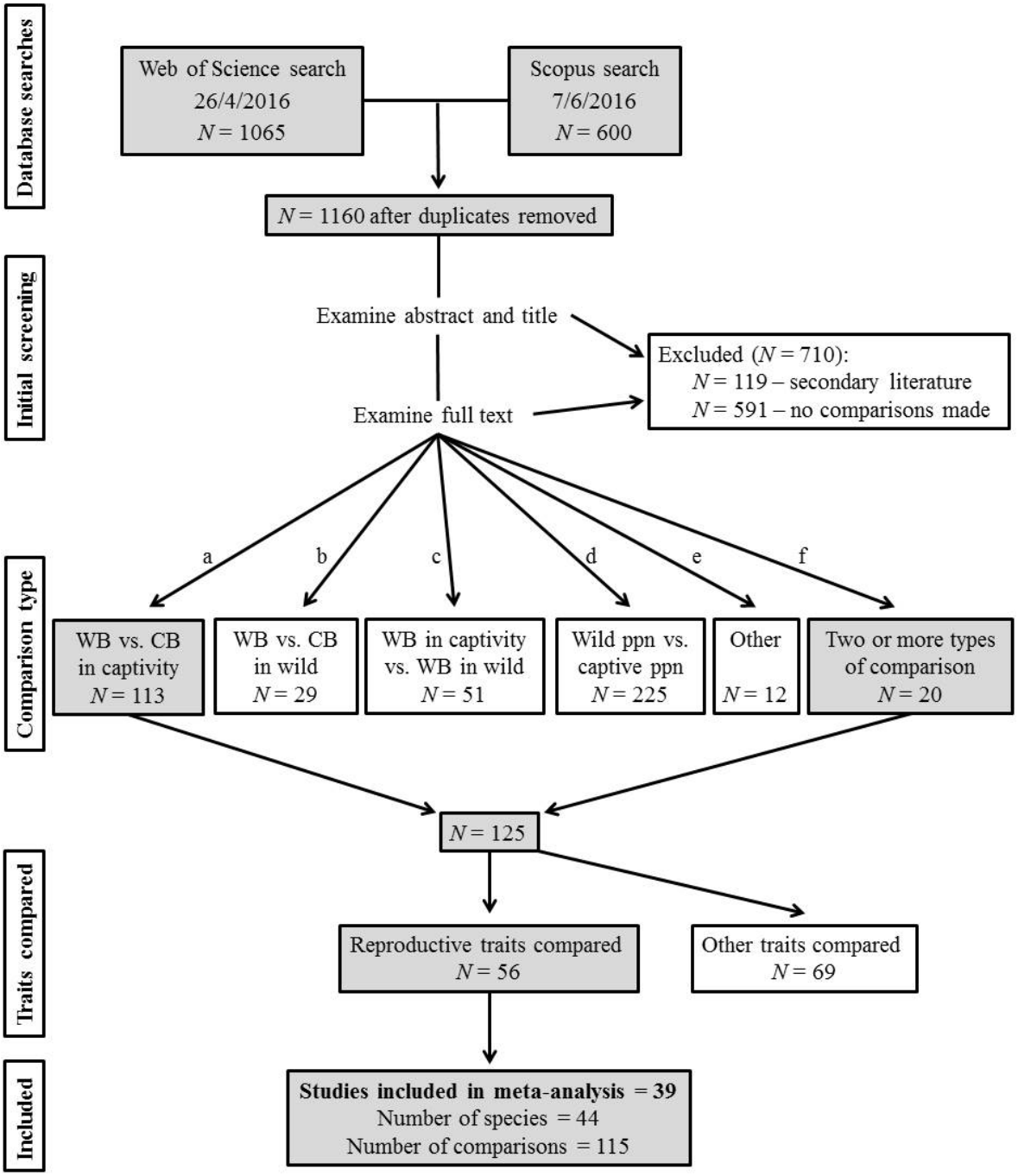

Figure A1.3.2: PRISMA flowchart of the overall literature filtering strategy.
N refers to the number of papers included at each stage of filtering. Shaded boxes represent the papers under consideration for inclusion in the systematic review and meta-analysis at each stage of filtering. a – f are the categories of comparison type, referred to in Chapter 2 Methods. (WB = wild-born, CB = captive-born, ppn = population). PRISMA guidelines from Moher *et al.* (2009).

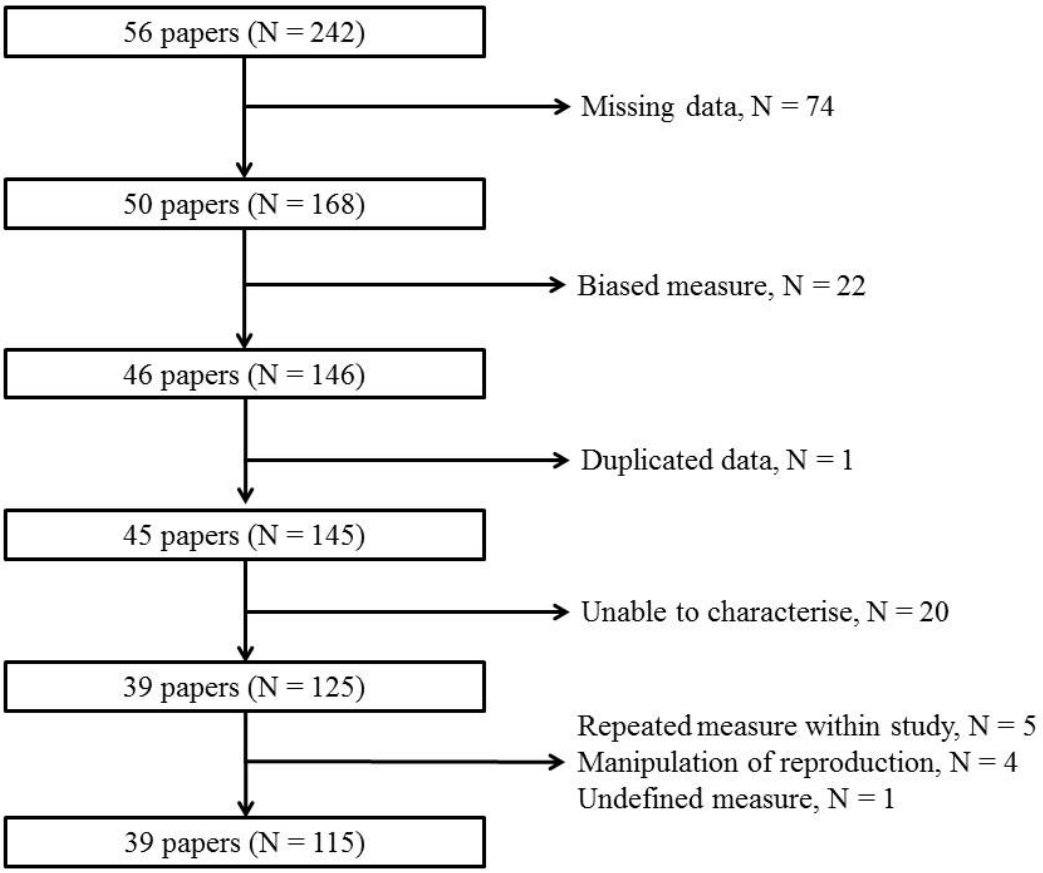

Figure A1.3.3: Filtering strategy with reasons for excluding comparisons and therefore papers from the analysis.
N = number of comparisons.

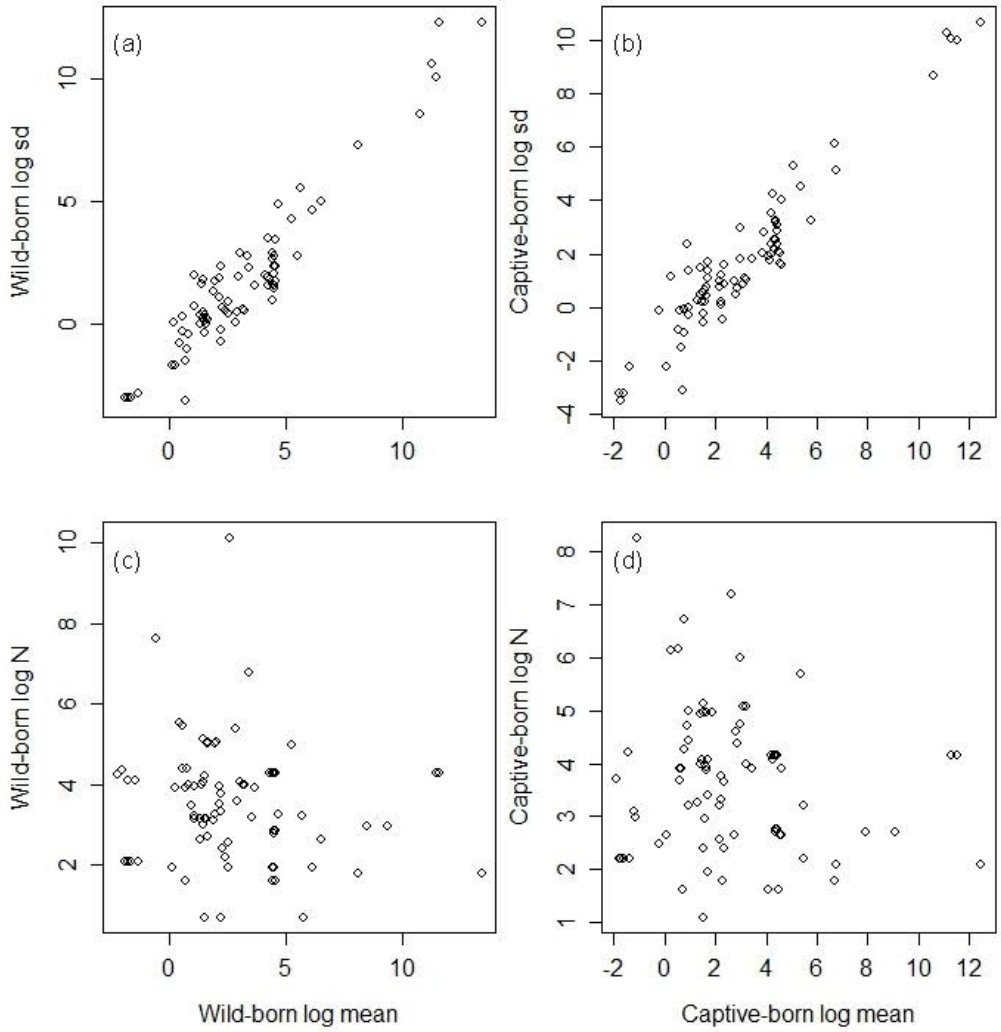

Figure A1.3.4: Mean, standard deviation, and sample size correlations.
Relationships between the mean and standard deviation (*sd*) or sample size (*N*) of wild-born and captive-born continuous comparisons (*N* = 67) on the natural log scale. Relationships between the log standard deviation and log mean of (a) wild-born and (b) captive-born continuous comparisons. Relationships between log sample size and log mean of (c) wild-born and (d) captive-born continuous comparisons.

A1.4 SUPPLEMENTARY TABLES

Table A1.4.1: Heterogeneity statistics.
Extended heterogeneity (I^2) statistics for the overall non-phylogenetic model and the overall model + phylogeny.

	Heterogeneity (%)			
	Total	Phylogeny	Study ID	Residual variance
Overall model	93.736	-	70.185	19.332
Overall model + phylogeny	94.339	0.290	67.307	24.235

Table A1.4.2: Reproductive trait type categories.
Assignment of captive-born to wild-born comparisons (as defined by original authors of publications) to reproductive trait type categories used in the meta-analyses, and direction of each effect on overall reproductive success. Positive effect (+) results in increased overall reproductive success, negative effect (-) results in decreased reproductive success. *N* is number of comparisons within each trait type category (total *N* = 115).

Trait type	Comparisons	Direction of effect	N
Fertility/ hatchability	Fertility (of the egg, clutch, or spawn; across years; live born offspring/female/reproductive year)	+	8
	Proportion of successful hatching (out of total incubated eggs, fertile eggs, clutch, or spawning; across years)	+	6
	Reproductive success (i.e. binary statistic indicating producing at least one offspring for males, females, or pairs, or of these within a given time frame from pairing e.g. 6 months)	+	13
	Proportion of population with reproductive abnormalities (e.g. pathological lesions of reproductive tract)	-	1
	% normal sperm (visually) or % reactive sperm (undergo changes when in contact with egg)	+	2
Reproductive yield	Number of litters per pair	+	1
	Number of offspring (e.g. per female; or per individual in given time frame)	+	12
	Clutch/litter size (of $1^{st}/2^{nd}/3^{rd}/4^{th}$ litter; or litter size at weaning)	+	12
	Number of offspring surviving to a given time point (e.g. 5 years) per female per year	+	2
	Number of offspring produced per gram of body weight of female	+	1
Offspring quality	Proportion of offspring birth abnormalities (e.g. chondrodystrophy)	-	1
	Egg morphometric traits (e.g. mass, volume)	+	2
	Offspring size (hatch weight or length; body weight at weaning)	+	5
Offspring survival	Mortality rate (of embryos, neonates, or infants; also described as prenatal, perinatal or postnatal mortality)	-	8
	Juvenile mortality rate at a given time point (e.g. 1 week, 2 weeks, 6 months)	-	11
	Juvenile survival (to a given time point or developmental stage)	+	5
	Incidence of cannibalism/abandonment of young by parent	-	3
	Proportion of young successfully reared (live offspring out of total)	+	3
	Stillbirth/abortion rate	-	3
Reproductive phenology	Breeding interval (between pairing and first litter; interbirth or spawning interval)	-	7
	Rate of production of offspring (spawning rate)	+	3
	Mating rate (e.g. matings per female per month)	+	2
	Age at first parturition	-	4

Table A1.4.3: Generation (F) of the captive-born population compared to the wild-born population.
Generation as specified within the study grouped for each study environment category. Data are the number of comparisons/effect sizes within each group.

	Aquaculture	Conservation	Research	Other	Total
No generation specified	7	47	25	1	80
F1	12	0	5	0	17
F1-F2	1	0	8	0	9
F1-F3	3	2	1	0	6
F1-F4	0	2	1	0	3
Total	23	51	40	1	115

Table A1.4.4: Meta-analytic effect size estimates of differences in reproductive success between wild-born and captive-born animals in captive environments with imputed data.
Effect size estimates for the dataset include the original comparisons (*N* = 115) and the additional imputed comparisons (*N* = 17). Posterior mode gives the meta-analytic log odds ratio (lnOR) estimate from the MCMCglmm models, with lower and upper 95% higher posterior density credible intervals given. Estimates with the 95% HPD CI excluding zero are marked with *.

	Posterior mode (lnOR)	Lower 95% HPD CI	Upper 95% HPD CI	N
Overall model*	-0.67	-1.83	-0.04	132
Overall model + phylogeny	-0.96	-2.85	0.42	132
Captive environment				
Aquaculture*	-1.70	-3.78	-0.18	25
Conservation	-0.11	-1.38	1.24	59
Research	-0.78	-2.97	0.17	47
Other	2.00	-3.50	7.04	1
Trait type				
Fertility & hatchability	-0.92	-2.23	0.03	31
Reproductive yield	-0.84	-1.95	0.13	38
Offspring quality*	-1.59	-3.27	-0.32	8
Offspring survival*	-1.21	-2.49	-0.29	33
Reproductive phenology	0.14	-1.10	1.04	22

Table A1.4.5: Excluded studies.
Publications comparing reproductive traits in wild-born and captive-born animals in captive environments excluded from main analysis and reasons for their exclusion. See Chapter 2 Methods for details of inclusion/exclusion criteria. Note that comparing captive-born and wild-born animals may not have been the primary aim of some studies with missing data.

Publication	Species	Reason(s) for exclusion
Clubb *et al.*, 2008	African elephant, *Loxodonta africana*, Asian elephant, Elephas maximus	Missing data (analysis of juvenile mortality not conducive to calculation of effect sizes)
Curry *et al.*, 2015	Polar bear, Ursus maritimus	Comparison biased by opportunity to breed (e.g. total lifetime number of litters produced) Direction of effect on productivity can't be characterized for day of parturition or offspring sex ratio Missing data (no raw data or only P-values reported for litter size, incidence of stillbirths, neonatal mortality, juvenile survival and inter-birth interval)
Gupta, 1994	Round Island gecko, *Phelsuma guentheri*	Direction of effect cannot be characterized for age-specific fecundity
Ikeda *et al.*, 2009	Oval squid, Sepioteuthis lessoniana	Missing data (no error or sample size reported for age at first spawning or number of egg cases/female)
Keeley *et al.*, 2012	Tasmanian devil, Sarcophilus harrisii	Data are encompassed in a more recent and larger sample size study [Hogg et al. (2015)]
Kirkland & Linzey, 1973	Deer mouse, Peromyscus maniculatus	Missing data (no error or sample size reported for litter size)
Levallois & de Marigny, 2015	Cynomolgus macaque, *Macaca fascicularis*	Missing data (no error reported for inter-birth interval, no sample size reported for neonatal mortality, stillbirth incidence or proportional birth rate)
Mace, 1988	Western lowland gorilla, *Gorilla gorilla*	Comparison biased by opportunity to breed (e.g. total offspring produced per male/female)

Publication	Species	Reason(s) for exclusion
Mar, 2013	Asian elephant, *Elephas maximus*	Direction of effect cannot be characterized for age-specific fecundity or offspring sex ratio Missing data (analysis of interbirth interval not conducive to effect size calculation)
Marker-Kraus, 1997	Cheetah, Acinonyx jubatus	Missing data (no error or sample size for age at first or last parturition for males and females) Comparison biased by opportunity to breed (e.g. total number of litters and total number of offspring produced in a lifetime)
Meng *et al.*, 2003	Alpine musk deer, *Moschus sifanicus*	Direction of effect cannot be characterized for mating date
Mooney & Lee, 1999	Woolly monkey, Lagothrix lagotricha	Missing data (only *P*-value reported for infant mortality, analysis of age at first birth and interbirth interval not conducive to effect size calculation) Biased measures of reproductive success (e.g. number of population having more than one reproductive event is biased by opportunity to breed)
Rasweiler & Badwaik, 1997	Short-tailed fruit bat, *Carollia perspicillata*	Direction of effect cannot be characterized for gestation length
Stuermer *et al.*, 2003	Mongolian gerbil, Meriones unguiculatus	Missing data (no error or sample size reported for litter size)
Talbot *et al.*, 1984	American lobster, *Homarus americanus*	Comparison biased by opportunity to breed (e.g. total egg production and total number of eggs attached) Missing data (no error reported for number of eggs extruded/female or number of eggs attached/female)
Vermeer & Devreese, 2015	Western lowland gorilla, *Gorilla gorilla*	Missing data (only *P*-value reported for infant mortality) Direction of effect cannot be characterized for offspring sex ratio
Yu, 2004	Golden monkey, Rhinopithecus roxellanae	Missing data (no sample size reported for reproductive rate)

Appendix 2: Supplementary Dataset to Chapter 2

This appendix relates the Chapter 2: A meta-analysis of birth origin effects on reproduction in diverse captive environments.

An Excel file containing the data extracted and used for the meta-analysis is available at:

https://static-content.springer.com/esm/art%3A10.1038%2Fs41467-018-03500-9/MediaObjects/41467_2018_3500_MOESM4_ESM.xlsx

The first sheet contains the main dataset: data extracted from the 39 studies (115 comparisons), comprising 33 columns and 115 rows. The second sheet contains data extracted from the additional 41 comparisons with missing data that could be recovered by multiple imputation, comprising 33 columns and 41 rows. Metadata describing the column headings is provided on the following page. Not all columns were collected for each row.

Column name	Description
ES.ID	Unique identifier for each effect size
ID	Unique identifier for each publication
First.author	First author of publication
Year	Year of publication
Journal	Journal of publication
Species	Common name of species
Scientific.name	Scientific name of species
Major.taxon	Major taxon grouping of species
Captive.environment	Captive environment of study (aquaculture, research, conservation or other)
Generation	Generation (F) of captive-breeding of the captive-born population. (No = not specified)
Reproductive. measurement	Reproductive trait measured between captive-born and wild-born animals in captivity
Direction	Expected direction of the effect of the reproductive trait measured on overall productivity (+ = increase in trait increases productivity)
Trait	Type of reproductive trait measured (1 = fertility/hatchability, 2 = reproductive yield, 3 = offspring quality, 4 = offspring survival, 5 = reproductive phenology)
WB.Percentage	Percentage of wild-born animals experiencing reproductive trait (where reported)
WB.n1	Number of wild-born animals out of total (calculated from percentage) with reported trait
WB.n2	Number of wild-born animals out of total (reported, or rounded from WB.n1)
WB.success	Number of wild-born animals experiencing reproductive trait in positive direction of productivity
WB.failure	Number of wild-born animals experiencing reproductive trait in negative direction of effect on productivity
WB.N	Total number of wild-born animals
CB.Percentage	Percentage of captive-born animals experiencing reproductive trait (where reported)
CB.n1	Number of captive-born animals out of total (calculated from percentage) with reported trait
CB.n2	Number of captive-born animals out of total (reported, or rounded from CB.n1)
CB.success	Number of captive-born animals experiencing reproductive trait in positive direction of productivity
CB.failure	Number of captive-born animals experiencing reproductive trait in negative direction of effect on productivity
CB.N	Total number of captive-born animals
WB.Mean	Mean of wild-born animals experiencing reproductive trait
WB.C.Mean	WB.Mean corrected for direction of effect on productivity (multiplied by -1 if trait has negative effect on productivity)
WB.SD	Standard deviation (wild-born)
WB.SEM	Standard error of the mean (wild-born)
CB.Mean	Mean of captive-born animals experiencing reproductive trait
CB.C.Mean	CB.Mean corrected for direction of effect on productivity (multiplied by -1 if trait has negative effect on productivity)
CB.SD	Standard deviation (captive-born)
CB.SEM	Standard error of the mean (captive-born)

Appendix 3: Supplementary Code to Chapter 2

This appendix relates to Chapter 2: A meta-analysis of birth origin effects on reproduction in diverse captive environments.

The following R code was used to perform the meta-analysis in Chapter 2. The code is annotated with the # symbol.

```
##########################################################################
# Annotated code for Farquharson, Hogg, Grueber "A meta-analysis of birth-
origin effects on reproduction in diverse captive environments"
# This code written by Katherine Farquharson, The University of Sydney,
2017
# Some code adapted from:
# Winter (2013) rotl tutorial:
# https://cran.r-project.org/web/packages/rotl/vignettes/data_mashups.html
# Moatt et al. (2016):
# http://dx.doi.org/10.5061/dryad.3fc02
# Kamiya et al. (2014):
#https://datadryad.org/bitstream/handle/10255/dryad.71107/R%20code.R?sequen
ce=1
##########################################################################

rm(list=ls())
##Load packages
library(rotl)
library(Hmisc)
library(MCMCglmm)
library(metafor)
library(ape)
library(mice)
library(lattice)
library(HDInterval)

############################
## Calculate effect sizes ##
############################
es <- read.csv('data file.csv')
#use log odds ratio as effect size (with captive-born input as control,
wild-born as treatment)

#two kinds of data - continuous (mean, sd/SEM & N), and proportional (n/N)
#for proportional data
es.proportional <- subset(es, (!is.na(es$WB.n1)))
lnor.proportional <- escalc(measure="OR", ai=es.proportional$CB.success,
bi=es.proportional$CB.failure,
                            ci=es.proportional$WB.success,
di=es.proportional$WB.failure,
                            n1i=es.proportional$CB.N,
n2i=es.proportional$WB.N, vtype='UB', add=0.5,
                            to = 'only0', drop00=FALSE)
es.proportional$yi <- lnor.proportional$yi
es.proportional$vi <- lnor.proportional$vi

#for continuous data
es.continuous <- subset(es, (!is.na(es$WB.Mean)))
```

```
colnames(es.continuous)
lnor.continuous <- escalc(measure='D2ORN', m1i=es.continuous$CB.C.Mean,
m2i=es.continuous$WB.C.Mean,
                          sd1i=es.continuous$CB.SD,
sd2i=es.continuous$WB.SD, n1i=es.continuous$CB.N,
                          n2i=es.continuous$WB.N, vtype='UB')
es.continuous$yi <- lnor.continuous$yi
es.continuous$vi <- lnor.continuous$vi
es <- rbind(es.proportional, es.continuous)

############################
##Create phylogenetic tree ##
############################
es$Scientific.name
es$Scientific.name <- as.character(es$Scientific.name)

#change species names in es to match those of rotl tree
es$Scientific.name[es$Scientific.name == "Cersus eldi thamin"] <- "Rucervus
eldii"
es$Scientific.name[es$Scientific.name == "Choreopsis liberiensis"] <-
"Hexaprotodon liberiensis"
es$Scientific.name[es$Scientific.name == "Clethrionymus glareolus"] <-
"Myodes glareolus"
es$Scientific.name[es$Scientific.name == "Equus burchelli"] <- "Equus
burchelii"
es$Scientific.name[es$Scientific.name == "Fenneropenaeus merguiensis"] <-
"Penaeus merguiensis"
es$Scientific.name[es$Scientific.name == "Gorilla gorilla gorilla"] <-
"Gorilla gorilla"
es$Scientific.name[es$Scientific.name == "Pan troglodytes"] <- "Pan
troglodytes troglodytes"
es$Scientific.name[es$Scientific.name == "Saguinus fuscicollis illigeri"]
<- "Saguinus fuscicollis"
es$Scientific.name[es$Scientific.name == "Eulemur mongoz, formerly Lemur
mongoz "] <- "Eulemur mongoz"
species <- unique(es$Scientific.name)
species <- as.data.frame(species)

species[,1] <- as.character(species[,1])
#Add outgroup (box jellyfish) to root tree
outgroup <- 'Chironex fleckeri'
species[45,1] <- outgroup

taxa <- tnrs_match_names(names=species[,1], context_name = "All life")
tr <- tol_induced_subtree(taxa$ott_id)
taxon_map <- structure(taxa$search_string,
names=as.character(taxa$unique_name))

#tree contains node labels for nodes that match a higher taxonomic group
#remove extra information from tip labels:
tr$tip.label
otl_tips <- strip_ott_ids(tr$tip.label, remove_underscores = TRUE)
tr$tip.label

#map names to tree
tr$tip.label <- taxon_map[otl_tips]
tr$tip.label <- capitalize(tr$tip.label)

#remove node labels
any(duplicated(tr$node.label))
tr$node.label <- NULL
```

```
##############################
### Phylogenetic VCV matrix ###
##############################

#rename column to 'animal' for input to MCMCglmm
es$animal <- es$Scientific.name

#compute branch lengths using ape package default based on topology
CorMatrix <- vcv(compute.brlen(tr, corr = T))

#match matrix to dataset
CorExt <- as.matrix(CorMatrix[match(es$animal, rownames(CorMatrix)),
match(es$animal, colnames(CorMatrix))])
levelplot(CorMatrix, xlab = '', ylab = 'Species')

#remove outgroup from CorMatrix
CorMatrix1 <- CorMatrix[-45, -45]
levelplot(CorMatrix1)

#MCMCglmm requires inverse matrix for input
Cinv <- solve(CorMatrix1)
Cinv2 <- as(Cinv, "dgCMatrix")

##################################
## Meta-analysis: overall model ##
##################################

#MCMCglmm also requires variances as input - we have no random structure
specified for variances
MEV <- es$vi

prior <- list(R=list(V=1,nu=0.002),G=list(G1=list(V=1, nu=0.002)))

#model will take a few minutes to run
o.model <- MCMCglmm(yi ~ 1, random = ~ ID, mev = MEV, data = es, verbose=T,
nitt=5000000,
                    thin=3000, burnin=150000, prior=prior, pr=T)

#results of all models may vary slightly from reported results (iterative
Bayesian models)
summary(o.model)

#model diagnostics
effectiveSize(o.model$Sol) # >1000
effectiveSize(o.model$VCV) # >1000
autocorr.diag(o.model$VCV) #all below 0.1
plot(o.model$Sol[,1])
plot(o.model$VCV) #convergence looks good
heidel.diag(o.model$VCV) #all passed

#run same model again 2x to check convergence of results (Gelman-Rubin
statistic < 1.1)
o.model_1 <- MCMCglmm(yi ~ 1, random = ~ ID, mev = MEV, data = es,
verbose=T, nitt=5000000,
                      thin=3000, burnin=150000, prior=prior, pr=T)
o.model_2 <- MCMCglmm(yi ~ 1, random = ~ ID, mev = MEV, data = es,
verbose=T, nitt=5000000,
                      thin=3000, burnin=150000, prior=prior, pr=T)
summary(o.model_1)
summary(o.model_2)
```

```
gelman.diag(mcmc.list(o.model$Sol, o.model_1$Sol, o.model_2$Sol)) #all <
1.1
o.model$DIC
o.model_1$DIC #use lowest DIC - o.model_1 in our case (though all are very
similar)
o.model_2$DIC

################################################
### Meta-analysis: Overall model + phylogeny ###
################################################

prior.phylo <- list(R=list(V=1,nu=0.002),G=list(G1=list(V=1, nu=0.002),
G2=list(V=1, nu=0.002)))

o.modelphylo <- MCMCglmm(yi ~ 1, random = ~animal + ID, mev = MEV, data =
es, verbose=T,
                    nitt=5000000, thin=3000, burnin=150000,
prior=prior.phylo, pr=T,
                    ginverse=list(animal = Cinv2))
summary(o.modelphylo)

#model diagnostics:
effectiveSize(o.modelphylo$Sol) # >1000
effectiveSize(o.modelphylo$VCV) #>1000
autocorr.diag(o.modelphylo$VCV) #all below 0.1
plot(o.modelphylo$Sol[,1])
plot(o.modelphylo$VCV)
heidel.diag(o.modelphylo$VCV) #all passed

#run same model again 2x to check convergence of results (Gelman-Rubin
statistic < 1.1)
o.modelphylo_1 <- MCMCglmm(yi ~ 1, random = ~animal + ID, mev = MEV, data =
es, verbose=T,
                      nitt=5000000, thin=3000, burnin=150000,
prior=prior.phylo, pr=T,
                      ginverse=list(animal = Cinv2))
o.modelphylo_2 <- MCMCglmm(yi ~ 1, random = ~animal + ID, mev = MEV, data =
es, verbose=T,
                      nitt=5000000, thin=3000, burnin=150000,
prior=prior.phylo, pr=T,
                      ginverse=list(animal = Cinv2))
summary(o.modelphylo_1)
summary(o.modelphylo_2)

o.modelphylo$DIC #317.6251
o.modelphylo_1$DIC #317.5601
o.modelphylo_2$DIC #317.5375 <- lowest DIC

gelman.diag(mcmc.list(o.modelphylo$Sol, o.modelphylo_1$Sol,
o.modelphylo_2$Sol)) #all <1.1

############################################################
### Comparing overall model to overall model + phylogeny ###
############################################################

summary(o.model_1)$DIC #317.3776
summary(o.modelphylo_2)$DIC #317.5375
#the non-phylogenetic model has a slightly lower DIC so is the better model

## Heterogeneity statistics
```

```
#for multilevel meta-analysis, extended heterogeneity statistic suggested
(Nakagawa)

k <- 115 #number of effect sizes
#sampling error:
s2m <- sum(1/es$vi) * (k-1) / (sum(1/es$vi)^2 - sum((1/es$vi)^2))

#For overall model (without phylogeny)
s2t <- o.model_1$VCV[,"ID"]+o.model_1$VCV[,"units"]+s2m
#total heterogeneity:
I2t <- 100* ((o.model_1$VCV[,"units"]+o.model_1$VCV[,"ID"])/s2t)
posterior.mode(I2t)
#study ID heterogeneity:
I2s <- 100* ((o.model_1$VCV[,"ID"])/s2t)
posterior.mode(I2s)
#residual variance
I2r <- 100* ((o.model_1$VCV[,"units"])/s2t)
posterior.mode(I2r) #19.3%

#For overall model + phylogeny
#total variance:
s2t.p <-
o.modelphylo_2$VCV[,"animal"]+o.modelphylo_2$VCV[,"ID"]+o.modelphylo_2$VCV[
,"units"]+s2m
#total heterogeneity:
I2t.p <- 100*
((o.modelphylo_2$VCV[,"units"]+o.modelphylo_2$VCV[,"animal"]+o.modelphylo_2
$VCV[,"ID"])/s2t.p)
posterior.mode(I2t.p)
#phylogeny heterogeneity:
I2p.p <- 100* ((o.modelphylo_2$VCV[,"animal"])/s2t.p)
posterior.mode(I2p.p)
HPDinterval(I2p.p)
#study ID heterogeneity:
I2s.p <- 100* ((o.modelphylo_2$VCV[,"ID"])/s2t.p)
posterior.mode(I2s.p)
#residual variance:
I2r.p <- 100* ((o.modelphylo_2$VCV[,"units"])/s2t.p)
posterior.mode(I2r.p) #24.2%

##Calculate phylogenetic heritability (lamda, phylogenetic signal)
lamda <- o.modelphylo_2$VCV[,"animal"]/
  (o.modelphylo_2$VCV[,"animal"] + o.modelphylo_2$VCV[,"ID"] +
o.modelphylo_2$VCV[,'units'])
posterior.mode(lamda)

#Heterogeneity is high in both models
#Phylogenetic signal is low, and DIC is lower in non-phylogenetic model
#Proceed with non-phylogenetic meta-regression

############################################
### Meta-regression: Captive Environment  ###
############################################

mr.model.ce <- MCMCglmm(yi ~ factor(Captive.environment)-1, random = ~ ID,
mev = MEV, data = es,
                    verbose=T, nitt=5000000, thin=3000, burnin=150000,
prior=prior, pr=T)
summary(mr.model.ce)

#model diagnostics:
```

```
effectiveSize(mr.model.ce$Sol) # >1000
effectiveSize(mr.model.ce$VCV) #>1000
autocorr.diag(mr.model.ce$VCV) #all below 0.1
plot(mr.model.ce$Sol[,1])
plot(mr.model.ce$VCV)
heidel.diag(mr.model.ce$VCV) #all passed

#run same model again 2x to check convergence of results (Gelman-Rubin
statistic < 1.1)
mr.model.ce_1 <- MCMCglmm(yi ~ factor(Captive.environment)-1, random = ~
ID, mev = MEV, data = es,
                      verbose=T, nitt=5000000, thin=3000, burnin=150000,
prior=prior, pr=T)
mr.model.ce_2 <- MCMCglmm(yi ~ factor(Captive.environment)-1, random = ~
ID, mev = MEV, data = es,
                      verbose=T, nitt=5000000, thin=3000, burnin=150000,
prior=prior, pr=T)
summary(mr.model.ce_1)
summary(mr.model.ce_2)

mr.model.ce$DIC #317.0837
mr.model.ce_1$DIC #317.0486
mr.model.ce_2$DIC #317.0434 <- lowest DIC model

gelman.diag(mcmc.list(mr.model.ce$Sol, mr.model.ce_1$Sol,
mr.model.ce_2$Sol)) #all < 1.1

###################################
### Meta-regression - Trait type ###
###################################

mr.model.t <- MCMCglmm(yi ~ factor(Trait)-1, random = ~ ID, mev = MEV, data
= es, verbose=T,
                    nitt=5000000, thin=3000, burnin=150000, prior=prior,
pr=T)
summary(mr.model.t)

#model diagnostics:
effectiveSize(mr.model.t$Sol) # >1000
effectiveSize(mr.model.t$VCV) #>1000
autocorr.diag(mr.model.t$VCV) #all below 0.1
plot(mr.model.t$Sol[,1])
plot(mr.model.t$VCV)
heidel.diag(mr.model.t$VCV) #all passed

#run same model again 2x to check convergence of results (Gelman-Rubin
statistic < 1.1)
mr.model.t_1 <- MCMCglmm(yi ~ factor(Trait)-1, random = ~ ID, mev = MEV,
data = es,
                      verbose=T, nitt=5000000, thin=3000,burnin=150000,
prior=prior, pr=T)
mr.model.t_2 <- MCMCglmm(yi ~ factor(Trait)-1, random = ~ ID, mev = MEV,
data = es,
                      verbose=T, nitt=5000000, thin=3000, burnin=150000,
prior=prior, pr=T)
summary(mr.model.t_1)
summary(mr.model.t_2)

mr.model.t$DIC #286.5063
mr.model.t_1$DIC #286.5062 <- lowest DIC
mr.model.t_2$DIC #286.5142
```

```
gelman.diag(mcmc.list(mr.model.t$Sol, mr.model.t_1$Sol, mr.model.t_2$Sol))
#all < 1.1

###################################
### Measures of publication bias ###
###################################

##Time-lag bias
#meta-regression with year of publication to see if effect sizes change
over time
mr.model.y <- MCMCglmm(yi ~ Year, random = ~ID, mev = MEV, data = es,
verbose=T, nitt=5000000,
                   thin=3000, burnin=150000, prior=prior, pr=T)
summary(mr.model.y)

#model diagnostics:
effectiveSize(mr.model.y$Sol) # >1000
effectiveSize(mr.model.y$VCV) #>1000
autocorr.diag(mr.model.y$VCV) #all below 0.1
plot(mr.model.y$Sol[,1])
plot(mr.model.y$VCV)
heidel.diag(mr.model.y$VCV) #all passed

#run same model again 2x to check convergence of results (Gelman-Rubin
statistic < 1.1)
mr.model.y_1 <- MCMCglmm(yi ~ Year, random = ~ID, mev = MEV, data = es,
verbose=T,
                     nitt=5000000, thin=3000, burnin=150000, prior=prior,
pr=T)
mr.model.y_2 <- MCMCglmm(yi ~ Year, random = ~ID, mev = MEV, data = es,
verbose=T,
                     nitt=5000000, thin=3000, burnin=150000, prior=prior,
pr=T)
summary(mr.model.y_1)
summary(mr.model.y_2)

gelman.diag(mcmc.list(mr.model.y$Sol, mr.model.y_1$Sol, mr.model.y_2$Sol))
#all < 1.1
#year is not significant - no evidence of publication bias over time

mr.model.y$DIC #316.5641
mr.model.y_1$DIC #316.5887
mr.model.y_2$DIC #316.495 <-- lowest DIC

##Egger's regression
#use meta-analytic residuals
#meta-analytic residuals = raw data - predictions
o.model_1$Random$formula <- update(o.model_1$Random$formula, ~.+leg(mev, -
1, FALSE):units)
prediction <- predict(o.model_1, marginal= ~leg(mev, -1, FALSE):units)
precision <- sqrt(1/es$vi)
MR <- es$yi - prediction
zMR <- MR*precision
egger<-lm(zMR ~ precision)
summary(egger)
#intercept is significant - evidence of possible publication bias (though
correction is small)
summary(egger)$coefficients

##Funnel plot
```

```
graphics.off()
par(mfrow=c(1,2))
plot(es$yi, precision, xlab='Log odds ratio', ylab = 'Precision')
abline(v=posterior.mode(o.model_1$Sol)[1],lwd=1) #v is the meta-analytic
mean
plot(MR, precision, xlab = 'Meta-analytic residuals', ylab = 'Precision')
abline(v=0,lwd=1,lty=2)

##Trim-and-fill analysis
#for multi-level meta-analysis, can only be performed on meta-analytic
residuals
#removes smaller studies causing funnel plot asymmetry,
#uses trimmed funnel plot to estimate the true centre of the funnel,
#replaces omitted studies and their missing counterparts around the centre
(filling)
model.tf <- rma(yi=MR,sei=1/precision)
summary(model.tf)
TFL <- trimfill(model.tf,side="left",estimator="R0")
TFR <- trimfill(model.tf, side='right', estimator = 'R0')
summary(TFL) #no studies estimated to be missing on left side
summary(TFR) #2 studies estimated to be missing on right side, but P =
0.125, estimate is small
graphics.off()
range(MR)
funnel(TFL,xlab="Meta-analytic residuals",xlim=c(-6,6))
funnel(TFR,xlab="Meta-analytic residuals",xlim=c(-6, 6))

#apparent outlier (with odds ratio = -10) could be affecting results
#this comes from ES.ID 75 (Murugan et al. 2013)

## Rerun all models without outlier
es2 <- es[c(-90),]
MEV2 <- es2$vi

o.model_out <- MCMCglmm(yi ~ 1, random = ~ ID, mev = MEV2, data = es2,
verbose=T, nitt=5000000,
                    thin=3000, burnin=150000, prior=prior, pr=T)
o.modelphylo_out <- MCMCglmm(yi ~ 1, random = ~animal + ID, mev = MEV2,
data = es2, verbose=T,
                    nitt=5000000, thin=3000, burnin=150000,
prior=prior.phylo, pr=T,
                    ginverse=list(animal = Cinv2))
mr.model.ce_out <- MCMCglmm(yi ~ factor(Captive.environment)-1, random = ~
ID, mev = MEV2,
                            data = es2, verbose=T, nitt=5000000, thin=3000,
burnin=150000,
                            prior=prior, pr=T)
mr.model.t_out <- MCMCglmm(yi ~ factor(Trait)-1, random = ~ ID, mev = MEV2,
data = es2,
                    verbose=T, nitt=5000000, thin=3000, burnin=150000,
prior=prior, pr=T)

#compare models with & without outlier
summary(o.model)
summary(o.model_out)

summary(o.modelphylo)
summary(o.modelphylo_out)

summary(mr.model.ce)
summary(mr.model.ce_out)
```

```
summary(mr.model.t)
summary(mr.model.t_out)
#qualitatively the same - does not alter conclusions

##Publication bias (with outlier removed)
##Time-lag bias
mr.model.y_out <- MCMCglmm(yi ~ Year, random = ~ID, mev = MEV2, data = es2,
verbose=T, nitt=5000000,
                        thin=3000, burnin=150000, prior=prior, pr=T)
summary(mr.model.y_out)

##Egger's regression
o.model_out$Random$formula <- update(o.model_out$Random$formula,
~.+leg(mev, -1, FALSE):units)
prediction_out <- predict(o.model_out, marginal= ~leg(mev, -1,
FALSE):units)
precision_out <- sqrt(1/es2$vi)
MR_out <- es2$yi - prediction_out
zMR_out <- MR_out*precision_out
egger_out <-lm(zMR_out ~ precision_out)
summary(egger_out)
#intercept is significant, correction still small

##Funnel plot
graphics.off()
par(mfrow=c(1,2))
plot(es2$yi, precision_out, xlab='Log odds ratio', ylab = 'Precision')
abline(v=posterior.mode(o.model_out$Sol)[1],lwd=1) #v is the meta-analytic
mean
plot(MR_out, precision_out, xlab = 'Meta-analytic residuals', ylab =
'Precision')
abline(v=0,lwd=2)

##Trim-and-fill analysis
model.tf_out <- rma(yi=MR_out,sei=1/precision_out)
summary(model.tf_out)
TFL_out <- trimfill(model.tf_out,side="left",estimator="R0")
TFR_out <- trimfill(model.tf_out, side='right', estimator = 'R0')
summary(TFL_out) #no studies estimated to be missing on left side
summary(TFR_out) #now 0 studies are estimated to be missing on right side
graphics.off()
range(MR_out)
funnel(TFL_out,xlab="Meta-analytic residuals",xlim=c(-4,4))
funnel(TFR_out,xlab="Meta-analytic residuals",xlim=c(-4, 4))
#no substantial differences by excluding outlier

##MI to recover missing standard deviations (N = 17 additional comparisons)

complete <- read.csv('completecases.csv', header=T)
# as the data we are imputing is continuous (mean, sd, N) use this subset
of data to form correlation matrix
#to impute data from
complete.c <- complete[49:115,]

#load missing data
missing <- read.csv('missing data imputation no variance without sample
size.csv', header=T)
tail(missing)
colnames(complete.c)
colnames(missing)
```

```
combined <- rbind(complete.c, missing)
colnames(combined)

#using columns 27, 28, 31, 32 to impute missing data
#plot missing data using md.pattern (1 = observed, 0 = missing)
md.pattern(combined[, c(27, 28, 31, 32)]) #mean known for all,
#SD missing for 17 effect sizes.

#check correlations between variables - MI uses this matrix
cor(combined[,c(27, 28, 31, 32)], use = "pairwise.complete.obs")

#use log scale to impute data as relationship is not linear otherwise
plot(combined$CB.Mean, combined$CB.SD)
plot(log(combined$CB.Mean), log(combined$CB.SD))

plot(combined$WB.Mean, combined$WB.SD)
plot(log(combined$WB.Mean), log(combined$WB.SD))

#can't do log of negative numbers - identify negative values (direction of
effect on reproductive success is negative)
CB.negatives <- which(combined$CB.C.Mean <0)
WB.negatives <- which(combined$WB.C.Mean <0)

which(combined$WB.SD == 0) #one WB.SD = 0, so can't be logged. Add 0.00001
(measurement is litter size) to this estimate?

combinedlog <- combined
combinedlog[66,28] <- 0.5
combinedlog$CB.C.Mean[CB.negatives] <- combined$CB.C.Mean[CB.negatives]*-1
combinedlog$WB.C.Mean[WB.negatives] <- combined$WB.C.Mean[WB.negatives]*-1

combinedlog$CB.C.Mean <- log(combinedlog$CB.C.Mean)
combinedlog$WB.C.Mean <- log(combinedlog$WB.C.Mean)
combinedlog$CB.SD <- log(combinedlog$CB.SD)
combinedlog$WB.SD <- log(combinedlog$WB.SD)
combinedlog$CB.N <- log(combinedlog$CB.N)
combinedlog$WB.N <- log(combinedlog$WB.N)

#######################################################
### Multiple imputation (MI); creating 20 datasets ###
#######################################################
#rerun all analyses with imputed data

#load missing data to be imputed
missing <- read.csv('data file 2.csv', header=T)
#17 comparisons have missing standard deviation, 24 have missing sample
size

#reload original data (used above):
complete <- read.csv('data file.csv', header=T)
#as the data we are imputing is continuous (mean, sd, N) use this subset of
data (continuous traits) to form correlation matrix
#to impute data from
complete.c <- complete[49:115,]

#impute missing standard deviations (N = 17 comparisons)
missing2 <- missing[!is.na(missing$WB.N),]
combined <- rbind(complete.c, missing2)

colnames(combined)
#using columns 19, 25, 27, 28, 31, 32 to impute missing data
```

```
#plot missing data using md.pattern (1 = observed, 0 = missing)
md.pattern(combined[, c(19, 25, 27, 28, 31, 32)]) #mean & N known for all,
SD missing for 17

#check correlations between variables - MI uses this matrix
cor(combined[,c(19, 25, 27, 28, 31, 32)], use = "pairwise.complete.obs")

#use log scale to impute data as relationship is not linear otherwise
plot(combined$CB.Mean, combined$CB.SD)
plot(log(combined$CB.Mean), log(combined$CB.SD))
plot(combined$WB.Mean, combined$WB.SD)
plot(log(combined$WB.Mean), log(combined$WB.SD))
plot(combined$CB.Mean, combined$CB.N)
plot(log(combined$CB.Mean), log(combined$CB.N))
plot(combined$WB.Mean, combined$WB.N)
plot(log(combined$WB.Mean), log(combined$WB.N))

#can't do log of negative numbers - identify negative values (direction of
effect on reproductive success is negative)
CB.negatives <- which(combined$CB.C.Mean <0)
WB.negatives <- which(combined$WB.C.Mean <0)

which(combined$WB.SD == 0) #one WB.SD = 0, so can't be logged. Add 0.5
(measurement is litter size) to this estimate

combinedlog <- combined
combinedlog[66,28] <- 0.5
combinedlog$CB.C.Mean[CB.negatives] <- combined$CB.C.Mean[CB.negatives]*-1
combinedlog$WB.C.Mean[WB.negatives] <- combined$WB.C.Mean[WB.negatives]*-1

combinedlog$CB.C.Mean <- log(combinedlog$CB.C.Mean)
combinedlog$WB.C.Mean <- log(combinedlog$WB.C.Mean)
combinedlog$CB.SD <- log(combinedlog$CB.SD)
combinedlog$WB.SD <- log(combinedlog$WB.SD)
combinedlog$CB.N <- log(combinedlog$CB.N)
combinedlog$WB.N <- log(combinedlog$WB.N)

#  multiple imputation (MI); creating 20 datasets
nos.log.Imp <- mice(combinedlog[,c(19, 25, 27, 28, 31, 32)], m = 20)

####check imputations (Imp)
names(nos.log.Imp)
nos.log.Imp$imp$WB.SD
nos.log.Imp$imp$CB.SD

#see if imputations seem reasonable given data:
stripplot(nos.log.Imp, pch = 20, cex = 1.2) #blue is observed values, red
is imputed missing values
densityplot(nos.log.Imp)

#check convergence of imputations
plot(nos.log.Imp)

##Create function to backtransform, make negative values negative again
(where direction
#of effect on reproductive success is negative), calculate effect sizes and
run meta-analysis,
#then run for each of 20 imputations and combine as one MCMClist

MImodel <- function(i) {
```

```
  data <- cbind(combinedlog[,1:13], combinedlog[,c(19, 25)],
complete(nos.log.Imp, action = i))
  data$CB.C.Mean <- exp(data$CB.C.Mean)
  data$WB.C.Mean <- exp(data$WB.C.Mean)
  data$CB.SD <- exp(data$CB.SD)
  data$WB.SD <- exp(data$WB.SD)
  data$CB.N <- exp(data$CB.N)
  data$WB.N <- exp(data$WB.N)
  negs <- which(data$Direction == '-')
  data$WB.C.Mean[negs] <- data$WB.C.Mean[negs]*-1
  data$CB.C.Mean[negs] <- data$CB.C.Mean[negs]*-1
  lnor.continuous <- escalc(measure='D2ORN', m1i=data$CB.C.Mean,
m2i=data$WB.C.Mean,
                            sd1i=data$CB.SD, sd2i=data$WB.SD,
n1i=data$CB.N,
                            n2i=data$WB.N, vtype='UB')
  data$yi <- lnor.continuous$yi
  data$vi <- lnor.continuous$vi
  data2 <- complete[1:48,]
  lnor.proportional <- escalc(measure="OR", ai=data2$CB.success,
bi=data2$CB.failure,
                              ci=data2$WB.success, di=data2$WB.failure,
                              n1i=data2$CB.N, n2i=data2$WB.N, vtype='UB',
add=0.5,
                              to = 'only0', drop00=FALSE)
  data2$yi <- lnor.proportional$yi
  data2$vi <- lnor.proportional$vi
  data1 <- data[,c(1:13,22:23)]
  data3 <- data2[,c(1:13,34:35)]
  data4 <<- rbind(data1, data3)
  MEV <<- data4$vi
  prior <<- list(R=list(V=1,nu=0.002),G=list(G1=list(V=1, nu=0.002)))
}

o.model <- function(i) {
  MCMCglmm(yi ~ 1, random = ~ ID, mev = MEV, data = data4, verbose=T,
nitt=5000000,
           thin=3000, burnin=150000, prior=prior, pr=T)
}

MImodel(1)
nos.log.model1 <- o.model(1)
MImodel(2)
nos.log.model2 <- o.model(2)
MImodel(3)
nos.log.model3 <- o.model(3)
MImodel(4)
nos.log.model4 <- o.model(4)
MImodel(5)
nos.log.model5 <- o.model(5)
MImodel(6)
nos.log.model6 <- o.model(6)
MImodel(7)
nos.log.model7 <- o.model(7)
MImodel(8)
nos.log.model8 <- o.model(8)
MImodel(9)
nos.log.model9 <- o.model(9)
MImodel(10)
nos.log.model10 <- o.model(10)
MImodel(11)
```

```
nos.log.model11 <- o.model(11)
MImodel(12)
nos.log.model12 <- o.model(12)
MImodel(13)
nos.log.model13 <- o.model(13)
MImodel(14)
nos.log.model14 <- o.model(14)
MImodel(15)
nos.log.model15 <- o.model(15)
MImodel(16)
nos.log.model16 <- o.model(16)
MImodel(17)
nos.log.model17 <- o.model(17)
MImodel(18)
nos.log.model18 <- o.model(18)
MImodel(19)
nos.log.model19 <- o.model(19)
MImodel(20)
nos.log.model20 <- o.model(20)

#VCV = variance components
nos.log.MI.comb.VCV <- mcmc.list(nos.log.model1$VCV, nos.log.model2$VCV,
nos.log.model3$VCV, nos.log.model4$VCV, nos.log.model5$VCV,
nos.log.model6$VCV, nos.log.model7$VCV,
                                 nos.log.model8$VCV, nos.log.model9$VCV,
nos.log.model10$VCV, nos.log.model11$VCV, nos.log.model12$VCV,
nos.log.model13$VCV,
                                 nos.log.model14$VCV, nos.log.model15$VCV,
nos.log.model16$VCV, nos.log.model17$VCV, nos.log.model18$VCV,
nos.log.model19$VCV,
                                 nos.log.model20$VCV)
summary(nos.log.MI.comb.VCV)

#Sol = solutions
nos.log.MI.comb.Sol <- mcmc.list(nos.log.model1$Sol, nos.log.model2$Sol,
nos.log.model3$Sol, nos.log.model4$Sol, nos.log.model5$Sol,
nos.log.model6$Sol, nos.log.model7$Sol, nos.log.model8$Sol,
                                 nos.log.model9$Sol, nos.log.model10$Sol,
nos.log.model11$Sol, nos.log.model12$Sol, nos.log.model13$Sol,
nos.log.model14$Sol, nos.log.model15$Sol,
                                 nos.log.model16$Sol, nos.log.model17$Sol,
nos.log.model18$Sol, nos.log.model19$Sol, nos.log.model20$Sol)
plot(mcmc.list(nos.log.model1$Sol[,1], nos.log.model2$Sol[,1],
nos.log.model11$Sol[,1]))
#check plot with different models for visual convergence

autocorr.diag(nos.log.MI.comb.VCV) #passes autocorrelation
heidel.diag(nos.log.MI.comb.VCV) #all passed

nos.log.MI.sol1 <- as.data.frame(nos.log.model1$Sol[,1])
nos.log.MI.sol2 <- as.data.frame(nos.log.model2$Sol[,1])
nos.log.MI.sol3 <- as.data.frame(nos.log.model3$Sol[,1])
nos.log.MI.sol4 <- as.data.frame(nos.log.model4$Sol[,1])
nos.log.MI.sol5 <- as.data.frame(nos.log.model5$Sol[,1])
nos.log.MI.sol6 <- as.data.frame(nos.log.model6$Sol[,1])
nos.log.MI.sol7 <- as.data.frame(nos.log.model7$Sol[,1])
nos.log.MI.sol8 <- as.data.frame(nos.log.model8$Sol[,1])
nos.log.MI.sol9 <- as.data.frame(nos.log.model9$Sol[,1])
nos.log.MI.sol10 <- as.data.frame(nos.log.model10$Sol[,1])
nos.log.MI.sol11 <- as.data.frame(nos.log.model11$Sol[,1])
nos.log.MI.sol12 <- as.data.frame(nos.log.model12$Sol[,1])
```

```
nos.log.MI.sol13 <- as.data.frame(nos.log.model13$Sol[,1])
nos.log.MI.sol14 <- as.data.frame(nos.log.model14$Sol[,1])
nos.log.MI.sol15 <- as.data.frame(nos.log.model15$Sol[,1])
nos.log.MI.sol16 <- as.data.frame(nos.log.model16$Sol[,1])
nos.log.MI.sol17 <- as.data.frame(nos.log.model17$Sol[,1])
nos.log.MI.sol18 <- as.data.frame(nos.log.model18$Sol[,1])
nos.log.MI.sol19 <- as.data.frame(nos.log.model19$Sol[,1])
nos.log.MI.sol20 <- as.data.frame(nos.log.model20$Sol[,1])

#pool posterior estimates
nos.log.MI.comb.sol1 <- rbind(nos.log.MI.sol1, nos.log.MI.sol2,
nos.log.MI.sol3, nos.log.MI.sol4, nos.log.MI.sol5, nos.log.MI.sol6,
nos.log.MI.sol7, nos.log.MI.sol8, nos.log.MI.sol9, nos.log.MI.sol10,
                              nos.log.MI.sol11, nos.log.MI.sol12,
nos.log.MI.sol13, nos.log.MI.sol14, nos.log.MI.sol15, nos.log.MI.sol16,
nos.log.MI.sol17, nos.log.MI.sol18, nos.log.MI.sol19,
                              nos.log.MI.sol20)
posterior.mode(nos.log.MI.comb.sol1) #posterior mode of pooled estimates
hdi(nos.log.MI.comb.sol1, credMass=0.95) #95% HPD CI of pooled estimates

##### Phylogenetic model (with MI data)

#update tree - extra species included with MI data
data4$Scientific.name <- as.character(data4$Scientific.name)

#change species names in es to match those of rotl tree
data4$Scientific.name[data4$Scientific.name == "Cersus eldi thamin"] <-
"Rucervus eldii"
data4$Scientific.name[data4$Scientific.name == "Choreopsis liberiensis"] <-
"Hexaprotodon liberiensis"
data4$Scientific.name[data4$Scientific.name == "Clethrionymus glareolus"]
<- "Myodes glareolus"
data4$Scientific.name[data4$Scientific.name == "Equus burchelli"] <- "Equus
burchelii"
data4$Scientific.name[data4$Scientific.name == "Fenneropenaeus
merguiensis"] <- "Penaeus merguiensis"
data4$Scientific.name[data4$Scientific.name == "Gorilla gorilla gorilla"]
<- "Gorilla gorilla"
data4$Scientific.name[data4$Scientific.name == "Pan troglodytes"] <- "Pan
troglodytes troglodytes"
data4$Scientific.name[data4$Scientific.name == "Saguinus fuscicollis
illigeri"] <- "Saguinus fuscicollis"
data4$Scientific.name[data4$Scientific.name == "Eulemur mongoz, formerly
Lemur mongoz "] <- "Eulemur mongoz"

species <- unique(data4$Scientific.name)
species <- as.data.frame(species)
species[,1] <- as.character(species[,1]) #48 unique species with missing
data included

#Add outgroup (box jellyfish) to root tree
outgroup <- 'Chironex fleckeri'
species[49,1] <- outgroup

taxa <- tnrs_match_names(names=species[,1], context_name = "All life")
tr <- tol_induced_subtree(taxa$ott_id)
taxon_map <- structure(taxa$search_string,
names=as.character(taxa$unique_name))
tr$tip.label
otl_tips <- strip_ott_ids(tr$tip.label, remove_underscores = TRUE)
tr$tip.label
```

```
tr$tip.label <- taxon_map[otl_tips]
tr$tip.label <- capitalize(tr$tip.label)
any(duplicated(tr$node.label))
tr$node.label <- NULL
plot(tr, show.tip.label=TRUE, show.node.label = FALSE)

#Phylogenetic VCV matrix (with MI data)

data4$animal <- data4$Scientific.name
CorMatrix <- vcv(compute.brlen(tr, corr = T))
CorExt <- as.matrix(CorMatrix[match(data4$animal, rownames(CorMatrix)),
match(data4$animal, colnames(CorMatrix))])
levelplot(CorMatrix, xlab = '', ylab = 'Species')
CorMatrix1 <- CorMatrix[-49, -49]
levelplot(CorMatrix1)
Cinv <- solve(CorMatrix1)
Cinv2 <- as(Cinv, "dgCMatrix")

prior.phylo <- list(R=list(V=1,nu=0.002),G=list(G1=list(V=1, nu=0.002),
G2=list(V=1, nu=0.002)))

o.modelphylo <- function(i) {
  data4$Scientific.name <- as.character(data4$Scientific.name)
  data4$Scientific.name[data4$Scientific.name == "Cersus eldi thamin"] <-
"Rucervus eldii"
  data4$Scientific.name[data4$Scientific.name == "Choreopsis liberiensis"]
<- "Hexaprotodon liberiensis"
  data4$Scientific.name[data4$Scientific.name == "Clethrionymus glareolus"]
<- "Myodes glareolus"
  data4$Scientific.name[data4$Scientific.name == "Equus burchelli"] <-
"Equus burchelii"
  data4$Scientific.name[data4$Scientific.name == "Fenneropenaeus
merguiensis"] <- "Penaeus merguiensis"
  data4$Scientific.name[data4$Scientific.name == "Gorilla gorilla gorilla"]
<- "Gorilla gorilla"
  data4$Scientific.name[data4$Scientific.name == "Pan troglodytes"] <- "Pan
troglodytes troglodytes"
  data4$Scientific.name[data4$Scientific.name == "Saguinus fuscicollis
illigeri"] <- "Saguinus fuscicollis"
  data4$Scientific.name[data4$Scientific.name == "Eulemur mongoz, formerly
Lemur mongoz "] <- "Eulemur mongoz"
  data4$animal <- data4$Scientific.name
  MCMCglmm(yi ~ 1, random = ~animal + ID, mev = MEV, data = data4,
verbose=T,
           nitt=5000000, thin=3000, burnin=150000, prior=prior.phylo, pr=T,
           ginverse=list(animal = Cinv2))
}

MImodel(1)
nos.log.phylomodel1 <- o.modelphylo(1)
MImodel(2)
nos.log.phylomodel2 <- o.modelphylo(2)
MImodel(3)
nos.log.phylomodel3 <- o.modelphylo(3)
MImodel(4)
nos.log.phylomodel4 <- o.modelphylo(4)
MImodel(5)
nos.log.phylomodel5 <- o.modelphylo(5)
MImodel(6)
nos.log.phylomodel6 <- o.modelphylo(6)
MImodel(7)
```

```
nos.log.phylomodel7 <- o.modelphylo(7)
MImodel(8)
nos.log.phylomodel8 <- o.modelphylo(8)
MImodel(9)
nos.log.phylomodel9 <- o.modelphylo(9)
MImodel(10)
nos.log.phylomodel10 <- o.modelphylo(10)
MImodel(11)
nos.log.phylomodel11 <- o.modelphylo(11)
MImodel(12)
nos.log.phylomodel12 <- o.modelphylo(12)
MImodel(13)
nos.log.phylomodel13 <- o.modelphylo(13)
MImodel(14)
nos.log.phylomodel14 <- o.modelphylo(14)
MImodel(15)
nos.log.phylomodel15 <- o.modelphylo(15)
MImodel(16)
nos.log.phylomodel16 <- o.modelphylo(16)
MImodel(17)
nos.log.phylomodel17 <- o.modelphylo(17)
MImodel(18)
nos.log.phylomodel18 <- o.modelphylo(18)
MImodel(19)
nos.log.phylomodel19 <- o.modelphylo(19)
MImodel(20)
nos.log.phylomodel20 <- o.modelphylo(20)

#VCV = variance components
nos.log.phylo.MI.comb.VCV <- mcmc.list(nos.log.phylomodel1$VCV,
nos.log.phylomodel2$VCV, nos.log.phylomodel3$VCV, nos.log.phylomodel4$VCV,
nos.log.phylomodel5$VCV, nos.log.phylomodel6$VCV, nos.log.phylomodel7$VCV,
                                        nos.log.phylomodel8$VCV,
nos.log.phylomodel9$VCV, nos.log.phylomodel10$VCV,
nos.log.phylomodel11$VCV, nos.log.phylomodel12$VCV,
nos.log.phylomodel13$VCV,
                                        nos.log.phylomodel14$VCV,
nos.log.phylomodel15$VCV, nos.log.phylomodel16$VCV,
nos.log.phylomodel17$VCV, nos.log.phylomodel18$VCV,
nos.log.phylomodel19$VCV,
                                        nos.log.phylomodel20$VCV)
summary(nos.log.phylo.MI.comb.VCV)

#Sol = solutions
nos.log.phylo.MI.comb.Sol <- mcmc.list(nos.log.phylomodel1$Sol,
nos.log.phylomodel2$Sol, nos.log.phylomodel3$Sol, nos.log.phylomodel4$Sol,
nos.log.phylomodel5$Sol, nos.log.phylomodel6$Sol, nos.log.phylomodel7$Sol,
nos.log.phylomodel8$Sol,
                                        nos.log.phylomodel9$Sol,
nos.log.phylomodel10$Sol, nos.log.phylomodel11$Sol,
nos.log.phylomodel12$Sol, nos.log.phylomodel13$Sol,
nos.log.phylomodel14$Sol, nos.log.phylomodel15$Sol,
                                        nos.log.phylomodel16$Sol,
nos.log.phylomodel17$Sol, nos.log.phylomodel18$Sol,
nos.log.phylomodel19$Sol, nos.log.phylomodel20$Sol)
plot(mcmc.list(nos.log.phylomodel1$Sol[,1], nos.log.phylomodel2$Sol[,1],
nos.log.phylomodel3$Sol[,1]))

autocorr.diag(nos.log.phylo.MI.comb.VCV) #passes autocorrelation
heidel.diag(nos.log.phylo.MI.comb.VCV) #all passed
```

```
nos.log.phylo.MI.sol1 <- as.data.frame(nos.log.phylomodel1$Sol[,1])
nos.log.phylo.MI.sol2 <- as.data.frame(nos.log.phylomodel2$Sol[,1])
nos.log.phylo.MI.sol3 <- as.data.frame(nos.log.phylomodel3$Sol[,1])
nos.log.phylo.MI.sol4 <- as.data.frame(nos.log.phylomodel4$Sol[,1])
nos.log.phylo.MI.sol5 <- as.data.frame(nos.log.phylomodel5$Sol[,1])
nos.log.phylo.MI.sol6 <- as.data.frame(nos.log.phylomodel6$Sol[,1])
nos.log.phylo.MI.sol7 <- as.data.frame(nos.log.phylomodel7$Sol[,1])
nos.log.phylo.MI.sol8 <- as.data.frame(nos.log.phylomodel8$Sol[,1])
nos.log.phylo.MI.sol9 <- as.data.frame(nos.log.phylomodel9$Sol[,1])
nos.log.phylo.MI.sol10 <- as.data.frame(nos.log.phylomodel10$Sol[,1])
nos.log.phylo.MI.sol11 <- as.data.frame(nos.log.phylomodel11$Sol[,1])
nos.log.phylo.MI.sol12 <- as.data.frame(nos.log.phylomodel12$Sol[,1])
nos.log.phylo.MI.sol13 <- as.data.frame(nos.log.phylomodel13$Sol[,1])
nos.log.phylo.MI.sol14 <- as.data.frame(nos.log.phylomodel14$Sol[,1])
nos.log.phylo.MI.sol15 <- as.data.frame(nos.log.phylomodel15$Sol[,1])
nos.log.phylo.MI.sol16 <- as.data.frame(nos.log.phylomodel16$Sol[,1])
nos.log.phylo.MI.sol17 <- as.data.frame(nos.log.phylomodel17$Sol[,1])
nos.log.phylo.MI.sol18 <- as.data.frame(nos.log.phylomodel18$Sol[,1])
nos.log.phylo.MI.sol19 <- as.data.frame(nos.log.phylomodel19$Sol[,1])
nos.log.phylo.MI.sol20 <- as.data.frame(nos.log.phylomodel20$Sol[,1])

nos.log.phylo.MI.comb.sol1 <- rbind(nos.log.phylo.MI.sol1,
nos.log.phylo.MI.sol2, nos.log.phylo.MI.sol3, nos.log.phylo.MI.sol4,
nos.log.phylo.MI.sol5, nos.log.phylo.MI.sol6, nos.log.phylo.MI.sol7,
nos.log.phylo.MI.sol8, nos.log.phylo.MI.sol9, nos.log.phylo.MI.sol10,
                                    nos.log.phylo.MI.sol11,
nos.log.phylo.MI.sol12, nos.log.phylo.MI.sol13, nos.log.phylo.MI.sol14,
nos.log.phylo.MI.sol15, nos.log.phylo.MI.sol16, nos.log.phylo.MI.sol17,
nos.log.phylo.MI.sol18, nos.log.phylo.MI.sol19,
                                    nos.log.phylo.MI.sol20)
posterior.mode(nos.log.phylo.MI.comb.sol1)
hdi(nos.log.phylo.MI.comb.sol1, credMass=0.95)

### Study environment model (with MI data)
ce.model <- function(i) {
  MCMCglmm(yi ~ factor(Captive.environment)-1, random = ~ ID, mev = MEV,
           data = data4, verbose=T, nitt=5000000, thin=3000, burnin=150000,
           prior=prior, pr=T)
}

MImodel(1)
nos.log.cemodel1 <- ce.model(1)
MImodel(2)
nos.log.cemodel2 <- ce.model(2)
MImodel(3)
nos.log.cemodel3 <- ce.model(3)
MImodel(4)
nos.log.cemodel4 <- ce.model(4)
MImodel(5)
nos.log.cemodel5 <- ce.model(5)
MImodel(6)
nos.log.cemodel6 <- ce.model(6)
MImodel(7)
nos.log.cemodel7 <- ce.model(7)
MImodel(8)
nos.log.cemodel8 <- ce.model(8)
MImodel(9)
nos.log.cemodel9 <- ce.model(9)
MImodel(10)
nos.log.cemodel10 <- ce.model(10)
MImodel(11)
```

```
nos.log.cemodel11 <- ce.model(11)
MImodel(12)
nos.log.cemodel12 <- ce.model(12)
MImodel(13)
nos.log.cemodel13 <- ce.model(13)
MImodel(14)
nos.log.cemodel14 <- ce.model(14)
MImodel(15)
nos.log.cemodel15 <- ce.model(15)
MImodel(16)
nos.log.cemodel16 <- ce.model(16)
MImodel(17)
nos.log.cemodel17 <- ce.model(17)
MImodel(18)
nos.log.cemodel18 <- ce.model(18)
MImodel(19)
nos.log.cemodel19 <- ce.model(19)
MImodel(20)
nos.log.cemodel20 <- ce.model(20)

#VCV = variance components
nos.log.ce.MI.comb.VCV <- mcmc.list(nos.log.cemodel1$VCV,
nos.log.cemodel2$VCV, nos.log.cemodel3$VCV, nos.log.cemodel4$VCV,
nos.log.cemodel5$VCV, nos.log.cemodel6$VCV, nos.log.cemodel7$VCV,
                                    nos.log.cemodel8$VCV,
nos.log.cemodel9$VCV, nos.log.cemodel10$VCV, nos.log.cemodel11$VCV,
nos.log.cemodel12$VCV, nos.log.cemodel13$VCV,
                                    nos.log.cemodel14$VCV,
nos.log.cemodel15$VCV, nos.log.cemodel16$VCV, nos.log.cemodel17$VCV,
nos.log.cemodel18$VCV, nos.log.cemodel19$VCV,
                                    nos.log.cemodel20$VCV)
summary(nos.log.ce.MI.comb.VCV)

#Sol = solutions
nos.log.ce.MI.comb.Sol <- mcmc.list(nos.log.cemodel1$Sol,
nos.log.cemodel2$Sol, nos.log.cemodel3$Sol, nos.log.cemodel4$Sol,
nos.log.cemodel5$Sol, nos.log.cemodel6$Sol, nos.log.cemodel7$Sol,
nos.log.cemodel8$Sol,
                                    nos.log.cemodel9$Sol,
nos.log.cemodel10$Sol, nos.log.cemodel11$Sol, nos.log.cemodel12$Sol,
nos.log.cemodel13$Sol, nos.log.cemodel14$Sol, nos.log.cemodel15$Sol,
                                    nos.log.cemodel16$Sol,
nos.log.cemodel17$Sol, nos.log.cemodel18$Sol, nos.log.cemodel19$Sol,
nos.log.cemodel20$Sol)
plot(mcmc.list(nos.log.cemodel1$Sol[,1], nos.log.cemodel2$Sol[,1],
nos.log.cemodel3$Sol[,1]))

autocorr.diag(nos.log.ce.MI.comb.VCV) #passes autocorrelation
heidel.diag(nos.log.ce.MI.comb.VCV) #all passed

nos.log.ce.MI.sol1 <- as.data.frame(nos.log.cemodel1$Sol[,1:4])
nos.log.ce.MI.sol2 <- as.data.frame(nos.log.cemodel2$Sol[,1:4])
nos.log.ce.MI.sol3 <- as.data.frame(nos.log.cemodel3$Sol[,1:4])
nos.log.ce.MI.sol4 <- as.data.frame(nos.log.cemodel4$Sol[,1:4])
nos.log.ce.MI.sol5 <- as.data.frame(nos.log.cemodel5$Sol[,1:4])
nos.log.ce.MI.sol6 <- as.data.frame(nos.log.cemodel6$Sol[,1:4])
nos.log.ce.MI.sol7 <- as.data.frame(nos.log.cemodel7$Sol[,1:4])
nos.log.ce.MI.sol8 <- as.data.frame(nos.log.cemodel8$Sol[,1:4])
nos.log.ce.MI.sol9 <- as.data.frame(nos.log.cemodel9$Sol[,1:4])
nos.log.ce.MI.sol10 <- as.data.frame(nos.log.cemodel10$Sol[,1:4])
nos.log.ce.MI.sol11 <- as.data.frame(nos.log.cemodel11$Sol[,1:4])
```

```
nos.log.ce.MI.sol12 <- as.data.frame(nos.log.cemodel12$Sol[,1:4])
nos.log.ce.MI.sol13 <- as.data.frame(nos.log.cemodel13$Sol[,1:4])
nos.log.ce.MI.sol14 <- as.data.frame(nos.log.cemodel14$Sol[,1:4])
nos.log.ce.MI.sol15 <- as.data.frame(nos.log.cemodel15$Sol[,1:4])
nos.log.ce.MI.sol16 <- as.data.frame(nos.log.cemodel16$Sol[,1:4])
nos.log.ce.MI.sol17 <- as.data.frame(nos.log.cemodel17$Sol[,1:4])
nos.log.ce.MI.sol18 <- as.data.frame(nos.log.cemodel18$Sol[,1:4])
nos.log.ce.MI.sol19 <- as.data.frame(nos.log.cemodel19$Sol[,1:4])
nos.log.ce.MI.sol20 <- as.data.frame(nos.log.cemodel20$Sol[,1:4])

nos.log.ce.MI.comb.sol1 <- rbind(nos.log.ce.MI.sol1, nos.log.ce.MI.sol2,
nos.log.ce.MI.sol3, nos.log.ce.MI.sol4, nos.log.ce.MI.sol5,
nos.log.ce.MI.sol6, nos.log.ce.MI.sol7, nos.log.ce.MI.sol8,
nos.log.ce.MI.sol9, nos.log.ce.MI.sol10,
                                nos.log.ce.MI.sol11, nos.log.ce.MI.sol12,
nos.log.ce.MI.sol13, nos.log.ce.MI.sol14, nos.log.ce.MI.sol15,
nos.log.ce.MI.sol16, nos.log.ce.MI.sol17, nos.log.ce.MI.sol18,
nos.log.ce.MI.sol19,
                                nos.log.ce.MI.sol20)
posterior.mode(nos.log.ce.MI.comb.sol1)
hdi(nos.log.ce.MI.comb.sol1, credMass=0.95)

#### Trait type (with MI data)
t.model <- function(i) {
  MCMCglmm(yi ~ factor(Trait)-1, random = ~ ID, mev = MEV,
           data = data4, verbose=T, nitt=5000000, thin=3000, burnin=150000,
           prior=prior, pr=T)
}

MImodel(1)
nos.log.tmodel1 <- t.model(1)
MImodel(2)
nos.log.tmodel2 <- t.model(2)
MImodel(3)
nos.log.tmodel3 <- t.model(3)
MImodel(4)
nos.log.tmodel4 <- t.model(4)
MImodel(5)
nos.log.tmodel5 <- t.model(5)
MImodel(6)
nos.log.tmodel6 <- t.model(6)
MImodel(7)
nos.log.tmodel7 <- t.model(7)
MImodel(8)
nos.log.tmodel8 <- t.model(8)
MImodel(9)
nos.log.tmodel9 <- t.model(9)
MImodel(10)
nos.log.tmodel10 <- t.model(10)
MImodel(11)
nos.log.tmodel11 <- t.model(11)
MImodel(12)
nos.log.tmodel12 <- t.model(12)
MImodel(13)
nos.log.tmodel13 <- t.model(13)
MImodel(14)
nos.log.tmodel14 <- t.model(14)
MImodel(15)
nos.log.tmodel15 <- t.model(15)
MImodel(16)
nos.log.tmodel16 <- t.model(16)
```

```
MImodel(17)
nos.log.tmodel17 <- t.model(17)
MImodel(18)
nos.log.tmodel18 <- t.model(18)
MImodel(19)
nos.log.tmodel19 <- t.model(19)
MImodel(20)
nos.log.tmodel20 <- t.model(20)

#VCV = variance components
nos.log.t.MI.comb.VCV <- mcmc.list(nos.log.tmodel1$VCV,
nos.log.tmodel2$VCV, nos.log.tmodel3$VCV, nos.log.tmodel4$VCV,
nos.log.tmodel5$VCV, nos.log.tmodel6$VCV, nos.log.tmodel7$VCV,
                                   nos.log.tmodel8$VCV,
nos.log.tmodel9$VCV, nos.log.tmodel10$VCV, nos.log.tmodel11$VCV,
nos.log.tmodel12$VCV, nos.log.tmodel13$VCV,
                                   nos.log.tmodel14$VCV,
nos.log.tmodel15$VCV, nos.log.tmodel16$VCV, nos.log.tmodel17$VCV,
nos.log.tmodel18$VCV, nos.log.tmodel19$VCV,
                                   nos.log.tmodel20$VCV)
summary(nos.log.t.MI.comb.VCV)

#Sol = solutions
nos.log.t.MI.comb.Sol <- mcmc.list(nos.log.tmodel1$Sol,
nos.log.tmodel2$Sol, nos.log.tmodel3$Sol, nos.log.tmodel4$Sol,
nos.log.tmodel5$Sol, nos.log.tmodel6$Sol, nos.log.tmodel7$Sol,
nos.log.tmodel8$Sol,
                                   nos.log.tmodel9$Sol,
nos.log.tmodel10$Sol, nos.log.tmodel11$Sol, nos.log.tmodel12$Sol,
nos.log.tmodel13$Sol, nos.log.tmodel14$Sol, nos.log.tmodel15$Sol,
                                   nos.log.tmodel16$Sol,
nos.log.tmodel17$Sol, nos.log.tmodel18$Sol, nos.log.tmodel19$Sol,
nos.log.tmodel20$Sol)
plot(mcmc.list(nos.log.tmodel1$Sol[,1], nos.log.tmodel2$Sol[,1],
nos.log.tmodel3$Sol[,1]))

autocorr.diag(nos.log.t.MI.comb.VCV) #passes autocorrelation
heidel.diag(nos.log.t.MI.comb.VCV) #all passed

nos.log.t.MI.sol1 <- as.data.frame(nos.log.tmodel1$Sol[,1:5])
nos.log.t.MI.sol2 <- as.data.frame(nos.log.tmodel2$Sol[,1:5])
nos.log.t.MI.sol3 <- as.data.frame(nos.log.tmodel3$Sol[,1:5])
nos.log.t.MI.sol4 <- as.data.frame(nos.log.tmodel4$Sol[,1:5])
nos.log.t.MI.sol5 <- as.data.frame(nos.log.tmodel5$Sol[,1:5])
nos.log.t.MI.sol6 <- as.data.frame(nos.log.tmodel6$Sol[,1:5])
nos.log.t.MI.sol7 <- as.data.frame(nos.log.tmodel7$Sol[,1:5])
nos.log.t.MI.sol8 <- as.data.frame(nos.log.tmodel8$Sol[,1:5])
nos.log.t.MI.sol9 <- as.data.frame(nos.log.tmodel9$Sol[,1:5])
nos.log.t.MI.sol10 <- as.data.frame(nos.log.tmodel10$Sol[,1:5])
nos.log.t.MI.sol11 <- as.data.frame(nos.log.tmodel11$Sol[,1:5])
nos.log.t.MI.sol12 <- as.data.frame(nos.log.tmodel12$Sol[,1:5])
nos.log.t.MI.sol13 <- as.data.frame(nos.log.tmodel13$Sol[,1:5])
nos.log.t.MI.sol14 <- as.data.frame(nos.log.tmodel14$Sol[,1:5])
nos.log.t.MI.sol15 <- as.data.frame(nos.log.tmodel15$Sol[,1:5])
nos.log.t.MI.sol16 <- as.data.frame(nos.log.tmodel16$Sol[,1:5])
nos.log.t.MI.sol17 <- as.data.frame(nos.log.tmodel17$Sol[,1:5])
nos.log.t.MI.sol18 <- as.data.frame(nos.log.tmodel18$Sol[,1:5])
nos.log.t.MI.sol19 <- as.data.frame(nos.log.tmodel19$Sol[,1:5])
nos.log.t.MI.sol20 <- as.data.frame(nos.log.tmodel20$Sol[,1:5])
```

```
nos.log.t.MI.comb.sol1 <- rbind(nos.log.t.MI.sol1, nos.log.t.MI.sol2,
nos.log.t.MI.sol3, nos.log.t.MI.sol4, nos.log.t.MI.sol5, nos.log.t.MI.sol6,
nos.log.t.MI.sol7, nos.log.t.MI.sol8, nos.log.t.MI.sol9,
nos.log.t.MI.sol10,
                                nos.log.t.MI.sol11, nos.log.t.MI.sol12,
nos.log.t.MI.sol13, nos.log.t.MI.sol14, nos.log.t.MI.sol15,
nos.log.t.MI.sol16, nos.log.t.MI.sol17, nos.log.t.MI.sol18,
nos.log.t.MI.sol19,
                                nos.log.t.MI.sol20)
posterior.mode(nos.log.t.MI.comb.sol1)
hdi(nos.log.t.MI.comb.sol1, credMass=0.95)

#compare MI model results to original analysis model results
```

Appendix 4: Supplementary Material to Chapter 3

This appendix relates to Chapter 3: Changes in fitness over generations in captivity in conservation breeding programs.

A4.1 SUPPLEMENTARY TABLES

Table A4.1.1: Modelled results for offspring survival.
A) Results of offspring survival for the extended dataset analysis (*N* = 37,484 individuals) after model averaging (conditional average). B) Results of offspring survival for the extended dataset F_{2+} analysis when individuals with one or both wild-born parents are excluded (*N* = 27,734 individuals). Estimates presented are after model averaging (conditional average).

Predictor	Estimate	Adjusted SE	95% CI	RI
A) All offspring				
Intercept	0.5387	0.2039	0.1391, 0.9382	
Dam generation	-0.0367	0.0188	-0.0735, 0.0002	0.70
Sire generation	0.0350	0.0192	-0.0026, 0.0726	0.83
Dam age at breeding	-0.0755	0.0130	-0.1010, -0.0499	1
Sire age at breeding	0.0522	0.0133	0.0261, 0.0782	1
Dam *f*	0.0172	0.0134	-0.0090, 0.0435	0.32
Sire *f*	-0.0073	0.0135	-0.0337, 0.0192	0.21
Offspring *f*	-0.1526	0.0128	-0.1776, -0.1276	1
B) F_{2+}				
Intercept	0.5280	0.1814	0.1725, 0.8834	
Dam generation	-0.0741	0.0211	-0.1155, -0.0328	1
Sire generation	0.0585	0.0205	0.0182, 0.0988	1
Dam age at breeding	-0.1088	0.0148	-0.1378, -0.0797	1
Sire age at breeding	0.0735	0.0148	0.0445, 0.1026	1
Dam *f*	0.0163	0.0150	-0.0132, 0.0457	0.32
Sire *f*	0.0052	0.0152	-0.0245, 0.0349	0.19
Offspring *f*	-0.1884	0.0144	-0.2167, -0.1602	1

Table A4.1.2: Top model sets.

Top model set (top 2 AIC_C) of generalised linear mixed models for each of the five models where one offspring per litter/clutch has been selected. All parameters were retained in each of the five top model sets. Subset 3 was used as a representative model to fit random slopes.

Subset	Model statement	AIC_C	Δ_i[a]	w_i[b]
1	β_0 + Dam age + Sire age + Offspring *f*	27526.9		0.226
	β_0 + Sire generation + Dam age + Sire age + Offspring *f*	27528.1	1.20	0.124
	β_0 + Dam generation + Dam age + Sire age + Offspring *f*	27528.2	1.33	0.116
	β_0 + Dam age + Sire age + Sire *f* + Offspring *f*	27528.6	1.77	0.093
	β_0 + Dam age + Sire age + Dam *f* + Offspring *f*	27528.7	1.81	0.091
2	β_0 + Dam age + Sire age + Offspring *f*	27516.3		0.243
	β_0 + Sire generation + Dam age + Sire age + Offspring *f*	27517.6	1.29	0.127
	β_0 + Dam age + Sire age + Sire *f* + Offspring *f*	27518.3	1.95	0.091
	β_0 + Dam age + Sire age + Dam *f* + Offspring *f*	27518.3	1.97	0.090
	β_0 + Dam generation + Dam age + Sire age + Offspring *f*	27518.3	1.99	0.090
3	β_0 + Dam age + Sire age + Offspring *f*	27527.7		0.224
	β_0 + Sire generation + Dam age + Sire age + Offspring *f*	27528.7	0.99	0.136
	β_0 + Dam generation + Dam age + Sire age + Offspring *f*	27529.3	1.61	0.100
	β_0 + Dam age + Sire age + Dam *f* + Offspring *f*	27529.5	1.81	0.091
	β_0 + Dam age + Sire age + Sire *f* + Offspring *f*	27529.5	1.81	0.090
4	β_0 + Sire generation + Dam age + Sire age + Offspring *f*	27559.7		0.207
	β_0 + Dam age + Sire age + Offspring *f*	27560.7	0.97	0.127
	β_0 + Sire generation + Dam age + Sire age + Sire *f* + Offspring *f*	27561.4	1.69	0.089
	β_0 + Dam age + Sire age + Sire *f* + Offspring *f*	27561.6	1.87	0.081
	β_0 + Dam generation + Sire generation + Dam age + Sire age + Offspring *f*	27561.7	1.96	0.078
5	β_0 + Sire generation + Dam age + Sire age + Offspring *f*	27514.2		0.175
	β_0 + Dam age + Sire age + Offspring *f*	27514.3	0.07	0.169
	β_0 + Dam age + Sire age + Sire *f* + Offspring *f*	27515.6	1.31	0.091
	β_0 + Dam generation + Dam age + Sire age + Offspring *f*	27515.8	1.51	0.082
	β_0 + Sire generation + Dam age + Sire age + Sire *f* + Offspring *f*	27516.0	1.79	0.072
	β_0 + Dam generation + Sire generation + Dam age + Sire age + Offspring *f*	27516.2	1.94	0.066
	β_0 + Sire generation + Dam age + Sire age + Dam *f* + Offspring *f*	27516.2	1.98	0.065

[a] Change in AIC_C from the best model.

[b] Akaike model weight.

A4.2 SUPPLEMENTARY FIGURES

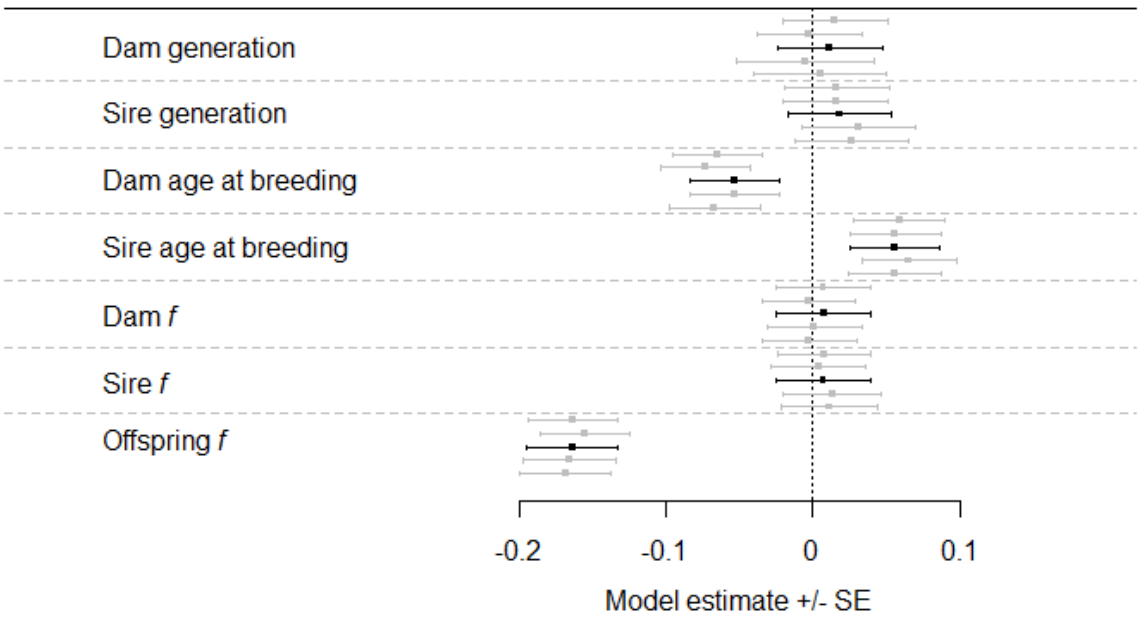

Figure A4.2.1: Model estimates for offspring survival analysis with five random subsets. Estimates (square +/- SE interval) using the five random selections of one offspring per litter/clutch (*N* = 21,282 individuals). The third subset (black) was selected as the representative dataset to model random slopes.

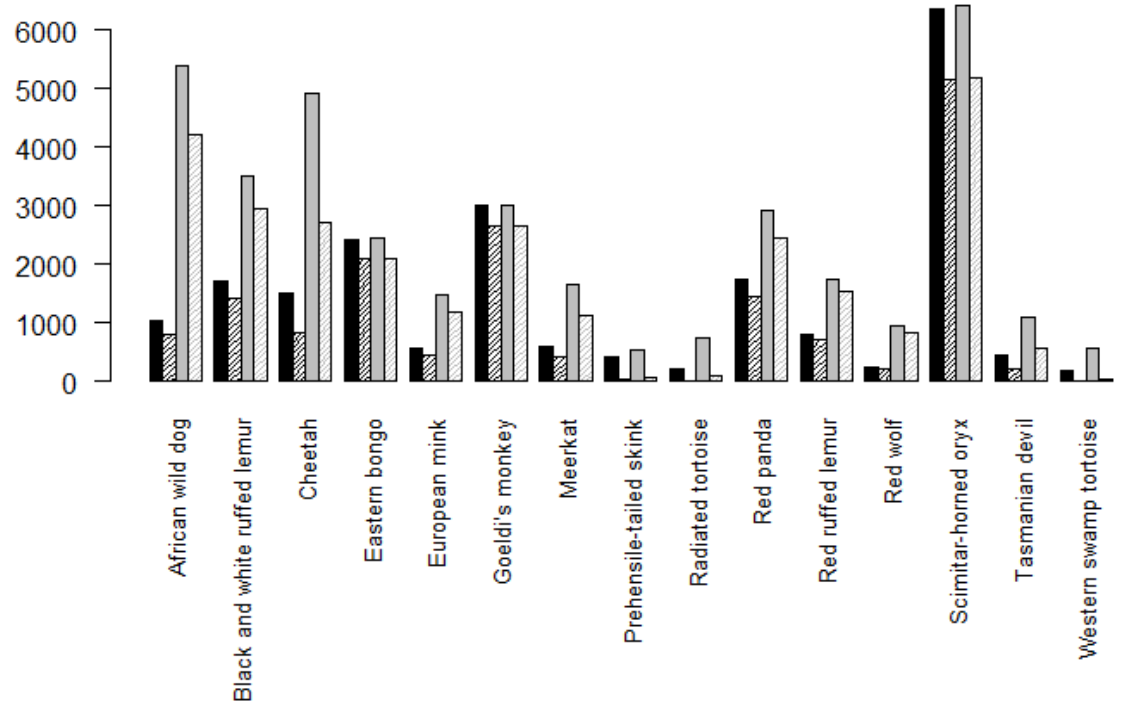

Figure A4.2.2: Number of each species in each dataset.
Number of individuals of each species in the main random litter-mate analysis (black, total *N* = 21,282), representative random litter-mate F_{2+} analysis (black hatched, total *N* = 16,516), extended dataset model (grey, total *N* = 37,484), and extended dataset F_{2+} model (grey hatched, total *N* = 27,734).

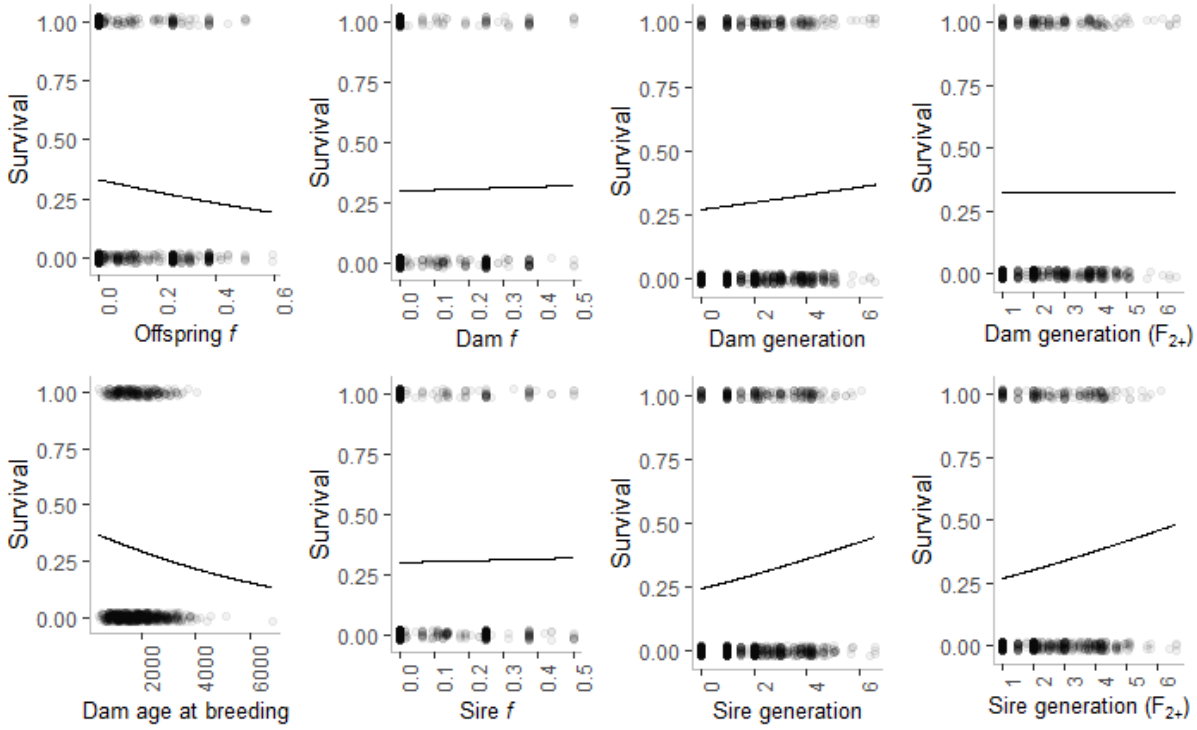

Figure A4.2.3: Random slope estimates for African wild dog.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

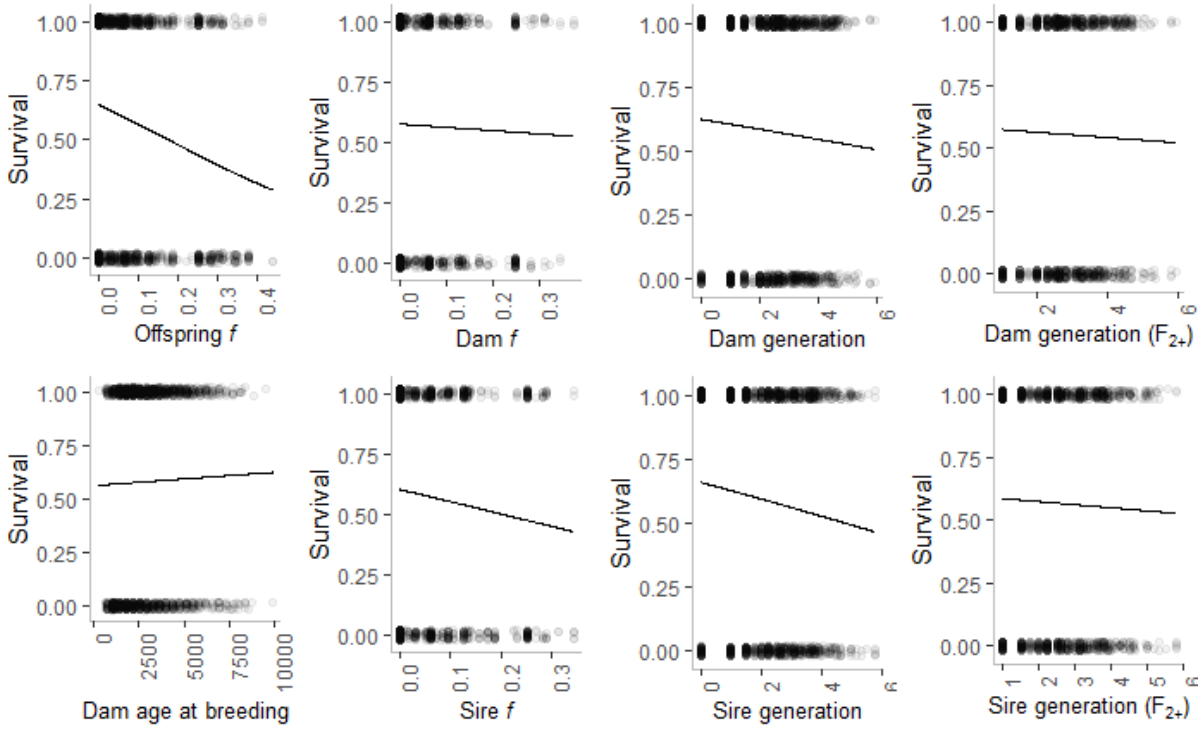

Figure A4.2.4: Random slope estimates for black-and-white ruffed lemur.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

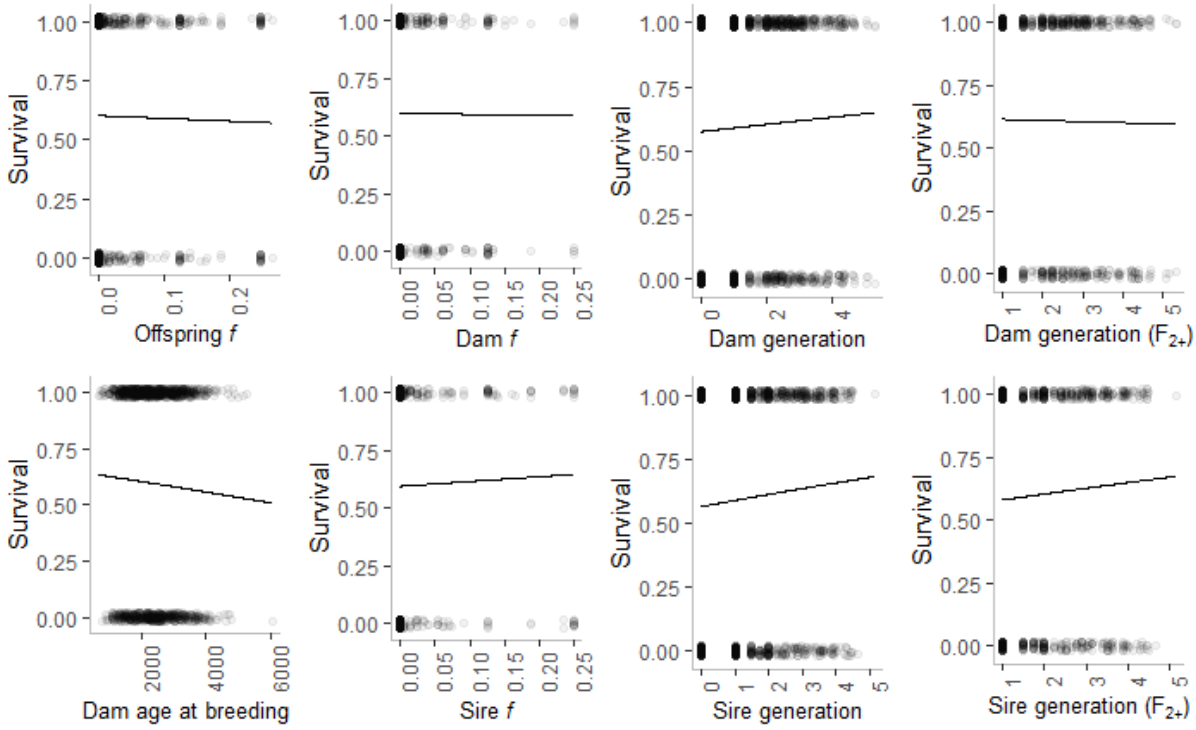

Figure A4.2.5: Random slope estimates for cheetah.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

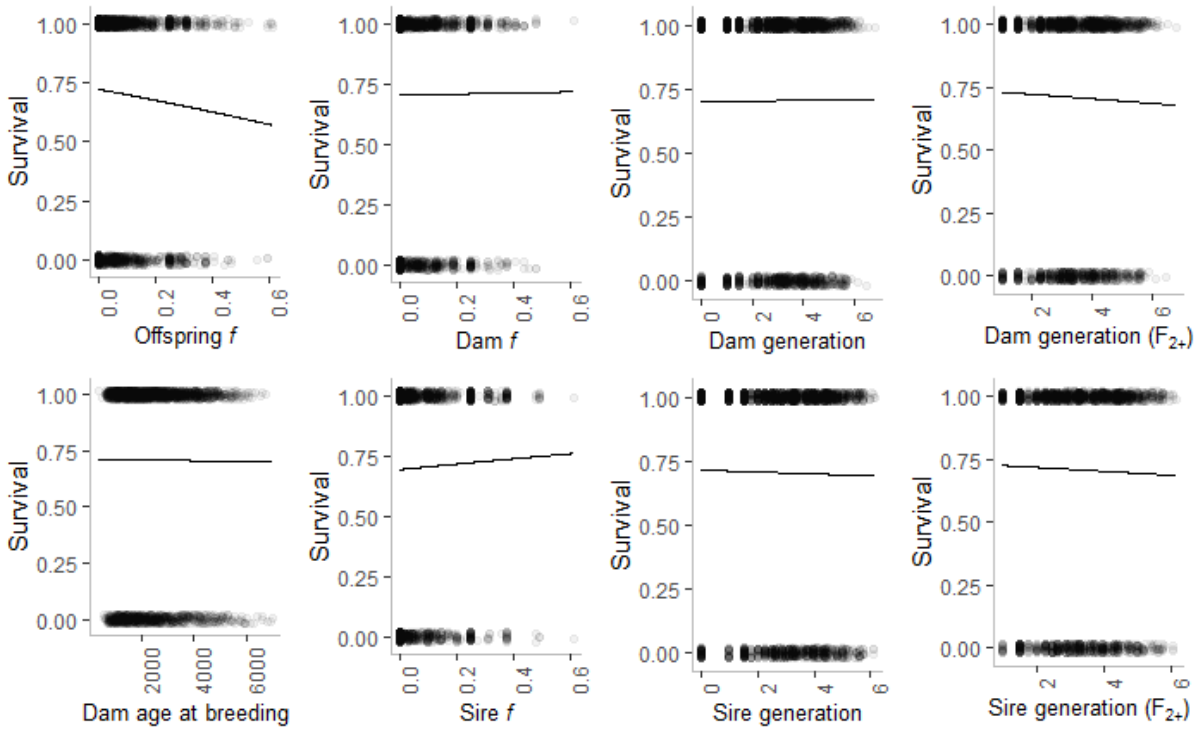

Figure A4.2.6: Random slope estimates for Eastern bongo.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

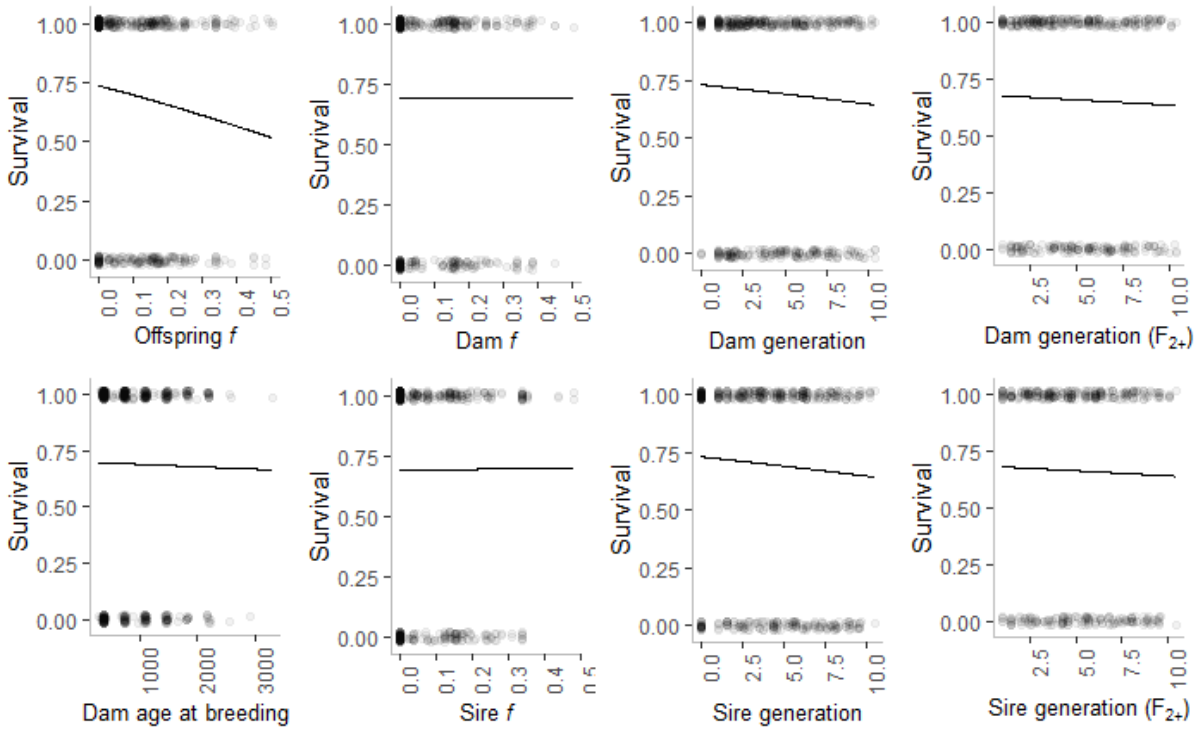

Figure A4.2.7: Random slope estimates for European mink.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

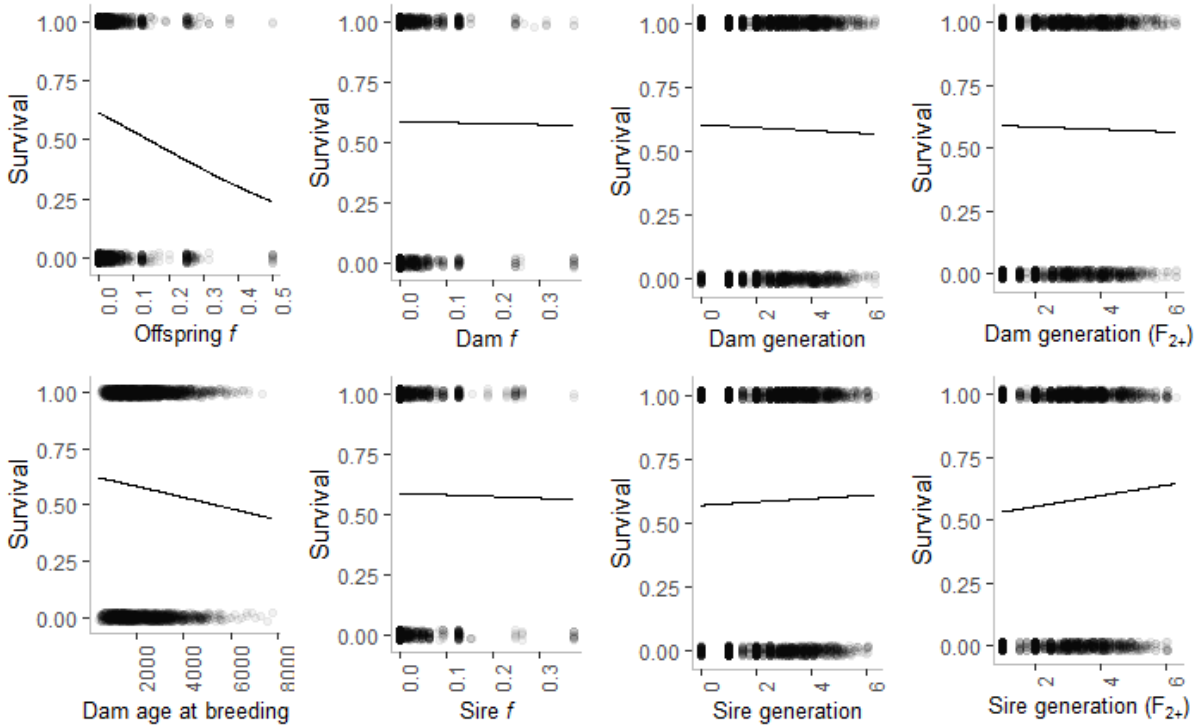

Figure A4.2.8: Random slope estimates for Goeldi's monkey.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

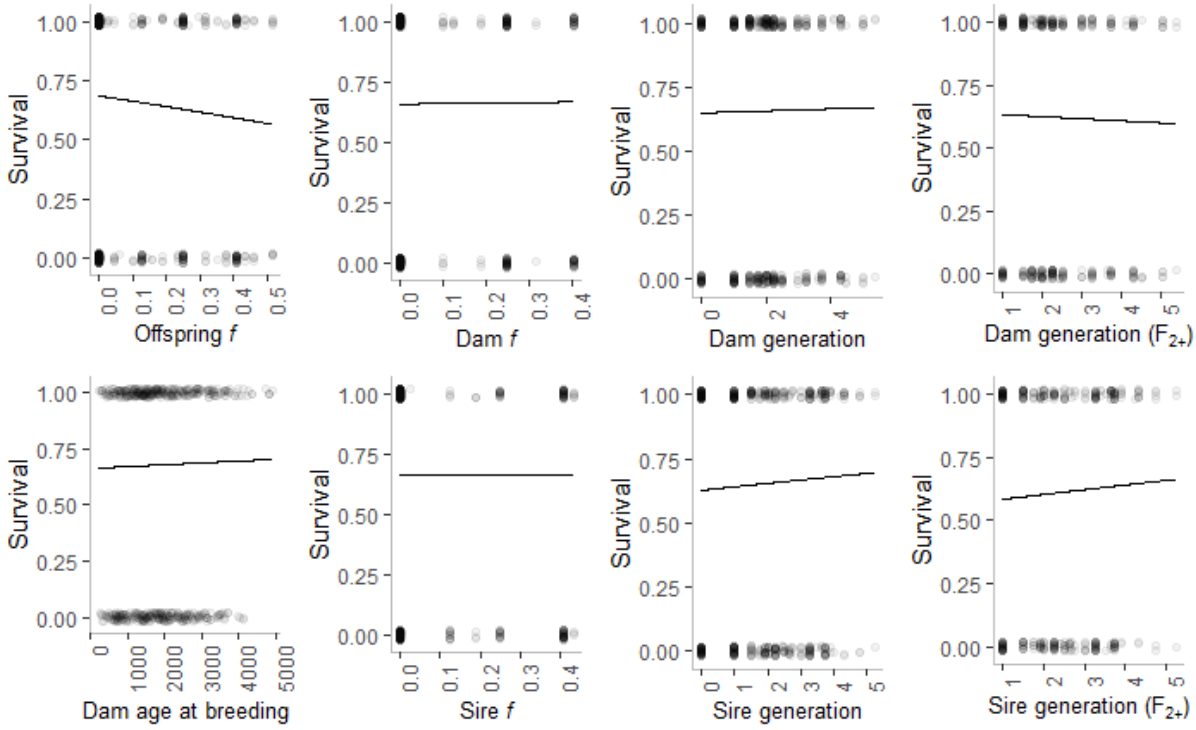

Figure A4.2.9: Random slope estimates for meerkat.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

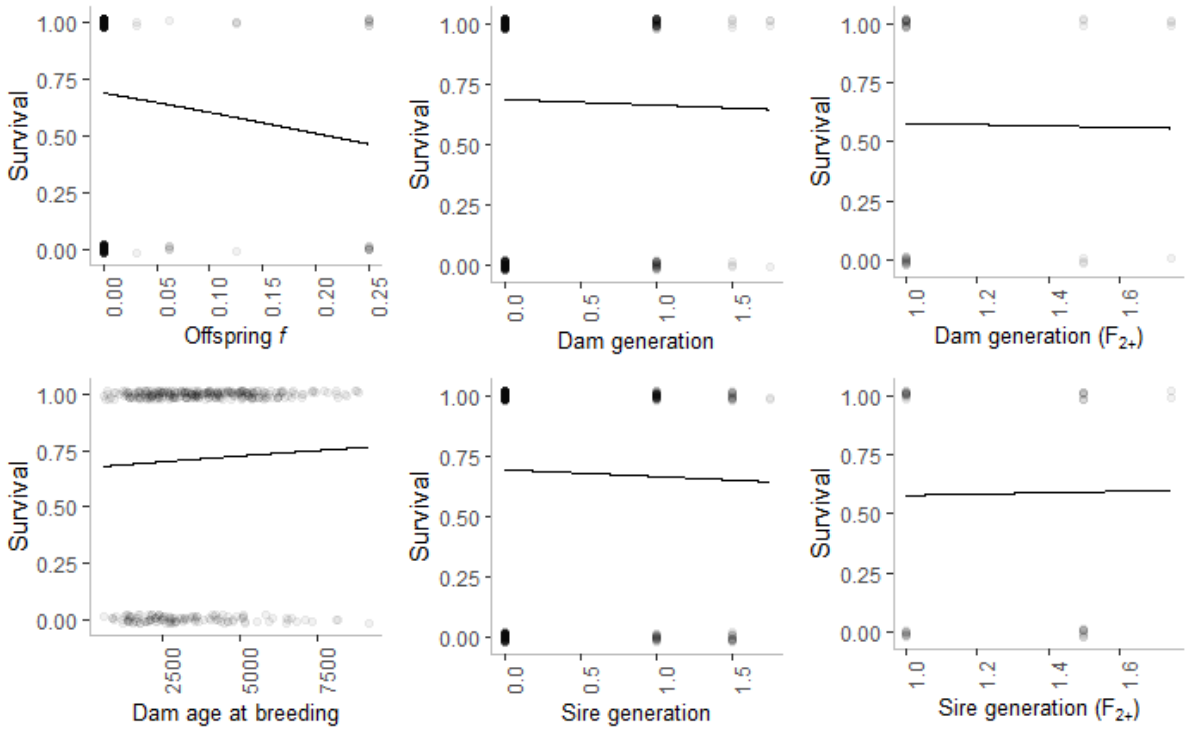

Figure A4.2.10: Random slope estimates for prehensile-tailed skink.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density. There was not enough variation in dam or sire inbreeding to fit random slopes for these parameters (Table 3.1).

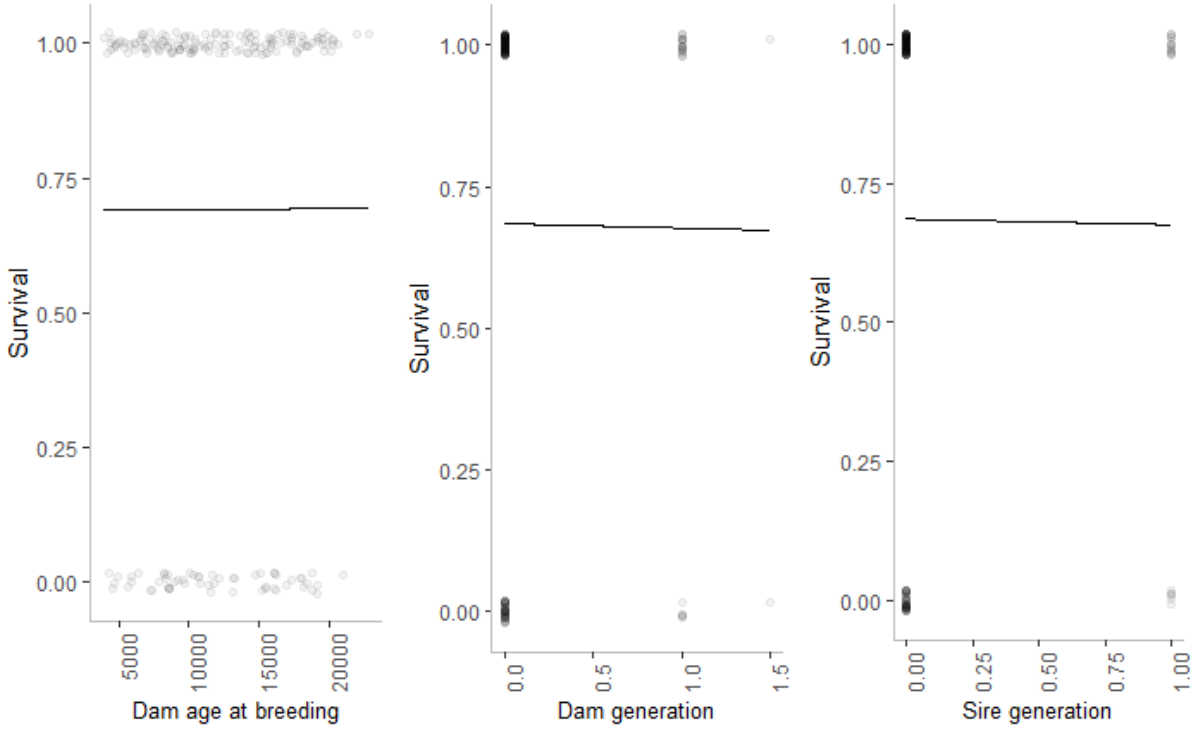

Figure A4.2.11: Random slope estimates for radiated tortoise.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282). Points represent raw data, shaded by density. There was not enough variation in dam sire or offspring inbreeding to fit random slopes for these parameters, nor were there F_{2+} offspring (Table 3.1).

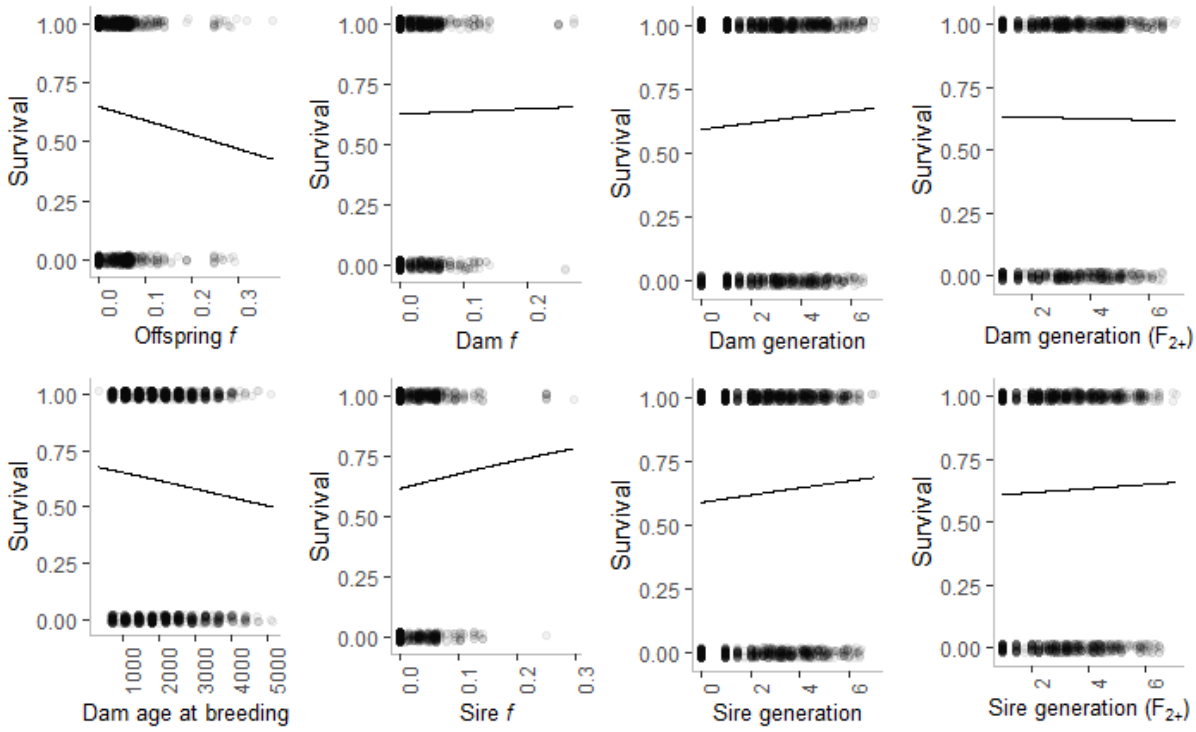

Figure A4.2.12: Random slope estimates for red panda.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

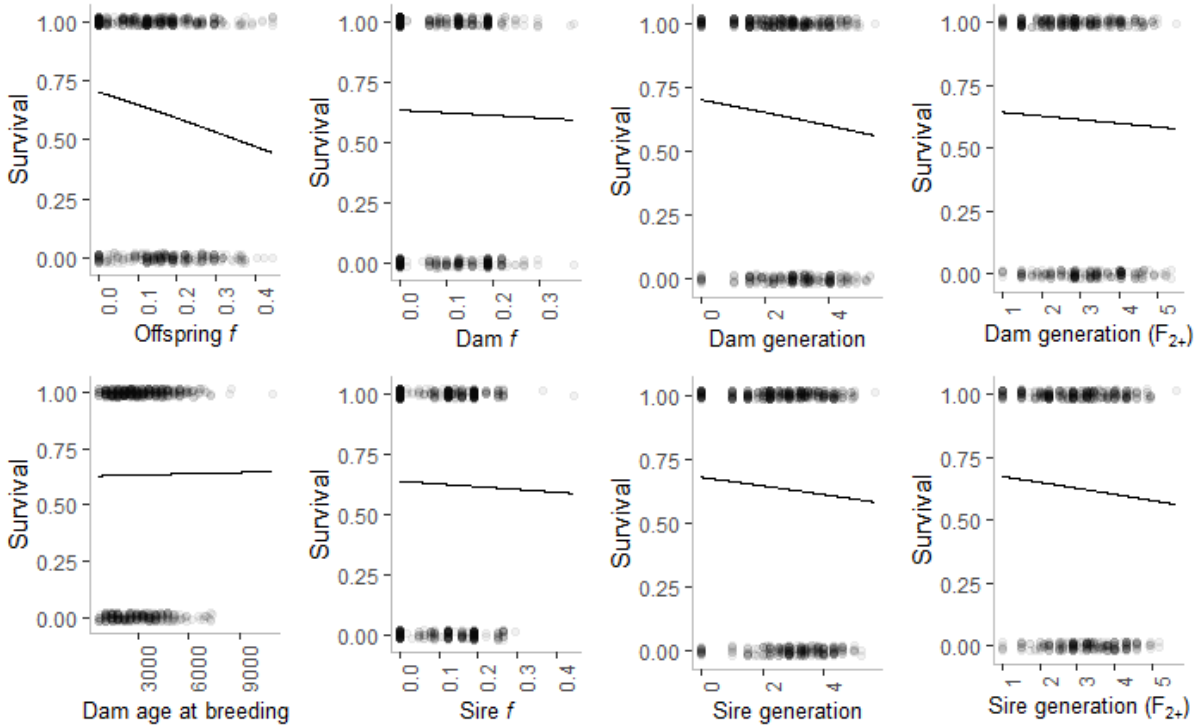

Figure A4.2.13: Random slope estimates for red ruffed lemur.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

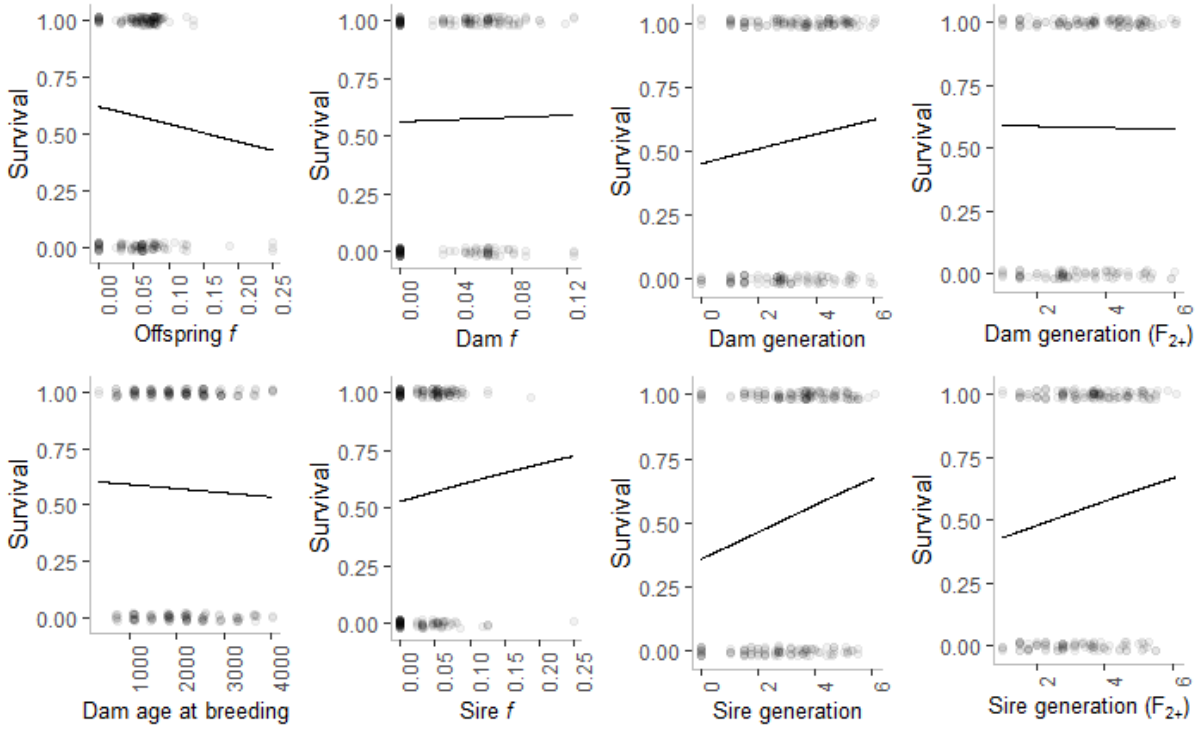

Figure A4.2.14: Random slope estimates for red wolf.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

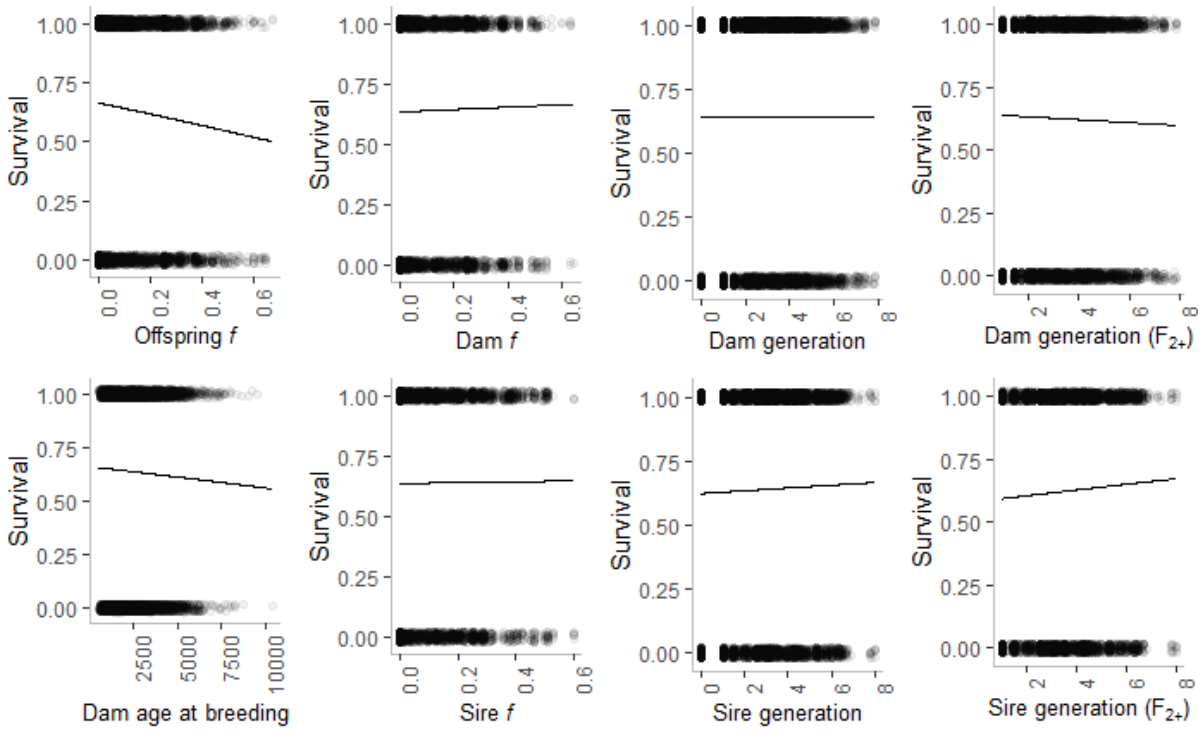

Figure A4.2.15: Random slope estimates for scimitar-horned oryx.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

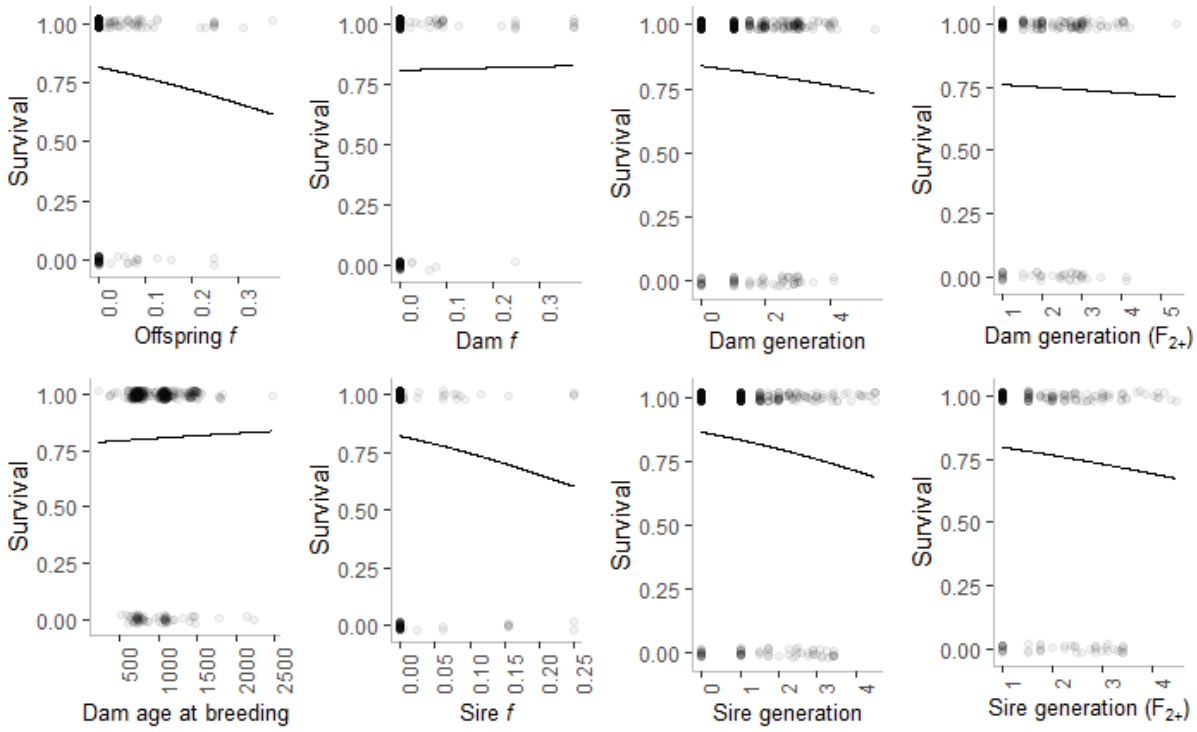

Figure A4.2.16: Random slope estimates for Tasmanian devil.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282), or the dataset with only one individual per litter/clutch and F_{2+} offspring only (*N* = 16,516) for dam and sire generation F_{2+}. Points represent raw data, shaded by density.

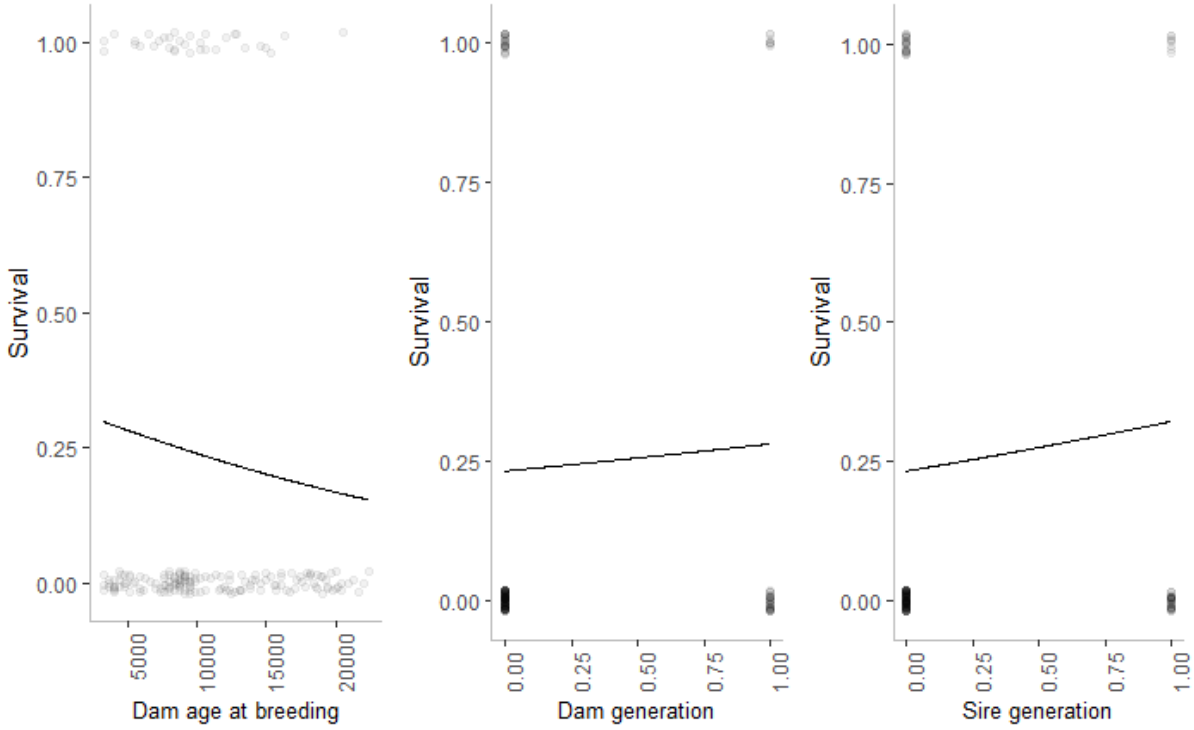

Figure A4.2.17: Random slope estimates for Western swamp tortoise.
Random slopes estimated using a dataset with only one individual per litter/clutch (*N* = 21,282). There was not enough variation in dam sire or inbreeding to fit random slopes for these parameters, nor were there F_{2+} offspring (Table 3.1).

Appendix 5: Supplementary Material to Chapter 4

This appendix relates to Chapter 4: From reference genomes to population genomics: comparing three reference-aligned reduced representation sequencing pipelines in two wildlife species.

A5.1 SUPPLEMENTARY METHODS

Tasmanian devil reduced-representation sequencing

In response to concerns about the persistence of the devil in the wild, a captive insurance population was established in 2006 with the intake of 122 founders from across Tasmania (Hogg *et al.*, 2015). Due to the progression of the disease, founding devils were only obtained from limited locations on the east coast or from the north-western region of Tasmania, causing distinct population structuring among the founding individuals (Hogg *et al.*, 2015). To overcome concerns of potential inbreeding, founders were regularly paired with individuals from the opposing provenance resulting in mixed lineages among the insurance population (Hogg *et al.*, 2015). The insurance population now consists of over 700 devils across 37 zoo-based facilities and free-range enclosures, one island (Maria Island) and a fenced peninsula (Forestier Peninsula) (Hogg *et al.*, 2017b). For this study, we selected 131 Tasmanian devil samples from our genomic DNA database, including 65 wild-caught individuals of both eastern and western origins, and 66 captive-born individuals from the Tasmanian devil insurance population with mixed lineages (Hogg *et al.*, 2015). The wild-caught individuals included here were a subset of those used to establish the insurance population (i.e. population "founders"), for which sufficient archival DNA was available for analysis.

Ear biopsies in 70% ethanol, or 1 mL whole blood in EDTA, have been collected from Tasmanian devils by the Save the Tasmanian Devil Program, or participating zoos, for management purposes since the commencement of the insurance population. These archival samples varied in quality, assessed visually via gel electrophoresis (see below). DNA was extracted using either a modified phenol/chloroform protocol (Sambrook & Russell, 2006) or commercial DNA extraction kit (Qiagen DNeasy Blood & Tissue Kit). DNA sample quality was assessed using a NanoDrop to measure DNA concentration, and by visualisation via agarose gel (0.8%, 90 V for 30 minutes) to measure concentration and fragmentation. Extractions

were scored from 1 - 8, with a strong, clear band on the gel given a ranking of 1 - 2 'high quality', 3 - 4 is a moderate-strength band, 5 - 6 is a weak fragmented 'poor quality' band and 7 - 8 is no evidence of a band or DNA 'very poor quality' (see Figure A5.3.5a for examples). Of our 131 unique samples, 26 (19.8%) were rated 'high' quality, 49 (28.6%) 'moderate' quality, and 56 (32.6%) 'poor' quality. None were rated 'very poor'. There was no clear trend in sample quality vs. sequencing quality, measured as proportion of missing data/total SNPs (Figure A5.3.5b-d) across the three pipelines.

Of the many RRS techniques available, DArTseq™ (Diversity Arrays Technology Pty Ltd, hereafter DArT PL) is particularly well-used in Australia for varied applications including management of selective breeding programs, genetic mapping, and population genetics studies (Ren *et al.*, 2015; Lambert *et al.*, 2016; Baloch *et al.*, 2017). Initially developed for use in commercial crop species, the approach has since been applied to diverse wildlife including mammals (Schultz *et al.*, 2018), reptiles (Melville *et al.*, 2017), amphibians (Lambert *et al.*, 2016), and fish (Donnellan *et al.*, 2015; DiBattista *et al.*, 2017; Pazmiño *et al.*, 2017). The restriction enzyme combination used by DArT PL for our dataset was PstI-SphI, with fragments sequenced on a HiSeq 2500 as 77-bp single-end reads. DArT PL also performed technical sample replicates, resulting in raw sequences from 166 samples for analysis. Following sequencing, DArT PL returns results from their proprietary data filtering pipeline, DArTSoft14, as a Microsoft Excel spreadsheet. Recently, the dartR package (Gruber *et al.*, 2018) in R (R Core Team, 2018) has been developed for filtering and analysis of the DArT PL spreadsheets. In this study however, we processed the raw sequencing reads also provided by DArT PL.

Raw data were processed using the 'process_radtags' module of Stacks v2.0b (Catchen *et al.*, 2013) with the flags --disable_rad_check and --inline_null (as a single inline barcode was used) to remove barcodes (4bp - 8bp) for each sequencing lane, check for adapter contamination and clean data of reads containing uncalled bases. We performed checks of the log files to ensure all samples had a reasonable number of reads. Cleaned reads were then investigated using FastQC (Andrews, 2010) to visually check for sequencing errors and to determine if reads needed to be trimmed. These cleaned reads were used as input for further processing and analysis in all three pipelines.

A5.2 SUPPLEMENTARY TABLES

Table A5.2.1: Summary statistics for the resultant SNP loci datasets of three pipelines.
Data filtered less stringently at a higher allowable missing data (30% call rate; *cf* Table 4.1), for Tasmanian devil (*N* = 131) and pink-footed goose (*N* = 40), including the total number of loci (total loci), the average number of loci sequenced across individuals (mean loci), the amount of missing data (%), the calculated error rates (%), the mean observed heterozygosity across loci (H_O), the mean expected heterozygosity across loci (H_E), and the average multilocus heterozygosity of individuals (MLH).

Dataset	Pipeline	Total loci	Mean loci (min; max)	% missing	Error rate[1] (%)	H_O (± SD)	H_E (± SD)	MLH (± SD)
Devils	Stacks	2,537	1,773.4 (680; 2,186)	30.1	1.9	0.215 (0.168)	0.260 (0.167)	0.208 (0.042)
	SAMtools	786	479.4 (172; 569)	39.0	5.8	0.342 (0.186)	0.357 (0.121)	0.328 (0.090)
	GATK	2,450	1,834.8 (682; 2,192)	25.1	4.9	0.163 (0.147)	0.251 (0.163)	0.167 (0.033)
Geese	Stacks	139,979	86,844.2 (1,616; 128,458)	38.0	NA	0.163 (0.157)	0.207 (0.153)	0.134 (0.036)
	SAMtools	146,599	79,440.6 (3,407; 109,403)	45.8	NA	0.290 (0.181)	0.351 (0.134)	0.286 (0.117)
	GATK	601,707	412,978.5 (10,584; 553,941)	31.4	NA	0.137 (0.125)	0.216 (0.155)	0.126 (0.041)

[1] Error rates could not be calculated for the pink-footed goose dataset as no replicates were included in the current analysis. Error rate is calculated after filtering on SNPs with > 85% reproducibility, so is lower than initial error rates.

A5.3 SUPPLEMENTARY FIGURES

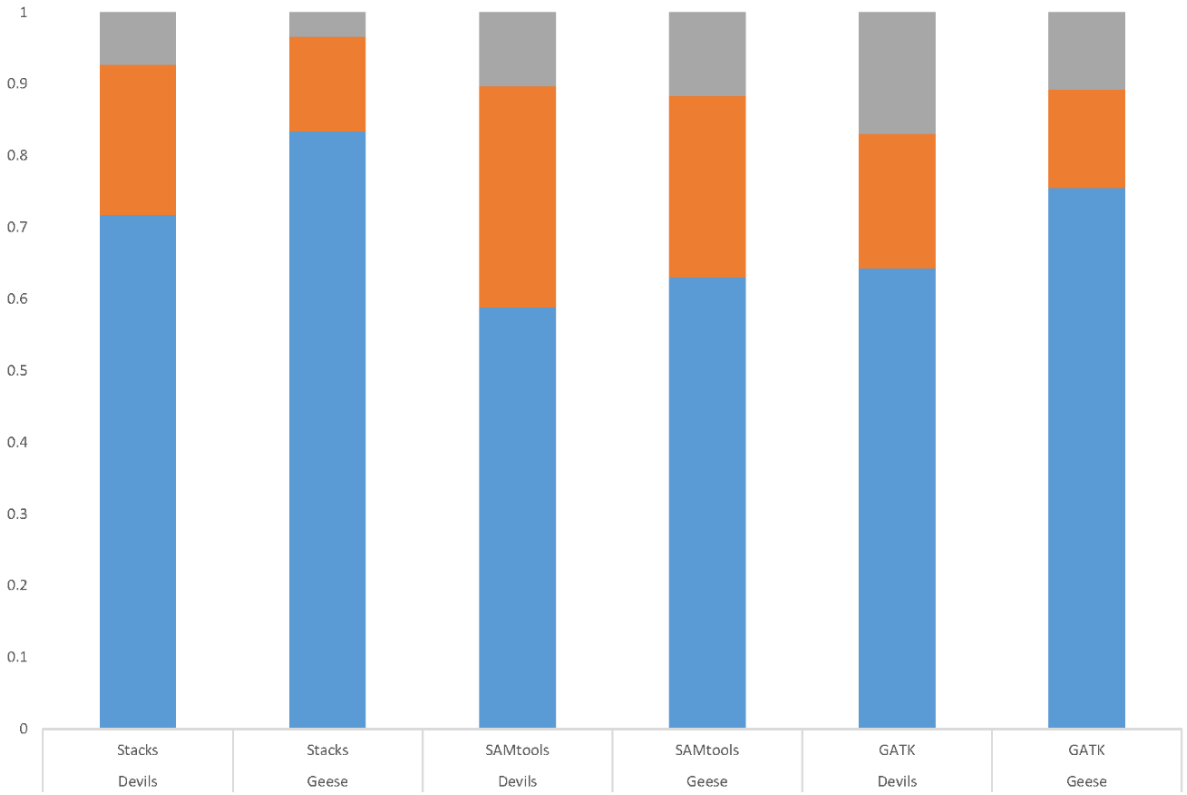

Figure A5.3.1: Ratios of genotype calls between the three different pipelines for devils and geese.
Blue indicates the most frequent homozygotes, orange indicates heterozygotes and grey the least frequent homozygotes. Note that SAMtools and GATK are able to report those homozygotes that match the reference or alternate allele but Stacks assigns the most frequent allele as the reference allele, hence how these genotypes are referred to here.

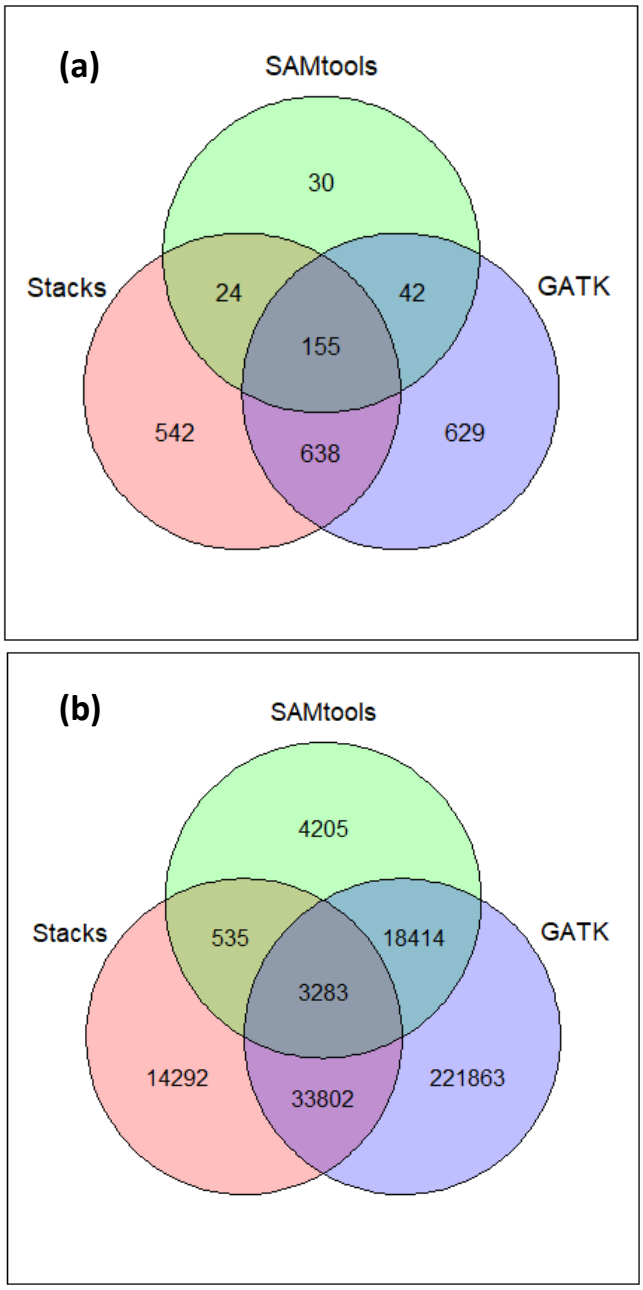

Figure A5.3.2: Venn diagram depicting number of shared loci between the three different pipelines for (a) devil and (b) goose.

Figure A5.3.3: PCoA of the devil dataset only for the three pipelines, considering all three populations.
"West" (red) and "east" (blue) are the wild-born founding individuals (*N* = 65). "IP" (green) are the captive-born insurance population individuals. Row one shows data processed with a call rate of 70%, row two shows data processed less stringently with a call rate of 30%. Inertia ellipses illustrate groupings and do not necessarily indicate confidence.

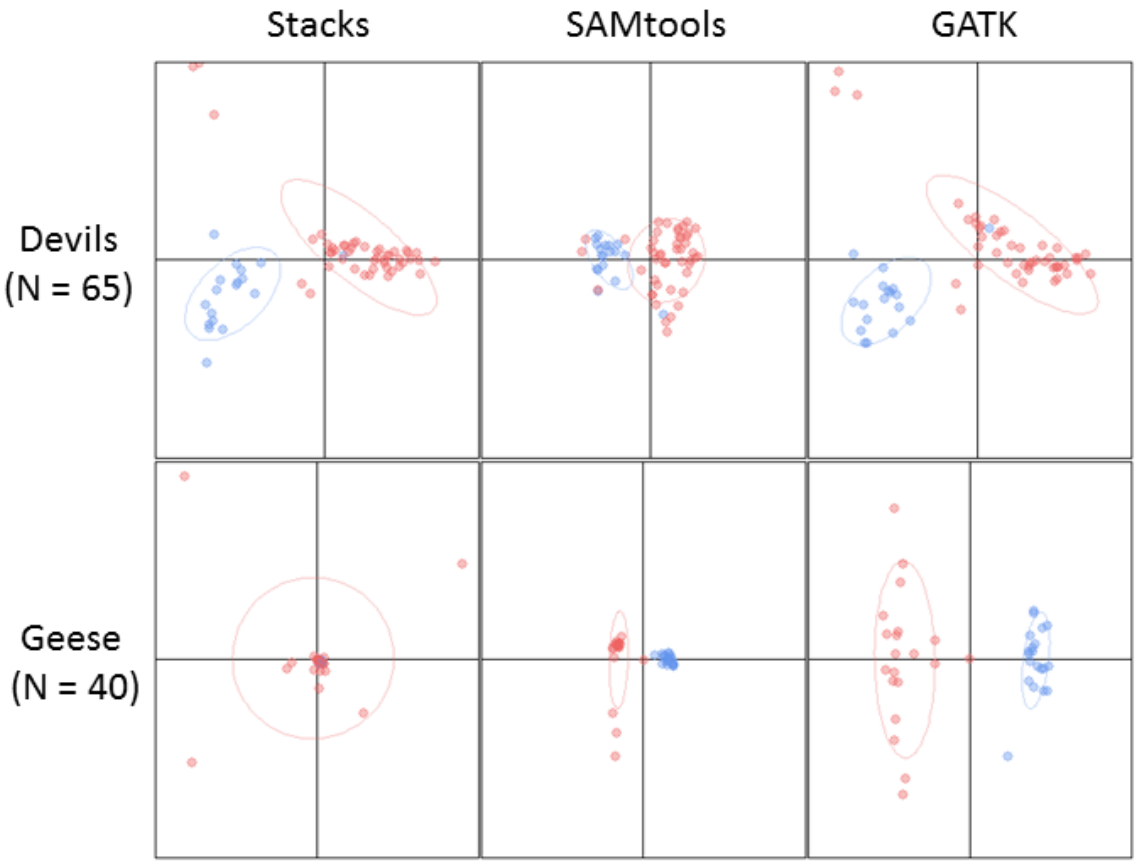

Figure A5.3.4: PCoAs of the two datasets after processing through three pipelines.
Data filtered less stringently, allowing more missing data (30% call rate). For devils, red is the "west" and blue is the "east" population. For goose, red is the "Iceland" and blue is the "Denmark" population. Inertia ellipses illustrate groupings and do not necessarily indicate confidence.

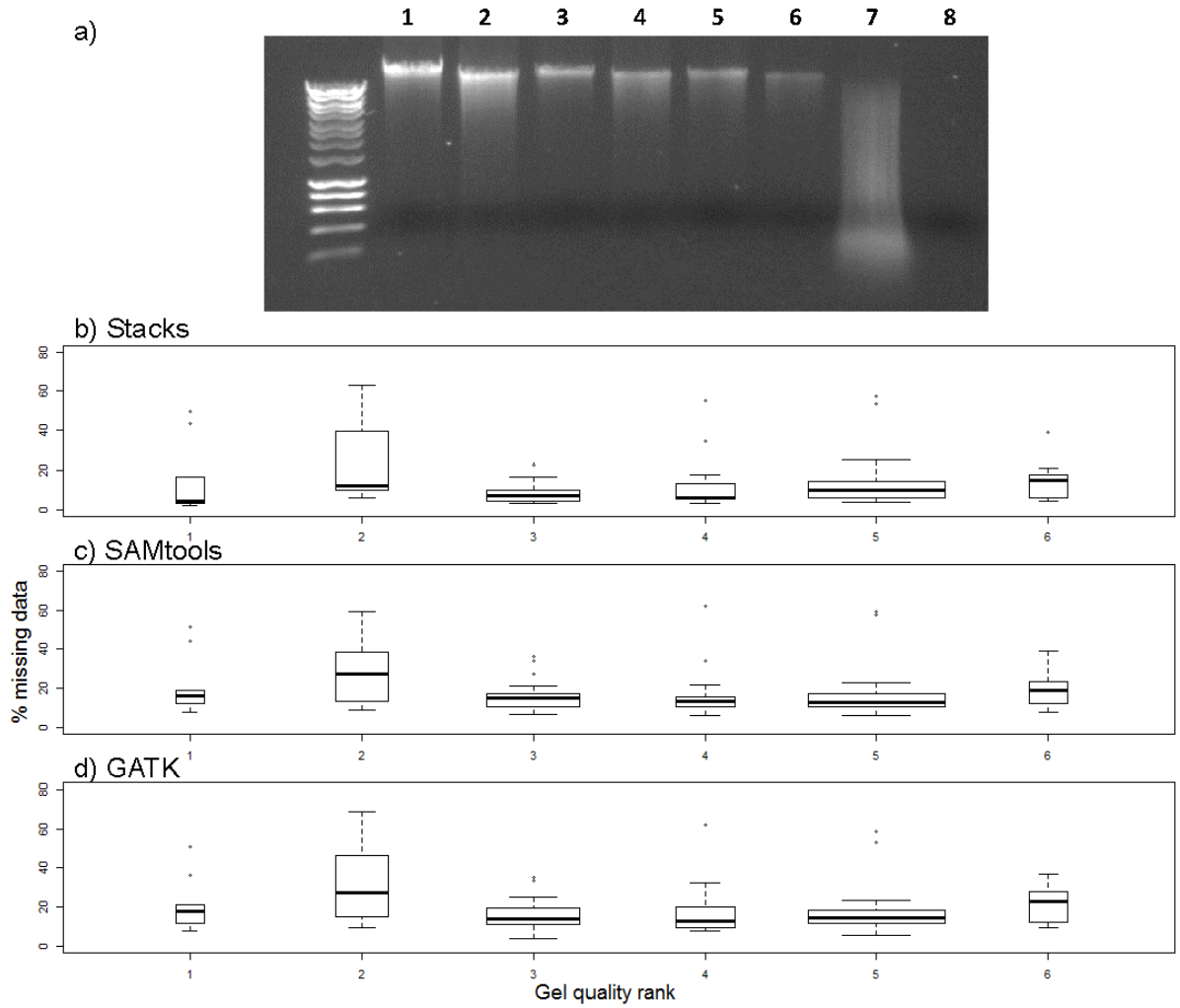

Figure A5.3.5: Gel quality vs. missing data.
a) Gel image example of sample quality from 1 (highest) to 8 (no apparent DNA); b) - d) Gel quality rank (rank 7 and 8 not included as too low quality to include in study) vs. the amount of missing data of a sample for the b) Stacks, c) SAMtools and d) GATK pipelines. Boxplots are scaled with the width proportional to the number of samples within the gel quality rank (range 9 to 44).

Appendix 6: Supplementary Code to Chapter 4

This appendix relates to Chapter 4: From reference genomes to population genomics: comparing three reference-aligned reduced representation sequencing pipelines in two wildlife species.

The following R code has been published alongside the article presented in Chapter 4 as the custom R script to process reduced representation sequencing SNPs. The R script contains functions to read in VCF files; filter on allelic depth, coverage difference, call rate, minor allele frequency, heterozygosity, reproducibility; identify possible sex-linked SNPs; and calculate error rates post-filtering. Comments are annotated with #.

```
#This document has been created to process reduced representation
sequencing output in VCF format (e.g. from Stacks/2.0b pipeline with output
generated from the "populations" module specified with the -vcf flag to
obtain populations.snps.vcf file). We provide code to reproduce metrics
provided by DArT PL (DArTseq) from user-processed data for downstream
filtering.

#For information about the vcfR package in R, see online
[tutorial](https://knausb.github.io/vcfR_documentation/index.html) from
package creators. Also see this [pdf](https://samtools.github.io/hts-
specs/VCFv4.2.pdf) for how to interpret vcf format.

#Set working directory first.

rm(list=ls())

#Need to install "vcfR" package in R if not already installed. Once
installed, load package:

library(vcfR)

##Read in data
#Read in and check populations.snps.vcf file:

vcf <- read.vcfR("populations.snps.vcf")
head(vcf)
queryMETA(vcf)

#The vcf file has a format with three sections:
#1. Meta information - defines the abbreviations used elsewhere in the
file.
#2. Fixed information - first 8 columns, contain chromosomal position & ID,
REF and SNP allele, and SNP quality info:
#  + allelic depths for the ref and alt alleles in the order listed (AD)
#  + allele frequency (AF)
#  + combined depth across samples (DP)
#  + number of samples with data (NS)
```

```
#3. Genotype information - one column per sample, includes:
#  + genotype encoded as alleles separated by /. Alleles are "0" for the
reference allele, "1" for alternate, or "." if missing (GT). Heterozygote :
0/1
#  + read depth at this position for this sample (DP)
#  + haplotype qualities (HQ)
#  + genotype likelihoods (GL)
#  + conditional genotype quality (GQ)

#Reformat the dataset to obtain SNP data by combining the chromosome
position information and the genotype information in a new object.
'extract.gt' function extracts genotype information, 'getFIX' function
extracts fixed data for each row.

gt <- extract.gt(vcf, IDtoRowNames = F)
fixed <- getFIX(vcf)
snps <- cbind(fixed[,1:5], gt)
head(snps)[,1:10]
snps.1 <- as.data.frame(as.matrix(snps))
snps.1$CHROM <- as.character(as.factor(snps.1$CHROM))
snps.1$POS <- as.character(as.factor(snps.1$POS))
snps.1$ID <- as.character(as.factor(snps.1$ID))
snps.1$identifier <- with(snps.1, paste0(CHROM, POS, ID))
snps.3 <- snps.1[,1:(ncol(snps.1)-1)]

##Data filtering

#Our raw data has been filtered in Stacks/2.0b "populations" module with:
#  * __-r 0.70__ (must be sequenced at >= 70% of samples in a population to
process loci). This is equivalent to "CallRate" column in DArTseq filtered
data, although it is calculated including technical replicates in Stacks
data.
#  * __--min_maf 0.01__
#  * __--max_obs_het 0.70__
#  * __--write_random_snp__ (restrict data analysis to one random SNP per
locus) - filters linked SNPs. However, any filtering after this step in R
uses data already reduced to one SNP per locus, not necessarily the best
SNP in the locus if further filtering is needed, so better to change
filtering parameters in Stacks rather than using R post-Stacks.

####Read Depth
#Filter on read depth to remove SNPs with low read depth at ref and/or alt
allele. This is equivalent to the AvgCountRef and AvgCountSNP columns in
the DArT data.
#This step can be time consuming with many samples.

read.depth <- extract.gt(vcf, element="AD")
length(unique(rownames(read.depth)))
nrow(read.depth)
read.depth.ref <- masplit(read.depth, record = 1, sort=0)
read.depth.snp <- masplit(read.depth, record = 2, sort=0)

#for reference allele coverage:
head(read.depth.ref)[1:6,1:6]
read.depth.ref.count<- rowSums(read.depth.ref, na.rm=T)
head(read.depth.ref.count)
```

```
read.depth.ref <- as.data.frame(read.depth.ref)
read.depth.ref$length <- rep(NA)

n <- ncol(read.depth.ref)-1
for (r in 1:nrow(read.depth.ref)) {
  read.depth.ref$length[r] <- length(which(read.depth.ref[r,1:n] !=0))
}

read.depth.ref.avg <- read.depth.ref.count/read.depth.ref$length
head(read.depth.ref.avg)
summary(read.depth.ref.avg)

#for snp allele coverage:
read.depth.snp.count<- rowSums(read.depth.snp, na.rm=T)
head(read.depth.snp.count)

read.depth.snp <- as.data.frame(read.depth.snp)
read.depth.snp$length <- rep(NA)

n2 <- ncol(read.depth.snp)-1
for (r in 1:nrow(read.depth.snp)) {
  read.depth.snp$length[r] <- length(which(read.depth.snp[r,1:n2] !=0))
}

read.depth.snp.avg <- read.depth.snp.count/read.depth.snp$length
summary(read.depth.snp.avg)

#We have selected a minimum read depth of 2.5 for our data. Histograms can
be used to visualise appropriate thresholds for other types of data.

length(which(read.depth.ref.avg > 2.5))
length(which(read.depth.snp.avg > 2.5))

coverage.rd <- cbind(read.depth.ref.avg, read.depth.snp.avg)
coverage.rd <- as.data.frame(coverage.rd)
coverage.rd$snp.index <- 1:nrow(coverage.rd)
coverage.rd1 <- coverage.rd[which(coverage.rd$read.depth.ref.avg > 2.5),]
nrow(coverage.rd)
nrow(coverage.rd1)
coverage.rd2 <- coverage.rd1[which(coverage.rd1$read.depth.snp.avg > 2.5),]
nrow(coverage.rd2)

par(mfrow=c(2,2))
hist(read.depth.ref.avg, main="Read depth of ref allele", xlab="Read
depth")
hist(read.depth.snp.avg, main="Read depth of snp allele", xlab="Read
depth")
hist(coverage.rd2$read.depth.ref.avg, main="Read depth of ref allele >
2.5", xlab="Read depth")
hist(coverage.rd2$read.depth.snp.avg, main="Read depth of snp allele >
2.5", xlab="Read depth")

index <- 1:nrow(snps.3)
snps.index <- cbind(index, snps.3)
snps.rd <- snps.index[which(snps.index[,1] %in% coverage.rd2$snp.index),]
nrow(snps.rd)
```

```
####Coverage
#We also need to filter by __coverage__. If the reference and SNP allele do
not amplify at the same rate, this may indicate potential bias (errors in
calling). DArT PL includes AvgCountRef as the sum of the tag read counts
for all samples, divided by the number of samples with non-zero tag read
counts for the reference allele row, AvgCountSNP is the same but for the
SNP allele row.
#Coverage can be calculated as the absolute percentage difference between
the AvgCountRef and AvgCountSNP columns. vcf output gives Allele Depth,
with coverage of reference, then coverage of SNP allele e.g. AD = 4,3 means
reference allele has coverage = 4, snp allele coverage = 3. Count only non-
zero reads (already filtered out anyway from above).

#Need to extract AD (allelic depth) for each sample and loci.

coverage.rd2$max <- pmax(coverage.rd2$read.depth.ref.avg,
coverage.rd2$read.depth.snp.avg)
coverage.rd2$diff <- ((abs(coverage.rd2$read.depth.ref.avg -
coverage.rd2$read.depth.snp.avg))/(coverage.rd2$max))*100
hist(coverage.rd2$diff, main="Coverage difference", xlab="% diff in
coverage")

#Histogram can be used to determine appropriate thresholds for filtering.
We aim for SNPs with a low coverage difference. The following check will
give an idea as to how many SNPs will be retained with varying coverage
difference, but is not filtering.

length(which(coverage.rd2$diff <= 80))

####Call Rate

#Histogram of Call Rate:

callrate <- apply(snps.rd, 1, function(x) 100-
(sum(is.na(x))/(ncol(snps.rd)-5))*100)
hist(callrate, main="Call Rate", xlab="Call Rate")

#Number of SNPs retained with call rate threshold:
length(which(callrate >= 30))


####Minor Allele Frequency (MAF)
#SNPs have already been filtered on MAF in Stacks (using --min_maf 0.01
flag in populations module). We may wish to further filter on MAF for
downstream purposes, e.g. parentage analysis is optimised with only a few
hundred SNPs at high MAF.

snps.rd1 <- snps.rd
snpsrd1 <- as.data.frame(snps.rd1)
snps.rd1$refcount <- rep(NA)
n3 <- ncol(snps.rd)
for (r in 1:nrow(snps.rd1)) {
  snps.rd1$refcount[r] <- 2*(length(which(snps.rd1[r,7:n3] == "0/0"))) +
    length(which(snps.rd1[r,7:n3] == "0/1"))
}

snps.rd1$altcount <- rep(NA)
```

```
for (r in 1:nrow(snps.rd1)) {
  snps.rd1$altcount[r] <- 2*(length(which(snps.rd1[r,7:n3] == "1/1"))) +
    length(which(snps.rd1[r,7:n3] == "0/1"))
}

snps.rd1$minor <- pmin(snps.rd1$refcount, snps.rd1$altcount)
snps.rd1$total <- snps.rd1$refcount + snps.rd1$altcount
snps.rd1$maf <- snps.rd1$minor/snps.rd1$total
hist(snps.rd1$maf, main="Minor Allele Frequency", xlab="MAF")

#Number of SNPs retained with MAF threshold:

length(which(snps.rd1$maf > 0.01))

###Heterozygosity
#SNP calling may result in an excess of heterozygotes. We filtered on --
max_obs_het of 0.7 in the "populations" module of Stacks already. For other
pipelines where heterozygosity filtering is not easily achieved, or if the
user would like to visualise data to choose appropriate threshold:

#Heterozygosity
snps.rd2 <- snps.rd
snps.rd2$na <- rep(NA)
snps.rd2$na <- apply(snps.rd2, 1, function(x) sum(is.na(x)))
snps.rd2$seq <- rep(NA)
snps.rd2$seq <- ncol(snps.rd2) - snps.rd2$na - 8
snps.rd2$hets <- apply(snps.rd2, 1, function(x) length(which(x == "0/1")))
het_count.rd <- 100*(snps.rd2$hets/snps.rd2$seq)
hist(het_count.rd, main="Heterozygosity", xlab="Proportion of heterozygotes
at SNP")

####Reproducibility
#Additionally, we can filter on __reproducibility__ by comparing technical
replicates (performed by DArTseq). DArTseq defines reproducibility as the
proportion of technical replicate assay pairs for which the marker score is
consistent.
#For other sequencing methods, this code can be applied to other forms of
replicates (e.g. within-plate or between-plate replicates) to filter on
reproducibility and/or calculate error rates.

#Use replicate pairs (we have named them T1 & T2 to distinguish). Create a
.csv file with one column listing all samples and their technical
replicates (this section only works for pairs of replicates). Column should
be ordered by sample e.g. sample1_T1, sample1_T2, sample2_T1, sample2_T2
(though will be reordered regardless).

tech.reps <- read.csv('technical replicates - pairs.csv', header=F)
snp.tech.reps.rd <- snps.rd[,which(colnames(snps.rd) %in% tech.reps$V1)]
head(colnames(snp.tech.reps.rd))
snp.tech.reps.rd <- snp.tech.reps.rd[,order(names(snp.tech.reps.rd))]
head(colnames(snp.tech.reps.rd))
snp.tech.match.rd <- matrix(nrow=nrow(snp.tech.reps.rd),
ncol=ncol(snp.tech.reps.rd))

for(r in 1:nrow(snp.tech.reps.rd)){
  for(c in seq(1,ncol(snp.tech.reps.rd), 2)) {
```

```
      if(!is.na(snp.tech.reps.rd[r,c]) && !is.na(snp.tech.reps.rd[r,(c+1)])
&& snp.tech.reps.rd[r,c] != snp.tech.reps.rd[r,c+1]){
  snp.tech.match.rd[r,c] <- "ERROR"
      } else {
    snp.tech.match.rd[r,c] <- NA
      }
  }
}
snp.tech.match.rd <- as.data.frame(as.matrix(snp.tech.match.rd))
snp.tech.match.rd$error <- rep(NA)
n4 <- ncol(snp.tech.match.rd) - 1
for (r in 1:nrow(snp.tech.match.rd)) {
  snp.tech.match.rd$error[r] <- length(which(snp.tech.match.rd[r,1:n4] ==
"ERROR"))
}

snp.tech.match.rd$reproducibility <- rep(NA)
n5 <- (ncol(snp.tech.match.rd) - 2)/2
snp.tech.match.rd$reproducibility <- 100-((snp.tech.match.rd$error/n5)*100)
summary(snp.tech.match.rd$reproducibility)
summary(snp.tech.match.rd$error)
error.rate <- 100-snp.tech.match.rd$reproducibility
summary(error.rate) #mean error rate pre-filtering (aside from read depth
filtering)
hist(snp.tech.match.rd$reproducibility, main="Reproducibility between
technical replicates (pairs)", cex.main=0.8, xlab="Reproducibility %")

####Identifying possible sex-linked SNPs
#It may also be necessary to remove possibly __sex-linked SNPs__. If it is
not clear how to sort sex-linked SNPs based on genotype position, we can
instead  filter by possibly sex-linked SNPs. If the SNP is heterozygous in
at least one female but homozygous in all males, SNP may be sex-linked and
should be removed from analysis.
#__NOTE__: This is for XX/XY systems - for ZZ/ZW systems code can be easily
altered. This filtering step should be performed with the whole dataset to
increase power of detection.

#First, seperate male and female samples. We are able to do this based on
our naming system with F_samplename or M_samplename, where unknown sexes
will be ignored. If sample naming system does not follow, an additional
file could be provided with sex information.

snps.rd.f <- snps.rd
ids <- colnames(snps.rd.f)
str(ids)
males <- ids[which(startsWith(ids, "M_") == T)]
females <- ids[which(startsWith(ids, "F_") == T)]
snp.males <- snps.rd.f[,(which(colnames(snps.rd.f) %in% males))]
snp.females <- snps.rd.f[,(which(colnames(snps.rd.f) %in% females))]

#Next, identify SNPs that are heterozygous in at least one female. vcf
format encodes heterozygotes as "0/1":

snp.females <- cbind(snps.rd.f[,1:6], snp.females)
het.females <- rep(NA)
for (r in 1:nrow(snp.females)) {
```

```
  het.females[r] <- ifelse("0/1" %in% snp.females[r,7:ncol(snp.females)],
"HET", "FALSE")
}
het.females <- cbind(snp.females[,1], het.females)

#Now identify SNPs where there are no male heterozygotes:
snp.males <- cbind(snps.rd.f[,1:6], snp.males)
het.males <- rep(NA)
for (r in 1:nrow(snp.males)) {
  het.males[r] <- ifelse("0/1" %in% snp.males[r,7:ncol(snp.males)], "HET",
"FALSE")
}

#Now find SNP index for when het.females = "HET" but het.males = "FALSE":
str(het.females)
het.females <- as.data.frame(het.females)
het.males <- as.data.frame(het.males)
hets <- cbind(het.females, het.males)
colnames(hets) <- c("index", "het.females", "het.males" )
sex.linked.a <- hets[which(hets$het.females == "HET"),]
sex.linked <- sex.linked.a[which(sex.linked.a$het.males == "FALSE"),]

#Number of possibly sex-linked SNPs:
nrow(sex.linked)

#Now filter data to remove these SNPs.
snps.rd.g <- snps.rd.f[which(!(snps.rd.f[,1] %in% sex.linked$index)),]
nrow(snps.rd.g)
nrow(snps.rd.f) - nrow(snps.rd.g) #should equal number of possibly sex-
linked SNPs

##Filter data
#Now filter data with the thresholds decided above.
filter.rd <- cbind(coverage.rd2, snp.tech.match.rd$reproducibility,
callrate, het_count.rd, snps.rd1$maf)
filter.rd <- as.data.frame(as.matrix(filter.rd))
filter.rd$snp.index <- 1:nrow(filter.rd)
colnames(filter.rd) <- c("read.depth.ref.avg", "read.depth.snp.avg",
"snp.index", "max", "diff", "Reproducibility", "Callrate",
"Heterozygosity", "maf")
nrow(filter.rd)

filter.1.rd <- filter.rd[which(filter.rd$diff <=80),]
nrow(filter.1.rd)

filter.2.rd <- filter.1.rd[which(filter.1.rd$Reproducibility > 85),]
nrow(filter.2.rd)

###we filtered on call rate, heterozygosity and MAF in Stacks, so do not
need to perform this additional filtering. Unhash following code and set
thresholds to filter on these parameters.
#filter.3.rd <- filter.2.rd[which(filter.2.rd$Callrate > 70),]
#nrow(filter.3.rd)
#filter.4.rd <- filter.3.rd[which(filter.3.rd$Heterozygosity <= 70),]
#nrow(filter.4.rd)
#filter.5.rd <- filter.4.rd[which(filter.4.rd$maf >= 0.01),]
#nrow(filter.5.rd)
```

```
index <- 1:nrow(snps.rd.g)
snps.index <- cbind(index, snps.rd.g)
snps.filter.rd <- snps.index[which(snps.index[,1] %in%
filter.2.rd$snp.index),] #update filter.2.rd to appropriate selection if
call rate, heterozygosity, MAF filtering is performed.
nrow(snps.filter.rd)
#Write out file of filtered SNPs (unhash code):
#write.csv(snps.filter.rd, file="filteredsnps.csv")

##Recalculate reproducibility/error rates after filtering
#Previous calculations of reproducibility were made on data only filtered
by read depth > 2.5.
#Can re-calculate reproducibility to obtain error rate after the downstream
filtering pipeline has been run.

#Only calculate error rates for complete cases, so when genotype recorded
for both replicates (not when recorded for one but NA for other).
snp.tech.reps.filter <- snps.filter.rd[,which(colnames(snps.filter.rd) %in%
tech.reps$V1)]
head(colnames(snp.tech.reps.filter))
snp.tech.reps.filter <-
snp.tech.reps.filter[,order(names(snp.tech.reps.filter))]
head(colnames(snp.tech.reps.filter))
snp.tech.match.filter <- matrix(nrow=nrow(snp.tech.reps.filter),
ncol=ncol(snp.tech.reps.filter))

for(r in 1:nrow(snp.tech.reps.filter)){
  for(c in seq(1,ncol(snp.tech.reps.filter), 2)) {
      if(!is.na(snp.tech.reps.filter[r,c]) &&
!is.na(snp.tech.reps.filter[r,(c+1)]) && snp.tech.reps.filter[r,c] !=
snp.tech.reps.filter[r,c+1]){
  snp.tech.match.filter[r,c] <- "ERROR"
      } else {
    snp.tech.match.filter[r,c] <- NA
      }
  }
}
snp.tech.match.filter <- as.data.frame(as.matrix(snp.tech.match.filter))
snp.tech.match.filter$error <- rep(NA)
n4.f <- ncol(snp.tech.match.filter) - 1
for (r in 1:nrow(snp.tech.match.filter)) {
  snp.tech.match.filter$error[r] <-
length(which(snp.tech.match.filter[r,1:n4.f] == "ERROR"))
}

snp.tech.match.filter$reproducibility <- rep(NA)
n5.f <- (ncol(snp.tech.match.filter) - 2)/2
snp.tech.match.filter$reproducibility <- 100-
((snp.tech.match.filter$error/n5.f)*100)
summary(snp.tech.match.filter$reproducibility)
summary(snp.tech.match.filter$error)
error.rate.filter <- 100-snp.tech.match.filter$reproducibility
summary(error.rate.filter) #error rate
sd(error.rate.filter)
hist(snp.tech.match.filter$reproducibility, main="Reproducibility between
technical replicates (pairs)", cex.main=0.8, xlab="Reproducibility %")
```

Appendix 7: Supplementary Code to Chapter 5

This appendix relates to Chapter 5: A case for genetic parentage assignment in captive group housing.

The following R code has been published alongside the article presented in Chapter 5. The first part of this script is adapted from the code in Appendix 6 to process reduced representation sequencing SNPs as per the method presented in Chapter 4, with modifications described in Chapter 5. The second part of this script is to perform parentage analysis. Comments are annotated with #.

```
rm(list=ls())

#Need to install "vcfR" package in R if not already installed. Load
package:
library(vcfR)

##Read in data
#Read in and check populations.snps.vcf file:
vcf <- read.vcfR("populations.snps.vcf")
head(vcf)
queryMETA(vcf)

#Reformat the dataset to obtain SNP data by combining the chromosome
position information and the genotype information in a new object.
'extract.gt' function extracts genotype information, 'getFIX' function
extracts fixed data for each row.
gt <- extract.gt(vcf, IDtoRowNames = F)
fixed <- getFIX(vcf)
snps <- cbind(fixed[,1:5], gt)
head(snps)[,1:10]
snps.1 <- as.data.frame(as.matrix(snps))
snps.1$CHROM <- as.character(as.factor(snps.1$CHROM))
snps.1$POS <- as.character(as.factor(snps.1$POS))
snps.1$ID <- as.character(as.factor(snps.1$ID))
snps.1$identifier <- with(snps.1, paste0(CHROM, POS, ID))
snps.3 <- snps.1[,1:(ncol(snps.1)-1)]

##Data filtering

#Our raw data has been filtered in Stacks/2.0b "populations" module with:
#  * __-r 0.20__ (must be sequenced at >= 20% of samples in a population to
process loci)
#  * __--write_random_snp__ (restrict data analysis to one random SNP per
locus) - filters linked SNPs
#  * __--min_maf 0.01__
#  * __--max_obs_het 0.70__

####Read Depth
```

```
#Filter on allelic read depth to remove SNPs with low coverage at ref or
snp("alt") allele.
read.depth <- extract.gt(vcf, element="AD")
length(unique(rownames(read.depth)))
nrow(read.depth)
read.depth.ref <- masplit(read.depth, record = 1, sort=0)
read.depth.snp <- masplit(read.depth, record = 2, sort=0)

#for reference allele depth:
head(read.depth.ref)[1:6,1:6]
read.depth.ref.count<- rowSums(read.depth.ref, na.rm=T)
head(read.depth.ref.count)
read.depth.ref <- as.data.frame(read.depth.ref)
read.depth.ref$length <- rep(NA)

n <- ncol(read.depth.ref)-1
for (r in 1:nrow(read.depth.ref)) {
  read.depth.ref$length[r] <- length(which(read.depth.ref[r,1:n] !=0))
}

read.depth.ref.avg <- read.depth.ref.count/read.depth.ref$length
head(read.depth.ref.avg)
summary(read.depth.ref.avg)

#for snp allele depth:
read.depth.snp.count<- rowSums(read.depth.snp, na.rm=T)
head(read.depth.snp.count)

read.depth.snp <- as.data.frame(read.depth.snp)
read.depth.snp$length <- rep(NA)

n2 <- ncol(read.depth.snp)-1
for (r in 1:nrow(read.depth.snp)) {
  read.depth.snp$length[r] <- length(which(read.depth.snp[r,1:n2] !=0))
}

read.depth.snp.avg <- read.depth.snp.count/read.depth.snp$length
summary(read.depth.snp.avg)

length(which(read.depth.ref.avg > 2.5)) #can change this number to see how
many SNPs will be retained
length(which(read.depth.snp.avg > 2.5))

coverage.rd <- cbind(read.depth.ref.avg, read.depth.snp.avg)
coverage.rd <- as.data.frame(coverage.rd)
coverage.rd$snp.index <- 1:nrow(coverage.rd)
coverage.rd1 <- coverage.rd[which(coverage.rd$read.depth.ref.avg > 2.5),]
#decide on minimum allelic depth here
nrow(coverage.rd)
nrow(coverage.rd1)
coverage.rd2 <- coverage.rd1[which(coverage.rd1$read.depth.snp.avg > 2.5),]
#decide on minimum allelic depth here
nrow(coverage.rd2)

#visually compare distribution of SNPs pre- and post- filtering
par(mfrow=c(2,2))
```

```
hist(read.depth.ref.avg, main="Read depth of ref allele", xlab="Read
depth")
hist(read.depth.snp.avg, main="Read depth of snp allele", xlab="Read
depth")
hist(coverage.rd2$read.depth.ref.avg, main="Read depth of ref allele >
2.5", xlab="Read depth")
hist(coverage.rd2$read.depth.snp.avg, main="Read depth of snp allele >
2.5", xlab="Read depth")

index <- 1:nrow(snps.3)
snps.index <- cbind(index, snps.3)
snps.rd <- snps.index[which(snps.index[,1] %in% coverage.rd2$snp.index),]
nrow(snps.rd)

####Coverage Difference
#We also need to filter by __coverage difference__. If the reference and
SNP allele do not amplify at the same rate, this may indicate potential
bias (errors in calling).
#Coverage can be calculated as the absolute percentage difference between
the REF alllelic depth and ALT allelic depth. vcf output gives Allele
Depth, with coverage of reference, then coverage of SNP allele e.g. AD =
4,3 means reference allele has coverage = 4, snp allele coverage = 3. Count
only non-zero reads.

coverage.rd2$max <- pmax(coverage.rd2$read.depth.ref.avg,
coverage.rd2$read.depth.snp.avg)
coverage.rd2$diff <- ((abs(coverage.rd2$read.depth.ref.avg -
coverage.rd2$read.depth.snp.avg))/(coverage.rd2$max))*100
hist(coverage.rd2$diff, main="Coverage difference", xlab="% diff in
coverage")
length(which(coverage.rd2$diff < 60)) #edit this to see how many SNPs will
be retained

####Call Rate

#Histogram of Call Rate/Genotyping rate:
callrate <- apply(snps.rd, 1, function(x) 100-
(sum(is.na(x))/(ncol(snps.rd)-5))*100)
hist(callrate, main="Call Rate", xlab="Call Rate")
#We have already filtered on call rate in Stacks, however could filter
within this script if desired.
length(which(callrate >= 20)) #edit to see how many SNPs would be retained

####Minor Allele Frequency (MAF)
#SNPs have already been filtered on MAF in Stacks (using --min_maf 0.01
flag in populations module). We may wish to further filter on MAF for
downstream purposes, e.g. parentage analysis with Sequoia recommends higher
MAF > 0.3, and only requires a few hundred SNPs so we can reduce the SNP
set.

snps.rd1 <- snps.rd
snpsrd1 <- as.data.frame(snps.rd1)
snps.rd1$refcount <- rep(NA)
n3 <- ncol(snps.rd)
for (r in 1:nrow(snps.rd1)) {
  snps.rd1$refcount[r] <- 2*(length(which(snps.rd1[r,7:n3] == "0/0"))) +
    length(which(snps.rd1[r,7:n3] == "0/1"))
```

```
}

snps.rd1$altcount <- rep(NA)
for (r in 1:nrow(snps.rd1)) {
  snps.rd1$altcount[r] <- 2*(length(which(snps.rd1[r,7:n3] == "1/1"))) +
    length(which(snps.rd1[r,7:n3] == "0/1"))
}

snps.rd1$minor <- pmin(snps.rd1$refcount, snps.rd1$altcount)
snps.rd1$total <- snps.rd1$refcount + snps.rd1$altcount
snps.rd1$maf <- snps.rd1$minor/snps.rd1$total
hist(snps.rd1$maf, main="Minor Allele Frequency", xlab="MAF")
length(which(snps.rd1$maf > 0.05)) #edit this to see how many SNPs would be
retained

###Heterozygosity
#Excess heterozygotes in data may indicate problems with SNP-calling
software. We have already filtered on --max_obs_het (maximum observed
heterozygosity = 70%) in Stacks, however could be applied in this script if
needed. Calculate proportion of heterozygous genotypes:
#Heterozygosity
snps.rd2 <- snps.rd
snps.rd2$na <- rep(NA)
snps.rd2$na <- apply(snps.rd2, 1, function(x) sum(is.na(x)))
snps.rd2$seq <- rep(NA)
snps.rd2$seq <- ncol(snps.rd2) - snps.rd2$na - 8
snps.rd2$hets <- apply(snps.rd2, 1, function(x) length(which(x == "0/1")))
het_count.rd <- 100*(snps.rd2$hets/snps.rd2$seq)
hist(het_count.rd, main="Heterozygosity", xlab="Proportion of heterozygotes
at SNP")

####Reproducibility
#Additionally, we can filter on __reproducibility__ by comparing technical
replicates (performed by DArTseq). DArTseq defines reproducibility as the
proportion of technical replicate assay pairs for which the marker score is
consistent.
#We can also use this to calculate error rate with all technical replicates
for each individual SNP so that we can filter on reproducibility. Use
samples with 2 technical replicates (we have named them T1 & T2, e.g.
F_1224_ChinaGirl_T1, F_1224_ChinaGirl_T2). Only calculate error rates for
complete cases, so when genotype recorded for both replicates (not when
recorded for one but NA for other).
#Create a .csv file with one column listing all samples and their technical
replicates (this section of code only works for pairs of replicates).
Column must be ordered by sample e.g. sample1_T1, sample1_T2, sample2_T1,
sample2_T2

tech.reps <- read.csv('technical replicates - pairs.csv', header=F)
snp.tech.reps.rd <- snps.rd[,which(colnames(snps.rd) %in% tech.reps$V1)]
head(colnames(snp.tech.reps.rd))
snp.tech.reps.rd <- snp.tech.reps.rd[,order(names(snp.tech.reps.rd))]
head(colnames(snp.tech.reps.rd))
snp.tech.match.rd <- matrix(nrow=nrow(snp.tech.reps.rd),
ncol=ncol(snp.tech.reps.rd))

for(r in 1:nrow(snp.tech.reps.rd)){
  for(c in seq(1,ncol(snp.tech.reps.rd), 2)) {
```

```
      if(!is.na(snp.tech.reps.rd[r,c]) && !is.na(snp.tech.reps.rd[r,(c+1)])
&& snp.tech.reps.rd[r,c] != snp.tech.reps.rd[r,c+1]){
  snp.tech.match.rd[r,c] <- "ERROR"
      } else {
    snp.tech.match.rd[r,c] <- NA
      }
  }
}
snp.tech.match.rd <- as.data.frame(as.matrix(snp.tech.match.rd))
snp.tech.match.rd$error <- rep(NA)
n4 <- ncol(snp.tech.match.rd) - 1
for (r in 1:nrow(snp.tech.match.rd)) {
  snp.tech.match.rd$error[r] <- length(which(snp.tech.match.rd[r,1:n4] ==
"ERROR"))
}

snp.tech.match.rd$reproducibility <- rep(NA)
n5 <- (ncol(snp.tech.match.rd) - 2)/2
snp.tech.match.rd$reproducibility <- 100-((snp.tech.match.rd$error/n5)*100)
summary(snp.tech.match.rd$reproducibility)
summary(snp.tech.match.rd$error)
error.rate <- 100-snp.tech.match.rd$reproducibility
summary(error.rate)
hist(snp.tech.match.rd$reproducibility, main="Reproducibility between
technical replicates (pairs)", cex.main=0.8, xlab="Reproducibility %")

#We can then combine information from technical replicates:
#  * first choose best quality technical replicate, based on highest call
rate (least amount of missing data)
#  * then, where higher quality sample is missing data, add genotype
information from lower quality (lower call rate) sample where possible

#Step 1:
na_count <- function(x) sapply(x, function(y) sum(is.na(y)))
nocalls <- na_count(snps.rd[7:ncol(snps.rd)])
nocalls2 <- cbind(nocalls, colnames(snps.rd[7:ncol(snps.rd)]))
nocalls3 <- nocalls2[which(rownames(nocalls2) %in% tech.reps$V1),]
nrow(nocalls3)
tech.reps$nocalls <- rep(NA)
tech.reps2 <- tech.reps[which(tech.reps$V1 %in% colnames(snps.rd)),]
nrow(tech.reps2)

for (r in 1:nrow(tech.reps2)) {
  tech.reps2[r,2] <- nocalls2[which(tech.reps2[r,1] == nocalls2[,2]),1]
}

tech.reps3 <- tech.reps2
tech.reps3$max <- rep(NA)
for (r in seq(1, nrow(tech.reps3), 2)){
  if(tech.reps3[r,2] > tech.reps3[(r+1),2]) {
    tech.reps3$max[r] <- "MAX"
    tech.reps3$max[r+1] <- "MIN"
  } else {
    tech.reps3$max[r] <- "MIN"
    tech.reps3$max[r+1] <- "MAX"
  }
}
```

```
tech.reps4 <- tech.reps3[which(tech.reps3$max == "MAX"),] #these samples
have highest number of no genotypes
nrow(tech.reps4) #should be half of nrow(tech.reps3)
tech.reps.min <- tech.reps3[which(tech.reps3$max == "MIN"),] #these samples
have lowest number of no calls (best)

#Step 2: Combine technical replicates into one composite sample for better
call rate. Rather than just using the sample with the highest call rate, we
can combine multiple technical replicates to improve Sequoia results. Do
this by taking the technical replicate with the highest call rate, then
adding any genotypes from the lower call rate replicate where the highest
call rate replicate is not already sequenced.

max.snps <- snps.rd[,(which(colnames(snps.rd) %in% tech.reps4$V1))]
ncol(max.snps)
min.snps <- snps.rd[,(which(colnames(snps.rd) %in% tech.reps.min$V1))]
ncol(min.snps)
#if not ordered pairs will not be in same row between min.snps and max.snps
head(colnames(max.snps))
head(colnames(min.snps))
max.snps <- max.snps[,order(names(max.snps))] #order to be same
min.snps <- min.snps[,order(names(min.snps))] #order to be same
head(colnames(max.snps))
head(colnames(min.snps))

combined <- min.snps
for (r in 1:nrow(combined)){
  for (c in 1:ncol(combined)){
    if (is.na(min.snps[r,c])){
      combined[r,c] <- max.snps[r,c]
    }
  }
}

snps.rd.a <- snps.rd
ncol(snps.rd.a)
snps.rd.a <- snps.rd.a[,-(which(colnames(snps.rd.a) %in% tech.reps2$V1))]
ncol(snps.rd.a)
snps.rd.b <- cbind(snps.rd.a[1:ncol(snps.rd.a)], combined)
ncol(snps.rd.b)

#We can also do this technical replicate combination for samples with three
technical replicates, as we have in our dataset. Find replicate with
highest call rate, then add genotype information from second highest call
rate replicate, and finally lowest call rate.
tech.reps.trios <- read.csv('technical replicates - trios.csv', header=F)
#first get snp data for trios
snp.tech.reps.trios <- snps.rd[,which(colnames(snps.rd) %in%
tech.reps.trios$V1)]
ncol(snp.tech.reps.trios)
head(colnames(snp.tech.reps.trios))

##must be in right order
nocalls3.t <- nocalls2[which(rownames(nocalls2) %in% tech.reps.trios$V1),]
nrow(nocalls3.t)
tech.reps.trios$nocalls <- rep(NA)
```

```
tech.reps.trios2 <- tech.reps.trios[which(tech.reps.trios$V1 %in%
colnames(snps.rd)),]
nrow(tech.reps.trios2)

for (r in 1:nrow(tech.reps.trios2)) {
  tech.reps.trios2[r,2] <- nocalls2[which(tech.reps.trios2[r,1] ==
nocalls2[,2]),1]
}

tech.reps.trios3 <- tech.reps.trios2
tech.reps.trios3$max <- rep(NA)
for (r in seq(1, nrow(tech.reps.trios3), 3)){
  if((tech.reps.trios3[r,2] > tech.reps.trios3[(r+1),2]) &&
(tech.reps.trios3[r,2] > tech.reps.trios3[(r+2),2])) {
    tech.reps.trios3$max[r] <- "MAX.A"
    tech.reps.trios3$max[r+1] <- "MIN.A"
    tech.reps.trios3$max[r+2] <- "MIN.A"
  }
  if((tech.reps.trios3[(r+1),2] > tech.reps.trios3[r,2]) &&
(tech.reps.trios3[(r+1),2] > tech.reps.trios3[(r+2),2])) {
    tech.reps.trios3$max[r] <- "MIN.B"
    tech.reps.trios3$max[r+1] <- "MAX.B"
    tech.reps.trios3$max[r+2] <- "MIN.B"
  } else {
    tech.reps.trios3$max[r] <- "MIN.C"
    tech.reps.trios3$max[r+1] <- "MIN.C"
    tech.reps.trios3$max[r+2] <- "MAX.C"
  }
}

tech.reps.trios.max <- tech.reps.trios3[which(tech.reps.trios3$max ==
c("MAX.A", "MAX.B", "MAX.C")),] #these samples have highest number of no
genotypes, and should be combined last
nrow(tech.reps.trios.max) #should be third of nrow(tech.reps.trios3)

tech.reps.trios3a <- rbind(tech.reps.trios3[which(tech.reps.trios3$max ==
"MIN.B"),], tech.reps.trios3[which(tech.reps.trios3$max == "MIN.C"),])
#these samples have lowest number of no calls (best)
nrow(tech.reps.trios3a)

#now find minimum from these two:
for (r in seq(1, nrow(tech.reps.trios3a),2)){
  if((tech.reps.trios3a[r,2] > tech.reps.trios3a[(r+1),2])){
    tech.reps.trios3a$max[r] <- "MED"
    tech.reps.trios3a$max[r+1] <- "MIN"
  } else {
    tech.reps.trios3a$max[r] <- "MIN"
    tech.reps.trios3a$max[r+1] <- "MED"
  }
}

tech.reps.trios.min <- tech.reps.trios3a[which(tech.reps.trios3a$max ==
"MIN"),]
nrow(tech.reps.trios.min) #should be one third of nrow(tech.reps.trios3)

tech.reps.trios.med <- tech.reps.trios3a[which(tech.reps.trios3a$max ==
"MED"),]
```

```
nrow(tech.reps.trios.med) #should be one third of nrow(tech.reps.trios3)

#Combine technical replicates, starting with highest call rate ("MIN"),
then next ("MED"), finally lowest ("MAX")
max.trios <- snps.rd[,(which(colnames(snps.rd) %in%
tech.reps.trios.max$V1))]
ncol(max.trios)
med.trios <- snps.rd[,(which(colnames(snps.rd) %in%
tech.reps.trios.med$V1))]
ncol(med.trios)
min.trios <- snps.rd[,(which(colnames(snps.rd) %in%
tech.reps.trios.min$V1))]
ncol(min.trios)

#is not ordered so pairs will not be in same row between min.snps and
max.snps
head(colnames(max.trios))
head(colnames(med.trios))
head(colnames(min.trios))

max.trios <- max.trios[,order(names(max.trios))]
med.trios <- med.trios[,order(names(med.trios))]
min.trios <- min.trios[,order(names(min.trios))]

combined.trios <- min.trios
for (r in 1:nrow(combined.trios)){
  for (c in 1:ncol(combined.trios)){
    if (is.na(min.trios[r,c])){
      combined.trios[r,c] <- med.trios[r,c]
    }
  }
}

combined.trios2 <- combined.trios
for (r in 1:nrow(combined.trios2)){
  for (c in 1:ncol(combined.trios2)){
    if (is.na(combined.trios[r,c])){
      combined.trios2[r,c] <- max.trios[r,c]
    }
  }
}

snps.rd.c <- snps.rd.b[,-(which(colnames(snps.rd.a) %in%
tech.reps.trios$V1))]
ncol(snps.rd.c)
snps.rd.d <- cbind(snps.rd.c[1:ncol(snps.rd.c)], combined.trios2)
ncol(snps.rd.d)
ncol(snps.rd.b) - ncol(snps.rd.d)

##Choose between replicates
#If there are within or between plate replicates (not technical replicates,
instead replicates provided by researcher), we can only use one for
downstream purposes such as Sequoia. Therefore choose the replicate with
the highest call rate. This code works for replicates where there are pairs
(i.e. M_948_Jimmy_R1 vs. M_948_Jimmy_T1/T2)
reps <- read.csv('plate replicates.csv', header=F)
nrow(reps)
```

```
snp.reps <- snps.rd.d[,which(colnames(snps.rd.d) %in% reps$V1)]
ncol(snp.reps) #once technical replicate has been chosen, now choosing
between plate replicates
na_count <- function(x) sapply(x, function(y) sum(is.na(y)))
nocalls.reps <- na_count(snp.reps)
nocalls3.reps <- cbind(nocalls.reps, colnames(snp.reps))
nrow(nocalls3.reps)
reps$nocalls <- rep(NA)
reps2 <- reps[which(reps$V1 %in% colnames(snps.rd.d)),]
nrow(reps2)

for (r in 1:nrow(reps2)) {
  reps2[r,2] <- nocalls3.reps[which(reps2[r,1] == nocalls3.reps[,2]),1]
}

reps3 <- reps2
str(reps3)
reps3$nocalls <- as.numeric(as.character(reps3$nocalls))
reps3$max <- rep(NA)
for (r in seq(1, nrow(reps3), 2)){
  if(reps3[r,2] > reps3[(r+1),2]) {
    reps3$max[r] <- "MAX"
    reps3$max[r+1] <- "MIN"
  } else {
    reps3$max[r] <- "MIN"
    reps3$max[r+1] <- "MAX"
  }
}

reps4 <- reps3[which(reps3$max == "MAX"),] #these samples have highest
number of no genotypes
nrow(reps4) #should be half of nrow(tech.reps3)
reps.min <- reps3[which(reps3$max == "MIN"),] #these samples have lowest
number of no calls (best)

#now remove other replicates so that only replicates with highest call rate
are included
min.snps.rep <- snps.rd.d[,(which(colnames(snps.rd.d) %in% reps.min$V1))]
combined.reps <- min.snps.rep
snps.rd.rep <- snps.rd.d[,-(which(colnames(snps.rd.d) %in% reps2$V1))]
ncol(snps.rd.rep)
snps.rd.rep2 <- cbind(snps.rd.rep[1:ncol(snps.rd.rep)], combined.reps)
ncol(snps.rd.rep2)
ncol(snps.rd.d) - ncol(snps.rd.rep2) #should equal ncol(snp.reps)/2
ncol(snp.reps)/2

#There may also be some that have more than one replicate, e.g.
M_1460_Malt, M_1460_Malt_R, M_1460_Malt_R1
reps.npairs <- read.csv("catalogue_805//plate replicates trios.csv",
header=F)
nrow(reps.npairs)
snp.reps.npairs <- snps.rd.rep2[,which(colnames(snps.rd.rep2) %in%
reps.npairs$V1)]
ncol(snp.reps.npairs) #3 replicates for each sample to choose from

nocalls.reps.npairs <- na_count(snp.reps.npairs)
```

```
nocalls3.reps.npairs <- cbind(nocalls.reps.npairs,
colnames(snp.reps.npairs))
nrow(nocalls3.reps.npairs)
reps.npairs$nocalls <- rep(NA)
reps.npairs2 <- reps.npairs[which(reps.npairs$V1 %in%
colnames(snps.rd.rep2)),]
nrow(reps.npairs2)

for (r in 1:nrow(reps.npairs2)) {
  reps.npairs2[r,2] <- nocalls3.reps.npairs[which(reps.npairs2[r,1] ==
nocalls3.reps.npairs[,2]),1]
}

reps.npairs3 <- reps.npairs2
str(reps.npairs3)
reps.npairs3$nocalls <- as.numeric(as.character(reps.npairs3$nocalls))
reps.npairs3$max <- rep(NA)
#compare R to R1/R2
for (r in seq(1, nrow(reps.npairs3), 3)){
  if((reps.npairs3[r,2] > reps.npairs3[(r+1),2]) && (reps.npairs3[r,2] >
reps.npairs3[(r+2),2])) {
    reps.npairs3$max[r] <- "MAX.A"
    reps.npairs3$max[r+1] <- "MIN.A"
    reps.npairs3$max[r+2] <- "MIN.A"
  } else {
      if((reps.npairs3[(r+1),2] > reps.npairs3[r,2]) &&
(reps.npairs3[(r+1),2] > reps.npairs3[(r+2),2])) {
    reps.npairs3$max[r] <- "MIN.B"
    reps.npairs3$max[r+1] <- "MAX.B"
    reps.npairs3$max[r+2] <- "MIN.B"
  } else {
    reps.npairs3$max[r] <- "MIN.C"
    reps.npairs3$max[r+1] <- "MIN.C"
    reps.npairs3$max[r+2] <- "MAX.C"
  }
  }
}

reps.npairs3.max <- reps.npairs3[which(reps.npairs3$max == c("MAX.A",
"MAX.B", "MAX.C")),] #these samples have highest missing data
nrow(reps.npairs3.max) #should be third of nrow(reps.npairs3)

reps.npairs3a <- rbind(reps.npairs3[which(reps.npairs3$max == "MIN.B"),],
reps.npairs3[which(reps.npairs3$max == "MIN.C"),],
reps.npairs3[which(reps.npairs3$max == "MIN.A"),]) #these samples have
lowest number of no calls (best)
nrow(reps.npairs3a)

#now find minimum from these two:
for (r in seq(1, nrow(reps.npairs3a),2)){
  if((reps.npairs3a[r,2] > reps.npairs3a[(r+1),2])){
    reps.npairs3a$max[r] <- "MED"
    reps.npairs3a$max[r+1] <- "MIN"
  } else {
    reps.npairs3a$max[r] <- "MIN"
    reps.npairs3a$max[r+1] <- "MED"
  }
```

```
}

reps.npairs.min <- reps.npairs3a[which(reps.npairs3a$max == "MIN"),] #these
samples have lowest number of no calls (best)
nrow(reps.npairs.min)
#now remove other replicates so that only replicates with highest call rate
are included
min.snps.reps.npairs <- snps.rd.rep2[,(which(colnames(snps.rd.rep2) %in%
reps.npairs.min$V1))]
combined.reps.npairs <- min.snps.reps.npairs
snps.rd.reps.npairs <- snps.rd.rep2[,-(which(colnames(snps.rd.rep2) %in%
reps.npairs$V1))]
ncol(snps.rd.reps.npairs)
snps.rd.reps.npairs2 <-
cbind(snps.rd.reps.npairs[1:ncol(snps.rd.reps.npairs)],
combined.reps.npairs)
ncol(snps.rd.reps.npairs2)
ncol(snps.rd.rep2) - ncol(snps.rd.reps.npairs2) #should equal
(ncol(snp.reps)/3)*2
ncol(snp.reps.npairs)/3

snps.rd.f <- snps.rd.reps.npairs2
dim(snps.rd.f)

####Identifying possible sex-linked SNPs
#It is also necessary to remove possibly __sex-linked SNPs__. If it is not
clear how to sort sex-linked SNPs based on genotype position, we can
instead  filter by possibly sex-linked SNPs. If the SNP is heterozygous in
at least one female but homozygous in all males, SNP may be sex-linked and
should be removed from analysis. NOTE: This is for XY systems - for birds
or others may be the other way around. Make sure this filtering step is
done with whole dataset to increase power of detection.
#First, seperate male and female samples (based on naming system with
F_SB_name or M_SB_name). Unknown sexes will be ignored.
males <- ids[which(startsWith(ids, "M_") == T)]
females <- ids[which(startsWith(ids, "F_") == T)]
snp.males <- snps.rd.f[,(which(colnames(snps.rd.f) %in% males))]
snp.females <- snps.rd.f[,(which(colnames(snps.rd.f) %in% females))]

#Next, identify SNPs that are heterozygous in at least one female. vcf
format encodes heterozygotes as "0/1":
snp.females <- cbind(snps.rd.f[,1:6], snp.females)
het.females <- rep(NA)
for (r in 1:nrow(snp.females)) {
  het.females[r] <- ifelse("0/1" %in% snp.females[r,7:ncol(snp.females)],
"HET", "FALSE")
}
het.females <- cbind(snp.females[,1], het.females)

#Now identify SNPs where there are no male heterozygotes:
snp.males <- cbind(snps.rd.f[,1:6], snp.males)
het.males <- rep(NA)
for (r in 1:nrow(snp.males)) {
  het.males[r] <- ifelse("0/1" %in% snp.males[r,7:ncol(snp.males)], "HET",
"FALSE")
}
```

```
#Now find SNP index for when het.females = "HET" but het.males = "FALSE":
str(het.females)
het.females <- as.data.frame(het.females)
het.males <- as.data.frame(het.males)
hets <- cbind(het.females, het.males)
colnames(hets) <- c("index", "het.females", "het.males" )
sex.linked.a <- hets[which(hets$het.females == "HET"),]
sex.linked <- sex.linked.a[which(sex.linked.a$het.males == "FALSE"),]

#Number of possibly sex-linked SNPs:
nrow(sex.linked)

#Now filter data to remove these SNPs.
snps.rd.g <- snps.rd.f[which(!(snps.rd.f[,1] %in% sex.linked$index)),]
nrow(snps.rd.g)
nrow(snps.rd.f) - nrow(snps.rd.g) #should equal number of possibly sex-
linked SNPs

#Now filter data based on parameters decided above.
filter.rd <- cbind(coverage.rd2, snp.tech.match.rd$reproducibility,
callrate, het_count.rd, snps.rd1$maf)
filter.rd <- as.data.frame(as.matrix(filter.rd))
filter.rd$snp.index <- 1:nrow(filter.rd)
colnames(filter.rd) <- c("read.depth.ref.avg", "read.depth.snp.avg",
"snp.index", "max", "diff", "Reproducibility", "Callrate",
"Heterozygosity", "maf")
nrow(filter.rd)

#filter on coverage difference:
filter.1.rd <- filter.rd[which(filter.rd$diff <=60),]
nrow(filter.1.rd)

#filter on reproducibility:
filter.2.rd <- filter.1.rd[which(filter.1.rd$Reproducibility >= 90),]
nrow(filter.2.rd)

#filter on call rate (if needed, we have already filtered in Stacks):
filter.3.rd <- filter.2.rd[which(filter.2.rd$Callrate >= 20),]
nrow(filter.3.rd)

#filter on heterozygosity (if needed, we have already filtered in Stacks):
filter.4.rd <- filter.3.rd[which(filter.3.rd$Heterozygosity <= 70),]
nrow(filter.4.rd)

#filter on MAF:
filter.5.rd <- filter.4.rd[which(filter.4.rd$maf >= 0.05),]
nrow(filter.5.rd)

index <- 1:nrow(snps.rd.g)
snps.index <- cbind(index, snps.rd.g)
snps.filter.rd <- snps.index[which(snps.index[,1] %in%
filter.5.rd$snp.index),]
nrow(snps.filter.rd)
#write.csv(snps.filter.rd, file="FREs_filteredsnps.csv")

##Re-filter on higher MAF for Sequoia
```

```
#We have used our entire catalogue of devil samples for the initial
filtering steps, as larger sample sizes improve confidence of SNP calls. We
are only performing parentage analysis for the free-range enclosure, so we
can now refilter more stringently using our population of interest.
#Sequoia requires higher MAF to obtain informative SNPs. Using higher MAF
across whole catalogue may not be useful if the population of interest
(Bridport & Freycinet) have high relatedness and therefore similar
genotypes. So filter on MAF just within population of interest now. Should
also filter on call rate so that there is not too much missing data for
parentage analysis with Sequoia.

dim(snps.filter.rd)

#provide list of samples of relevance
ppn <- read.csv('FREs lifehistory all reps.csv', header=T)
nrow(ppn)

fre.snps <- snps.filter.rd[,which(colnames(snps.filter.rd) %in% ppn$ID)]
fre.snps2 <- cbind(snps.filter.rd[,2:7], fre.snps)
ncol(fre.snps)

fre.snps2 <- as.data.frame(fre.snps2)
n3 <- ncol(fre.snps2)
fre.snps2$refcount <- rep(NA)

for (r in 1:nrow(fre.snps2)) {
  fre.snps2$refcount[r] <- 2*(length(which(fre.snps2[r,8:n3] == "0/0"))) +
    length(which(snps.rd1[r,7:n3] == "0/1"))
}

fre.snps2$altcount <- rep(NA)
for (r in 1:nrow(fre.snps2)) {
  fre.snps2$altcount[r] <- 2*(length(which(fre.snps2[r,7:n3] == "1/1"))) +
    length(which(fre.snps2[r,7:n3] == "0/1"))
}

fre.snps2$minor <- pmin(fre.snps2$refcount, fre.snps2$altcount)
fre.snps2$total <- fre.snps2$refcount + fre.snps2$altcount
fre.snps2$maf <- fre.snps2$minor/fre.snps2$total
hist(fre.snps2$maf, main="Minor Allele Frequency", xlab="MAF")
length(which(fre.snps2$maf > 0.01)) #can edit to see how many SNPs would be
retained
filter.fre.snps <- fre.snps2[which(fre.snps2$maf > 0.01),1:n3] #filter on
MAF here
dim(filter.fre.snps)

#Also filter on call rate for this population:
callrate.fre <- apply(filter.fre.snps, 1, function(x) 100-
(sum(is.na(x))/(ncol(filter.fre.snps)-6))*100)
hist(callrate.fre, main="Call Rate", xlab="Call Rate")
length(which(callrate.fre > 80)) #can edit to see how many SNPs would be
retained
filter.fre.snps$callrate.fre <- callrate.fre
summary(filter.fre.snps$callrate.fre)

filter.fre.snps2 <- filter.fre.snps[which(filter.fre.snps$callrate.fre >
80), 1:(ncol(filter.fre.snps)-1)] #filter on call rate here
```

```
dim(filter.fre.snps2)

##Format data for parentage analysis in Sequoia
#Once we have filtered the data to get a subset of quality SNPs, we can use
these SNPs to determine parentage using the 'sequoia' package in R.
#Sequoia requires genotype data in 1 row per individual (with individual as
row name), 1 column per SNP format (with SNP as column name). Data from vcf
is the other way around. Sequoia can use replicates, as it allows a few
mismatches (so can use technical replicates etc.), however they must have
identical names. Genotypes coded as:
#  * 0 = zero copies of reference allele (currently "1/1")
#  * 1 = one copy of reference allele (currently "0/1")
#  * 2 = two copies of reference allele (currently "0/0")
#  * -9 = missing data (currently "./." or NA)

filter.fre.snps2$CHROM <- as.character(as.factor(filter.fre.snps2$CHROM))
filter.fre.snps2$POS <- as.character(as.factor(filter.fre.snps2$POS))
filter.fre.snps2$ID <- as.character(as.factor(filter.fre.snps2$ID))
filter.fre.snps2$identifier <- with(filter.fre.snps2, paste0(CHROM, POS,
ID))

#Recode genotypes for Sequoia format (same as PLINK format):
filter.fre.snps3 <- filter.fre.snps2[,7:(ncol(filter.fre.snps2) -1)]
genotypes <- t(filter.fre.snps3)
genotypes2 <- as.data.frame(as.matrix(genotypes))
colnames(genotypes2) <- filter.fre.snps2$identifier

#replace "1/1" with "0"
genotypes3 <- matrix(nrow=nrow(genotypes2), ncol=ncol(genotypes2))
nrow(genotypes3) #number of samples
ncol(genotypes3) #number of snps

for (r in 1:nrow(genotypes2)) {
  for (c in 1:ncol(genotypes2)) {
      genotypes3[r,c] <- ifelse(genotypes2[r,c] == "1/1", "0", NA)
    }
}

#replace "0/0" with "2"
for (r in 1:nrow(genotypes2)) {
  for (c in 1:ncol(genotypes2)) {
    genotypes3[r,c] <- ifelse(genotypes2[r,c] == "0/0", "2",
genotypes3[r,c])
  }
}

#replace "0/1" with "1"
for (r in 1:nrow(genotypes2)) {
  for (c in 1:ncol(genotypes2)) {
    genotypes3[r,c] <- ifelse(genotypes2[r,c] == "0/1", "1",
genotypes3[r,c])
  }
}

#replace "NA" with "-9"
genotypes3 <- replace(genotypes3, is.na(genotypes3), "-9")
colnames(genotypes3) <- colnames(genotypes2)
```

```
rownames(genotypes3) <- rownames(genotypes2)

####Export for Sequoia
#"genotypes3" is now in correct format for Sequoia. Export as .csv for
input to Sequoia script.
#Unhash code to run this (to avoid replacing file), and update with file
directory.
#write.csv(genotypes3, file="genotypes for sequoia_fres.csv")

#Run parentage analysis with Sequoia. Requires life history file with
sample name, sex and year of birth. See sequoia information for ability to
deal with unknown life-history information.
library(sequoia)
s.genotypes<- as.matrix(read.csv('genotypes for sequoia_fres.csv',
header=TRUE, row.names=1))
lifehist <- read.csv('FREs lifehistory all reps.csv', header=T) #input
life-history file
lifehist$ID <- as.character(as.factor(lifehist$ID))

lifehist2 <- lifehist[which(lifehist$ID %in% rownames(s.genotypes)),]
nrow(lifehist2)
s.genotypes2 <- s.genotypes[which(rownames(s.genotypes) %in%
lifehist2$ID),]
nrow(s.genotypes2)
#first run without MaxSibIter to assign P-O (parent offspring)
relationships
ParOUT <- sequoia(GenoM = s.genotypes2,
                  LifeHistData = lifehist2,
                  MaxSibIter = 0,
                  Err = 0.01) #can vary error rate depending on error of
sequencing data
#now run with MaxSibIter to include sibling information
SeqOUT <- sequoia(GenoM = s.genotypes2,
                  SeqList = ParOUT,
                  MaxSibIter = 5,
                  Err = 0.01)

ped <- SeqOUT$Pedigree
may <- SeqOUT$MaybeParent

#write.csv(SeqOUT$Pedigree, file='ped.csv')
```

Appendix 8: Supplementary Material to Chapter 6

This appendix relates to Chapter 6: Deciphering genetic mate choice: not so simple in group-housed conservation breeding programs.

A8.1 SUPPLEMENTARY TABLES

Table A8.1.1: Characteristics of 12 MHC-linked microsatellites.
All markers developed by Cheng and Belov (2014), with the exception of MHCI12 (Day *et al.*, 2019).

Locus	MHC class linked	Multiplex	Fluorescent tag
MHCI06	I	MHC 1	NED
MHCI09	I	MHC 1	6-FAM
MHCI08	I	MHC 1	PET
MHCI07	I	MHC 2	NED
MHCI02	I	MHC 2	PET
MHCI10	I	MHC 2	NED
MHCII02	II	MHC 2	VIC
MHCI05	I	MHC 3	6-FAM
MHCI11	II	MHC 3	VIC
MHCII03	II	MHC 3	PET
MHCI01	I	MHC 3	NED
MHCI12	I	N/A	6-FAM

Table A8.1.2: Top model set (top 2 AIC_C) of generalised linear mixed models for overall breeding success and relative breeding success (standardised across competitive breeding enclosure).

Female models were fitted with a random intercept for "enclosure.year", male models were fitted with a random intercept for "ID". The final models are provided in Table 6.1 and 6.2.

Model statement	AIC_C	Δ_i[1]	w_i[2]
Females (overall)			
β_0 + Age	103.8		0.262
β_0 + Age + H_{GW}[3]	105.4	1.59	0.118
β_0 + Age + H_{MHC}[3]	105.8	1.98	0.097
Males (overall)			
β_0 + Average weight + H_{GW}[3]	92.0		0.191
β_0 + Age + Average weight + H_{GW}[3]	93.0	1.02	0.115
β_0 + Average weight	93.1	1.08	0.111
β_0 + Average weight + H_{GW}[3] + H_{MHC}[3]	93.6	1.61	0.085
Females (relative)[3]			
β_0 + z.Age	103.3		0.275
β_0 + z.Age + z.Average weight	104.8	1.50	0.130
β_0 + z.Age + z.H_{GW}[3]	105.2	1.90	0.106
Males (relative)[4]			
β_0 + z.Age + z.H_{GW}[3]	92.7		0.214
β_0 + z.Age	93.0	0.30	0.184

[1] Change in AIC_C from the best model.

[2] Akaike model weight.

[3] Genome-wide heterozygosity (H_{GW}) and MHC heterozygosity (H_{MHC}) were standardised across all loci for which an individual was genotyped to reduce the influence of missing data on the analysis.

[4] All predictors were converted to z-scores within each enclosure year and sex before input to models to reflect competition amongst individuals.

Appendix 9: Supplementary to Chapter 7

This appendix relates to Chapter 7: First empirical evidence of selection in captivity in an endangered vertebrate.

A9.1 SUPPLEMENTARY METHODS

Parentage confirmation

For high-intensity triads, the studbook (Srb, 2018) and keeper records were examined to confirm the cohabitation of the sire and dam during the relevant breeding season and verify unambiguous attribution of offspring to the dam. For devils housed in groups at medium- and low-intensity sites, parentage was determined using up to 33 microsatellite markers (following Jones *et al.*, 2003; Gooley *et al.*, 2017), and the software Cervus (Kalinowski *et al.*, 2007), and/or with ~ 1,126 SNPs generated by restriction-site associated DNA sequencing (Diversity Arrays Technology Pty. Ltd.) and the R package 'sequoia' (Huisman, 2017), using the method developed in Chapter 4. We also verified our parentage results against biological knowledge of the species, such as maximal number of offspring per female of four (Guiler, 1970), and known cohabitation of males and females in a given breeding year.

SNP mismatch handling

Mismatches between offspring and parental genotypes can occur due to mutation (likely rare), sequencing error, or incorrect triad assignment. We used Cervus to check for mismatches using the presumed sire and dam as fixed candidate parents of each given offspring and found 25/184 (13.6%) triads had at least one SNP mismatching. Triads varied in the number of SNP loci that they were sequenced at, so we calculated the mismatch percentage at both the sire, dam and triad level. Triads with > 5% mismatches at either the sire, dam or triad level were assumed to be incorrect and excluded from the analysis (18 triads removed). Of the 18 triads removed, 8 had been assigned using breeding records only, and 10 using molecular methods, 9 of which were triads from Maria Island, where it is possible that an untrapped but closely related individual is the true parent. The remaining 7 triads with ≤ 5% mismatches did not have more than 2 SNPs mismatching at either the sire, dam or triad level. These were assumed to be sequencing errors or possible mutations, so the triads were retained for analysis, with the mismatching data masked out.

Fitness modelling

We used logistic regression where the response variable litter size was fitted as a binomial two-column matrix of successes and failures, such that the number of successes was the number of observed offspring, and the number of failures was four minus the number of offspring (as the maximum litter size is four; Guiler, 1970). Because the devil IP is intensively managed, past breeding success of an individual may influence the likelihood of that individual being given further opportunities to breed, and so multiple breeding attempts from a given female are not necessarily independent. Thus, we included only the first breeding attempt of each female. In addition to genotype score, regression modelling also included breeding year, dam age and enclosure type (medium/high intensity versus low intensity housing) as fixed predictors (Farquharson *et al.*, 2017; Appendix 10). Models were fitted in R using 'lme4' (Bates *et al.*, 2015), standardised with 'arm' (Gelman & Su, 2015), and model selection performed via information theory (Grueber *et al.*, 2011) using 'MuMIn' (Barton, 2018). Regression results are reported on the standardised scale (data were standardised by subtracting the mean and dividing by 2 SD, [Gelman, 2008]). Parameters of interest are converted back to the original scale for interpretation on figures; this was only performed for genotype predictors that showed very strong support for their inclusion in the final model (i.e. they had a relative importance, also known as the sum of Akaike weights, greater than 0.9). Confidence intervals around fitted values were evaluated via parametric bootstrapping.

A9.2 SUPPLEMENTARY TABLES

Table A9.2.1: Research activity on adaptation to captivity.

We conducted a literature survey to generate an overview of research activity in the field of conservation genetics, and determine how much research was being undertaken on adaptation to captivity. On 4 December 2018, we used Scopus to conduct a "cited search" of all papers that reference Frankham's 2010a review paper of critical issues in conservation genetics, which included adaptation to captivity as a priority research challenge. Although our method is not intended to exhaustively identify all published works in conservation genetics, we expect that the resulting 215 works are representative of academic focus in the field, potentially capturing papers that target the conservation genetic research challenges identified therein (Frankham, 2010a). All papers were downloaded to an Endnote library, and sorted into broad categories based on the title and abstract (in the first instance), and full text (if necessary). A total of 13 studies that had an *ex situ* (e.g. captive) focus were further subdivided into more-specific categories.

Topic	N	References
Reviews and other secondary literature (such as book chapters)	33	Mobley *et al.*, 2011; Uller & Leimu, 2011; Angeloni *et al.*, 2012; Braby *et al.*, 2012; Palstra & Fraser, 2012; Segelbacher, 2012; Taylor & Friesen, 2012; Van Andel & Aronson, 2012b; Van Andel & Aronson, 2012a; Steiner *et al.*, 2013; Duarte *et al.*, 2015; Fisch *et al.*, 2015; Grueber, 2015; Larson *et al.*, 2015; Parlato *et al.*, 2015; Wilkening *et al.*, 2015; Clulow & Clulow, 2016; Figiel, 2016; Olivieri *et al.*, 2016; Schierenbeck, 2016; Shepard *et al.*, 2016; Taylor & Soanes, 2016; Anson, 2017; Fuentes-Pardo & Ruzzante, 2017; Nualart *et al.*, 2017; Ornosa *et al.*, 2017; Pérez-Espona, 2017; Sheth & Thaker, 2017; Sork, 2017; Tingley *et al.*, 2017; Fan *et al.*, 2018; Perez *et al.*, 2018; Torres-Florez *et al.*, 2018
Policy comment/ management review	24	Dulloo *et al.*, 2010; Haig *et al.*, 2011; Funk *et al.*, 2012; Caro & Sherman, 2013; Dulloo, 2013; Hoban *et al.*, 2013a; Hoban *et al.*, 2013b; Koskela *et al.*, 2013; Lu *et al.*, 2013; Paz-Vinas *et al.*, 2013; Jensen *et al.*, 2014; Pauls *et al.*, 2014; Carbonell, 2015; Braby & Williams, 2016; Fady *et al.*, 2016; Galla *et al.*, 2016; Hermoso *et al.*, 2016; Ledoux *et al.*, 2016; Ottewell *et al.*, 2016; Ovenden *et al.*, 2016; Cook & Sgrò, 2017; von der Heyden, 2017; Cook & Sgrò, 2018; Lange *et al.*, 2018

Topic	N	References
Genetic diversity and population structure	105	Beatty & Provan, 2011; Birt *et al.*, 2011; Dalianis *et al.*, 2011; Lawton *et al.*, 2011; Zanetti *et al.*, 2011; Zschokke *et al.*, 2011; Brütting *et al.*, 2012; Gömöry *et al.*, 2012; Huang & Zhou, 2012; Kaya *et al.*, 2012; Munshi-South, 2012; Perry *et al.*, 2012; Sapir & Mazzucco, 2012; Spencer *et al.*, 2012; Terracciano *et al.*, 2012; Tnah *et al.*, 2012; Wenzel *et al.*, 2012; Brauer *et al.*, 2013; Díez-del-Molino *et al.*, 2013; Lesser *et al.*, 2013; Lopez & Barriero, 2013; Perry *et al.*, 2013; Sanz *et al.*, 2013; Tsykun *et al.*, 2013; Baalsrud *et al.*, 2014; Baden *et al.*, 2014; Baillie *et al.*, 2014; Fernández-Mazuecos *et al.*, 2014; Halbur *et al.*, 2014; Katz *et al.*, 2014; Li *et al.*, 2014b; Roy *et al.*, 2014; Sanchez *et al.*, 2014; Schwalm *et al.*, 2014; Torres-Florez *et al.*, 2014; Turlure *et al.*, 2014; Yao *et al.*, 2014; Álvarez *et al.*, 2015; Benavente *et al.*, 2015; Bian *et al.*, 2015a; Bian *et al.*, 2015b; de Camargo *et al.*, 2015; Di Giacomo *et al.*, 2015; Farias *et al.*, 2015; García-Navas *et al.*, 2015; Gradish *et al.*, 2015; Guo *et al.*, 2015; Hadas *et al.*, 2015; Larriera *et al.*, 2015; Li *et al.*, 2015; Malekian *et al.*, 2015; Najbar *et al.*, 2015; Oakley, 2015; Psaroudaki *et al.*, 2015; Rianti *et al.*, 2015; Suni & Whiteley, 2015; Wang *et al.*, 2015; Ahrens & James, 2016; Al-Janabi *et al.*, 2016; Baillie *et al.*, 2016; Basto *et al.*, 2016; Bohling *et al.*, 2016; Carlson *et al.*, 2016; Chong *et al.*, 2016; Fourcade *et al.*, 2016; Gebiola *et al.*, 2016; Geue *et al.*, 2016; Goossens *et al.*, 2016; Jing *et al.*, 2016; Lopes-Lima *et al.*, 2016; Lumibao *et al.*, 2016; MacLeod & Steinfartz, 2016; Priolli *et al.*, 2016; Rico *et al.*, 2016; Riesgo *et al.*, 2016; Rodríguez-Quilón *et al.*, 2016; Solano *et al.*, 2016; Sousa-Santos *et al.*, 2016; Yardeni *et al.*, 2016; Ayala-Burbano *et al.*, 2017; Carreira *et al.*, 2017; Cox *et al.*, 2017; Domínguez *et al.*, 2017; Faulks *et al.*, 2017; Fonseca *et al.*, 2017; Honka *et al.*, 2017; Jinga & Ashley, 2017; Mouton *et al.*, 2017; Nowland *et al.*, 2017; Rico, 2017; Roitman *et al.*, 2017; Amaike *et al.*, 2018; Amirchakhmaghi *et al.*, 2018; Angelone *et al.*, 2018; Bradshaw *et al.*, 2018; Coates *et al.*, 2018; Do Prado *et al.*, 2018; Domingues *et al.*, 2018; Fountain *et al.*, 2018; Gugger *et al.*, 2018; Ismail *et al.*, 2018; Kangas *et al.*, 2018; Padró *et al.*, 2018; Sutton *et al.*, 2018; Mahboob *et al.*, 2019
In situ ecology and evolution, including taxonomy	23	Mariani *et al.*, 2012; Ni *et al.*, 2012; Page *et al.*, 2012; Pertoldi *et al.*, 2012; Rydgren *et al.*, 2012; Fourcade *et al.*, 2013; Pluess *et al.*, 2013; Solano *et al.*, 2013; Beltran *et al.*, 2014; Wan *et al.*, 2014; Wordley *et al.*, 2014; Jaramillo-Correa *et al.*, 2015; Ramírez-Valiente & Robledo-Arnuncio, 2015; Silva *et al.*, 2015; Stapley *et al.*, 2015; Brüniche-Olsen *et al.*, 2016; Hasbún *et al.*, 2016; Cobben *et al.*, 2017; de Groot *et al.*, 2017; Lima *et al.*, 2017; Bangs *et al.*, 2018; Capitani *et al.*, 2018; Truettner *et al.*, 2018

Topic	N	References
Reintroductions	12	Huff *et al.*, 2010; Weeks *et al.*, 2011; Lintermans, 2013; Tollington *et al.*, 2013; Li *et al.*, 2014a; Liu *et al.*, 2014; Lumibao & McLachlan, 2014; Frankham, 2016; Richards *et al.*, 2016; Kronenberger *et al.*, 2017; Jensen *et al.*, 2018; Shemesh *et al.*, 2018
Inbreeding depression	3	Angeloni *et al.*, 2011; Reed *et al.*, 2012; Angeloni *et al.*, 2014
Disease	2	Criscione, 2013; Cornwall *et al.*, 2018
Ex situ conservation	13	(see below)
Wild vs. captive genetic diversity	6	Brütting *et al.*, 2013; Ozer & Ashley, 2013; Shan *et al.*, 2014; Witzenberger & Hochkirch, 2014; Castellanos-Morales *et al.*, 2016; Anderson *et al.*, 2017
Captive management	3	Leus *et al.*, 2011; Rodewald *et al.*, 2011; Sandoval-Castillo *et al.*, 2017
Sampling design	2	Hoban & Schlarbaum, 2014; Hoban & Strand, 2015
Genetic rescue experiment	1	Weisenberger *et al.*, 2014
Reproductive success	1	Kiik *et al.*, 2017

Table A9.2.2: Details of the five sequencing amplicons used in this study.

Amplicon ID	Genomic location of amplicon (UCSC)	Nearby genes (UCSC)	Location of SNPs	Function notes	Reference
IL17B	chr1_GL834622:506838-509979	*IL17B*:chr1_GL834622506838-509979	The first few SNPs are predicted to fall just outside the gene with most other SNPs falling in introns. Two SNPs (506759 and 509694) are predicted to fall in exons at either end of the gene.	Immune region of interest-interleukin.	Morris *et al.*, 2015
UNC13B	chr2_GL841539:2046503-2056190	*UNC13B*:chr2_GL841539:1873970-2205813	Amplicon is largely in intronic regions although one SNP (2050495) is predicted to fall in an exon	This amplicon was designed as it came up as significant in a preliminary GWAS analysis for resilience to devil facial tumour disease, although this finding was not upheld with the addition of further samples (Wright *et al.*, 2017).	Wright *et al.*, 2015
NF2	chr2_GL842060:28389-37701	*NF2*:chr2_GL842060:81458-149966	SNPs are 44 kb from the NF2 gene.	*NF2* is involved in progression of schwannomas (Hadfield *et al.*, 2010), of which DFTD is a type (Murchison *et al.*, 2010). This amplicon is considered putatively neutral as the amplified region does not lie within the gene and has not displayed signs of selection in past analyses (BW unpubl. data).	Wright *et al.*, 2015

Amplicon ID	Genomic location of amplicon (UCSC)	Nearby genes (UCSC)	Location of SNPs	Function notes	Reference
DIG12	chr3_GL850167: 29343-35668	*DIG12*:chr3_GL850 167:29387-35520	Only one SNP is expected to fall in an exon.	Immune region of interest: devil immunoglobulin. Note, this gene is annotated as *LILRA5/6* on the UCSC genome.	Morris *et al.*, 2015
AGA	chr6_GL864793:3 14413-323784	*AGA*:chr6_GL86479 3:242456-261095 *NEIL3*:chr6_GL8647 93:343085-410581	SNPs are in intergenic regions: 53.4 kb from AGA and 19.6 kb from NEIL3	Originally chosen as a non-coding region, representative on chromosome 6.	Wright *et al.*, 2015

Table A9.2.3: Linear modelling of the effects of genotype on litter size for female Tasmanian devils.

Litter size modelled as a proportion of the maximum four joeys, showing model averaged effect sizes with adjusted standard error in parentheses and relative importance (RI; sum of Akaike weights) in brackets (statistics pertaining to top model sets are provided in Table A9.2.4). Blanks indicate predictors that were tested, but which did not appear in the top model set. Predictors with both a strong effect size (relative to error) and low model selection uncertainty (i.e. high RI) are in bold (see Chapter 7 Methods for more information). Effects are standardised following Gelman (2008), and models are fitted with a logit link.

Selection	Gene	N	Intercept	Enclosure type[1]	Gene	Breeding year	Dam age[2]
Directional	IL17B	102	-0.463 (0.106)	-0.193 (0.211) [0.34]	0.479 (0.227) [1.00]		-0.812 (0.234) [1.00]
	UNC13B	95	-0.410 (0.109)	-0.256 (0.219) [0.41]			-0.849 (0.238) [1.00]
	NF2	86	-0.465 (0.117)	-0.210 (0.234) [0.23]		0.277 (0.231) [0.32]	-1.047 (0.264) [1.00]
	DIG12	88	-0.472 (0.117)	-0.342 (0.302) [0.49]	-0.578 (0.233) [1.00]	-0.378 (0.310) [0.20]	-1.160 (0.274) [1.00]
	AGA	72	-0.338 (0.129)	-0.299 (0.308) [0.26]	-0.486 (0.262) [0.76]	0.865 (0.293) [1.00]	-1.006 (0.295) [1.00]
Heterozygosity	IL17B	102	-0.456 (0.106)	-0.206 (0.210) [0.36]	0.298 (0.248) [0.42]		-0.837 (0.232) [1.00]
	UNC13B	95	-0.410 (0.109)	-0.256 (0.219) [0.41]			-0.849 (0.238) [1.00]
	NF2	86	-0.466 (0.117)	-0.210 (0.234) [0.20]	-0.122 (0.247) [0.15]	0.277 (0.231) [0.27]	-1.051 (0.265) [1.00]
	DIG12	88	-0.487 (0.119)	-0.397 (0.317) [0.54]	-0.972 (0.262) [1.00]	-0.424 (0.314) [0.25]	-1.266 (0.285) [1.00]
	AGA	72	-0.330 (0.128)	-0.342 (0.305) [0.29]	0.240 (0.261) [0.24]	0.840 (0.298) [1.00]	-1.066 (0.292) [1.00]

[1] Medium/high intensity housing versus low intensity housing.

[2] Only first breeding attempts of individual females were included.

Table A9.2.4: Top model sets for effect of genotype on litter size.
Information theoretic statistics for the component models (top model sets) that contributed to the final (averaged) models shown in Table A9.2.3. An "X" indicates that a predictor was included in each model. See the caption to Table A9.2.3 for more information on model structure.

Selection	Gene	ET[1]	Gene	Year[2]	Age[3]	df	logLik	AICc	delta	weight
Directional	IL17B		X		X	3	-205.9	418.0		0.66
		X	X		X	4	-205.5	419.3	1.31	0.34
	UNC13B				X	2	-191.7	387.6		0.59
		X			X	3	-191.0	388.3	0.72	0.41
	NF2				X	2	-168.3	340.8		0.45
				X	X	3	-167.6	341.5	0.65	0.32
		X			X	3	-167.9	342.1	1.32	0.23
	DIG12		X		X	3	-173.1	352.4		0.51
		X	X		X	4	-172.5	353.5	1.16	0.29
		X	X	X	X	5	-171.8	354.2	1.85	0.20
	AGA		X	X	X	4	-137.1	282.7		0.50
		X	X	X	X	5	-136.6	284.1	1.33	0.26
				X	X	3	-138.9	284.2	1.49	0.24
Heterozygosity	IL17B				X	2	-208.3	420.7		0.37
			X		X	3	-207.5	421.3	0.60	0.27
		X			X	3	-207.8	421.8	1.11	0.21
		X	X		X	4	-207.1	422.5	1.80	0.15
	UNC13B				X	2	-191.7	387.6		0.59
		X			X	3	-191.0	388.3	0.72	0.41
	NF2				X	2	-168.3	340.8		0.38
				X	X	3	-167.6	341.5	0.65	0.27
		X			X	3	-167.9	342.1	1.32	0.20
			X		X	3	-168.2	342.7	1.90	0.15
	DIG12		X		X	3	-169.0	344.4		0.46
		X	X		X	4	-168.4	345.3	0.92	0.29
		X	X	X	X	5	-167.5	345.6	1.26	0.25
	AGA			X	X	3	-138.9	284.2		0.47
		X		X	X	4	-138.3	285.1	0.93	0.29
			X	X	X	4	-138.5	285.6	1.36	0.24

[1] Enclosure type (medium/high intensity housing versus low intensity housing).
[2] Breeding year.
[3] Dam age; only first breeding attempts of individual females were included.

A9.3 SUPPLEMENTARY FIGURES

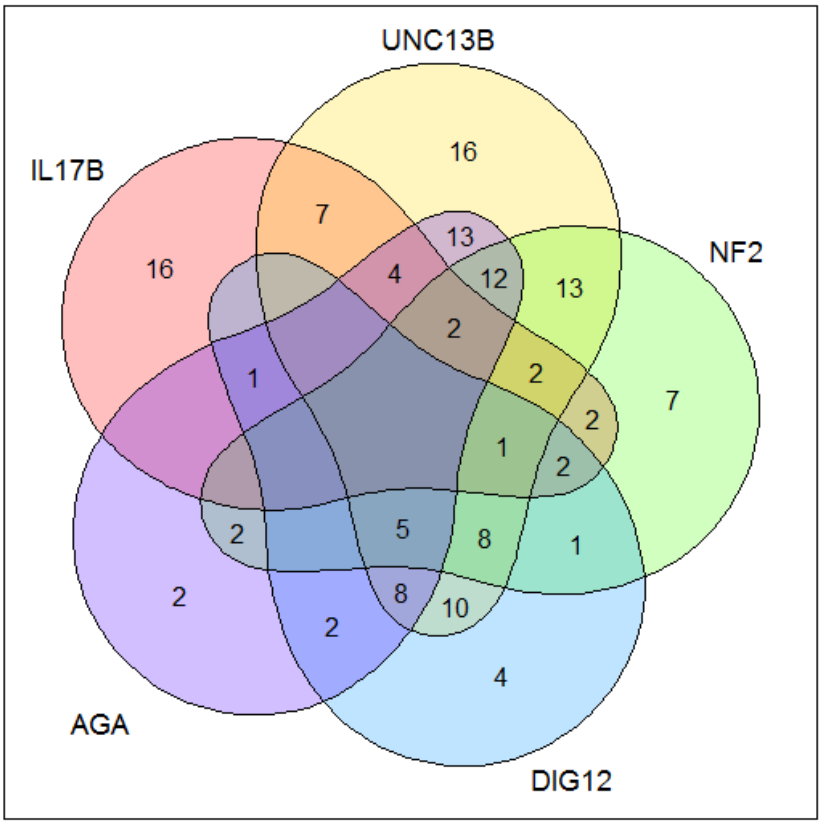

Figure A9.3.1: Venn diagram showing the number of triads sequenced at each amplicon or combination of amplicons.
The distribution of values across the diagram indicates that many trios were largely sequenced at different loci, and therefore that patterns across loci are unlikely to result from structure in the dataset.

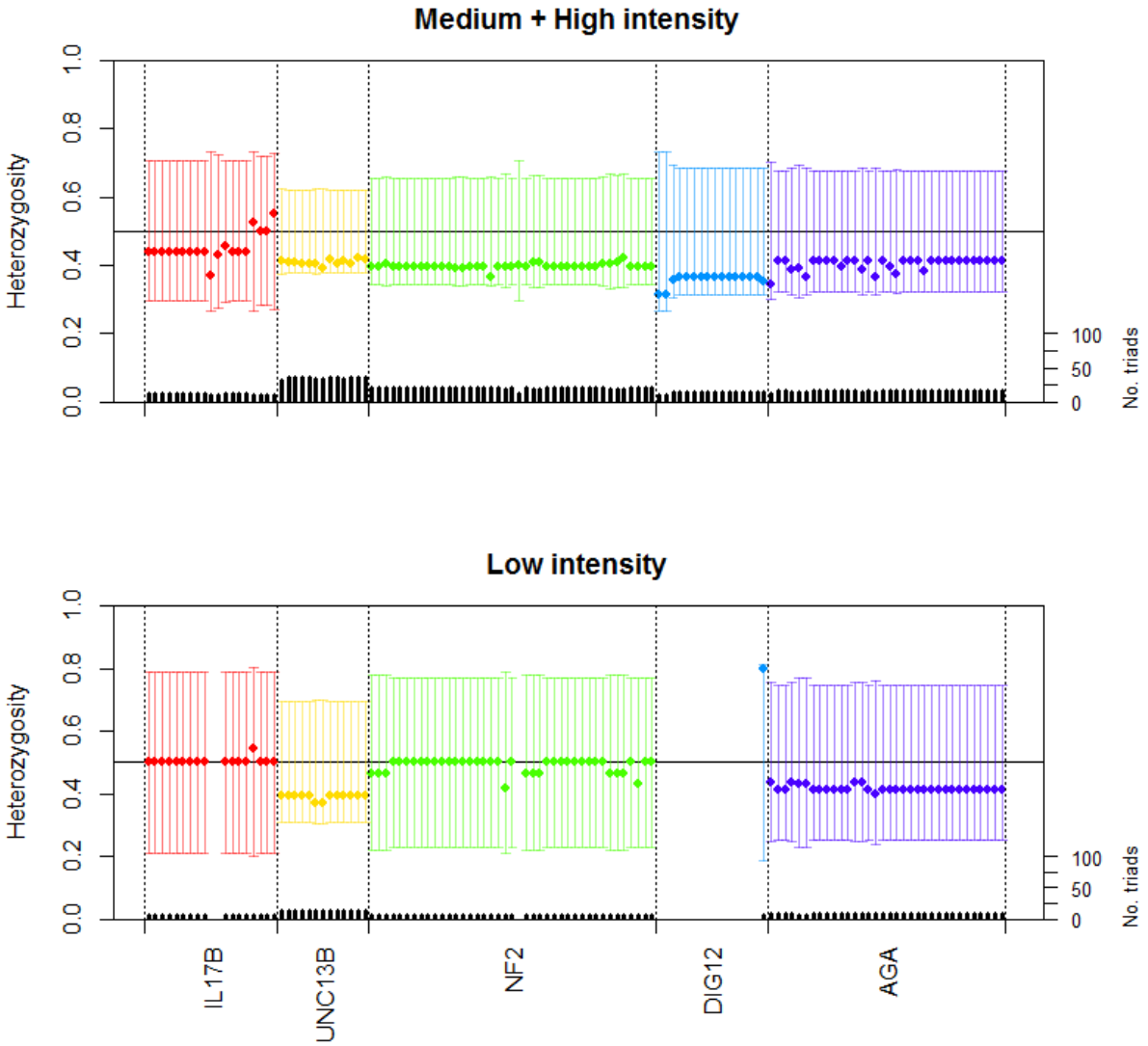

Figure A9.3.2: Comparison of observed and expected heterozygosity between sites with varying environmental variation.

Populations are buffered against environmental variation in both the high and medium-intensity housing, while individual exposure to natural variation in food availability, weather, etc., is greater at the low-intensity Maria Island site.

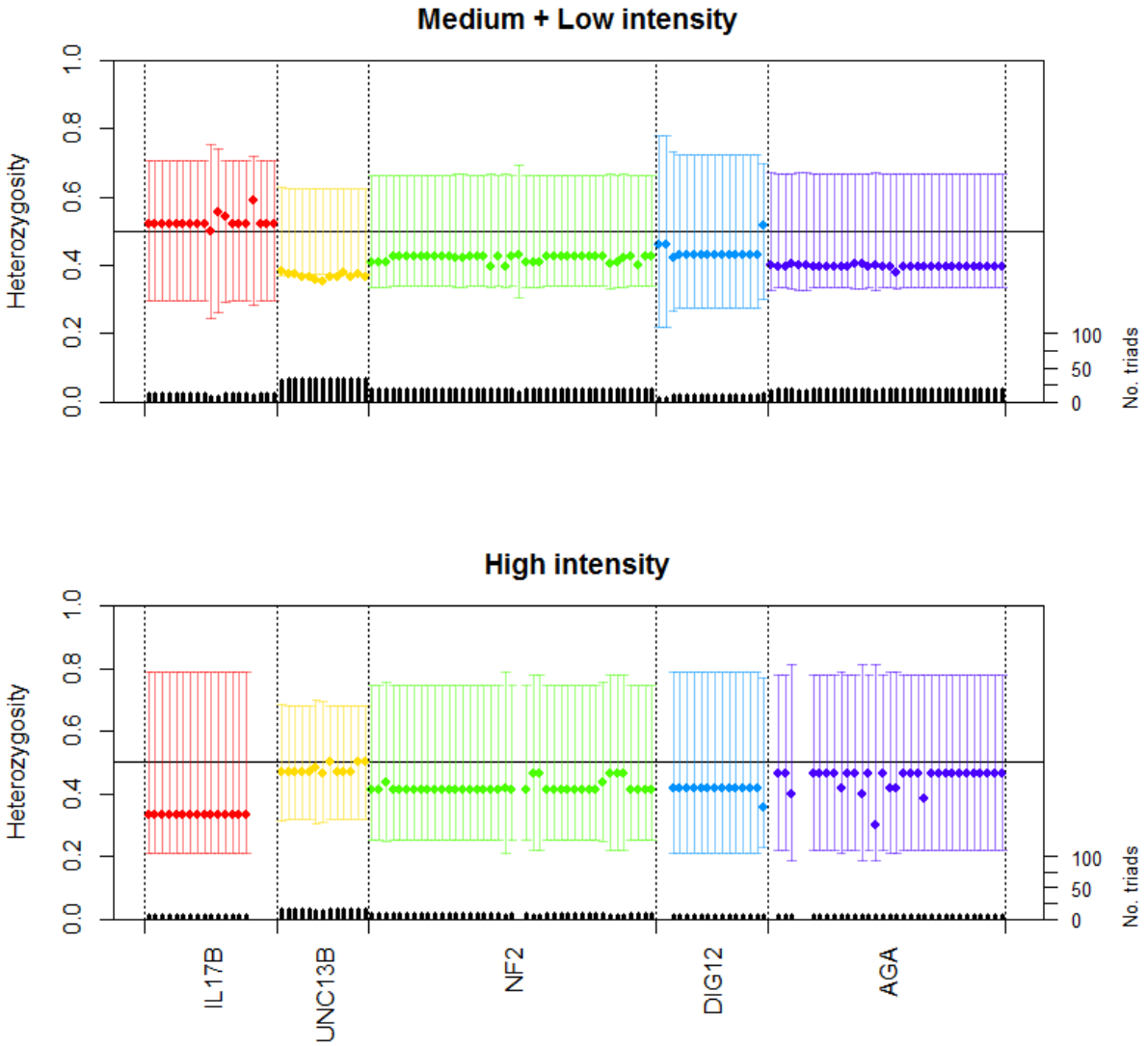

Figure A9.3.3: Comparison of observed and expected heterozygosity between sites with varying opportunity for mate choice.
Mate choice may occur at both the medium-intensity group housing, and the low intensity island population, while forced monogamy is used at high-intensity sites.

Appendix 10: Pedigree analysis reveals a generational decline in reproductive success of captive Tasmanian devil (*Sarcophilus harrisii*): implications for captive management of threatened species

A10.1 BACKGROUND

The following article provides an investigation into reproductive success in the Tasmanian devil insurance population. Modelling the factors contributing to reproductive success revealed a generational decline in the probability of a female producing offspring. Reproductive success increased with the number of years an institution had held devils, suggesting husbandry expertise that could be shared to improve breeding at newer zoos. Dam age also had a strong negative effect on reproductive success, as per Chapter 6.

This paper was revised and published during the course of my PhD research, incorporating results that I obtained during my AVBS(Hons) degree. I collated the data, performed the analysis, prepared figures and tables and drafted the manuscript, with the support and guidance of Carolyn Hogg and Catherine Grueber, who obtained funding for the research.

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Original Article

Pedigree analysis reveals a generational decline in reproductive success of captive Tasmanian devil (*Sarcophilus harrisii*): implications for captive management of threatened species

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Abstract

Captive breeding programs are an increasingly popular tool to augment the conservation of threatened wild populations. Many programs keep detailed pedigrees, which are used to prescribe breeding targets to meet demographic and genetic goals. Annual breeding targets are based on previous productivity, but do not account for changes in reproductive success that may occur over generations in captivity and which may impair the ability of a program to meet its goals. We utilize a large studbook from the Tasmanian devil (*Sarcophilus harrisii*) captive breeding program to investigate biological, genetic, and environmental factors that affect variation in reproductive success among individuals and over generations of captive breeding. Reproductive success declined with increasing generations in captivity: wild-born females had a 56.5% chance of producing a litter compared to a 2.8% chance for generation 5 captive-born females ($N = 182$) and when they did, wild-born females produced more offspring (3.1 joeys, 95% CI: 2.76–3.38, compared to 2.7 joeys, 95% CI: 2.55–2.90, in captive-born females [$N = 105$]). Reproductive success also declined as dam age at first breeding increased. Our results reveal a conflict with the widely cited conservation strategy to limit opportunity for selection by extending generation length through delaying reproduction, as captive breeding programs that delay female breeding with this goal in mind risk reduced productivity. Our data demonstrate the benefit of pedigree analysis to identify biological processes that reveal crucial trade-offs with conservation best-practice.

Subject area: Conservation genetics and biodiversity
Key words: adaptation to captivity, captive breeding, inbreeding, reproduction, senescence

Introduction

The world is facing an extinction crisis, with 26% of mammalian species threatened globally (IUCN 2014). In response, conservation management is becoming increasingly intensive, utilizing translocations, reintroductions, metapopulation management, and captive breeding to support wild populations (Pritchard et al. 2012; Seddon

et al. 2012; Lacy 2013; Canessa et al. 2016). The International Union for the Conservation of Nature (IUCN) has recognized the value of breeding threatened species in captivity until reintroductions to the wild are possible (Conde et al. 2011), and so reintroduction is a stated goal of many "insurance populations" today (Conway 2011). *Ex situ* management (i.e., captive breeding for reintroduction) aims to minimize genetic diversity loss, inbreeding and adaptation to captivity, in order to retain founder diversity, avoid inbreeding depression, and maximize evolutionary potential (Ballou et al. 2010). Quantitative goals are used, such as retaining over 90% of wild-sourced genetic diversity over 100 years (Ballou et al. 2010). Demographically, programs aim to rapidly increase population size to avoid extinction and maintain reliable reproduction (Frankham et al. 2004).

The World Zoo and Aquarium Conservation Strategy recommends the use of highly controlled breeding strategies to achieve population goals (WAZA 2005). Global management practices involve the use of "breeding recommendations": specific plans indicating which individual animals should be paired to breed. The widely-accepted method for retaining genetic diversity in managed populations is to provide breeding recommendations that pair animals with low and similar mean kinships (average relatedness to the rest of the population) (Lacy 1995; Frankham et al. 2010). Software programs, such as PMx (Lacy et al. 2012), are used to calculate the total population size required to meet the program's goals and therefore the number of breeding recommendations required each year to produce sufficient offspring, either to offset deaths or for release. Annual breeding targets, however, are always based on a given population's historical productivity and do not account for changes over generations in captivity. As a result, breeding programs may underestimate the number of breeding recommendations required if productivity has declined, limiting the effectiveness of intensive population management.

Differences between the wild and captive environment may be responsible for changes in productivity. The World Association of Zoos and Aquariums has highlighted adaptation to captivity as a key area for future research, in order to better understand the effects of captive breeding on the long-term viability of captive populations and reintroduction success (WAZA 2005). Genetic adaptation to captivity has been described in fish, insect models, and laboratory mice (Frankham and Loebel 1992; Araki et al. 2007; Lacy et al. 2013), though it is yet to be examined in captive populations of wild mammals (i.e., zoos and other similar intensive management settings). Genetic adaptation to captivity has been experimentally determined as the cause of reduced reproduction in reintroduced steelhead trout after controlling for environmental factors such as rearing environment (Araki et al. 2007; Christie et al. 2012). Species characterized by short generation lengths have a higher risk of adaptation to captivity over time than species with long generation lengths (Frankham 2008). Adaptation to captivity is likely to be a serious problem facing captive species, and "deserves a much higher priority than it is currently receiving" (Frankham 2008, p. 325).

Zoos keep detailed, long-term datasets that are available for analysis (Pelletier et al. 2009; Conde et al. 2011). Our study is the first to statistically model the effect of multiple generations in captivity in a multiple regression framework that controls for other parameters, such as age and institutional experience, that may affect productivity. We examined data from an Australian endemic marsupial, Tasmanian devil (*Sarcophilus harrisii*). The Tasmanian devil insurance population was established in 2006 in response to the catastrophic decline of the wild population due to devil facial tumor disease (Grueber, Peel et al. 2015; Hogg et al. 2015). This insurance population is ideal for answering broad questions about genetic impacts of captive breeding in conservation for two main reasons. First, it is one of the largest conservation breeding programs in the region, so a large dataset is available for analysis. The studbook currently contains over 1685 animals, and pedigree depth ranges from 0–5.625 generations, with 296 animals ≥3rd generation (including breeding data collected from captive devils prior to the establishment of the formal insurance population [Srb 2015]). Note that generation number is defined as 0 for founder animals or as the average generation of the parents plus one for captive offspring (e.g., the offspring of a founder and a first-generation captive-born animal would be considered generation 1.5). Second, the devil insurance population as a whole is distributed over a variety of holding types: from semi-natural island sites with basic monitoring only, to large managed enclosures, and intensive zoo facilities, with a range of human intervention from little-to-no contact to full veterinary treatment (DPIPWE 2010; Hogg et al. 2016). Data from all 20 sites holding the devil insurance population were available for this analysis. The devil program therefore represents the diversity of conservation practices in place today, and acts as an exemplar for the impacts of intensive management on population productivity in conservation. In addition, the devil has a short generation length of 4 years (Woinarski et al. 2014), making it particularly vulnerable to adaptation to captivity. Captive adaptations that affect fecundity could severely limit the ability of released animals to contribute to wild populations (Frankham 2008). Like many other captive breeding programs around the world, the ultimate goal of the devil recovery program is to release devils to ensure an ecologically functional population in the wild (STDP 2014).

In this study, we evaluated seven biological, genetic, and environmental factors thought to influence reproductive success of captive devils using nine years of pedigree-based studbook data and breeding records. Our results have broad implications for the management of threatened species worldwide, and illustrate the value of long-term conservation datasets for the analysis of genetic processes associated with captive breeding.

Methods

The devil is the world's largest marsupial carnivore (up to 12 kg) (Owen and Pemberton 2011), and is listed as endangered by the IUCN and under the Australian *Environmental Protection and Biodiversity Conservation Act 1999* (EPBC Act) (Woinarski et al. 2014). The Tasmanian devil insurance population is managed by the Zoo and Aquarium Association Australasia (ZAA), the peak industry body in Australasia responsible for the management of 121 captive programs across 90 institutions (Hogg 2013). The Tasmanian devil program is one of the conservation programs managed by the ZAA (Hogg 2013), where monthly data is inputted into the studbook using SPARKS and ZIMS software (Species360 2017). The ZAA uses pedigree data to generate annual breeding recommendations for devils using the mean kinship strategy (Hogg 2013). The full insurance population studbook (from 2006 to 2014) and associated breeding recommendations comprise the starting point for our analysis.

The Tasmanian devil is polyovular and monoestrus, with spontaneous ovulation. Sexual maturity is reached at 1 year, though most females breed at 2 years. Devils have a life expectancy of up to 6 years in the wild (disease-free areas) and 8 years in captivity (Woinarski et al. 2014); and live to approximately 4 years in diseased

areas (Jones et al. 2008). Devils are seasonal breeders, with females capable of producing one litter each year. Mating occurs in March with births in April after a short gestation period of 31 days, and weaning 250 days after birth (Woinarski et al. 2014). Females can give birth to over 20 early development stage young (Guiler 1970). However, females have only four teats with which to nurse attached immature offspring, so the devil is biologically limited to producing 4 offspring with litter sizes ranging from 1 to 4 (Guiler 1970). The small size of early offspring and female denning behavior (Owen and Pemberton 2011) present challenges for captive managers in accurately determining litter size, and as such offspring accessioned in the studbook (Srb 2015) are commonly those identified at weaning.

Not all breeding recommendations made by ZAA are necessarily attempted by holding institutions, for a variety of logistical and operational reasons. Therefore, our dataset only included animals that were housed together during the breeding season (i.e., breeding was attempted). Our dataset included all 20 insurance population breeding facilities for which data were available, classified into two broad categories: intensive (15) and free-range (5) (Hogg et al. 2015). At intensive sites, breeding recommendations are given to a particular male–female pair, which are housed together when the female comes into estrus; here, the pair is the statistical unit for analysis. At free-range sites, breeding recommendations are given for a group of devils that are housed together and can breed freely. Female reproductive status (number of pouch young) is determined by direct observations at both intensive and free-range sites, but at free-range sites the sire of weaned young cannot always be determined (Hogg et al. 2015); we could not use pairs as the statistical unit for data from the free-range sites. Therefore, we performed 2 analyses: one for "intensive pairs" (based on breeding pairs at intensive sites only) and one for "all females" (encompassing both intensive and free-range females).

Factors thought to influence reproductive success in the devil insurance population were modelled using two response variables: whether an attempted breeding recommendation was achieved or not (i.e., produced at least one offspring; referred to herein as "breeding success") and number of offspring produced from successful breeding events (referred to herein as "litter size"). Both responses were analyzed for both the intensive pairs and all-females datasets, giving a total of four main analyses. Data were collected for a total of 11 predictor variables across the four analyses, where applicable (Table 1). Sire age, dam age, sire origin (captive-born or wild-born), and dam origin were obtained from the studbook (Srb 2015). PMx (version 1.2.20140905) (Lacy et al. 2012) was used to determine sire and dam inbreeding coefficients, and sire and dam generation. Annual ZAA census data from 2006–2014 were used to obtain number of years since each site first held the species in any given year, and number of holdings of devils at each site each year. "Management type" was also included in our "all females" analyses as a categorical predictor, referring to intensive or free-range sites. In 2007–2008 (the early stages of the program [Hogg et al. 2015]), many pairs were given multiple chances to breed over consecutive breeding seasons, but in more recent years very few devils were given multiple opportunities; we therefore only included the first breeding attempt of a given pair or female (in their lifetime). Data from the first year of the program, 2006, was excluded as most devils were quarantined with only limited breeding (Hogg et al. 2015). Inbreeding parameters could not be included in the analyses as each parameter had a median of 0 with a limited range (only 3/130 females and 4/105 males at intensive sites, and 3/233 females in the all-females analysis had a non-zero inbreeding coefficient, Table 1). The use of pedigree-based management strategies to avoid breeding closely related individuals accounts for the low level of inbreeding in the population.

We evaluated the relationships between our predictor and response variables using generalized linear mixed models, with site and year included in all models as random factors to account for mean differences in reproductive success between institutions and

Table 1. Summary statistics for the 2 random effects, 11 fixed predictors of interest and 2 response variables of the 2 datasets [range (mean/median)]

	Intensive pairs	All females
Number of breeding records	239	500
Unique pairs	182	233[a]
Years	2006–2014	2006–2014
Sites	14[b]	17[c]
Sire age	2–6 (3.39/3.00)[d]	
Dam age	1–5 (2.85/3.00)[d]	1–4 (2.32/2.00)[d]
Sire generation	0.00–4.50 (0.78/1.00)[d]	
Dam generation	0.00–5.20 (0.90/1.00)[d]	0.00–5.20 (1.04/1.00)[d]
Sire origin (captive; wild)	92; 90[d]	
Dam origin (captive; wild)	110; 72[d]	155; 78[d]
Sire F	0.00–0.25 (<0.01/0.00)	
Dam F	0.00–0.25 (<0.01/0.00)	0.00–0.25 (<0.01/0.00)
No. years since site held species	0–8 (3.10/3.00)[d]	
No. holdings per site per year	2–140 (36.21/23)[d]	
Management (intensive; free-range)		149; 84[d]
Breeding success; failure	81; 101 (44.5%; 55.5%)	105; 128 (45.1%; 54.9%)
Litter size	1–4 (2.72/3)	1–4 (2.71/3)

Entries left blank indicate data were not available, not collected or not applicable.

[a]Only the first record per female was used; sample size includes $N = 149$ females from intensive dataset, and $N = 84$ females from free-range enclosures.

[b]Excludes one site, see Results.

[c]Excludes three sites, see Results.

[d]Indicates parameters that were included as fixed effects in the respective global model.

years. Correlations between variables of interest were calculated in R (R Core Team 2015) to determine variable independence and suitability for generalized linear modelling. All correlation coefficients between parameters were below 0.42, so no parameters were discarded at this stage. Global models were generated with the "glmer" function of the *lme4* package (Bates et al. 2015) with a binomial logit link. The breeding success/failure response was coded as a binary 1/0. Litter size was modelled as a binomial response (proportion of the maximum litter size of 4) using a 2-column matrix where successes = number of offspring produced and failures = 4 − successes. Because devils are biologically limited to carrying 4 offspring in the pouch (Guiler 1970), this modelling approach prevented our model from predicting biologically impossible litter sizes greater than 4.

To improve comparison of effect sizes within and between models, regression inputs were standardized by subtracting the mean and dividing by 2 standard deviations using the "standardize" function in the *arm* package (Gelman 2008; Gelman and Su 2015). Model selection and inference were conducted under information theory (Burnham and Anderson 2003). The "dredge" function in the *MuMIn* package (Barton 2015) was used to obtain all submodels of each global model. Top models were identified as those within 2 AICc (Akaike's information criterion corrected for small sample size) of the best model (Burnham and Anderson 2003). Model averaging using the natural average method was performed with "model.avg" in the *MuMIn* package (following Grueber et al. 2011). We evaluated 95% CIs for our final parameter estimates as the point estimate ± 1.96 × the unconditional SE. Inference was based on the relative magnitude of effect sizes and their SEs, 95% CIs, and relative importance (RI) values for each parameter. RI values indicate the probability that a parameter is included in the best model, using the sum of Akaike weights from models containing that parameter (Burnham and Anderson 2003). Examining CIs gives the magnitude, direction, and precision of the parameter (Gelman and Hill 2007). Where parameters demonstrated strong effects (see Results), these were further investigated on the natural scale by destandardizing and back-transforming fitted values using "invlogit" in *arm*. Parametric bootstrapping with 1000 iterations was used to estimate 95% CIs for back-transformed fitted values.

Results

For the intensive pairs analyses, of the 300 records in the dataset, 61 pairs were excluded due to missing sire information; however, these data were included in the all-females analysis (below). Age, origin, generation, and the two husbandry parameters were included in global models for both responses. One site was excluded from analysis as the dataset included only successful breeding events. The final intensive pairs dataset included the first breeding attempt of 182 unique pairs (comprising 130 females, 105 males). Dam age, dam generation, and number of years since the site held insurance population devils all had strong effects on breeding success with RIs of 1 (Table 2). As dam generation increased, the probability of breeding success declined steadily from an estimated 56.5% in the wild-born generation to 2.8% at generation 5 (Figure 1a). Our results also

Table 2. Summary results of the final models after model averaging[a]: effects of each predictor on breeding success and on litter size in each analysis

Predictor	Intensive pairs				All females			
	Breeding success (N = 182)		Litter size (N = 81)		Breeding success (N = 233)		Litter size (N = 105)	
	Estimate[b] (SE)[c] *95% CI*	RI[d]	Estimate (SE) *95% CI*	RI	Estimate (SE) *95% CI*	RI	Estimate (SE) *95% CI*	RI
Intercept	−0.43 (0.33) *−1.08, 0.23*		0.76 (0.12) *0.52, 1.00*		−0.49 (0.27) *−1.03, 0.04*		0.76 (0.11) *0.55, 0.96*	
Dam age	−1.87 (0.45) *−2.74, −0.99*	1	—		−0.28 (0.30) *−0.86, 0.30*	0.23	−0.22 (0.21) *−0.63, 0.18*	0.29
Sire age	—		—					
Dam origin (wild)[e]	0.37 (0.65) *−0.90, 1.64*	0.13	0.48 (0.30) *−0.11, 1.08*	0.56	0.18 (0.31) *−0.42, 0.79*	0.11	0.48 (0.23) *0.04, 0.93*	1
Sire origin (wild)[f]	−0.34 (0.47) *−1.25, 0.58*	0.27	—					
Dam generation	−1.48 (0.61) *−2.68, −0.28*	1	−0.47 (0.29) *−1.04, 0.09*	0.57	−0.37 (0.31) *−0.97, 0.22*	0.39	—	
Sire generation	—		—					
Years since site held species	1.31 (0.57) *0.20, 2.42*	1	—					
Holdings/site/year	0.86 (0.61) *−0.33, 2.06*	0.40	−0.19 (0.24) *−0.66, 0.28*	0.27				
Management (intensive)[g]					−0.50 (0.57) *−1.62, 0.61*	0.24	−0.15 (0.22) *−0.57, 0.28*	0.21

Entries left blank indicate predictors not tested (see Methods); dashes indicate global model predictors that did not appear in the top model set. Italic values correspond with 95% CI.

[a]Top models before model averaging are presented in Supporting Information: Table S2, Table S4, Table S6, and Table S8.

[b]Effect sizes have been standardized on 2 SD following Gelman (2008).

[c]SE represents the unconditional standard error.

[d]RI is the relative importance of the parameter in the final model.

[e]Dam origin (captive) was the reference category.

[f]Sire origin (captive) was the reference category.

[g]Management (intensive) was the reference category.

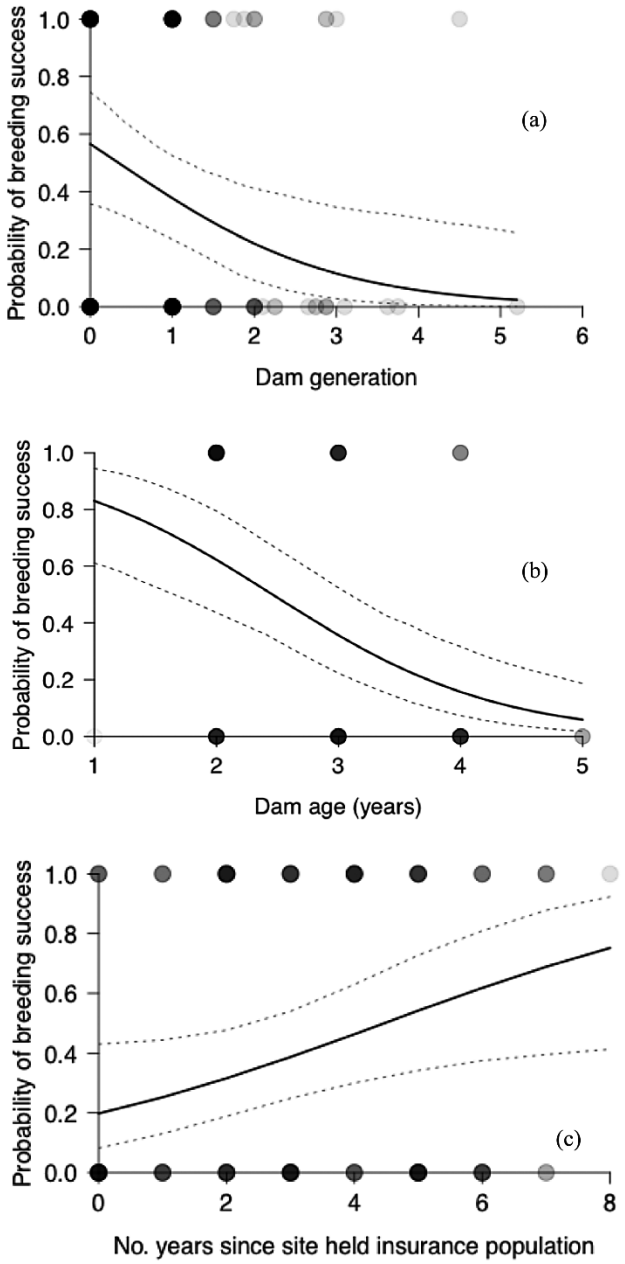

Figure 1. The effect of (**a**) dam generation, (**b**) dam age, and (**c**) number of years since the site first held insurance population devils on the probability of breeding success in the Tasmanian devil at intensive sites. Solid line shows the fitted conditional effect, based on the modelling results shown in Table 2; dashed lines show 95% CIs obtained by parametric bootstrapping (all model predictions have been back-transformed onto the natural scale). Dots represent data points, with higher density dots representing multiple overlaid points.

showed that an increase in dam age at first breeding decreased breeding success at intensive sites with only a 5.9% chance of breeding success by age 5 in our model (Figure 1b). Breeding success increased with number of years since the site first held insurance population devils from an estimated 19.8% in the first year of holding devils to 75.2% by the eighth year (Figure 1c). In the intensive pairs analysis, the model of litter size included 81 litters; after model averaging, none of the investigated parameters demonstrated strong effects on litter size (Table 2).

For the all-females analysis, which encompassed females from intensive and free-range sites, global model parameters for both breeding success and litter size were dam age, origin, and generation, and management type (intensive/free-range). Three sites were excluded from analysis, one as above, the second as it held devils mainly for quarantine and a third as it has no control over breeding. The breeding success model included the first breeding attempt of 233 females. None of the parameters we investigated demonstrated strong effects after model averaging, although all effect sizes were in the same direction as those of the intensive pairs analysis (Table 2). The litter size model contained 105 litters. Dam origin was the only strong predictor of litter size, with RI of 1 (Table 2). The fitted mean litter size of wild-born female devils was 3.1 joeys (95% CI: 2.76–3.38), much higher than the fitted mean for captive-born females of 2.7 joeys (95% CI: 2.55–2.90) (note that female devils are biologically limited to a maximum litter size of 4, see Methods).

The global models for all four analyses before model dredging and averaging are presented in the Supplementary Materials (Tables S1, S3, S5, and S7). For the top model sets after model dredging was performed see Supplementary Tables S2, S4, S6, and S8.

Discussion

We have demonstrated the value of long-term captive breeding datasets for revealing biological processes that may impact species conservation. The Tasmanian devil insurance population represents an ideal opportunity to evaluate the processes impacting reproductive success in captivity, due to the large size and varied nature of the program. When we analyzed the devil studbook, we observed a substantial decline in devil breeding success as generations in captivity increased (Figure 1a), as well as declining reproductive success of females as age at first breeding increased (Figure 1b). Our observation of larger litter sizes from wild-born females, relative to captive-born females, is consistent with a previous analysis, conducted on a subset of the data used here (Hogg et al. [2015]; using Anova). Overall, our observations present important implications for the intensive management of devils and other threatened species in captivity.

Husbandry Experience

We were able to investigate effects of breeding animals in different sites by including 2 novel measures of institutional experience. Our analysis showed that husbandry experience does influence the reproductive success of devils, as breeding success increased with number of years since the site first held insurance devils (Figure 1c). This trend can be used to inform program productivity going forward: for example, placing "high value" animals such as founders in experienced sites will improve the probability of successful breeding. On the other hand, the number of holdings of devils at a site did not demonstrate strong effects on breeding success or litter size (Table 2). Conde et al. (2011) and Conway (2011) advise that zoos specialize in breeding a few threatened species rather than diversifying, assuming that specialization increases breeding success—our results provide empirical support to this assumption.

Dam Age and Dam Generation

We found that, amongst intensively managed female devils, those that were not given their first opportunity to breed soon after maturity showed greatly reduced probability of breeding successfully (Figure 1b), with only a less than 6% chance of breeding success

by age 5 in our model. Dam generation also had a strong effect on breeding success, with only a 2.8% chance of breeding success by the fifth generation of captive breeding. Although dam age and dam generation were important predictors of breeding success in intensively managed devils, in the all-females analysis of breeding success (including all intensive and free-range female devils) these parameters did not demonstrate strong effects (Table 2). In fact, none of the parameters we investigated were of importance in the all-females analysis. It is unclear why such differences were observed between our two datasets, although it is possible that differences in husbandry style may be a contributing factor. Females in free-range enclosures are housed in groups instead of pairs and have some level of mate choice. It is also possible that factors such as body weight, density of animals in the enclosure, and behavior may be more important predictors of breeding success in free-range enclosures than the parameters we investigated. Although our pedigree analysis was unable to include these factors, the direction of the effects for dam generation and age was the same across all models (Table 2), suggesting an overall trend.

Adaptation to Captivity

While differences in productivity between wild and captive animals have been documented in many species, including the European mink (*Mustela lutreola*) (Kiik et al. 2013), island fox (*Urocyon littoralis*) (Calkins et al. 2013), black stilt (*Himantopus novaezelandiae*) (van Heezik et al. 2005), black-and-white ruffed lemur (*Varecia variegata*) (Schwitzer and Kaumanns 2009) and Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants (Clubb et al. 2009), we are not aware of any other studies that have investigated whether productivity in zoo species continues to decline after the first generation in captivity in a multiple regression framework. First-generation declines in the productivity of captive animals relative to wild-born counterparts have been attributed to a variety of factors, including stress, poor body condition and lack of appropriate social development (Levallois and de Marigny 2015). In devils, it is possible that the continual declines we observed, over multiple generations in captivity, may be attributable to similar processes, or other factors. For example, inbreeding can contribute to declines in breeding success in conservation programs (Boakes et al. 2007), although variation in pedigree-based inbreeding metrics was too low to include these parameters in our analyses of devil. Nevertheless, the low variability in pedigree-based inbreeding (Table 1) suggests that conventional measures of inbreeding are unlikely to explain the patterns we observed. That is not to say inbreeding is unimportant for devils, only that current pedigrees are uninformative for predicting the role of inbreeding. For example, at this time insurance population founders are assumed to be unrelated, although it is known that some founders were trapped at least 2 km apart (Hogg et al. 2015), and devils disperse an average of 20 km (Lachish et al. 2011). If some founders were closely related, reduced breeding success with increasing dam generation could result from inbreeding that is unaccounted for in the current pedigree (Rudnick and Lacy 2008). Therefore, generation may be a better predictor of breeding success than inbreeding where molecular inbreeding coefficients are unavailable in the early stages of the pedigree. Molecular resolution of founder relationships, using high-throughput genotyping, may inform these hypotheses and is ongoing (Hogg et al. 2015; Wright et al. 2015).

A further possible cause of the decline in devil productivity with increasing generation times may be selective processes, both in a management and an evolutionary sense. Minimizing adaptation to captivity is one of the key goals of captive breeding programs, along with retaining genetic diversity, however the former is often overlooked in favor of the latter (Montgomery et al. 2010). Under a mean kinship breeding strategy, widely adopted across the zoo industry, animals with low and similar mean kinships are paired in order to equalize founder contributions and minimize inbreeding (see also Introduction; Frankham et al. 2010). This strategy means that, as a program progresses, breeding is increasingly targeted towards underrepresented lineages in order to equalize founder representation (Frankham et al. 2010). If particular lineages are underrepresented early on due to heritable characteristics that contribute to poor breeding success, productivity of the population overall is likely to continue to decline as these lineages are increasingly targeted for breeding. Ultimately, demographic considerations may start to outweigh genetic ones, and recalcitrant breeders provided with fewer breeding opportunities. Such a process could result in adaptation to captivity at the population level, especially during the early stages of program establishment, if poor-breeding lineages become progressively more difficult to breed, fail to contribute, and are ultimately out-competed by good-breeding lineages that become favored by population managers in order to ensure population demographic stability. Although our data do not allow us to directly infer whether genetic adaptation to captivity has occurred in the highly managed devil population, opportunities for selection do exist as a result of competitive breeding in group pens, and characteristics of the species reproductive biology (Grueber, Hogg et al. 2015). Ongoing research by our group is examining the genetic basis of these processes.

Conflicts with Strategies to Minimize Adaptation to Captivity

Reproductive senescence has been reported in the devil at about 5 years of age (Jones et al. 2008) and is confirmed in our analyses (Figure 1b). Long non-reproductive periods have also been seen to reduce fertility and shorten the reproductive life of captive elephants and rhinoceroses (Hermes et al. 2004), suggesting the generality of this pattern, in mammals at least. Extending generation length by delaying reproduction is a key strategy used to minimize adaptation to captivity over time and maintain genetic diversity (Williams and Hoffman 2009; Frankham et al. 2010). While there are good reasons to delay reproduction (slower rate of adaptation to captivity, lower risks upon reintroduction), our findings show that delays pose a risk of severely reduced productivity (Figure 1b), which may threaten the goals of the program if these productivity reductions translate to negative population growth and demographic instability. This crucial trade-off between avoiding adaptation to captivity, and maintaining productivity, should be more readily acknowledged and addressed in captive programs, using quantitative means to make informed management decisions about the age at first reproduction.

Regardless of the underlying cause of declining productivity, we have shown that pedigree analysis of captive breeding populations can improve species management, by enabling more-accurate predictions of annual breeding success based on the characteristics of individuals in the population (e.g., dam generations). Detailed modelling of the factors affecting reproductive success in the captive breeding program can provide valuable information for future management, such as new founding events. For example, as no strong sire effects were observed in any of our analyses, we recommend management to prioritize female founders (paired with

captive males), as this strategy will result in larger litter sizes than the reciprocal (pairing a founder male with a captive female), and will maximize founder contributions. We believe that our results are unlikely to be restricted to devils; further analyses in other species will test this hypothesis.

Conclusions

We have detailed the practical applications of our results to conservation in captivity and the wild; our results have already been incorporated into the ZAA breeding recommendations for the devil. Importantly, we have shown that the productivity of intensively managed populations changes over time: historical data are a poor predictor of future success. Our results also reveal biological impediments to conservation goals, such as the widely recommended strategy to extend generation length by delaying breeding, which we found limits productivity in the devil. Analysis of captive studbooks can provide valuable data to improve management, and reveal important trade-offs in genetic and demographic goals by implementing strategies to avoid adaptation to captivity. In the short-term, managers may compensate for these declines by planning to undertake more breeding than life-history based software (that does not take into account productivity changes over generations in captivity) predicts. Further research is required to determine whether generational decline in breeding success is correlated with true inbreeding levels or is a result of other factors resulting from the captive environment, such as genetic adaptation to captivity. It cannot be assumed that adaptation to captivity is not occurring in managed captive populations, and we urge further study of diverse species to determine whether this conservation issue is a general trend.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Conflict of Interest

The authors have no conflict of interest to declare regarding this manuscript.

Data Availability

Data deposited at Dryad: http://dx.doi.org/10.5061/dryad.58ff4.

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A10.3 SUPPLEMENTARY MATERIAL TO A10.2

Tables are numbered as per A10.2 Main article.

Table S1: Full model before model dredging and averaging for breeding success of the Tasmanian devil at intensive sites.
All models were fitted with the random factors 'Site' and 'Year'. The top models after model dredging are provided in Table S2, and the final model after model averaging is provided in Table 2.

Fixed effects	Estimate[a]	SE
Intercept	-0.30	0.32
Sire age	-0.13	0.50
Sire origin (wild)[b]	-0.59	0.72
Sire generation	-0.27	0.57
Dam age	-1.96	0.51
Dam origin (wild)[c]	0.30	0.68
Dam generation	-1.46	0.70
Years since site held species	1.23	0.60
Holdings	0.89	0.61

[a] Estimates are standardized on two SD following Gelman (2008).

[b] Sire origin (captive) was the reference category.

[c] Dam origin (captive) was the reference category.

Table S2: Top model set (top 2 AIC_C) of generalized linear mixed models for breeding success of the Tasmanian devil at intensive sites.
All models were fitted with the random factors 'Site' and 'Year'. The final model is provided in Table 2.

Model statement	AIC_C	Δ_i[a]	w_i[b]
β_0 + dam age + dam generation + years since site held insurance ppn	217.5		0.104
β_0 + dam age + dam generation + holdings + years since site held insurance ppn	218	0.45	0.083
β_0 + sire origin + dam age + dam generation + years since site held insurance ppn	219.3	1.80	0.042
β_0 + dam origin + dam age + dam generation + years since site held insurance ppn	219.4	1.84	0.042
β_0 + sire origin + dam age + dam generation + holdings + years since site held insurance ppn	219.4	1.86	0.041

[a] Change in AIC_C from the best model.

[b] Akaike model weight.

Table S3: Full model before model dredging and averaging for litter size of the Tasmanian devil at intensive sites.

All models were fitted with the random factors 'Site' and 'Year'. The top models after model dredging are provided in Table S4, and the final model after model averaging is provided in Table 2.

Fixed effects	Estimate[a]	SE
Intercept	0.77	0.12
Sire age	0.26	0.43
Sire origin (wild)[b]	-0.18	0.49
Sire generation	0.05	0.41
Dam age	-0.31	0.37
Dam origin (wild)[c]	0.67	0.54
Dam generation	-0.35	0.43
Years since site held species	0.22	0.36
Holdings	-0.26	0.30

[a] Estimates are standardised on two SD following Gelman (2008).

[b] Sire origin (captive) was the reference category.

[c] Dam origin (captive) was the reference category.

Table S4: Top model set (top 2 AIC_C) of generalized linear mixed models for litter size of the Tasmanian devil at intensive sites.

All models were fitted with the random factors 'Site' and 'Year'. The final model is provided in Table 2.

Model statement	AIC_C	Δ_i[a]	w_i[b]
β_0 + dam generation	231.1		0.061
β_0 + dam origin	231.3	0.15	0.056
β_0 + dam origin + holdings	232.6	1.48	0.029
β_0 + dam origin + dam generation	232.8	1.70	0.026
β_0 + dam generation + holdings	233.1	1.95	0.023

[a] Change in AIC_C from the best model.

[b] Akaike model weight.

Table S5: Full model before model dredging and averaging for breeding success of female Tasmanian devils.

All models were fitted with the random factors 'Site' and 'Year'. The top models after model dredging are provided in Table S6, and the final model after model averaging is provided in Table 2.

Fixed effects	Estimate[a]	SE
Intercept	-0.46	0.26
Dam age	-0.33	0.30
Dam origin (wild)[b]	-0.14	0.47
Dam generation	-0.54	0.47
Management (intensive)[c]	-0.58	0.57

[a] Estimates are standardised on two SD following Gelman (2008).

[b] Dam origin (captive) was the reference category.

[c] Management (free-range) was the reference category.

Table S6: Top model set (top 2 AIC_C) of generalized linear mixed models for the breeding success of female Tasmanian devils.

All models were fitted with the random factors 'Site' and 'Year'. The final model is provided in Table 2.

Model statement	AIC_C	Δ_i[a]	w_i[b]
β_0	315.2		0.172
β_0 + dam generation	315.9	0.67	0.123
β_0 + management	316.5	1.33	0.088
β_0 + dam age	316.6	1.36	0.087
β_0 + dam age + dam generation	316.8	1.63	0.076
β_0 + dam origin	316.9	1.72	0.073
β_0 + management + dam generation	317.1	1.90	0.066

[a] Change in AIC_C from the best model.

[b] Akaike model weight.

Table S7: Full model before model dredging and averaging for litter size of female Tasmanian devils.

All models were fitted with the random factors 'Site' and 'Year'. The top models after model dredging are provided in Table S8, and the final model after model averaging is provided in Table 2.

Fixed effects	Estimate[a]	SE
Intercept	0.76	0.11
Dam age	-0.29	0.22
Dam origin (wild)[b]	0.61	0.34
Dam generation	0.12	0.32
Management (intensive)[c]	-0.20	0.22

[a] Estimates are standardised on two SD following Gelman (2008).

[b] Dam origin (captive) was the reference category.

[c] Management (free-range) was the reference category.

Table S8: Top model set (top 2 AIC_C) of generalized linear mixed models for litter size of female Tasmanian devils (*Sarcophilus harrisii*).

All models were fitted with the random factors 'Site' and 'Year'. The final model is provided in Table 2.

Model statement	AIC_C	Δ_i[a]	w_i[b]
β_0 + dam origin	311.8		0.228
β_0 + dam origin + dam age	312.9	1.06	0.134
β_0 + dam origin + management	313.6	1.75	0.095

[a] Change in AIC_C from the best model.

[b] Akaike model weight.

Appendix 11: Are any populations 'safe'? Unexpected reproductive decline in a population of Tasmanian devils free of devil facial tumour disease

A11.1 BACKGROUND

Since the emergence of devil facial tumour disease in north-east Tasmania in 1996, it has spread westerly across Tasmania. Only two wild populations remain unaffected: a recently discovered population in south-west Tasmania, and a population in the north-western tip at Woolnorth. The Woolnorth population has long-term monitoring data available. In this article, we investigated temporal reproductive success in the Woolnorth population. Reproductive success substantially declined between the 2004-2009 and 2014-2016 time periods. The decline could not be attributed to changes in body condition over the same time. Our findings therefore demonstrate why long-term monitoring of disease-free populations is still essential.

This publication resulted from a collaboration between our research group and our conservation partners in the Save the Tasmanian Devil Program. The data analysis was led by myself and Rebecca Gooley (also completing her PhD in our group at the time of this study); we joint first-authored this article. I analysed reproductive success and body condition data, prepared figures, and drafted sections of the manuscript. Rebecca analysed reproductive success and Southern Oscillation Index data, and drafted sections of the manuscript. The size-adjusted body condition measure developed in this paper has since been applied by the Save the Tasmanian Devil Program to monitor body condition in wild populations including Maria Island.

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Are any populations 'safe'? Unexpected reproductive decline in a population of Tasmanian devils free of devil facial tumour disease

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Abstract

Context. Conservation management relies on baseline demographic data of natural populations. For Tasmanian devils (*Sarcophilus harrisii*), threatened in the wild by two fatal and transmissible cancers (devil facial tumour disease DFTD: DFT1 and DFT2), understanding the characteristics of healthy populations is crucial for developing adaptive management strategies to bolster populations in the wild.

Aims. Our analysis aims to evaluate contemporary reproductive rates for wild, DFTD-free Tasmanian devil populations, and to provide a baseline with which to compare the outcome of current translocation activities.

Methods. We analysed 8 years of field-trapping data, including demographics and reproductive rates, across 2004–16, from the largest known DFTD-free remnant population at Woolnorth, Tasmania.

Key results. Surprisingly, we found a dramatic and statistically significant decline in female breeding rate when comparing data collected from 2004–2009 with data from 2014–2016. Unfortunately we do not have any data from the intermediate years. This decline in breeding rate was accompanied by a subtle but statistically significant decline in litter sizes. These changes were not associated with a change in body condition over the same period. Furthermore, we could not attribute the decline in breeding to a change in population size or sex ratio. Preliminary analysis suggested a possible association between annual breeding rate and coarse measures of environmental variation (Southern Oscillation Index), but any mechanistic associations are yet to be determined.

Conclusions. The decline in breeding rates was unexpected, so further monitoring and investigation into potential environmental and/or biological reasons for the decline in breeding rate are recommended before the arrival of DFTD at Woolnorth.

Implications. Our results provide valuable data to support the conservation management of Tasmanian devils in their native range. They also highlight the importance of continued monitoring of 'safe' populations, in the face of significant threats elsewhere.

Additional keywords: demography, fecundity, inbreeding, population monitoring, reproduction, translocation.

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Introduction

The Tasmanian devil (*Sarcophilus harrisii*) is threatened with extinction due to emergence of a contagious cancer called devil facial tumour disease (DFTD). DFTD is a clonal, transmissible cancer (Murchison *et al.* 2010), spread among individuals by biting (Hamede *et al.* 2013), a common behavioural characteristic of the Tasmanian devil during feeding and mating (Hamede *et al.* 2008). DFTD was first detected in 1996 (Loh *et al.* 2006), and the disease has since spread to cover almost the entire Tasmanian devil distribution (Save the Tasmanian Devil Program 2014). In 2014, a second form of DFTD, DFT2, was documented in south-east Tasmania (Pye *et al.* 2016). Since 1996, localised population declines of 90% have been reported, with an overall population decline estimated at 85%

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(McCallum *et al.* 2007; Lazenby *et al.* 2018). Despite recent vaccine trials (Kreiss *et al.* 2015) and the ongoing management of a Tasmanian devil insurance meta-population (Hogg *et al.* 2017), wild Tasmanian devils face continuing population declines (Save the Tasmanian Devil Program 2014).

In the face of an emerging threat, such as DFTD in devils, understanding the ecological and demographic parameters of unaffected populations is invaluable to management strategy, especially translocations (IUCN/SSC 2013). In devils, multiple translocation strategies are used to prevent species extinction and to maintain an ecologically functioning population in the wild (Save the Tasmanian Devil Program 2014). Understanding demographic parameters in natural populations will provide information to assist managers in choosing among conservation strategies (IUCN/SSC 2013).

Due to the prevalence of DFTD, our latest understanding of Tasmanian devil population structure is heavily weighted towards DFTD-affected populations (Jones *et al.* 2008; Lachish *et al.* 2011; Keeley *et al.* 2012; Hollings *et al.* 2014). Following the emergence of DFTD, changes in life-history traits, dispersal patterns, reproductive physiology and trophic cascades have been the focus of intense research. For example, female Tasmanian devils have shown an increase in precocial breeding post-DFTD arrival in a population (Owen and Pemberton 2005; Jones *et al.* 2008), while male Tasmanian devils have shown limited evidence of reproductive inhibition or alteration in response to DFTD (Keeley *et al.* 2012). Lachish *et al.* (2009) observed no significant difference in the number of pouch young per breeding female between healthy and DFTD devils (3.42 versus 3.40) within a DFTD area; however, other data on the natural demographics of DFTD-free populations was largely collected before the emergence of DFTD (Guiler 1970; Pemberton 1990). Breeding success in pre-DFTD areas was reported across four locations in the late 1960s, three in the north-east (55% of females breeding ± 23.8%; range 33–87%) and one in the north-west (49% of females breeding ± 7.6%; range 40–54% (Guiler 1970). Recent data obtained before the emergence of DFTD indicated that breeding success in the north-east was reported to be 75% (Pemberton 1990). Published reports indicate low variation in the number of offspring produced per breeding female, across time and space. For example, the mean number of pouch young per female was reported as 2.88 ± 1.05 ($n = 53$) by Guiler (1970) and 2.96 ± 1.08 ($n = 109$) by Pemberton (1990).

In this study, we utilise long-term detailed trapping information from the largest known remaining DFTD-free Tasmanian devil population to evaluate reproductive rates and provide a baseline with which to compare current translocation activities. We report on female reproductive characteristics, age and sex structure in this disease-free population and variation in these across eight sampling years from 2004 to 2016. We identified a noticeable decline in female breeding rate from 2014 onwards, so we further investigated some potential causes for the decline, including measures of body condition and environmental data.

Materials and methods

We used field survey data collated from DPIPWE-led trapping surveys to Woolnorth, at the north-western tip of the state of Tasmania (40.77°S, 144.77°E), for species monitoring purposes. Woolnorth has been a working farm (sheep and cattle) since 1825 and remains a mosaic of open pasture and bushland (Bain 2010). As of 2016, Woolnorth was still DFTD-free (DPIPWE 2015; Lazenby *et al.* 2018). Trapping data were available for 8 years between 2004 and 2016 (comparable data was not available for some years). In total, the data included 1364 captures (mean 170.5 per year, s.d. 16.9, range 141–197). Of these, a unique individual identifier (typically a microchip number) was recorded for 1354 (99.3%) records.

Although Tasmanian devils are seasonally polyoestrous, and in captivity can exhibit up to three oestrous cycles annually between February and August (Hesterman *et al.* 2008; Keeley *et al.* 2012), observations from the wild indicate that most mating occurs in March (Guiler 1970; Keeley *et al.* 2017; DPIPWE, unpubl. data). We examined only data collected from June to January (inclusive), as these are the months during which evidence of breeding is detectable for female devils. Breeding status classifications had been recorded based on observations of pouch appearance, colour and secretions previously determined for Tasmanian devils (Hesterman *et al.* 2008). We used these data to classify females as breeders, non-breeders or not applicable (the latter including immature, pre-ovulatory or senescent individuals, which were excluded from the reproduction analysis; scoring classifications are described in Table S1, available as Supplementary material to this paper).

Individual sex was recorded for an average of 98.5% of uniquely trapped animals across years. The sex ratio averaged 56.6% female across years (s.d. 4.5%, range 53.1–66.4%). Age was recorded, or inferred from previous trapping events, for 845 of the 861 (98.1%) uniquely trapped animals across years. Demographic stability of the population varied year to year, with the 2004–07 age distributions suggesting a growing population, as compared with the 2009–16 distributions, which suggest lower birth rates or recruitment (Fig. S1, available as Supplementary material to this paper).

Pouch status was recorded for an average of 97.5% females trapped across years (s.d. 2.0%, range 93.2–100%). Breeding status (breeding success or failure to breed) could be determined for an average 51.4% of the females for which pouch status was recorded across years (i.e. after excluding those females with pouch status scores that do not allow for inference of breeding status; Table S1) (s.d. 11.5%, range 40.7–69.5%), resulting in 240 mature, non-senescent females in our breeding status dataset (Table 1). Of these, 96 breeding females also had sufficient data to estimate the litter size (based on the number of active teats).

By examining body condition, we evaluated whether changes in breeding success may result from changes in nutritional state of the population. We calculated a size-adjusted measure of bodyweight by dividing bodyweight (to the nearest 0.1 kg) by head width measurement (to the nearest 0.1 mm). Head width was appropriate to standardise weight because there was high variance in weight for a given head width, although the two measurements were positively correlated (details provided at Fig. S2). Head width was measured at the widest point of skull, the zygomatic arch, with a pair of

Table 1. Devil trapping data from 2004 to 2016
Total unique traps and rate of re-trapping across 8 years of Tasmanian devil surveys at Woolnorth. Note the large interval between the 2009 and 2014 surveys

Year	Total traps	New traps	Re-traps	Re-trap rate (%)	New traps rate (%)	No. breeding age females
2004	103	103	–	–	–	25
2005	89	76	13	14.6	85.4	23
2006	111	67	44	39.6	60.4	25
2007	111	63	48	43.2	56.8	24
2009	109	71	38	34.9	65.1	35
2014	114	110	4	3.5	96.5	29
2015	113	57	56	49.6	50.4	41
2016	110	50	60	54.5	45.5	38

Vernier callipers. Bodyweight was recorded by weighing the devil in a hessian sack with a 20-kg Pesola scale. Once the devil was released, the sack was weighed, and this weight removed to give the weight of the animal. Sack weight was not recorded for 73 of 240 females; for these animals the mean sack weight across the dataset (0.64 kg, range 0.6–0.7 kg) was used. Six females were excluded from this analysis, because head width was not recorded. Both body measurements (head width and weight) were recorded for a total of 234 females with breeding success data, and for 93 females with active teat data.

Finally, we tested whether individual reproductive success was influenced by annual changes in weather conditions by examining data on the Southern Oscillation Index (SOI). The SOI is a large-scale oceanic index that relates to multiple local weather variables, such as temperature, winds and rainfall (Hallett *et al*. 2004; van de Pol *et al*. 2013). We chose this coarse climate metric because we do not have definite predictions about the effects of specific weather variables (e.g. rainfall) on devil productivity at Woolnorth. Sustained periods of negative SOI values below −7 and positive SOI values above +7 indicate an El Niño and La Niña event, respectively (Bureau of Meteorology 2017). Generally, an El Niño event is associated with local warming, weaker winds and decreased rainfall in winter and spring and a La Niña event is associated with stronger winds and increased rainfall. Monthly SOI information was available from online archives of the Australian Bureau of Meteorology (Bureau of Meteorology 2017), spanning all years of this study. We summarised SOI as the average for each year, because monthly SOI values are subject to extreme variation and we have no *a priori* information regarding the most influential months of weather patterns on devil reproductive success.

We tested the predictors of breeding success over time by fitting a generalised linear model (binomial) where females with pouch status confirmed were classed as breeding = 1 or not = 0 and year, SOI and body condition were fitted as the fixed predictors, using the 'glm' function of R (R Core Team 2016). We tested the predictors of breeding rate per female (litter size) by fitting a generalised linear model (GLM) with year, SOI and body condition as the fixed predictors. Number of active teats was modelled as a binomial response (proportion of the biological maximum number of active teats of 4, Guiler 1970) using a two-column matrix where successes equal the number of active teats and failures equal 4 minus successes.

Results

Of the 1364 trapping events, many devils were re-trapped across multiple years (Table 1). Female breeding success during 2004–09 was much higher (mean 75.0%, range 56.0–85.7%) than during 2014–16 (mean 19.4% range 17.2–21.1%), a decline that was statistically significant (Fig. 1*a*, Table 2). Examining the number of active teats of breeding females over time indicated a weak but statistically significant decline in litter size over the study period (Fig. 1*b*, Table 2). Because size-adjusted body condition showed a statistically significant association with breeding success (but not number of active teats) (Table 2), we fitted a linear model to test whether size-adjusted bodyweight (a measure of body condition) changed over the study period, but found no such relationship (Fig. 1*c*, Table 2).

We found that annual SOI showed a positive relationship with breeding success (Table 2), where low SOI was associated with decreased reproductive success (Fig. 1; Fig. S3). We did not detect a statistically significant effect of annual SOI on the number of active teats recorded for breeding females (Table 2).

Discussion

Woolnorth, in north-west Tasmania, is one of the last remaining DFTD-free sites in the state; other possible DFTD-free sites are not monitored because of their remoteness (Save the Tasmanian Devil Program 2015). The reproductive rates that we observed between 2004 and 2009 are similar to historical data from other locations in Tasmania in the 1960s (Guiler 1970) and 1980s (Pemberton 1990) (see also Introduction). Surprisingly, we saw a dramatic decrease in the number of breeding females in 2014–2016. We considered whether this reduction results from population decline, lack of available food resources, changes to environmental conditions or from a combination of factors.

Although age distributions indicate a lack of recruitment in more recent years (Fig. S1), we have no evidence of a population decline at Woolnorth, with field teams reporting a constant trapping rate of devils across 2004 to 2009 compared with 2014 to 2016; similar trapping effort was deployed across years, and our sample sizes were constant; Table 1. In addition, a stable, resident population of Tasmanian devils appears to exist in the area, as evidenced by a high re-trap rate across years (Table 1), and a constant population density between 2004 and 2016, estimated around one devil ha^{-1} (Lazenby *et al*. 2018).

Our analysis of size-adjusted bodyweight indicated that there was no apparent change in condition across all years of the analysis, suggesting that a lack of food does not appear to be driving changes in reproductive rates. Tasmanian devils require suitable den sites, both for breeding and mating (Pemberton 1990). A decline in female reproductive activity in the June-to-January period (as represented by our data) may indicate poor mating opportunities between March and June (prime breeding season; Guiler 1970), which may be caused by a lack of suitable copulation dens (e.g. subtle changes in land use or other environmental factors may affect the availability of features such as fallen logs) and/or mating opportunities.

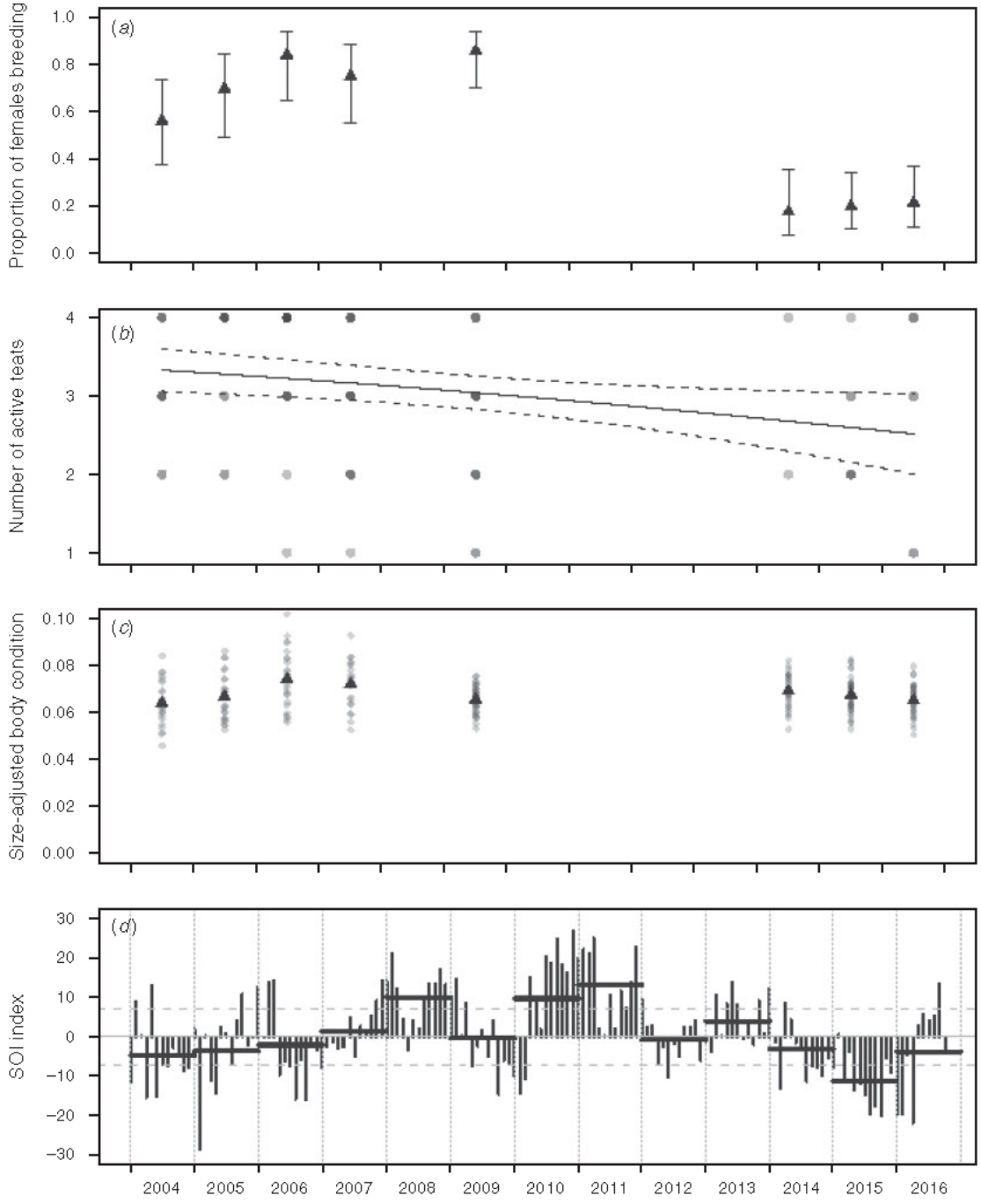

Fig. 1. Female breeding success and body condition over time. (*a*) triangles indicate the annual proportion of females that bred, out of the total number for which breeding status could be determined (sample sizes provided in Table 1). Error bars represent 95% confidence intervals for proportions (Agresti and Coull 1998). (*b*) round points indicate the number of active teats per individual breeding female per year (darker points indicate multiple overlaid values). The solid line is the fitted estimate based on logistic regression and at the mean of other parameters in the model (Table 2); dashed lines are 95% confidence intervals evaluated by parametric bootstrapping of the model (Table 2). (*c*) round points indicate the size-adjusted body condition of individual females for which breeding status was determined ($n = 234$, sample sizes as in (*a*)) each year. Triangles show the annual means. (*d*) Monthly Southern Oscillation Index (SOI) during the period of the study (vertical black bars), with vertical grey lines at January of each year. Sustained periods above +7 indicate a La Niña event, sustained periods below −7 indicate an El Niño event; horizontal dashed lines are at ±7. Heavy black dashes indicate the annual mean values used in our reproductive analyses. Redrawn using data from Bureau of Meteorology 2017.

Table 2. Modelling results

Associations between year, size-adjusted body condition, Southern Oscillation Index (SOI) and reproductive metrics. Reproductive traits were fitted as generalised linear models (binomial); size-adjusted body condition was fitted as a linear model. Breeding success was coded as success equals 1, failure equals 0. Number of active teats was coded as a two-column matrix where success equals number of active teats, failure equals 4 minus successes. The regression estimates and their standard errors (s.e., and *p*-value), as well as the sample size (*n*) of each model, are given

Response	Predictor	Estimate (s.e.)	*P*-value	*n*
Breeding success	Intercept	−7.301 (1.555)	<0.001	234
	Year	−0.238 (0.046)	<0.001	
	Size-adjusted body condition	143.455 (25.231)	<0.001	
	SOI	0.141 (0.054)	0.009	
Number of active teats	Intercept	0.746 (1.053)	0.479	93
	Year	−0.090 (0.036)	0.013	
	Size-adjusted body condition	12.661 (14.214)	0.373	
	SOI	−0.024 (0.042)	0.580	
Size-adjusted body condition[A]	Intercept	0.069 (0.001)	<0.001	234
	Year	< −0.001 (<0.001)	0.584	
	SOI	< −0.001 (<0.001)	0.386	

[A]Bodyweight (to the nearest 0.1 kg) divided by head width measurement (to the nearest 0.1 mm); see also Fig. S2.

Furthermore, a change in availability of suitable maternity (breeding) dens may be driving low fecundity, that is, females are breeding but losing their offspring. Our data provide limited support for the latter hypothesis, as we detected a small decline in litter size. However, we note that our data also suggest a negative impact earlier in the breeding season, as we saw a reduction in the proportion of females showing any evidence of breeding. We have no evidence that the availability of males is a limiting factor – sex ratios remained fairly constant over our study period (Fig. S1). The low observed fecundity in recent years may be driven by environmental factors, biological factors or a combination of these.

Significant fluctuations in weather patterns are known to influence breeding behaviour and fecundity in many species (e.g. Holmes and York 2003; Forcada and Hoffman 2014; Marchant *et al.* 2016). The Southern Oscillation has a significant influence on Australia's weather patterns (Allan *et al.* 1996). The Southern Oscillation Index (SOI) fluctuated greatly across the years of trapping, with a low in March 2005 and a high in December 2010, and a possible El Niño event between 2014 and 2016 (Fig. 1*d*). Although we detected a statistically significant association between annual SOI values and devil breeding success (Table 2), this effect may be largely driven by data from 2015, where breeding was low and the mean annual SOI was below −7 (Fig. 1*d*; Fig. S3). Breeding was similarly low in 2014 and 2016, when annual average SOI values were closer to zero (Fig. 1*d*), implying that SOI in the year of breeding is unlikely to be a strong predictor of devil productivity at Woolnorth. Unfortunately, we only have 3 years with poor breeding, and these occurred in succession. Future monitoring, when SOI returns to neutral or even positive values, is required to fully understand any possible impact of the Southern Oscillation on the breeding success of Tasmanian devils. Additional targets for further investigation include other environmental factors such as disturbance, influence of land-clearing and potential pollutants.

The severe reduction in female breeding rate at Woolnorth may also be the result of some inherent biological factor. For example, hormonal imbalances may be contributing to the low reproductive rate, or extensive population isolation may have led to declines in individual fitness due to increased inbreeding (Keller and Waller 2002). Inbreeding depression occurs when the mating of related individuals leads to a decrease in fitness, relative to non-inbred individuals (Keller and Waller 2002). The significant decrease in reproductive output at Woolnorth might result from an increase in inbreeding in this population. Immigration between populations can mitigate the effects of inbreeding (Brown and Kodric-Brown 1977; García-Navas *et al.* 2014), but we are unable to infer the level of migration into, or out of, Woolnorth without extensive genetic sampling of the population and surrounds. Nevertheless, inbred and isolated populations can be genetically rescued by augmenting gene flow (Frankham 2015). In a meta-analysis conducted on the benefits of supplementing gene flow in small populations, Frankham (2015) found that genetic rescue resulted in consistently beneficial improvements (92.9% of populations experienced a fitness increase). Frankham (2015) concluded that outcrossing will be beneficial to a population if, among other reasons, (1) there are signs of potential inbreeding depression, (2) there is a donor population available for outcrossing and (3) if the potential benefits outweigh the financial and time investments required to achieve the outcrossing process (for a full list of criteria see Frankham 2015).

The decline in breeding that we observed was unexpected, but it highlights the importance of continual monitoring of remote populations, even in the absence of disease or other immediate threats. Understanding natural fluctuations in Tasmanian devil populations is crucial for monitoring supplementation success. Ongoing monitoring and sampling will help to understand the potential causes of the observed fluctuations, whether the result of climate, changes in genetic diversity or inbreeding. The effects of inbreeding can be difficult to quantify in the wild for lack of pedigree information and detailed fitness information. However, in the absence of a detailed pedigree, molecular measures of genome-wide homozygosity can offer an alternative

to quantifying inbreeding in wild populations (Szulkin *et al.* 2010). Annual population monitoring at Woolnorth is planned for the coming years (DPIPWE, unpubl. data).

We undertook this analysis to evaluate contemporary population parameters in one of the last remaining DFTD-free populations in Tasmania, and provide guidelines for determining the success of future release events. The data between 2004 and 2009 yielded similar results to those noted in historical literature. More recent data, between 2014 and 2016, has highlighted a large reduction in fecundity at Woolnorth, and suggests avenues for future work to investigate the causes of this pattern in a DFTD-free population. Together, the results will better inform the ongoing conservation management of Tasmanian devil populations in their native range.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix 12: A demonstration of conservation genomics for threatened species management

A12.1 BACKGROUND

The following appendix contains the abstract of a manuscript that I contributed to during the course of my PhD research, led by Belinda Wright. The manuscript has been revised and is currently in preparation for resubmission to Molecular Ecology Resources. Resequenced whole genomes from 24 Tasmanian devils were used to conduct a genome-wide association study (GWAS) for breeding success. Reduced representation sequencing data is assumed to be representative of genome-wide heterozygosity, but this assumption is rarely tested. By comparing RRS data to the whole genome data, RRS was found to be a good proxy for genome-wide heterozygosity. However, marker density was not high enough to examine functional diversity such as through GWAS.

For this study, I processed RRS data using the method I developed in Chapter 4 (R script in Appendix 6), and critically revised the manuscript.

A12.2 ABSTRACT

A demonstration of conservation genomics for threatened species management

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As species extinction rates increase, genomics provides a powerful tool to support intensive management of threatened species. However, to date, few case studies have been published. We use the Tasmanian devil to demonstrate how conservation genomics can be implemented in threatened species management. We sequenced the genomes of 24 individuals from the captive breeding program, and conducted reduced-representation sequencing (RRS) of 98 founders from the same program. A subset of the genome-sequenced samples was also sequenced by RRS, so we are able to directly compare genome-wide heterozygosity with estimates from RRS data. We found good congruence between the two datasets, indicating that our RRS data reflects inter-individual variation well. Similarly, analysis of gene-ontology classifications showed further congruence between the two datasets, suggesting minimal bias in our RRS data. We also attempted genome-wide association studies with both datasets (regarding breeding success), but the genomic data suffered from small sample size, while the RRS data suffered from lack of precision, highlighting a key trade-off in the design of conservation genomic research. Nevertheless, we identified two genes possibly associated with variation in breeding success, both involved in sperm function. Individual RRS heterozygosity was not associated with the likelihood of a founder breeding in captivity but was negatively associated with litter sizes of breeding females. Our observation of high congruence between whole-genome and RRS datasets offers hope for conservation geneticists who target the latter due to its cost efficiency. We caution, however, that deep functional insights may be impaired by a lack of precision.