Nutrient Transport in the Male Pregnant Seahorse, *Hippocampus abdominalis*

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Statement of originality

I certify that to the best of my knowledge the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Zoe Skalkos

Date: 30.09.2024

Authorship attribution statement

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I carried out the sample collection and preparation for data collection. Mass Spectrometry was carried out by the University of New South Wales Bioanalytical Mass Spectrometry Facility service lab. I analysed the data and wrote the first draft of the manuscript. JUVD, SND and CMW and I contributed to the experimental design, data interpretation, manuscript revisions and approved the final version.

Chapter 4
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Should the status of this manuscript have changed before thesis submission, an update will be appended on the next page of this thesis. I carried out the light microscopy, immunohistochemistry, western blotting and wrote the first draft of the manuscript. CMW, JSD and I collected the samples. JUVD, JSD, SND, CMW and I contributed to experimental design, image interpretation, manuscript revisions and approved the final version.

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

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Abstract

Syngnathids offer a rare opportunity to study the complexity of male pregnancy in vertebrates and serve as a unique comparative model for the convergent evolution of live birth and parentotrophy. Male seahorses have the most complex brooding structure known among oviparous brooding vertebrates, where males internally fertilise and incubate developing embryos in an enclosed brood pouch until the birth of live young, a process analogous to viviparity. The inner tissue layer of the brood pouch represents the male component of a functional placenta, analogous to the female placenta seen in viviparous species. The aim of this thesis was to identify the range of nutrients supplied via patrotrophy in the male-pregnant pot-bellied seahorse, *H. abdominalis*, to test the hypothesis that wide-ranging parentotrophy has convergently evolved in male brooding and viviparous vertebrates, irrespective of sex or gestating organ. I introduce the thesis with a review of how teleost literature assigns provisioning strategies based on a parentotrophy index (PI) threshold. There are widespread methodological inconsistencies in the source and preservation of tissue, embryonic staging, and approaches to calculate or estimate the PI. Additionally, there is considerable variability in threshold values (ranging from 0.6-1) used to distinguish between lecithotrophy and incipient parentotrophy. I provide recommendations for consistent methodology in obtaining and interpreting a PI, along with alternative methods for identifying provisioning strategy. Next, protein, calcium, magnesium, and phosphorus content were measured in newly fertilised embryos (NFE) and neonates using mass spectrometry. A significant increase in all measured nutrients was observed in neonates compared to NFE, providing the first evidence that a male brooding vertebrate has convergently evolved wide-ranging mechanisms for patrotrophy, analogous to matrotrophy in viviparous vertebrates. Lastly, I characterise an acellular layer closely apposed to the pregnant brood pouch of *H. abdominalis*, using light microscopy and immunohistochemistry. The acellular layer is only present during pregnancy, is composed of acid mucins and is partially composed of oviductal glycoprotein 1 (OVGP1). This thesis provides new data that demonstrates pregnancy in the male pregnant seahorse is closely analogous to matrotrophic viviparous vertebrates. Using an extreme example of convergent evolution, my work further supports the hypothesis that viviparity, or the internal incubation and close association of parental and embryonic tissues with the release of live young, and parentotrophy consistently co-evolve across taxa and sex.

Chapter 1: General introduction

Reproductive modes in vertebrates

Parental care strategies are exceptionally diverse within and across animal taxa, and are a fundamental part of an organism's development, life-history, and reproduction (Royle *et al.*, 2012). In vertebrates, there are two general reproductive modes. Oviparity is defined as the release of eggs into the external environment, either after internal fertilisation (zygoparity) (Wourms and Lombardi, 1992; Blackburn, 2015), or preceding external fertilisation (ovuliparity) (Lodé, 2001; Furness *et al.*, 2015). Viviparity involves internal fertilisation and incubation of developing embryos within the female reproductive tract, with the release of live young (larvae or neonates) (Blackburn, 1992; Blackburn, 2000; Furness *et al.*, 2015). The transition from oviparity to viviparity has occurred in almost all major vertebrate groups including Mammalia, Lissamphibia, Reptilia, and the earliest origin, fishes (Osteichthyes and Chondrichthyes) (Long *et al.*, 2009; Blackburn, 2015). The independent transition to viviparity has occurred over 150 times in non-mammalian taxa, who provide a much broader range of reproductive and parental care strategies than mammals (Blackburn, 1992; Bainbridge, 2014; Blackburn, 2015). Viviparity has evolved convergently at least 11 times in chondrichthyans (sharks and rays), 8 times in amphibians, 12 times in teleosts, 115 times in squamates (snakes and lizards) and only once in mammals (Wourms, 1981; Blackburn, 2015; Blackburn and Hughes, 2024). Thus, anamniotes (fishes and amphibians) and squamates offer excellent opportunities to compare the diverse range of convergently evolved strategies that support embryonic development inside the parental body across various vertebrate lineages (Bainbridge, 2014).

Oviparity with brooding in vertebrates

Some oviparous species incubate their embryos on or inside the parental body until the release of live young, a complex form of parental care termed 'brooding', in both invertebrates (Ostrovsky *et al.*, 2016) and vertebrates (Whittington and Friesen, 2020). In vertebrates, female brooding has evolved in anurans in the form of gastric-brooding (e.g. the now extinct *Rheobatrachus silus* (Tyler and Carter, 1981) and skin brooding (also known as back-brooding or marsupium brooding) in two lineages of frogs e.g. *Gastrotheca* spp. (Wake, 2014; Warne and Catenazzi, 2016). Female brooding in fishes occurs as mouthbrooding e.g. Cichlidae spp. (Balshine-Earn and Earn, 1998), or skin brooding e.g. Aspredinidae spp. (e.g. *Playstachus* spp.), which carry embryos on cotylephore outgrowths of the abdomen or fin

(Wetzel *et al.*, 1997). Paternal care in teleost fishes is the most common form of parental care, as opposed to maternal care in most vertebrates (Gross and Shine, 1981; Gross and Sargent, 1985; Gross, 2005; Royle *et al.*, 2012). Paternal care has evolved at least 22 times in Actinopterygii (ray-finned fishes) (Mank *et al.*, 2005) and includes male brooding. Despite the prevalence of other forms of paternal care in teleosts, oviparity with male brooding is rare across vertebrates. In teleosts, male brooding has evolved multiple times in the form of mouth brooding e.g. Opistognathidae spp. (Hess, 1993), or skin/apendiculae brooding e.g. *Kurtus gulliveri* (Berra and Humphrey, 2002), which carry eggs on a vascularised male-only forehead hook, and Syngnathidae (Carcupino *et al.*, 2002), which carry embryos on or in a brood pouch. Male brooding has also evolved in anurans e.g. *Rhinoderma darwini* (Goicoechea *et al.,* 1986)*,* which incubate their embryos in their vocal sacks during gestation. Oviparity with male brooding can be analogous to viviparity (Ostrovsky *et al.*, 2016; Whittington and Friesen, 2020) when fertilisation and embryogenesis occur in or on the paternal tissue until the birth of live young. Brooding and viviparity are extreme forms of parental care that enable parental manipulation of embryonic development to significantly increase offspring fitness (Wourms and Lombardi, 1992; Shine and Harlow, 1993; Robert and Thompson, 2001; Royle *et al.*, 2012), and may allow greater parental and embryonic control over the timing of parental resource allocation compared to oviparity without brooding (Trexler and DeAngelis, 2003; Van Dyke *et al.*, 2014). Nonetheless, the lack of fundamental knowledge about the reproductive biology of oviparous brooding vertebrates, especially male brooders, greatly hinders the capacity to compare the relative parental role in provisioning embryos during internal incubation across a variety of convergently evolved vertebrates.

Vertebrate embryonic provisioning strategies

The source of nutritional provisioning of developing embryos occurs on a nourishment continuum whereby some species rely entirely on maternally-allocated yolk and others require varying degrees of post-fertilisation parental supplementation. Lecithotrophy refers to embryonic development that relies solely on the nutrients supplied by the maternally produced yolk and albumen (Wourms, 1981; Wourms *et al.*, 1988; Blackburn, 2000; Blackburn, 2015). Lecithotrophy is abundant in oviparous species (Blackburn, 1992). Parentotrophy refers to the post-fertilisation maternal (matrotrophy) or paternal (patrotrophy) nutrient provisioning of developing embryos (Blackburn, 2015; Skalkos *et al.*, 2023). These

terms were initially applied to piscines by John Wourms (1981) and have subsequently been used for vertebrates in general. In **Chapter 2** (Skalkos *et al.*, 2023), I discuss these two provisioning strategies, review the available teleost data that uses a popular approach for distinguishing between lecithotrophy and parentotrophy, discuss the limitations of the method, provide recommendations for improved consistency and accuracy, and suggest alternative approaches for establishing embryonic provisioning strategy.

Parentotrophy has independently evolved from lecithotrophy at least 33 times in viviparous clades, with the majority of origins (26), having occurred among anamniotes (Blackburn, 2015). The Trexler DeAngelis model hypothesises that the evolution of parentotrophy likely occurred in environments where there is consistently high availability of food resources during gestation (Thibault and Schultz, 1978; Wourms and Lombardi, 1992; Trexler and DeAngelis, 2003). A well-known adaptation to viviparity that facilitates parentotrophy is the placenta. The evolution of the placenta involved modification of parental and fetal tissues and is defined as an 'intimate apposition or fusion of the fetal organs to the maternal [or paternal] tissues for physiological exchange' (Mossman, 1937, p156). Placentae are present in all Therian mammals and have evolved alongside viviparity in multiple forms in Reptilia, Lissamphibia, and Osteichthyes and Chondrichthyes (Blackburn, 2015; Buddle *et al.*, 2019). Non-placental modes of matrotrophic viviparity are common in anamniotes (Wake, 2014; Blackburn, 2015). They include embryonic absorption (histotrophy) or ingestion (histophagy) of uterine secretions (histotroph); embryonic ingestion of maternal gestational tissues (matrophagy) or their eggs (oophagy) and/or siblings (adelophagy) (Wourms *et al.*, 1988; Blackburn, 2015; Ostrovsky *et al.*, 2016). Mechanisms for embryonic nourishment during gestation are not mutually exclusive (Blackburn, 2015; Ostrovsky *et al.*, 2016). Thus, developing embryos can be nourished by multiple methods simultaneously or within one gestational period (Blackburn, 2015). The diversity of modes of nutrient provisioning in viviparous and oviparous brooding anamniotes provides a unique opportunity to investigate the fundamental mechanisms underlying the independent convergent evolution of parentotrophy across vertebrate lineages (Blackburn, 2015; Whittington and Friesen, 2020). The preliminary step in discerning and comparing these convergently evolved mechanisms between male and female internal gestating species is to identify the respective roles of each parent in embryo provisioning and the array of nutrients each parent can supply. Furthermore, the convergent evolution of parentotrophy is hypothesised to have co-evolved with the transition of oviparity to viviparity (and thus brooding) (Blackburn, 1992). There is strong

evidence that supports this hypothesis among all studied squamates (Blackburn, 1992; Stewart and Thompson, 2000; Thompson *et al.*, 2004), and some chondrichthyans (Hamlett, 2005; Cotton *et al.*, 2015), caecilians (e.g. Wake, 1977; Wake, 1993), and teleost fishes (e.g. Morrison *et al.*, 2017). Thus, oviparous brooding vertebrates provide unusual and extreme comparative models that can test the convergent co-evolution hypothesis in different organs and sexes.

Syngnathids as a comparative model for the evolution of live birth and parentotrophy

Fishes contain the greatest diversity of reproductive strategies and parental-embryo relationships among vertebrates (Wourms and Lombardi, 1992), making them important models for the evolution of live birth. The Syngnathidae family (pipefish, seadragons, and seahorses) are oviparous male brooders, exhibiting one of the most specialised forms of parental care (Monteiro *et al.*, 2005), whereby males incubate developing embryos on or in a structure known as the brood pouch until the birth of live precocial young (Wetzel and Wourms, 2004; Stölting and Wilson, 2007; Sutton and Wilson, 2018). Syngnathid brood pouches vary in complexity from entirely exposed egg attachment in some pipefishes to a fully enclosed brood pouch in seahorses (*Hippocampus* spp.), with a single pore opening (reviewed in Whittington and Friesen, 2020). The syngnathid brood pouch is derived from outgrowths of the male abdominal epithelium and in *Hippocampus abdominalis*, is completely formed at 8 months post birth, when males are sexually mature (Kawaguchi *et al.*, 2017). Across the family, male syngnathids have many reproductive characteristics that are analogous to female viviparity (Blackburn, 2018), including a close apposition of embryonic and paternal tissues that likely facilitate physiological exchange, and the release of independent live young (Skalkos *et al.*, 2020; Dudley *et al.*, 2021). The variability in complexity of brooding structures in syngnathids (Whittington and Friesen, 2020), provides multiple comparative models to study the development of reproductive complexity and degree of physiological exchange in internally incubating vertebrates.

The evolution of syngnathid male pregnancy

Several non-exclusive hypotheses for how male pregnancy (oviparity with brooding) evolved in syngnathids have been proposed. Male parental care has evolved multiple times from external fertilisation with no parental care (Mank *et al.*, 2005) and thus, it is most likely that

the syngnathids' ancestor displayed external fertilisation and no parental care (Sutton and Wilson, 2019; Whittington and Friesen, 2020). If territorial, males may have transitioned to common forms of male parental care in teleosts including nest building and nest guarding (Mank *et al.*, 2005), to increase paternity assurance and embryonic survival (Wilson *et al.*, 2001). Syngnathid eggs contain large brightly coloured yolks that are not fully absorbed until late-stage embryogenesis (Sommer *et al.*, 2012; Novelli *et al.*, 2017; Skalkos *et al.*, 2020), likely making them more susceptible to predation when exposed (Whittington and Friesen, 2020). To increase offspring survival, male syngnathids may then have evolved nest-gluing, as seen in Gasterosteids (Gasterosteids and Syngnathids are related percomorphs) (Baylis, 1981; Small *et al.,* 2016; Whittington and Friesen, 2020). Male gasterosteids secrete a glue to aid in adherence during nest building (Baylis, 1981). To further increase paternity and reduce embryo predation, a behavioural shift from "gluing" nest material during nest building in ancestral syngnathids, combined with a selection for increased egg stickiness (Watanabe, 1999; Wilson *et al.*, 2001), may have resulted in egg adhesion to male external epithelium, which could have led to the origin of the simple open brood pouch (McCoy *et al.*, 2001). However, reproductive male *Nerophis ophidion*, who have a fully open brood pouch, are more susceptible to predation than non-reproductive males (Svensson, 1988). It is also a common feature in viviparous vertebrates that reproductive individuals are more vulnerable to predation than non-pregnant (Hussain *et al.*, 2024). As such, the evolution of the closure of the syngnathid brood pouch has the potential selective advantage of reducing parent predation mortality and increasing embryonic survival via increased crypsis and embryonic environmental control (Goncalves *et al.*, 2016; Whittington and Friesen, 2020). Two further selective advantages are hypothesised to have resulted in the closure of the brood pouch and include increased clutch size, which is associated with closed brood pouches (Monteiro *et al.*, 2005; Skalkos *et al.*, 2020), and paternal control over embryogenesis (Whittington and Friesen, 2020). Interestingly, increased assurance of paternity is unlikely to be a driver for the closure of the syngnathid brood pouch because full paternity does not depend on pouch type (Jones and Avise, 2001; McCoy *et al.*, 2001), as fertilisation is immediate upon egg deposition in all pouch types (Watanabe, 1999; Watanabe *et al.*, 2000; Monteiro *et al.*, 2002; Van Look *et al.*, 2007). Further discussion on why male pregnancy may have evolved is discussed in Whittington and Friesen (2020). The evolution of male pregnancy, specifically the closure of the syngnathid brood pouch, provides a valuable comparison for the evolution of parentotrophy and live birth, independent of the female reproductive tract. The morphology of the seahorse brood pouch has been somewhat studied (Harada *et al.*, 2022),

but little is known about the embryo-paternal interface and its morphological features that may influence patrotrophy. For example, an acellular layer, hypothesised to be comprised of mucins, was recently discovered in the pregnant brood pouch of *H. abdominalis* (Dudley *et al.*, 2021), but has yet to be characterised.

Seahorses as a comparative model for parentotrophy

Seahorses (*Hippocampus* spp.) have the most complex brood pouch known to oviparous male brooders. Like viviparity, fertilisation is internal and extensive morphological and physiological adaptations for the protection and provisioning of embryos occurs, but embryo development is within the male (Stölting and Wilson, 2007; Whittington and Friesen, 2020). The most complex closed brood pouch has independently evolved three times (in *Hippocampus* spp., *Acentronura* and *Idiotropiscis* spp., and *Apterygocampus eppinulatus* (Hamilton *et al.,* 2017; Whittington and Friesen, 2020), from the open syngnathid brood pouch, which exhibits less extensive morphological and physiological adaptations than seahorse brood pouches (Watanabe, 1999; Ripley *et al.*, 2010; Hamilton *et al.*, 2017; Dudley *et al.*, 2021). The inner tissue layer of the seahorse brood pouch makes up the paternal component of a functional placenta (Laksanawimol *et al.*, 2006; Kawaguchi *et al.*, 2017). Thus, the inner layer of the male seahorse brood pouch is analogous to the female placenta in viviparous vertebrates (Whittington and Friesen, 2020). The physiological and morphological changes required to support embryogenesis in an enclosed brood pouch, indicate specific regulation of the internal environment (Laksanawimol et al., 2006; Stölting and Wilson, 2007; Whittington *et al.,* 2015). The complex adaptations for a closed brood pouch include providing embryos with respiratory gas supply (Dudley *et al.*, 2021), osmoregulation (Linton and Soloff, 1964; Quast and Howe, 1980), and paternal nutritional provisioning (Skalkos *et al.* 2020), and putatively nitrogenous waste removal or storage (Linton and Soloff, 1964), and immunological protection (Melamed *et al.*, 2005; Roth *et al.*, 2012; Whittington *et al.*, 2015), across the brood pouch placenta (Whittington and Friesen, 2020). Seahorses are thus an essential comparative model to explore the convergent evolution of male brooding and internal gestation, placentae, and the likely co-evolution of parentotrophy. The pregnant brood pouch of *H. abdominalis* contains significantly upregulated nutrient transporters for lipid, protein, and inorganic molecules such as calcium, compared to the non-pregnant pouch (Whittington *et al.*, 2015; Skalkos *et al.*, 2020). Thus, the transcriptomic data of *H.*

abdominalis brood pouch provides multiple testable hypotheses for what other nutrients may be paternally provided to developing embryos during male pregnancy in seahorses.

Energetic demand during syngnathid embryogenesis is high (Faleiro and Narciso, 2010), and thus patrotrophic supply of nutrients during gestation could help meet the developmental requirements of embryos. Prior to the research contained in this thesis, patrotrophy had been confirmed in seahorses, whereby fathers transport lipids to developing embryos during gestation (Skalkos *et al.*, 2020). Other syngnathids with less complex brooding structures can provision embryos with protein during gestation (Haresign and Shumway, 1981; Ripley and Foran, 2009; Kvarnemo *et al.*, 2011). Thus, in addition to the evolution of internal embryonic incubation, syngnathids are valuable comparative models for the evolution of parentotrophy. Seahorses, with a functional male placenta, are likely to be capable of expansive paternal nutrient provisioning. Seahorses thus provide the opportunity to establish what adaptations that support reproductive success have convergently evolved in males and whether the physiological mechanisms underpinning female viviparity, placentation and parentotrophy are replicated in seahorse male pregnancy, or whether novel pathways have evolved. If similar mechanisms have evolved across various biological scales, organisms, and sexes to support parentotrophy, it implies a consistent evolutionary trajectory for this trait. Conversely, if alternative pathways have evolved in male pregnancy, there are multiple routes and possibly fewer developmental constraints by which a convergently similar trait can evolve. Furthermore, these implications extend beyond provisioning strategies, internal gestation, and even reproduction: the male placenta serves as a model for how complex organ systems can evolve to perform similar functions (Lau *et al.*, 2024).

Parental resource allocation and the implications of male pregnancy

Resource allocation refers to the distribution and management of an individual's available resources, including energy, time, and nutrients, towards maintenance, activity, growth, and reproductive functions. Here, I narrow our focus to the allocation of nutrients to oocytes pre-fertilisation and developing embryos post- fertilisation, including gamete production and nutrient provisioning of embryos by the carrying/gestating parent. The evolution of viviparity and parentotrophy provides a dramatic expansion in the possible magnitude and timing of allocation of resources to parental care (Trexler and DeAngelis, 2003). For example, in lecithotrophic oviparous and viviparous species, females undergo large energetic investment

in offspring during oogenesis and vitellogenesis. In contrast, parentotrophic oviparous and viviparous species can spread their investment both before fertilisation and across gestation until hatching/birth. Fishes and squamate reptiles represent ideal models for studying the evolution of embryonic nourishment because of the extensive continuum of lecithotrophic and parentotrophic forms of nutrient provisioning in both males and females (Wourms *et al.*, 1988; Stewart, 1989; Trexler, 1997; Skalkos *et al.*, 2020). Some teleost and squamate species display plasticity in the relative pre- and post-fertilisation investment, likely due to environmental factors such as resource availability (Thibault and Schultz, 1978; Stewart, 1989; Itonaga *et al.*, 2012; Van Dyke *et al.*, 2014), with some studies even classifying the same teleost species as both parentotrophic and lecithotrophic, which are mutually exclusive (**Chapter 2**: Skalkos *et al.* 2023). In female matrotrophic viviparous species, the ability to allocate nutritional resources towards reproduction both during vitellogenesis and embryogenesis is exclusively maternal. Conversely in syngnathids, the mother contributes nutritionally via the egg-yolk, and the father contributes via patrotrophy, sharing the allocation of nutritional resources required for embryonic development between parents. Interestingly, in these sex-role reversed fishes, the female transfers the eggs into the brood pouch via a viscous ovarian fluid (Watanabe, 1999), that is yet to be characterised. This medium may influence male pregnancy and embryonic provisioning, but such mechanisms have not yet been studied. These fishes raise interesting questions about relative parental nutrient allocation and how the division of roles has evolved over time. To lay the foundation for determining the role of the father in the nutrient provisioning of developing embryos, and how relative parental investment may have evolved in male seahorse pregnancy, identifying *which* nutrients are provided by the father during pregnancy is fundamental. However, the current understanding of nutrient transport across the male placenta in seahorses is limited to the transport of lipids to developing embryos during pregnancy (Skalkos *et al.*, 2020).

Study species

My study species, *Hippocampus abdominalis,* exhibits the most complex form of male vertebrate pregnancy (Whittington and Friesen, 2020). *Hippocampus abdominalis* was recently categorised as patrotrophic, whereby pregnant males transport lipids to developing embryos during pregnancy (Skalkos *et al.*, 2020). The extent to which the male seahorse provides other nutrients (organic and inorganic) to developing embryos during pregnancy remains unknown, despite knowledge of brood pouch physiology being an important

prerequisite for understanding the evolution of male pregnancy (Whittington and Friesen, 2020). The present thesis contributes new knowledge on patrotrophy by demonstrating the transport of a range of other nutrients for the first time. Seahorses provide a unique comparative model for studying the convergent evolution of parental investment strategies in internally incubating vertebrates, and can significantly contribute to our knowledge of the fundamental biology of pregnancy across taxa.

Thesis aim and outline

The broad aim of this thesis is to explore the extent of paternal nutrient transport and the potential mechanisms facilitating it in the male-pregnant Pot-bellied seahorse, *H. abdominalis*, as a comparative model to vertebrate viviparity. Given the current limited knowledge of the male placenta, my thesis primarily provides experimental evidence for what capabilities the male brood pouch possesses in relation to nutrient provisioning and parental resource allocation. I briefly touch upon potential mechanisms that may support these adaptations in syngnathids, but primarily provide a foundation and direction for future studies. Each of the following chapters was written as a separate manuscript and thus contain some repetition. Two of these chapters have been published, one is prepared for journal submission, and one is to be published in combination with further work.

In **Chapter 2** (Skalkos *et al.*, 2023) I review the data on assigned provisioning strategies in teleost fishes. I highlight the sources of variation in the methodology involved in using a parentotrophy index threshold to distinguish between and classify a provisioning strategy for individual species. I provide suggestions for future consistency in calculating parentotrophy indices and the resultant classification of provisioning strategy and suggest alternative approaches to measuring parentotrophy.

In **Chapter 3** (Skalkos *et al.*, 2024) I test the hypothesis that proteins are paternally transported from father to developing embryos during pregnancy in *H. abdominalis*. Protein content increases significantly over embryonic development, providing evidence that embryos receive protein supplementation from brooding fathers. This chapter provides further understanding of the diversity of paternal organic nutrient contributions to embryonic development.

In **Chapter 4**, I test the hypothesis that paternal transport of inorganic nutrients calcium, phosphorus, and magnesium to developing embryos occurs during pregnancy in *H. abdominalis*. I provide evidence that all three minerals are paternally transported, with the father being the primary source of neonate calcium and magnesium, while phosphorus is predominantly maternally derived. I explore the potential mechanisms through which fathers transport nutrients and their subsequent embryonic uptake.

In **Chapter 5,** I test the hypothesis that an acellular layer at the interface between paternal and embryonic tissue in the pouch of *H. abdominalis* is comprised of mucins and discuss its putative functions. The acellular layer is partially composed of oviductal glycoprotein 1 (Ovgp1) and is likely paternally derived. Both the external and internal brood pouch epithelium also stain positively for Ovgp1, suggesting the protein's function is not exclusive to reproduction. The layer may facilitate nutrient transport or may serve as a barrier that paternal nourishment mechanisms effectively navigate.

In **Chapter 6**, I provide a comprehensive discussion of my findings, explore the implications for the current field of nutrient provisioning and parental nutrient allocation in vertebrates, and propose direction for future research on the parental allocation and extent of nutrient transport in brooding vertebrates.

References

Bainbridge, D. R. (2014). The evolution of pregnancy. *Early Human Development* **90**, 741-745.

Balshine-Earn, S. & Earn, D. J. D. (1998). On the evolutionary pathway of parental care in mouth–brooding cichlid fishes. *Proceedings of the Royal Society, Part B* **265**, 2217-2222.

Baylis, J. R. (1981). The evolution of parental care in fishes, with reference to Darwin's rule of male sexual selection. *Environmental Biology of Fishes* **6**, 223-251.

Berra, T. M. & Humphrey, J. D. (2002). Gross anatomy and histology of the hook and skin of forehead brooding male Nurseryfish, *Kurtus gulliveri,* from Northern Australia. *Environmental Biology of Fishes* **65**, 263-270.

Blackburn, D. G. (1992). Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *American Zoologist* **32**, 313-321.

Blackburn, D. G. (2000). Classification of the reproductive patterns of Amniotes. *Herpetological Monographs* **14**, 371-377.

Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *Journal of Morphology* **276**, 961-990.

Blackburn, D. G. (2018). Viviparity in reptiles and amphibians. In: *Encyclopedia of Reproduction,* vol. 6*,* pp. 443-449: Elsevier Science & Technology.

Blackburn, D. G. & Hughes, D. F. (2024). Phylogenetic analysis of viviparity, matrotrophy, and other reproductive patterns in chondrichthyan fishes. *Biological Reviews of the Cambridge Philosophical Society* **99**, 1314-1356.

Buddle, A. L., Van Dyke, J. U., Thompson, M. B., Simpfendorfer, C. A. & Whittington, C. M. (2019). Evolution of placentotrophy: using viviparous sharks as a model to understand vertebrate placental evolution. *Marine and Freshwater Research* **70**, 908-924.

Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. (2002). Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *Journal of Fish Biology* **61**, 1465-1480.

Cotton, C. F., Dean Grubbs, R., Dyb, J. E., Fossen, I. & Musick, J. A. (2015). Reproduction and embryonic development in two species of squaliform sharks, *Centrophorus granulosus* and *Etmopterus princeps*: Evidence of matrotrophy? *Deep-sea Research, Part II* **115**, 41-54.

Dudley, J. S., Hannaford, P., Dowland, S. N., Lindsay, L. A., Thompson, M. B., Murphy, C. R., Van Dyke, J. U. & Whittington, C. M. (2021). Structural changes to the brood pouch of male pregnant seahorses (*Hippocampus abdominalis*) facilitate exchange between father and embryos. *Placenta* **114**, 115-123.

Faleiro, F. & Narciso, L. (2010). Lipid dynamics during early development of *Hippocampus guttulatus* seahorses: Searching for clues on fatty acid requirements. *Aquaculture* **307**, 56-64.

Furness, A., Morrison, K., Orr, T., Arendt, J. & Reznick, D. (2015). Reproductive mode and the shifting arenas of evolutionary conflict. *Annals of the New York Academy of Sciences* **1360**, 75-100.

Goicoechea, O., Garrido, O. & Jorquera, B. (1986). Evidence for a trophic paternal-larval relationship in the frog *Rhinoderma darwinii*. *Journal of Herpetology* **20**, 168-178.

Goncalves, I. B., Ahnesjö, I. & Kvarnemo, C. (2016). Evolutionary ecology of pipefish brooding structures: embryo survival and growth do not improve with a pouch. *Ecology and Evolution* **6**, 3608-3620.

Gross, Mart R. (2005). The evolution of parental care. *The Quarterly Review of Biology* **80**, 37-45.

Gross, M. R. & Sargent, R. C. (1985). The evolution of male and female parental care in fishes. *American Zoologist* **25**, 807-822.

Gross, M. R. & Shine, R. (1981). Parental care and mode of fertilization in ectothermic vertebrates. *Evolution* **35**, 775-793.

Hamilton, H., Saarman, N., Short, G., Sellas, A. B., Moore, B., Hoang, T., Grace, C. L., Gomon, M., Crow, K. & Brian Simison, W. (2017). Molecular phylogeny and patterns of diversification in syngnathid fishes. *Molecular Phylogenetics and Evolution* **107**, 388-403.

Hamlett, W. C. (2005). *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids, and Chimaeras,* vol 3: Taylor & Francis Group.

Harada, A., Shiota, R., Okubo, R., Yorifuji, M., Sogabe, A., Motomura, H., Hiroi, J., Yasumasu, S. & Kawaguchi, M. (2022). Brood pouch evolution in pipefish and seahorse based on histological observation. *Placenta* **120**, 88-96.

Haresign, T. W. & Shumway, S. E. (1981). Permeability of the marsupium of the pipefish *Syngnathus fuscus* to [14C]-alpha amino isobutyric acid. *Comparative Biochemistry and Physiology, Part A* **69**, 603-604.

Hess, H. C. (1993). Male mouth brooding in Jawfishes (Opistognathidae): Constraints on polygyny. *Bulletin of Marine Science* **52**, 806-818.

Hussain, R., Recknagel, H. & Elmer, K. R. (2024). Higher locomotor costs of pregnancy in viviparous compared to oviparous common lizards (*Zootoca vivipara*). *Ecology and Evolution* **14**, e70171.

Itonaga, K., Jones, S. M. & Wapstra, E. (2012). Effects of maternal basking and food quantity during gestation provide evidence for the selective advantage of matrotrophy in a viviparous lizard. *PloS one* **7**, e41835-e41835.

Jones, A. G. & Avise, J. C. (2001). Mating systems and sexual selection in male-pregnant pipefishes and seahorses: insights from microsatellite-based studies of maternity. *The Journal of Heredity* **92**, 150-158.

Kawaguchi, M., Okubo, R., Harada, A., Miyasaka, K., Takada, K., Hiroi, J. & Yasumasu, S. (2017). Morphology of brood pouch formation in the pot-bellied seahorse *Hippocampus abdominalis*. *Zoological Letters* **3**, 19-19.

Kvarnemo, C., Mobley, K. B., Partridge, C., Jones, A. G. & Ahnesjö, I. (2011). Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. *Journal of Fish Biology* **78**, 1725-1737.

Laksanawimol, P., Damrongphol, P. & Kruatrachue, M. (2006). Alteration of the brood pouch morphology during gestation of male seahorses, *Hippocampus kuda*. *Marine and Freshwater Research* **57**, 497-502.

Lau, E., Varney, R. & Goodheart, J. (2024). An integrative understanding of evolutionary convergence across organisms and biological scales. *Integrative and Comparative Biology*.

Linton, J. R. & Soloff, B. L. (1964). The physiology of the brood pouch of the male sea horse *Hippocampus erectus*. *Bulletin of Marine Science* **14**, 45-61.

Lodé, T. (2001). Reproductive strategies in animal kingdom (in French). *Dunod-Massion Sciences*.

Long, J. A., Trinajstic, K. & Johanson, Z. (2009). Devonian arthrodire embryos and the origin of internal fertilization in vertebrates. *Nature* **457**, 1124-1127.

Mank, J. E., Promislow, D. E. L. & Avise, J. C. (2005). Phylogenetic perspectives in the evolution of parental care in ray-finned fishes. *Evolution* **59**, 1570-1578.

McCoy, E. E., Jones, A. G. & Avise, J. C. (2001). The genetic mating system and tests for cuckoldry in a pipefish species in which males fertilize eggs and brood offspring externally. *Molecular Ecology* **10**, 1793-1800.

Melamed, P., Xue, Y., Poon, J. F. D., Wu, Q., Xie, H., Yeo, J., Foo, T. W. J. & Chua, H. K. (2005). The male seahorse synthesizes and secretes a novel C-type lectin into the brood pouch during early pregnancy. *The FEBS Journal* **272**, 1221-1235.

Monteiro, N., Da Natividade Vieira, M. & Almada, V. C. (2002). The courtship behaviour of the pipefish *Nerophis lumbriciformis*: Reflections of an adaptation to intertidal life. *Acta Ethologica* **4**, 109-111.

Monteiro, N. M., Almada, V. C. & Vieira, M. N. (2005). Implications of different brood pouch structures in syngnathid reproduction. *Journal of the Marine Biological Association of the United Kingdom* **85**, 1235-1241.

Morrison, K. R., Ngo, V., Cardullo, R. A. & Reznick, D. N. (2017). How fish eggs are preadapted for the evolution of matrotrophy. *Proceedings of the Royal Society, Part B* **284**, 20171342-20171342.

Mossman, H. W. (1937). Comparative morphogenesis of the fetal membranes and accessory uterine structures. *Placenta* **12**, 1-5.

Novelli, B., Otero-Ferrer, F., Socorro, J. A., Caballero, M. J., Segade-Botella, A. & Molina Domínguez, L. (2017). Development of short-snouted seahorse (*Hippocampus hippocampus,* L. 1758): osteological and morphological aspects. *Fish Physiology and Biochemistry* **43**, 833-848.

Ostrovsky, A. N., Lidgard, S., Gordon, D. P., Schwaha, T., Genikhovich, G. & Ereskovsky, A. V. (2016). Matrotrophy and placentation in invertebrates: a new paradigm. *Biological Reviews of the Cambridge Philosophical Society* **91**, 673-711.

Quast, W. D. & Howe, N. R. (1980). The osmotic role of the brood pouch in the pipefish *Syngnathus scovelli*. *Comparative Biochemistry and Physiology, Part A* **67**, 675-678.

Ripley, J., Cantrell, P. & Foran, C. (2010). Morphological and quantitative changes in paternal brood-pouch vasculature during embryonic development in two Syngnathus pipefishes. *Journal of Fish Biology* **77**, 67-79.

Ripley, J. L. & Foran, C. M. (2009). Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). *Journal of Comparative physiology, Part B* **179**, 325-333.

Robert, K. A. & Thompson, M. B. (2001). Sex determination: viviparous lizard selects sex of embryos. *Nature* **412**, 698-699.

Roth, O., Klein, V., Beemelmanns, A., Scharsack, J. P. & Reusch, T. B. H. (2012). Male pregnancy and biparental immune priming. *The American Naturalist* **180**, 802-814.

Royle, N. J., Smiseth, P. T. & Kölliker, M. (2012). *The evolution of parental care*: Oxford University Press.

Shine, R. & Harlow, P. (1993). Maternal thermoregulation influences offspring viability in a viviparous lizard. *Oecologia* **96**, 122-127.

Skalkos, Z., Van Dyke, J., Dowland, S. & Whittington, C. (2024). Paternal protein provisioning to embryos during male seahorse pregnancy. *Reproduction* **167**, e230420.
Skalkos, Z. M. G., Van Dyke, J. U. & Whittington, C. M. (2020). Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus abdominalis*. *Journal of Comparative Physiology, Part B* **190**, 547-556.
Skalkos, Z. M. G., Van Dyke, J. U. & Whittington, C. M. (2023). Distinguishing between embryonic provisioning strategies in Teleost fishes using a threshold value for parentotrophy. *Biomolecules* **13**, 166-180.
Small, C. M., Bassham, S., Catchen, J., Amores, A., Fuiten, A. M., Brown, R. S., Jones, A. G. and Cresko, W. A. (2016). The genome of the Gulf pipefish enables understanding of evolutionary innovations. *Genome Biology* **17**, 258.
Sommer, S., Whittington, C. M. & Wilson, A. B. (2012). Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family Syngnathidae). *Developmental Biology* **12**, 39-39.
Stewart, J. R. (1989). Facultative placentotrophy and the evolution of squamate placentation: quality of eggs and neonates in *Virginia striatula*. *The American Naturalist* **133**, 111-137.
Stewart, J. R. & Thompson, M. B. (2000). Evolution of placentation among squamate reptiles: recent research and future directions. *Comparative Biochemistry and Physiology, Part A* **127**, 411-431.
Stölting, K. N. & Wilson, A. B. (2007). Male pregnancy in seahorses and pipefish: beyond the mammalian model. *BioEssays* **29**, 884-896.
Sutton, F. B. & Wilson, A. B. (2018). Parental behavior in fish. In: *Encyclopedia of Reproduction*, pp. 106-114: Elsevier Science & Technology.
Sutton, F. B. & Wilson, A. B. (2019). Where are all the moms? External fertilization predicts the rise of male parental care in bony fishes. *Evolution* **73**, 2451-2460.
Svensson, I. (1988). Reproductive costs in two sex-role reversed pipefish species (Syngnathidae). *The Journal of Animal Ecology* **57**, 929-942.
Thibault, R. E. & Schultz, R. J. (1978). Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **32**, 320-333.
Thompson, M. B., Adams, S. M., Herbert, J. F., Biazik, J. M. & Murphy, C. R. (2004). Placental function in lizards. *International Congress Series* **1275**, 218-225.
Trexler, J. C. (1997). Resource availability and plasticity in offspring provisioning: embryo nourishment in Sailfin mollies. *Ecology* **78**, 1370-1381.

Trexler, J. C. & DeAngelis, D. L. (2003). Resource allocation in offspring provisioning: an evaluation of the conditions favoring the evolution of matrotrophy. *The American Naturalist* **162**, 574-585.

Tyler, M. J. & Carter, D. B. (1981). Oral birth of the young of the gastric brooding frog *Rheobatrachus silus*. *Animal Behaviour* **29**, 280-282.

Van Dyke, J., U. , Griffith, O., W. & Thompson, M., B. (2014). High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. *The American Naturalist* **184**, 198-210.

Van Look, K. J., Dzyuba, B., Cliffe, A., Koldewey, H. J. & Holt, W. V. (2007). Dimorphic sperm and the unlikely route to fertilisation in the yellow seahorse. *Journal of Experimental Biology* **210**, 432-437.

Wake, M. H. (1977). Fetal maintenance and its evolutionary significance in the Amphibia: Gymnophiona. *Journal of Herpetology* **11**, 379-386.

Wake, M. H. (1993). Evolution of oviductal gestation in amphibians. *The Journal of Experimental Zoology* **266**, 394-413.

Wake, M. H. (2014). Fetal adaptations for viviparity in amphibians: amphibian fetal adaptations. *Journal of Morphology* **276**, 941-960.

Warne, R. W. & Catenazzi, A. (2016). Pouch brooding marsupial frogs transfer nutrients to developing embryos. *Biology Letters* **12**, 20160673.

Watanabe, S. (1999). The role of male brood pouch in the reproduction of the seaweed pipefish, *Syngnathus schlegeli*. Univeristy of Tokyo.

Watanabe, S., Hara, M. & Watanabe, Y. (2000). Male internal fertilization and introsperm-like sperm of the Seaweed pipefish (*Syngnathus schlegeli*). *Zoological Science* **17**, 759-767.

Wetzel, J., Wourms, J. P. & Friel, J. (1997). Comparative morphology of cotylephores in *Platystacus* and *Solenostomus*: Modifications of the integument for egg attachment in skin-brooding fishes. *Environmental Biology of Fishes* **50**, 13-25.

Wetzel, J. E. & Wourms, J. P. (2004). Embryogenesis in the dwarf seahorse, *Hippocampus zosterae* (Syngnathidae). *Gulf and Caribbean Research* **16**, 27-35.

Whittington, C. M. & Friesen, C. R. (2020). The evolution and physiology of male pregnancy in syngnathid fishes. *Biological Reviews of the Cambridge Philosophical Society* **95**, 1252-1272.

Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B. & Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution* **32**, 3114-3131.

Wilson, A. B., Vincent, A., Ahnesjö, I. & Meyer, A. (2001). Male pregnancy in seahorses and pipefishes (Family Syngnathidae): Rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *Journal of Heredity* **92**, 159-166.
Wourms, J. P. (1981). Viviparity: the maternal-fetal relationship in fishes. *American Zoologist* **21**, 473-515.
Wourms, J. P., Grove, B. D. & Lombardi, J. (1988). The maternal-embryonic relationship in viviparous fishes. In: *Fish Physiology*, pp. 1-134: Elsevier Science & Technology.
Wourms, J. P. & Lombardi, J. (1992). Reflections on the evolution of piscine viviparity. *American Zoologist* **32**, 276-293.

Chapter 2: Distinguishing between embryonic provisioning strategies in Teleost fishes using a threshold value for parentotrophy

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Review

Distinguishing Between Embryonic Provisioning Strategies in Teleost Fishes Using a Threshold Value for Parentotrophy

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Abstract: The source of embryonic nutrition for development varies across teleost fishes. A parentotrophy index (ratio of neonate: ovulated egg dry mass) is often used to determine provisioning strategy, but the methodologies used vary across studies. The variation in source and preservation of tissue, staging of embryos, and estimation approach impedes our ability to discern between methodological and biological differences in parentotrophy indices inter- and intra-specifically. The threshold value used to distinguish between lecithotrophy and parentotrophy (0.6–1) differs considerably across studies. The lack of a standardised approach in definition and application of parentotrophy indices has contributed to inconsistent classifications of provisioning strategy. Consistency in both methodology used to obtain a parentotrophy index, and in the classification of provisioning strategy using a threshold value are essential to reliably distinguish between provisioning strategies in teleosts. We discuss alternative methods for determining parentotrophy and suggest consistent standards for obtaining and interpreting parentotrophy indices.

Keywords: matrotrophy; lecithotrophy; matrotrophy index; embryonic nutrition; viviparous; oviparous; patrotrophy; maternal–foetal interactions

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1. Introduction

Embryonic nutrition can be provided entirely from the maternally supplied yolk (lecithotrophy), or supplemented with post-fertilisation maternal (matrotrophy) [1,2], or paternal (patrotrophy) nutrient provisioning [1,3]. Lecithotrophy and parentotrophy represent endpoints of a complex continuum of embryonic nutritional patterns, with no definitive biological point of distinguishment [4,5], For example, small amounts of inorganic nutrient provisioning (calcium) occur in lecithotrophic-classified viviparous squamates, either mobilised from the eggshell, or via simple placentotrophy [6,7]. Squamate classification of provisioning strategy is often also associated with anatomy, i.e., simple vs. complex placentation for lecithotrophy and placentotrophy (parentotrophy via placenta), respectively, despite both capable of post-fertilisation nutrient provisioning [7]. The transport of inorganic molecules requires relatively simple mechanisms compared to the transport of organic molecules [8]. Interestingly though, small uptake of organic molecules (amino acids) from the external environment has also been observed in oviparous teleosts [4]. This suggests the ability of parentotrophic embryos to absorb nutrients from their environment may be retained from an egg-laying ancestor. This ability is a prerequisite for parentotrophy, when the absorbed nutrients are acquired from the gestating parent during pregnancy [1,2]. Applying two broad provisioning strategies to vertebrates that exhibit a continuum of nutrient provisioning abilities between lecithotrophy and parentotrophy, makes correctness and consistency difficult.

Here, we define parentotrophy as the paternal or maternal post-fertilisation supplementation of nutrients to developing embryos during gestation. The parentotrophy

provisioning continuum amongst viviparous (live-bearing) teleosts ranges from incipient (mostly yolk reliant with small amounts of supplementation) to substantial parental supplementation post-fertilisation (primarily provisioning) [1,9], and can be achieved via many physiological processes [10]. Viviparous animals can exhibit any provisioning strategy along this continuum. In contrast, oviparous (egg-laying) embryos are lecithotrophic, except for monotremes, and some species that brood their embryos [1,9,11]. Brooding involves egg incubation on or in the parental body [11,12], and provides an opportunity for additional nutrients to be supplied by the brooding parent.

Teleosts (modern bony fishes) demonstrate a diverse range of parental care and reproductive strategies [13], including oviparity, viviparity (incubation inside the female reproductive tract [14], and oviparity with brooding in either parent. Thus, brooding males can provide embryonic nutrition in some species. For example, the Syngnathidae family (pipefish, seadragons, and seahorses) exhibits a specialised form of parental care, whereby fertilisation and gestation occur inside the male, in a specialised structure called the brood pouch [13,15]. Some syngnathids are capable of post-fertilisation provisioning from the father [3,16]. Thus, in this review we use the term parentotrophy to encompass both maternal and paternal post-fertilisation provisioning [10,11]. Unlike viviparous amphibians, amniotes, and chondrichthyans, viviparous teleosts do not develop Müllerian ducts during embryogenesis, thus there is no development of oviducts or uteri in females [10,17–19]. Consequently, viviparous teleosts have intra-ovarian rather than intra-uterine gestation [10].

Parentotrophy has independently evolved from the ancestral state, lecithotrophy [9], in at least 33 clades of vertebrates, with substantial parentotrophy evolving 24 times, mostly in bony fishes [1,20]. Matrotrophy has been most extensively studied in the viviparous teleost families; Anablepidae [21], Goodeidae [22] and Poeciliidae [23,24]. Anablepidae and Goodeidae exhibit extreme matrotrophy, increasing in dry mass one thousandfold over embryonic development [21,22]. In contrast, poeciliid embryos receive varying amounts of post-fertilisation nutrition ranging from a decrease in dry mass of 57% to an increase of 625% [9,25,26]. Patrotrophy has evolved at least once in teleosts in the Syngnathidae family [3], but has not yet been studied in other male-brooding teleosts, for example, the forehead-brooding Kurtidae [27], or mouth-brooding Apogonidae [27] and Ariidae [28].

The degree of parentotrophy is commonly measured using a matrotrophy index (MI) (or patrotrophy index (PI) in male pregnant species) [3,23,29]. This ratio has been applied to reptiles [30,31], sharks [32] and bony fishes [3,33]. Wourms et al. [2] were the first to suggest that the degree of post-fertilisation nutrient provisioning could be measured by "the ratio of dry weight of the developed embryo to that of the fertilised egg". This notion has since evolved to the dry mass of the neonate or newborn at birth divided by the dry mass of the recently fertilised egg [24,34]. The ratio is referred to in this review as matrotrophy index (MI) in matrotrophic species and patrotrophy index (PI) in patrotrophic species. As weight is relative to gravity and the studies mentioned here measured mass, we use the term, mass, for accuracy and consistency.

The dry mass lost by embryos during development in oviparous species provides a threshold MI or PI value used to distinguish between lecithotrophic and parentotrophic nutrition. In oviparous teleosts, the catabolised portion of the yolk to provide energy for growth results in a dry mass loss; thus, the average ratio of an oviparous teleost is MI = 0.7, but always <1, as the embryonic nutrition is provided in the yolk or eggshell only [23,35]. A lecithotrophic viviparous MI is expected to be similar to oviparous teleosts, as neither receive post-fertilisation provisioning.

Despite the continuum of embryonic provisioning, the degree of dry mass lost in oviparous species is used as a threshold value above which a closely-related viviparous species is assumed parentotrophic. However, the threshold values used to distinguish between lecithotrophy and parentotrophy vary considerably across the teleost literature (anything between 0.6 and 1.0: see Section 2), resulting in inconsistent application of threshold values to classify viviparous or brooding species as lecithotrophic or parentotrophic.

The concerns about the use of a threshold MI/PI are: (1) it assumes a consistent dry mass loss across oviparous species, but available data are lacking for oviparous teleosts; (2) it assumes consistency in catabolic costs across lecithotrophic viviparous and oviparous species [36]; (3) parentotrophic species may catabolise nutrients at a higher rate if they are receiving additional parental supplementation [37]; and (4) there are varying methodologies for measuring MI/PI. A review of dry weight losses in chondrichthyans questions the current use of threshold MI value [38]. The authors found a large discrepancy in the standard chondrichthyan threshold MI = 0.8 and the MI 0.6 calculated for the oviparous *Heterodontus portusjacksoni* and discuss inconsistencies and inaccuracies in previous chondrichthyan research. Frazer et al. [38] strongly recommended the disuse of a threshold value to distinguish between lecithotrophy and incipient parentotrophy in chondrichthyans. Therefore, in this review, we evaluate the use of threshold values to classify parentotrophy in teleosts and discuss the variation in methodology used across studies. The goal of this review is to determine the applicability of a single threshold value indicating teleost parentotrophy, and to provide suggestions for methodological consistency to enable a meaningful threshold value to be determined.

2. Teleost Parentotrophy Indices: Methodology and Provisioning Strategy Classification

Teleost literature varies in its methodology of obtaining and interpreting a parentotrophy index for the purpose of classifying a provisioning strategy to a species (Table S1). It differs in the embryonic stages used, the source and preservation of samples, and the use of real or estimated means to calculate an MI/PI. This variation can result in parentotrophy indices not accurately representing real differences in dry mass between birth and fertilisation and eliminate the ability to compare results between studies. Furthermore, the interpretation of an MI/PI varies across studies using varying parentotrophy thresholds and statistical methods, resulting in an overlap of provisioning strategy classifications between and within species. Here, we explore sources of variation in calculating MI/PI.

2.1. Methodological Sources of Variation in Parentotrophy Indices

2.1.1. Raw vs. Regression Estimated Means

An MI/PI can be calculated from either raw (real) or estimated data for the dry mass of an offspring at birth divided by the raw or estimated dry mass of an embryo at fertilisation. Thus, dry masses are required from two embryonic developmental stages: "near-fertilisation" and "near-birth". Raw data are derived from measuring dry masses of either preserved or freshly collected samples. For these comparisons, mean dry masses from several to hundreds of embryos/offspring are calculated (see references in Table 1).

Studies using raw data to measure dry mass means do so through varying methods to pool offspring within a clutch. These methods include: whole brood total dry mass divided by the number of offspring per brood [39–41]; dividing the whole clutch into pools of two to ten individuals for dry mass measurement, dividing each result by the number of offspring in each pool, and then calculating a clutch average [3,42]; or measuring a predetermined number of pooled offspring per clutch (5-100) [33,43]. Some studies do not specify if or how they pooled, subsampled or used whole clutches to measure dry mass, and each study has varying numbers of replicates per stage of development.

In contrast, estimating mean dry mass data uses dry mass data from varying (often opportunistic) stages of embryonic development to code either a linear or quadratic regression model of the relationship between embryonic stage and dry mass. These models are used to estimate the dry masses of embryos at fertilisation and/or at birth and are suitable when embryos cannot be collected at those specific developmental stages. The predicted values, rather than raw measured values, are then used to calculate the parentotrophy index.

Using regression models to predict embryonic dry mass is flawed for two main reasons. Firstly, a regression assumes that the *x*-axis values are truly quantitative and continuous. Embryonic stage is a discrete variable and offers a qualitative descriptor of the progression

of embryogenesis [44,45]. Using embryonic stage as the independent variable assumes a consistent quantitative progression between each stage from beginning to end, which is unlikely [44]. Time after fertilisation or prior to birth, rather than embryonic stage, could be a better consistent quantitative metric, but is still not ideal as developmental rate is affected by environmental variables like temperature [46,47]. Furthermore, a regression of mass vs. time would demonstrate embryo mass gain or loss over time, which does not equate to a parentotrophy index.

The second problem with predicting *y*-axis values from a regression is that predictions are only valid for the range of data used to estimate the model. Thus, attempts to estimate fertilised egg or newborn dry mass from regressions based on datasets comprising developmental stages after fertilisation and prior to birth will accumulate error at the extremes of the developmental stages as predictions stray further from the available data on the *x*-axis. Regression models could be improved by including mature eggs prior to fertilization, and newborns. Yet, many teleost studies use a regression to estimate MI/PI but do not specify using samples that expands that full range (see references in Table 1). Furthermore, very few studies provide the sample sizes for each embryonic stage the regression was based on. For those that do, the sample sizes at either extreme of embryonic development are very low. For example, out of a sample size of ~48 clutches of reproductively active *Heterophallus milleri*, only two and three clutches were from the earliest and latest embryonic stages, respectively, with most data collected from mid-development [48]. The timing of parentotrophy during embryonic development is variable between species (see Section 2.2.3). Therefore, making predictions outside the existing data range, especially with small sample sizes or no samples at the two available ends of development, is discouraged.

Differences in real mean and estimated mean may result in incorrect provisioning strategy classification for species that are lecithotrophic or incipiently parentotrophic. Regressions are therefore likely not suitable for estimating dry masses using an MI/PI for many teleost species. In contrast, this approach is less likely to have a strong influence on conclusions drawn for highly parentotrophic species than for incipiently parentotrophic or lecithotrophic species, since the difference in fertilised egg and newborn dry mass is so large, e.g., *Dermogenys sumatrana*, which has an MI of 198.5 [49]. The regression approach is therefore potentially useful for such species, even when recently fertilised eggs or newborns are unavailable.

2.1.2. Embryonic Staging

The ideal MI calculation is estimated as the dry mass of the offspring at birth divided by the dry mass of the embryo at fertilisation [2,24]. As yolk formation is complete by the time fertilisation occurs, any additional nutrients present in the embryo must be due to post-fertilisation provisioning [2,50]. However, the embryonic stages used to estimate an MI/PI from raw data vary across teleost studies.

Obtaining samples exactly at fertilisation or birth is impossible in some viviparous teleosts without constant monitoring, thus the next best achievable stage is often used. However, these stages are not consistent across studies, which is problematic in two ways. Firstly, the post-fertilisation sampling delay can affect a calculated MI/PI as parentotrophy may have already started. For lecithotrophic species, the later the sampling occurs after fertilisation, the more likely the embryos have already lost dry mass and thus, the MI would be overestimated. For parentotrophic species, a longer time post-fertilisation before sampling may result in an underestimated MI/PI, as provisioning may have already begun. There is no consistent standard for "near fertilisation" sampling within or across teleost studies, and timing varies considerably across fresh data studies from minutes [50], to hours [3]. Secondly, the amount of time before or after birth that samples are collected can also affect a calculated MI, with no consistent standard for "near birth". Normal tables of development vary across species and thus, without consistent staging of both near fertilisation and near birth within a species or the equivalent between species, comparisons cannot be drawn. Most studies followed Haynes [44] embryonic staging, comparing stage

four (blastocyst) to stage eleven (mature) [51,52], or Reznick [45], comparing stage two (uneyed) to stage six (very-late eyed/mature) [42,53]. Stage four and eleven from Haynes [44], equate to stage one and six in Reznick [45], respectively. Using late-stage embryos is common for wild populations as we predict collection of this stage prior to birth, reduces the potential effect of catabolism or consumption of food from the external environment, on new-born dry mass and is more logistically achievable. In contrast, laboratory studies can sample immediately after birth [54], or within hours of parturition [3]. The point at which parental nutrient transport to embryos starts and the constant or fluctuating rate at which it occurs is unknown for most parentotrophic teleosts, so the measurable impacts of sampling post-fertilisation or pre-birth are unknown. Even so, using near birth and near fertilisation stages likely results in under- or over-estimated MIs, and studies using different methodology in their embryonic staging are not comparable.

2.1.3. Variation in Maternal Size

Ideally, a parentotrophy index comparison of embryos at fertilisation and new-borns would be obtained from the same female, for multiple individuals across multiple populations. This is possible for species with superfetation, where two or more broods are developing at the same time within the same female, but collecting multiple individuals containing both "near fertilisation" and "near-birth" embryonic stages is likely to be difficult. Thus, matrotrophy indices compare the eggs from multiple females to the offspring of multiple other females, assuming heterogeneity of initial egg size. However, larger egg [55,56] and new-born [56–60] dry mass/size is correlated with larger viviparous females in some species. Note that an increase in offspring size does not always equate to an increase in offspring dry mass [33]. Furthermore, the degree of parentotrophy may change with female size/age [61]. The influence of maternal size can be methodologically mitigated by selecting for same age/sized females across embryonic comparisons.

2.1.4. Source and Preservation of Samples

Dry mass measurements used to calculate both raw and estimated MI/PI can be derived from wild or captive, fresh or preserved samples, which can alter the dry mass of a sample. Only two studies within the viviparous teleost MI/PI range of 0.6 and 1.1 used fresh samples, both in laboratory conditions [3,54]. All oviparous teleost studies mentioned here measured dry mass changes from fresh samples, with liquid nitrogen used as temporary storage for *Danio rerio* [62], before drying (Table 1). However, no significant difference is observed between dry masses of demersal fish eggs frozen before drying and eggs dried fresh [63]. Most viviparous studies presented here use samples from wild populations with preserved specimens in Neutral Buffered Formalin (NBF) alone or followed by ethanol, in varying concentrations (Table 1). However, preservation in formaldehyde solutions can significantly increase [64] or decrease [63,65] fish egg dry mass. The use of NBF can also result in some loss of lipids from tissue [66]. Furthermore, as lipids are ethanol soluble [67], fixation in ethanol measures lean dry mass, which can be different to dry mass [68,69]. The use of these preservatives can result in a conservative or exaggerated parentotrophy index. Not all teleost parentotrophy research specifies or measures the effects of the method of preservation, except for Thibault and Shultz [43] (Table 1). For example, Olivera-Tlahuel et al. [70] obtained data from alternate sources to use in their MI regression formula when calculating MIs for some species. For two of those species, *Phalloceros caudiomaculatus* [68] and *Phalloceros anisophallos* [71], the data obtained were lean dry masses, but the MIs were presented with those derived from dry masses, without distinguishment. Studies in which samples were preserved in NBF or ethanol may not accurately represent the true MI/PI, unless validated by determining the effect of preservation on the embryo dry mass. This is important when distinguishing between facultative or incipient parentotrophy and lecithotrophy because any preservative effect on dry mass may result in the classification of an incorrect provisioning strategy.

Table 1. Parentotrophy indices calculated for teleost fishes indicate discrepant application of a threshold value for parentotrophy.

Species	MI/PI	Real v Estimated	Parentotrophy Threshold	Statistical Test for Significant Difference	Classification of Nutrient Provisioning Strategy	Resource
Viviparous						
Alfaro huberi	0.64	EEN	>1	NA	Lecithotrophy	[72] P
Belonesox belizanus	0.70	EEN	>1	NA	Lecithotrophy	* [72] P
Brachyrhaphis episcopi	0.78	R	Not stated	NA	Not specified	[73] PF
Brachyrhaphis holdridgei	0.66	EEN	>1	NA	Lecithotrophy	[72] P
Brachyrhaphis rhabdophora	0.77	EEN	>1	NA	Lecithotrophy	* [72] P
Dermogenys burmanica	0.67	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Dermogenys siamensis	0.64	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Gambusia affinis	0.62	EEN	>1	NA	Lecithotrophy	* [72] P
Gambusia aurata	0.82	EEN	≥0.8	NA	Matrotrophy	[70] PE
Gambusia holbrooki	0.70	R	Not stated	NA	Not specified	** [42]
	0.64	EEN	>1	NA	Lecithotrophy	* [72] P
Gambusia hubbsi	0.86	EN	>0.7	0.7	Both	** [74] PE
Gambusia punctata	0.78	EEN	>0.7	0.7	Lecithotrophy	[75] P
Gambusia sexradiata	0.73	EEN	>0.7	0.7	Lecithotrophy	[75] P
Gambusia vittata	0.77	EEN	>0.7	0.7	Lecithotrophy	** [75] P
	0.74	EEN	>1	NA	Lecithotrophy	[72] P
	1.29	EEN	≥1	NA	Both	** [41] PE
Gambusia wrayi	0.70	EEN	>0.7	0.7	Lecithotrophy	[75] P
Hemirhamphodon kapuasensis	0.61	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Hemirhamphodon pogonognathus	0.64	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Heterophallus milleri	0.74	EEN	>0.75	NA	Lecithotrophy	[48] PF
Hippocampus abdominalis$_p$	1	R	>0.7	Stage	Patrotrophy	[3] F
Hippocampus fuscus$_p$	0.75	R	Not stated	NA	Not specified	[54] F
Limia dominicensis	0.65	EEN	≥1	NA	Lecithotrophy	[76] P
	0.51	EEN	≥1	NA	Lecithotrophy	[77] N
Limia heterandria	0.67	EEN	≥1	NA	Lecithotrophy	[77] N
Limia melanogaster	0.71	EEN	≥1	NA	Lecithotrophy	** [77] N
	0.67	EEN	≥1	NA	Lecithotrophy	[76] P
Limia melanonotata	0.67	EEN	≥1	NA	Lecithotrophy	[77] N
Limia nigrofasciata	0.64	EEN	≥1	NA	Lecithotrophy	** [77] N
Limia pauciradiata	0.66	EEN	≥1	NA	Lecithotrophy	[77] N
Limia perugiae	0.90	EEN	≥1	NA	Lecithotrophy	[77] N
Limia tridens	0.90	EEN	≥1	NA	Lecithotrophy	[77] N
Limia versicolor	0.74	EEN	≥1	NA	Lecithotrophy	[77] N
Limia vittata	0.76	EEN	≥1	NA	Lecithotrophy	** [77] N
Limia zonata	0.91	EEN	≥1	NA	Lecithotrophy	[77] N
Micropoecilia picta	0.78	EEN	>0.7	0.7	Lecithotrophy	** [78] PF
Nomorhamphus kolonodalensis	0.66	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Nomorhamphus megarrhamphus	0.84	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Nomorhamphus weberi	0.77	EEN	>0.7	0.7	Lecithotrophy	[49] PE
Phallichthys fairweatheri	0.65	EEN	≥0.7	0.7	Lecithotrophy	[25] P
Phallichthys quadripunctatus	0.75	EEN	≥0.7	0.7	Lecithotrophy	** [25] N
Poecilia caucana	0.77	EEN	≥1	NA	Lecithotrophy	[76] P
Poecilia latipinna	0.92	EEN	Not stated	NA	Both	** [79] PF
Poecilia latipunctata	0.85	EEN	≥1	NA	Lecithotrophy	[80] PF

Table 1. *Cont.*

Species	MI/PI	Real v Estimated	Parentotrophy Threshold	Statistical Test for Significant Difference	Classification of Nutrient Provisioning Strategy	Resource
Poecilia mexicana	0.63	EN	>1	NA	Lecithotrophy	[72] ^{P}
	0.57	EEN	>0.7	NA	Lecithotrophy	** [81] ^{PF}
	0.68	EN	>0.65	NA	Lecithotrophy	** [34] ^{PF}
Poecilia reticulata	0.70	EEN	>0.7	0.7	Lecithotrophy	** [78] ^{PF}
Poecilia wingei	0.84	EEN	>0.7	0.7	Lecithotrophy	[78] ^{PF}
Poeciliopsis baenschi	0.98	EEN	≥0.8	NA	Matrotrophy	[70] ^{PE}
Poeciliopsis balsas	1.05	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
Poeciliopsis catemaco	0.68	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
Poeciliopsis fasciata	0.81	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
Poeciliopsis gracilis	0.69	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
	0.84	EEN	≥0.8	NA	Matrotrophy	[70] ^{PE}
	0.80	EEN	≥1	NA	Both	** [82] ^{PE}
	0.72	R	Not stated	NA	Lecithotrophy	[51] ^{PE}
Poeciliopsis hnilickai	0.86	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
Poeciliopsis infans	0.86	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
	1.05	EEN	≥0.8	NA	Matrotrophy	[70] ^{PE}
Poeciliopsis latidens	0.86	EN	>0.6	0.7	Matrotrophy	[23] ^{P}
Poeciliopsis monacha	0.61	R	Not stated	NA	Not specified	[43] ^{PE}
Poeciliopsis scarlli	0.87	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
Poeciliopsis turrubarensis	0.66	EN	>0.6	0.7	Lecithotrophy	[23] ^{P}
	1.05	R	Not stated	NA	Matrotrophy	** [52] ^{PE}
Poeciliopsis viriosa	0.93	EN	>0.6	0.7	Matrotrophy	[23] ^{P}
Priapella chamulae	0.71	EEN	>0.75	Stage	Lecithotrophy	[34] ^{PF}
Priapella intermedia	1.03	R	Not stated	NA	Matrotrophy	[51] ^{PE}
Priapella olmecae	0.76	EEN	≥0.8	NA	Lecithotrophy	[70]
Priapichthys festae	0.60	R	>0.65	NA	Lecithotrophy	[83] ^{PF}
Pseudoxhiphophorus jonesii	0.65	EEN	≥0.8	NA	Lecithotrophy	[70] ^{PE}
Syngnathus schlegeli$_{p}$	0.71	R	Not stated	Stage	Patrotrophy	[33] ^{PE}
Xiphophorus hellerii	0.61	EEN	>1	NA	Lecithotrophy	[72] ^{P}
Oviparous						
Clupea harengus	0.73	R	Not stated	NA	Not specified	[84] ^{F}
Danio rerio	0.77	R	Not stated	NA	Not specified	[62] ^{FL}
Salmo fario	0.63	R	Not stated	NA	Not specified	[50] ^{F}
Salmo salar	0.70	R	Not stated	NA	Not specified	[85] ^{F}
Salmo irideus	0.62	R	Not stated	NA	Not specified	[86] ^{F}
Salvelinus fontinalis	0.75	R	Not stated	NA	Not specified	[87] ^{F}

Only MI/PI between 0.6 and 1.1 were included to represent the species most likely affected by in-consistencies in methodology. The calculated matrotrophy index (MI) or patrotrophy index (PI) and the classification of nutrient provisioning strategy is shown for each study. Each of the included studies calculates at least one Parentotrophy Index (MI/PI) or % dry mass change during embryogenesis. For studies containing multiple populations, the average is displayed here (**). Estimations were performed using regression models. Statistics listed were parentotrophy index-specific in determining provisioning strategy. Parentotrophy index values were rounded to 2 decimal places. * = not original data source, but MI is reported in the publication. In the Test for Significant Difference column, "stage" indicates a test for significant difference between early and late/newborn stage and "0.7" indicates testing the MI/PI to be significantly different to 0.7. (NA) = no classifying statistics performed or no parentotrophy threshold given and thus no classification; (R) = raw mean embryo dry mass values from two embryonic stages (fresh and pre-served with 10% Neutral Buffered Formalin) were used to calculate the MI/PI; (EN) = regression used to estimate newborn dry mass; EEN = regression used to estimate embryo at fertilisation and newborn dry mass. Subscript (p) indicates a Patrotrophy Index for male gestating parents. Superscripts indicate whether samples were fresh (F), fresh but briefly stored in liquid nitrogen (FL), preserved in Neutral Buffered Formalin (PF), preserved in Ethanol with or without formalin fixation (PE), preserved but methodology not specified (P), or not specified whether fresh or preserved (N). All preservation types were included for oviparous due to the limited studies available. Ash-free (organic) masses were not included. Studies not specifying fresh or pre-served samples were not included. Studies that used unfertilised eggs as comparison were excluded unless they specified that they were mature. Thresholds and classifications were only included if specified in the original source.

2.2. Methodological Sources of Variation in Provisioning Strategy Classification

2.2.1. Discrepant Use of Threshold Value for Parentotrophy

The threshold value for parentotrophy used in teleost literature is highly variable, ranging from MI > 0.6 to MI > 1 (Table 1), resulting in overlap of classifications along the provisioning continuum (Figure 1). The most common MI/PI threshold values are ≥1 and >0.7 (Table 1). The lack of a consistent threshold applied to the continuum of embryonic provisioning, means that distinguishing lecithotrophy from incipient parentotrophy is difficult and has resulted in overlap of classifications (Figure 1). Multiple species are classified differently across studies due to varying threshold values, including *Gambusia vittata*, *Poeciliopsis gracilis*, *Poeciliopsis infans*, and *Poeciliopsis turrubarensis* (Table 1, Figure 1). For teleosts with moderate or substantial parentotrophy (Table 2), the use of a consistent methodology is not as essential because the methodological causes of variation should not change the provisioning strategy classification.

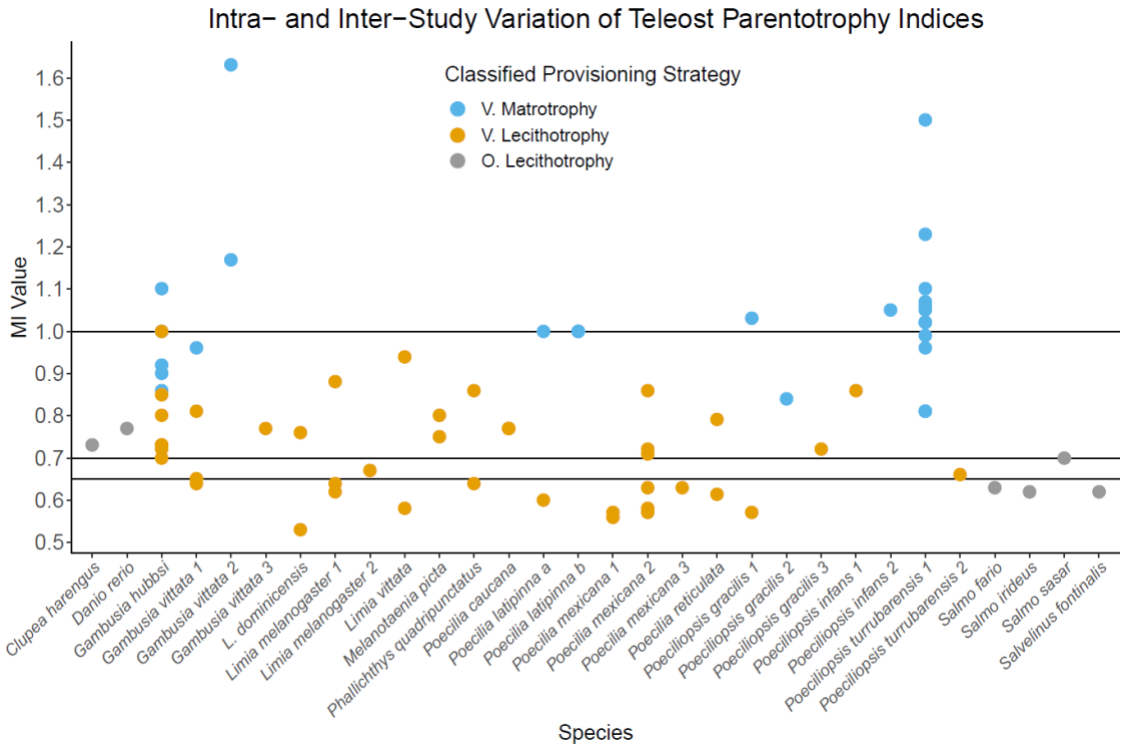

Figure 1. The variation in matrotrophy indices and parentotrophy classifications between populations of teleosts. Each species along the x-axis represents a single study, except for *Poecilia latipinna* (a & b), which come from a single study and represent two populations (population a & population b) one year apart. If there are multiple studies for a single species, each study is separated by species name followed by a number (1–3). Each plotted value for each species represents a separate population within a study, except for *Poeciliopsis gracilis* 1, which represents the same population across the wet season (classified as lecithotrophy) and dry season (classified as matrotrophy), and *Poecilia latipinna*. All viviparous MI values were estimated using regression models except for *Poeciliopsis turrubararensis* and all oviparous species, which used raw mean values. *Poecilia caucana* includes two populations with an MI of 0.77. The provisioning strategy presented was assigned by each paper based on their specified threshold and statistics. The varying threshold MI and PI values used to classify parentotrophy in different studies are >0.65, >0.7, or ≥1, represented by horizontal lines. There was either no statistics or confirmation of parentotrophy was done by determining whether the population MI was significantly different to 0.7 (refer to Table 1). (V.) represents viviparous species in which an MI has been calculated for at least two populations. (O.) represents oviparous, lecithotrophic species, which were included for comparison.

Table 2. A non-exhaustive list of published parentotrophy indices for teleost species with MI < 0.6 or MI > 1.1, including examples from all known matrotrophic teleost families in which MI/PI studies have been conducted; Anablepidae; Clinidae; Goodeidae; Poeciliidae; Zenarchopteridae. All data are presented as in the resource, to 2 decimal places.

Species	Matrotrophy Index	Classification of Nutrient Provisioning Strategy	Resource
Ameca splendens	150.00	Matrotrophy	[2]
Clinus superciliosus	35.60	Matrotrophy	[88]
Dermogenys bispina	152.00	Matrotrophy	[49]
Dermogenys orientalis	18.35	Matrotrophy	[49]
Dermogenys sumatrana	198.50	Matrotrophy	[49]
Gambusia rhizophorae	1.24	Matrotrophy	[75]
Gambusia speciosa	0.45	Lecithotrophy	[70]
Gambusia yucatana	0.53	Lecithotrophy	[70]
Hemirhamphodon keukenthali	0.58	Lecithotrophy	[49]
Heterandria formosa	14.18	Matrotrophy	[40]
Jenysia multidentata	606.14	Matrotrophy	[89]
Limia caymanensis	0.57	Lecithotrophy	[77]
Micropoecilia bifurca	55.06	Matrotrophy	[78]
Micropoecilia branneri	86.84	Matrotrophy	[78]
Micropoecilia parae	6.33	Matrotrophy	[78]
Nomorhamphus bakeri	3.44	Matrotrophy	[49]
Nomorhamphus brembachi	11.40	Matrotrophy	[49]
Nomorhamphus manifesta	15.80	Matrotrophy	[49]
Nomorhamphus rossi	22.00	Matrotrophy	[49]
Phallichthys amates	0.52	Lecithotrophy	[25]
Phallichthys tico	0.43	Lecithotrophy	[25]
Phalloceros anisophallos	2.80	Matrotrophy	[71]
	2.82	Matrotrophy	[90]
Phalloceros aspilos	2.50	Matrotrophy	[90]
Phalloceros enneaktinos	2.43	Matrotrophy	[90]
Phalloceros harpagos	3.33	Matrotrophy	[53]
	2.52	Matrotrophy	[90]
Phalloceros leptokeras	1.52	Matrotrophy	[90]
Phalloceros tupinamba	2.17	Matrotrophy	[90]
Phalloceros wai	2.64	Matrotrophy	[90]
Pamphorichthys araguaiensis	9.62	Matrotrophy	[76]
Pamphorichthys hasemani	36.37	Matrotrophy	[76]
Pamphorichthys hollandi	21.29	Matrotrophy	[76]
Pamphorichthys minor	1.63	Matrotrophy	[76]
Pamphorichthys scalpridens	16.58	Matrotrophy	[76]
Poecilia butleri	2.30	Matrotrophy	[91]
Poeciliopsis elongata	68.90	Matrotrophy	[23]
Poeciliopsis lucida	1.34	Matrotrophy	[43]
	1.79	Matrotrophy	[92]
Poeciliopsis occidentalis	1.12	Matrotrophy	[23]
	1.50	Matrotrophy	[39]
Poeciliopsis pleurospilus	0.50	Lecithotrophy	[70]
Poeciliopsis presidionis	21.5	Matrotrophy	[23]
Poeciliopsis prolifica	5.40	Matrotrophy	[23]
	7.01	Matrotrophy	[80]
Poeciliopsis retropinna	22.39	Matrotrophy	[93]
Poeciliopsis turneri	41.4	Matrotrophy	[23]
Xenodexia ctenolepis	3.86	Matrotrophy	[49]

2.2.2. Variation in Use of Statistical Tests

The lack of a standard method or statistical approach to assign a provisioning strategy based on an MI/PI can also account for some of the inconsistency in classification observed inter- and intraspecifically (Figure 1; Table 1). Most studies perform no parentotrophy

index-specific statistics while others tested if the MI/PI was significantly different from 0.7. This can result in species with MI/PI values larger than 0.7 being classified as lecithotrophic, e.g., *Gambusia punctata and Micropoecilia picta* (MI = 0.78), or *Nomorhamphus megarrhamphus* (MI = 0.84) (Table 1), likely due to large variance around the mean and/or statistically inadequate sample sizes. The remaining studies assessed if their near fertilisation and near birth developmental stages were significantly different from one another (Table 1).

2.2.3. Intra-Specific Variation in Parentotrophy Indices

Intra-Specific Variation in MI/PI Suggests Parentotrophy

In lecithotrophic species, a change in biological factors (e.g., temperature, resource availability) should result in no change in calculated MI/PI across study conditions of populations, providing methodology is consistent and sample size is sufficient. However, if the species is capable of parentotrophy, then variation in MI/PI is likely to occur, even with consistent methodology, due to environmental or genetic factors. This variation means that under certain contexts, an MI could fail to detect parentotrophy, despite the species being parentotrophic. For example, if a species is facultatively parentotrophic, it would be unlikely to be detected in systems where the gestating parent does not have the excess nutrients/energy to provide to developing embryos, like in resource limiting environments [94,95]. As also seen in reptiles, if a pregnant mother has sufficient resource availability for facultative parentotrophy, the MI should increase [96]. For some teleost species with MIs calculated for multiple populations within a study, some populations are classified as lecithotrophic and others parentotrophic, for example *Gambusia hubbsi*, *G. vittata*, *P. gracilis*, and *Poecilia latipinna* (Figure 1). However, if a species is capable of parentotrophy, but does not always exhibit parentotrophy, then it should be classified as parentotrophic (i.e., capable of parentotrophy). By this standard, assuming methodology is consistent within studies, *G. hubbsi*, *G. vittata*, *P. gracilis*, and *P. latipinna* should all be classified as potentially parentotrophic. For species with MI/PI between 0.6 and 1, if methodology is not consistent within or across studies, large variation in parentotrophy indices may be methodological and incorrectly imply parentotrophy. We suggest that a parentotrophy index from a single population may not accurately represent a species' ability for parentotrophy if its MI/PI falls within the range of 0.6 and 1, and that studies should look at multiple populations, age groups (or equal variation in parental ages across the two stages), and/or study conditions (e.g., resource availability).

Temporal Intra-Specific Variation in Parentotrophy

In parentotrophic teleosts, the timing, source, and quantity of embryonic nutrition across development is variable between species and can be non-linear. Species can receive parental provisioning at different stages of development, so the timing of sampling in species with varying metabolisms can heavily influence parentotrophy index results and provisioning conclusions. For example, Gambusia holbrooki is classified as lecithotrophic (Table 1), yet embryos increase in dry mass by almost 50% during embryogenesis before decreasing again before parturition [37]. This increase to ~150% of the original dry mass is significantly different to early and late embryonic masses. In contrast, in some Phalloceros species, the embryos decrease in dry mass at the beginning of gestation and then increase up to three-fold [90]. Similarly, Xenodexia ctenolepis embryos dry weights remain stable or decrease slightly until about half-way through gestation, where significant matrotrophy occurs, resulting in a three-to-four-fold increase in dry mass from fertilisation to birth [49]. Comparing two stages on either end of embryonic development without consideration for the intermediate stages, is a limitation to using the MI/PI to classify provisioning strategy and can result in incorrectly classifying a species as lecithotrophic.

3. Alternative Approaches to Measuring Parentotrophy

There are several alternative methods to determine provisioning strategies that have been used across vertebrate literature, including comparing the nutrient content of neonates

to that of eggs via nutrient extractions, mass spectrometry or chemical composition analysis. In lecithotrophic species, if the nutrient in question is catabolisable (e.g., lipids), its mass should decrease due to the catabolism of the embryo. Thus, if transport of a metabolised nutrient occurs, then the mass of that nutrient in the newborns should be more than or slightly less than that of the recently fertilised eggs [3]. For example, quantifying lipids [3,97,98] and proteins [97,98] has been used as evidence for matrotrophy.

To determine whether parentotrophy occurs, the transport of specifically labelled nutrients can be tracked between the parent and offspring, as commonly used in reptiles [98–100] and fish [16,94,101–103]. Currently, there are two direct labelling methods to track nutrient transport and determine the presence of parentotrophy: stable isotope labelled nutrients and radioactively labelled nutrients. These methods can be used with a range of nutrients including fatty acids [104] and amino acids [81,100,104] and consist of either feeding or directly injecting the labelled nutrients into pregnant individuals. The abundance of the labelled nutrients is then measured in the embryos to determine if they have taken up any of the labelled nutrients [104]. Labelled nutrient studies can offer opposite conclusions to a derived parentotrophy index. For example, an estimated MI of 0.56 and 0.57 for two separate populations of *Poecilia mexicana* suggests lecithotrophy [81]. However, the study also conducted a radio-tracer assay and found significant maternal nutrient transfer of labelled leucine to developing embryos, revealing parentotrophy had occurred. Respirometry by measurement of oxygen consumption, and calorimetry by measurement of heat loss, can indirectly measure embryonic metabolic rates and has been used to make inferences on the level of parentotrophy in the genus *Sebastes* [36]. Respirometry indicates that *Sebastes melanops* embryos catabolise 64% of the energy provided in the egg but offspring at birth contained 81% of the initial energy of the mature egg. Therefore, the total energy required for embryonic development is ~1.45 x the initial egg supply, suggesting that the species is parentotrophic [36]. Additionally, immunohistochemistry can localise proteins [105] involved in nutrient transport and can display how, when and/or where parentotrophy may be occurring.

4. Conclusions and Future Directions

Teleost nutrient provisioning strategies range from lecithotrophy to extreme parentotrophy [21,22], and distinguishing between the two is vital in gaining foundational biological understanding of a species' reproduction. Therefore, methodological consistency in obtaining an MI/PI and consistency in a parentotrophy threshold is essential for establishing a credible standard parentotrophy index comparison across species, populations, and studies. We argue that preserved specimens may not accurately estimate an MI/PI unless validated with a comparison of dried fresh and dried preserved specimens. We further argue that calculating an MI/PI using a regression to estimate dry mass based on embryonic staging can be problematic because embryonic stage is not quantitative and continuous, and predictions cannot be made outside the range of data used to make the regression. We therefore propose that a single threshold cannot be used to distinguish between lecithotrophy and incipient parentotrophy in every teleost species, similar to Frazer et al.'s [38] recommendations for chondrichthyans. For species with $0.6 \leq \text{MI} \leq 1$, we recommend the addition of another method to distinguish between lecithotrophy and parentotrophy (e.g., nutrient extractions or mass spectrometry of embryos/uterine or pouch fluid, radio-tracer assay, histology, and electron microscopy), to confirm a species' ability (whether realised in natural populations or not) to be parentotrophic. Alternatively, studies of similar sized/aged animals in multiple environmental treatments (e.g., feeding regimes) in which separate parentotrophy indices are calculated may be useful to determine any variability that may suggest parentotrophy. The number of oviparous teleost species for which MI has been calculated is limited and these studies are highly variable in their methodology. Thus, further research is required with consistent embryonic stage comparisons between species and a preference for the use of fresh rather than fixed tissues. With consistent

methodology, the biological and genetic causes for variation in parentotrophic species can then be compared across studies, species, and populations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/biom13010166/s1, Table S1: Inclusion Criteria.

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References

1. Blackburn, D.G. Evolution of vertebrate viviparity and specializations for fetal nutrition: A quantitative and qualitative analysis. *J. Morphol.* **2014**, *276*, 961–990. [CrossRef] [PubMed]
2. Wourms, J.P.; Grove, B.D.; Lombardi, J. The Maternal-Embryonic Relationship in Viviparous FishesHoar, W.S., Randall, D.J., Eds.; Fish Physiology1988; Volume 11, pp. 1–34.
3. Skalkos, Z.M.G.; Van Dyke, J.U.; Whittington, C.M. Paternal nutrient provisioning during male pregnancy in the seahorse Hippocampus abdominalis. *J. Comp. Physiol. B* **2020**, *190*, 547–556. [CrossRef]
4. Morrison, K.R.; Ngo, V.; Cardullo, A.R.; Reznick, D.N. How fish eggs are preadapted for the evolution of matrotrophy. *Proc. R. Soc.* **2017**, *284*, 20171342. [CrossRef]
5. Stewart, J.R. Fetal nutrition in lecithotrophic squamate reptiles: Toward a comprehensive model for evolution of viviparity and placentation. *J. Morphol.* **2013**, *274*, 824–843. [CrossRef]
6. Stewart, J.R.; Thompson, M. Evolution of placentation among squamate reptiles: Recent research and future directions. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2000**, *127*, 411–431. [CrossRef]
7. Thompson, M.B.; Speake, B.K. A review of the evolution of viviparity in lizards: Structure, function and physiology of the placenta. *J. Comp. Physiol. B* **2005**, *176*, 179–189. [CrossRef] [PubMed]
8. Silverthorn, D.U. Human Physiology: An Integrated Approach, 8th ed.Pearson Education Limited: Harlow, UK, 2018.
9. Blackburn, D.G. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *Am. Zool.* **1992**, *32*, 313–321. [CrossRef]
10. Wourms, J.P. Viviparity: The maternal-fetal relationship in fishes. *Am. Zool.* **1981**, *21*, 473–515. [CrossRef]
11. Whittington, C.M.; Friesen, C.R. The evolution and physiology of male pregnancy in syngnathid fishes. *Biol. Rev.* **2020**, *95*, 1252–1272. [CrossRef]
12. Ostrovsky, A.N.; Lidgard, S.; Gordon, D.P.; Schwaha, T.; Genikhovich, G.; Ereskovsky, A.V. Matrotrophy and placentation in invertebrates: A new paradigm. *Biol. Rev.* **2015**, *91*, 673–711. [CrossRef]
13. Sutton, F.B.; Wilson, A.B. Parental behavior in fish. In *Encyclopedia of Reproduction*, 2nd ed.; Skinner, M.K., Ed.; Elsevier Science & Technology: Amsterdam, The Netherlands, 2018; pp. 106–114.
14. Blackburn, D.G. Classification of vertebrate reproductive modes. *Am. Zool.* **2000**, *22*, 371–377.
15. Stölting, K.N.; Wilson, A.B. Male pregnancy in seahorses and pipefish: Beyond the mammalian model. *Bioessays* **2007**, *29*, 884–896. [CrossRef] [PubMed]
16. Kvarnemo, C.; Mobley, K.B.; Partridge, C.; Jones, A.; Ahnesjö, I. Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish Syngnathus typhle. *J. Fish Biol.* **2011**, *78*, 1725–1737. [CrossRef]
17. Turner, C.L. Viviparity in teleost fishes. *Sci. Mon.* **1947**, *65*, 508–518. [PubMed]
18. Uribe, M.C.; Cruz, G.D.L.R.; Alarcón, A.G.; Caballero, J.C.C.; Bárcenas, M.G.G. Structures Associated with Oogenesis and Embryonic Development during Intraovarian Gestation in *Viviparous teleosts* (Poeciliidae). *Fishes* **2019**, *4*, 35. [CrossRef]
19. Uribe, M.C.; Grier, H.J.; De la Rosa Cruz, G.; García Alarcón, A. Modifications in ovarian and testicular morphology associated with viviparity in teleosts. In *Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes)*; Jamieson, B.G.M., Ed.; CRC Press: Boca Raton, FL, USA, 2009; pp. 99–132.
20. Blackburn, D.G. The evolution of viviparity and matrotrophy in vertebrates, with special reference to reptiles. Ph.D. Dissertation, Cornell University, New York, NY, USA, 1985.
21. Knight, F.M.; Lombardi, J.; Wourms, J.P.; Burns, J.R. Follicular placenta and embryonic growth of the viviparous four-eyed fish (Anableps). *J. Morphol.* **1985**, *185*, 131–142. [CrossRef] [PubMed]

22. Hollenberg, F.; Wourms, J.P. Ultrastructure and protein uptake of the embryonic trophotaeniae of four species of goodeid fishes (Teleostei: Atheriniformes). *J. Morphol.* **1994**, *219*, 105–129. [CrossRef]
23. Reznick, D.N.; Mateos, M.; Springer, M.S. Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **2002**, *298*, 1018–1020. [CrossRef]
24. Pollux, B.; Pires, M.; Banet, A.; Reznick, D. Evolution of placentas in the fish family Poeciliidae: An empirical study of macroevolution. *Annu. Rev. Ecol. Evol. Syst.* **2009**, *40*, 271–289. [CrossRef]
25. Regus, J.U.; Johnson, J.B.; Webb, S.A.; Reznick, D.N. Comparative life histories of fishes in the genus *Phallichthys* (Pisces: Poeciliidae). *J. Fish Biol.* **2013**, *83*, 144–155. [CrossRef]
26. Scrimshaw, N.S. Embryonic growth in the viviparous Poeciliid, *Heterandria formosa*. *Biol. Bull.* **1944**, *87*, 37–51. [CrossRef]
27. Balon, E.K. Reproductive guilds of fishes: A proposal and definition. *J. Fish. Res. Board Can.* **1975**, *32*, 821–864. [CrossRef]
28. Lima, A.R.A.; Barletta, M.; Dantas, D.V.; Ramos, J.A.A.; Costa, M.F. Early development of marine catfishes (Ariidae): From mouth brooding to the release of juveniles in nursery habitats. *J. Fish Biol.* **2013**, *82*, 1990–2014. [CrossRef]
29. Whittington, C.M.; Buddle, A.L.; Griffith, O.W.; Carter, A.M. Embryonic specializations for vertebrate placentation. *Philosoophical Trans. R. Soc. B Biol. Sci.* **2022**, *377*, 20210261. [CrossRef] [PubMed]
30. Stewart, J.R.; Castillo, R.E. Nutritional provision of the yolk of two species of viviparous reptiles. *Physiol. Zool.* **1984**, *57*, 377–383. [CrossRef]
31. Stewart, J.R.; Thompson, M. A novel pattern of embryonic nutrition in a viviparous reptile. *J. Exp. Biol.* **1993**, *174*, 97–108. [CrossRef]
32. Paiva, R.B.; Neves, A.; Sequeira, V.; Nunes, M.L.; Gordo, L.S.; Bandarra, N. Reproductive strategy of the female deep-water shark birdbeak dogfish, *Deania calcea*: Lecithotrophy or matrotrophy? *J. Mar. Biol. Assoc. U. K.* **2011**, *92*, 387–394. [CrossRef]
33. Watanabe, S.; Watanabe, Y. Relationship between male size and newborn size in the Seaweed Pipefish, *Syngnathus schlegeli*. *J. Appl. Phycol.* **2002**, *65*, 319–325. [CrossRef]
34. Riesch, R.; Plath, M.; Schlupp, I. Toxic hydrogen sulfide and dark caves: Life-history adaptations in a livebearing fish (*Poecilia mexicana*, Poeciliidae). *Ecology* **2010**, *91*, 1494–1505. [CrossRef]
35. Huveneers, C.; Otway, N.M.; Harcourt, R.G.; Ellis, M. Quantification of the maternal-embryonal nutritional relationship of elasmobranchs: Case study of wobbegong sharks (genus *Orectolobus*). *J. Fish Biol.* **2011**, *78*, 1375–1389. [CrossRef]
36. Boehlert, G.W.; Yamada, J. *Rockfishes of the Genus Sebastes: Their Reproduction and Early Life History*; Springer: Berlin/Heidelberg, Germany, 1991. [CrossRef]
37. Edwards, T.M.; Miller, H.D.; Guillette, L.J. Water quality influences reproduction in female Mosquitofish (*Gambusia holbrooki*) from eight Florida springs. *Environ. Health Perspect.* **2006**, *114*, 69–75. [CrossRef] [PubMed]
38. Frazer, H.A.; Ellis, M.; Huveneers, C. Can a threshold value be used to classify Chondrichthyan reproductive modes: Systematic review and validation using an oviparous species. *PLoS ONE* **2012**, *7*, e50196. [CrossRef] [PubMed]
39. Constantz, G.D. Energetics of viviparity in the *Gila topminnow* (Pisces: Poeciliidae). *Copeia* **1980**, *1980*, 876. [CrossRef]
40. Schrader, M.; Travis, J. Do embryos influence maternal investment? Evaluating maternal-fetal coadaptation and the potential for parent-offspring conflict in a placental fish. *Evolution* **2009**, *63*, 2805–2815. [CrossRef]
41. Weldele, M.L.; Zúñiga-Vega, J.J.; Johnson, J.B. Life history of *Gambusia vittate* (Pisces: Poeciliidae). *Southwest. Nat.* **2014**, *59*, 449–460. [CrossRef]
42. Fernandez-Delgado, C.; Rossomanno, S. Reproductive biology of the mosquitofish in a permanent natural lagoon in south-west Spain: Two tactics for one species. *J. Fish Biol.* **1997**, *51*, 80–92. [CrossRef]
43. Thibault, R.E.; Schultz, R.J. Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **1978**, *32*, 320–333. [CrossRef]
44. Haynes, J.L. Standardized classification of Poeciliid development for life-history studies. *Copeia* **1995**, *1995*, 147. [CrossRef]
45. Reznick, D. "Grandfather effects": The genetics of interpopulation differences in offspring size in the Mosquito fish. *Evolution* **1981**, *35*, 941–953. [CrossRef]
46. Gillooly, J.F.; Charnov, E.L.; West, G.B.; Savage, V.M.; Brown, J.H. Effects of size and temperature on developmental time. *Nature* **2002**, *417*, 70–73. [CrossRef]
47. Mason, M.W.; Bertucci, E.M.; Leri, F.M.; Parrott, B.B. Transient copper exposure during embryogenesis and temperature affect developmental rate, survival, and fin regeneration in Japanese Medaka (*Oryzias latipes*). *Environ. Toxicol. Chem.* **2022**, *41*, 748–757. [CrossRef] [PubMed]
48. Riesch, R.; Colston, T.J.; Joachim, B.L.; Schlupp, I. Natural history and life history of the *Grijalva gambusia Heterophallus milleri* Radda, 1987 (Teleostei: Poeciliidae). *Aqua Int. J. Ichthyol.* **2011**, *17*, 95–102.
49. Reznick, D.; Hrbek, T.; Caura, S.; De Greef, J.; Roff, D. Life history of *Xenodexia ctenolepis*: Implications for life history evolution in the family Poeciliidae. *Biol. J. Linn. Soc.* **2007**, *92*, 77–85. [CrossRef]
50. Gray, J. The growth of fish: I. The relationship between embryo and yolk in *Salmo fario*. *J. Exp. Biol.* **1926**, *4*, 215–226. [CrossRef]
51. Saleh-Subaie, N.; Ramírez-Cruz, G.A.; Zúñiga-Vega, J.J. Examination of the Trexler-DeAngelis Model of maternal provisioning reveals that matrotrophy is costly. *Front. Ecol. Evol.* **2021**, *9*, 635. [CrossRef]
52. Zúñiga-Vega, J.J.; Reznick, D.N.; Johnson, J.B. Habitat predicts reproductive superfetation and body shape in the livebearing fish *Poeciliopsis turrubarensis*. *Oikos* **2007**, *116*, 995–1005. [CrossRef]

53. Gorini-Pacheco, B.; Zandonà, E.; Mazzoni, R. Predation effects on matrotrophy, superfetation and other life history traits in *Phalloceros harpagos*. *Ecol. Freshw. Fish* **2017**, *27*, 442–452. [CrossRef]
54. Vincent, A.C.J. Reproductive Ecology of Seahorses. Ph.D. Dissertation, Cambridge University, Cambridge, UK, 1990.
55. Kindsvater, H.K.; Bonsall, M.B.; Alonzo, S.H. Survival costs of reproduction predict age-dependent variation in maternal investment. *J. Evol. Biol.* **2011**, *24*, 2230–2240. [CrossRef]
56. Kindsvater, H.K.; Rosenthal, G.G.; Alonzo, S.H. Correction: Maternal size and age shape offspring size in a live-bearing fish, *Xiphophorus birchmanni*. *PLoS ONE* **2012**, *8*, e48473. [CrossRef]
57. Berkeley, S.A.; Chapman, C.; Sogard, S.M. Maternal age as a determinant of larval growth and survival in a marine fish, *Sebastes melanops*. *Ecology* **2004**, *85*, 1258–1264. [CrossRef]
58. Cheong, R.T.; Henrich, S.; Farr, J.A.; Travis, J. Variation in fecundity and its relationship to body size in a population of the Least Killifish, *Heterandria formosa* (Pisces: Poeciliidae). *Copeia* **1984**, *1984*, 720. [CrossRef]
59. Hagmayer, A.; Furness, A.I.; Reznick, D.N.; Pollux, B.J.A. Maternal size and body condition predict the amount of post-fertilization maternal provisioning in matrotrophic fish. *Ecol. Evol.* **2018**, *8*, 12386–12396. [CrossRef]
60. Marshall, D.J.; Heppell, S.S.; Munch, S.B.; Warner, R.R. The relationship between maternal phenotype and offspring quality: Do older mothers really produce the best offspring? *Ecology* **2010**, *91*, 2862–2873. [CrossRef] [PubMed]
61. Saleh-Subaie, N.; Johnson, J.B.; Zúñiga-Vega, J.J. Small sizes, big strategies: The relationship between female size, matrotrophy and superfetation throughout the reproductive lives of poeciliid fishes. *J. Zool.* **2021**, *315*, 261–275. [CrossRef]
62. Hachicho, N.; Reithel, S.; Miltner, A.; Heipieper, H.J.; Küster, E.; Luckenbach, T. Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-Dinitrophenol exposure. *PLoS ONE* **2015**, *10*, e0134755. [CrossRef] [PubMed]
63. Hislop, J.R.G.; Bell, M.A. Observations on the size, dry weight and energy content of the eggs of some demersal fish species from British marine waters. *J. Fish Biol.* **1987**, *31*, 1–20. [CrossRef]
64. Frimpong, E.A.; Henebry, M.L. Short-term effects of Formalin and Ethanol fixation and preservation techniques on weight and size of fish eggs. *Trans. Am. Fish. Soc.* **2012**, *141*, 1472–1479. [CrossRef]
65. Johnston, T.A.; Mathias, J.A. Length reduction and dry weight loss in frozen and formalin-preserved larval walleye, *Stizostedion vitreum* (Mitchill). *Aquac. Res.* **1993**, *24*, 365–371. [CrossRef]
66. Leist, D.P.; Nettleton, G.S.; Feldhoff, R.C. Determination of lipid loss during aqueous and phase partition fixation using formalin and glutaraldehyde. *J. Histochem. Cytochem.* **1986**, *34*, 437–441. [CrossRef]
67. Yang, F.; Xiang, W.; Sun, X.; Wu, H.; Li, T.; Long, L. A novel lipid extraction method from wet Microalga *Picochlorum* sp. at room temperature. *Mar. Drugs* **2014**, *12*, 1258–1270. [CrossRef]
68. Arias, A.-L.; Reznick, D. Life History of *Phalloceros caudiomaculatus*: A novel variation on the theme of livebearing in the family Poeciliidae. *Copeia* **2000**, *2000*, 792–798. [CrossRef]
69. Wetzel, M.A.; Leuchs, H.; Koop, J.H.E. Preservation effects on wet weight, dry weight, and ash-free dry weight biomass estimates of four common estuarine macro-invertebrates: No difference between ethanol and formalin. *Helgol. Mar. Res.* **2005**, *59*, 206–213. [CrossRef]
70. Olivera-Tlahuel, C.; Ossip-Klein, A.G.; Espinosa-Pérez, H.S.; Zúñiga-Vega, J.J. Have superfetation and matrotrophy facilitated the evolution of larger offspring in poeciliid fishes? *Biol. J. Linn. Soc.* **2015**, *116*, 787–804. [CrossRef]
71. Almeida-Silva, P.H.; Mazzoni, R. Life history aspects of *Phalloceros anisophallos* Lucinda, 2008 (Osteichthyes, Poeciliidae) from Córrego Andorinha, Ilha Grande (RJ, Brazil). *Stud. Neotrop. Fauna Environ.* **2014**, *49*, 191–198. [CrossRef]
72. Pollux, B.J.A.; Meredith, R.W.; Springer, M.S.; Garland, T.; Reznick, D.N. The evolution of the placenta drives a shift in sexual selection in livebearing fish. *Nature* **2014**, *513*, 233–236. [CrossRef] [PubMed]
73. Jennions, M.D.; Telford, S. Life-history phenotypes in populations of *Brachyrhaphis episcopi* (Poeciliidae) with different predator communities. *Oecologia* **2002**, *132*, 44–50. [CrossRef]
74. Riesch, R.; Martin, R.A.; Langerhans, R.B. Predation's role in life-history evolution of a livebearing fish and a test of the Trexler-DeAngelis Model of maternal provisioning. *Am. Nat.* **2013**, *181*, 78–93. [CrossRef]
75. Torres-Mejia, R.M. Ecomorphology of Body Shape and Life History in Females of the Genus *Gambusia* (Poeciliidae) and in Guppies (*Poecilia reticulata*, Poeciliidae). Ph.D. Thesis, University of California, Berkeley, CA, USA, 2012.
76. Pires, M.N.; Reznick, D.N. Life-history evolution in the fish genus *Poecilia* (Poeciliidae: Cyprinodontiformes: Subgenus *Pamphorichthys*): An evolutionary origin of extensive matrotrophy decoupled from superfetation. *Biol. J. Linn. Soc.* **2018**, *125*, 547–560. [CrossRef]
77. Cohen, S.N.; Regus, J.U.; Reynoso, Y.; Mastro, T.; Reznick, D.N. Comparative life histories of fishes in the subgenus *Limia* (Pisces: Poeciliidae). *J. Fish Biol.* **2015**, *87*, 100–114. [CrossRef]
78. Pires, M.N.; Arendt, J.; Reznick, D.N. The evolution of placentas and superfetation in the fish genus *Poecilia* (Cyprinodontiformes: Poeciliidae: Subgenera *Micropoecilia* and *Acanthophacelus*). *Biol. J. Linn. Soc.* **2010**, *99*, 784–796. [CrossRef]
79. Trexler, J.C. Variation in the degree of viviparity in the Sailfin Molly, *Poecilia latipinna*. *Copeia* **1985**, *1985*, 999. [CrossRef]
80. Pires, M.N. The evolution of placentas in Poeciliid fishes. Ph.D. Dissertation, University of California, Berkeley, CA, USA, 2007.
81. Riesch, R.; Plath, M.; Schlupp, I.; Marsh-Matthews, E. Matrotrophy in the cave molly: An unexpected provisioning strategy in an extreme environment. *Evol. Ecol.* **2009**, *24*, 789–801. [CrossRef]
82. Molina-Moctezuma, A.; Hernández-Rosas, A.L.; Zúñiga-Vega, J.J. Resource availability and its effects on mother to embryo nutrient transfer in two viviparous fish species. *J. Exp. Zool. Part A Ecol. Integr. Physiol.* **2020**, *333*, 181–193. [CrossRef] [PubMed]

83. Reznick, D.; Callahan, H.; Llauredo, R. Maternal effects on offspring quality in Poeciliid fishes. *Amer. Zool.* **1996**, *36*, 147–156. [CrossRef]
84. Paffenhöfer, G.A.; Rosenthal, H. Trockengewicht und Kaloriengehalt sich entwickelnder Heringseier. *Helgol. Mar. Res.* **1968**, *18*, 45–52. [CrossRef]
85. Hayes, F.R.; Armstrong, F.H. Physical changes in the constituent parts of developing salmon eggs. *Can. J. Res.* **1942**, *20d*, 99–114. [CrossRef]
86. Smith, S. Studies in the development of the rainbow trout (*Salmo irideus*) I. The heat production and nitrogenous excretion. *J. Exp. Biol.* **1947**, *23*, 357–378. [CrossRef]
87. Gortner, R.A. Studies on the chemistry of embryonic growth I. certain changes in the nitrogen ratios of developing trout eggs. *J. Am. Chem. Soc.* **1913**, *35*, 632–645. [CrossRef]
88. Veith, W. Reproduction in the live-bearing teleost *Clinus superciliosus*. *S. Afr. J. Zool.* **1979**, *14*, 208–211. [CrossRef]
89. López-Rodríguez, N.C.; de Barros, C.M.; Petry, A.C. A macroscopic classification of the embryonic development of the one-sided livebearer *Jenynsia multidentata* (Teleostei: Anablepidae). *Neotrop. Ichthyol.* **2017**, *15*, e160170. [CrossRef]
90. Zandonà, E.; Kajin, M.; Buckup, P.A.; Amaral, J.R.; Souto-Santos, I.C.A.; Reznick, D.N. Mode of maternal provisioning in the fish genus *Phalloceros*: A variation on the theme of matrotrophy. *Biol. J. Linn. Soc.* **2021**, *134*, 867–878. [CrossRef]
91. Zúñiga-Vega, J.J.; Suárez-Rodríguez, M.; Espinosa-Pérez, H.; Johnson, J.B. Morphological and reproductive variation among populations of the Pacific molly *Poecilia butleri*. *J. Fish Biol.* **2011**, *79*, 1029–1046. [CrossRef]
92. Turcotte, M.M.; Pires, M.N.; Vrijenhoek, R.C.; Reznick, D.N. Pre- and post-fertilization maternal provisioning in livebearing fish species and their hybrids (Poeciliidae: *Poeciliopsis*). *Funct. Ecol.* **2008**, *22*, 1118–1124. [CrossRef]
93. Hagmayer, A.; Furness, A.I.; Reznick, D.N.; Dekker, M.L.; Pollux, B.J.A. Predation risk shapes the degree of placentation in natural populations of live-bearing fish. *Ecol. Lett.* **2020**, *23*, 831–840. [CrossRef] [PubMed]
94. Marsh-Matthews, E.; Deaton, R. Resources and offspring provisioning: A test of the Trexler-DeAngelis model for matrotrophy evolution. *Ecology* **2006**, *87*, 3014–3020. [CrossRef]
95. Trexler, J.C. Resource availability and plasticity in offspring provisioning: Embryo nourishment in Sailfin mollies. *Ecology* **1997**, *78*, 1370–1381. [CrossRef]
96. Stewart, J.R. Facultative Placentotrophy and the Evolution of Squamate Placentation: Quality of Eggs and Neonates in *Virginia striatula*. *Am. Nat.* **1989**, *133*, 111–137. [CrossRef]
97. Van Dyke, J.U.; Griffith, O.W.; Thompson, M.B. High food abundance permits the evolution of placentotrophy: Evidence from a placental lizard, *Pseudemoia entrecasteauxii*. *Am. Nat.* **2014**, *184*, 198–210. [CrossRef]
98. Ramírez-Pinilla, M.P. Placental transfer of nutrients during gestation in an Andean population of the highly matrotrophic lizard genus *Mabuya* (Squamata: Scincidae). *Herpetol. Monogr.* **2006**, *20*, 194–204. [CrossRef]
99. Swain, R.; Jones, S.M. Maternal-fetal transfer of 3H-labelled leucine in the viviparous lizard *Niveoscincus metallicus* (scincidae: Lygosominae). *J. Exp. Zool.* **1997**, *277*, 139–145. [CrossRef]
100. Van Dyke, J.U.; Beaupre, S.J. Stable isotope tracer reveals that viviparous snakes transport amino acids to offspring during gestation. *J. Exp. Biol.* **2012**, *215*, 760–765. [CrossRef] [PubMed]
101. Haresign, T.W.; Shumway, S. Permeability of the marsupium of the pipefish *Syngnathus fuscus* to [^{14}C]-alpha amino isobutyric acid. *Comp. Biochem. Physiol. Part A Physiol.* **1981**, *69*, 603–604. [CrossRef]
102. Marsh-Matthews, E. Matrotrophy. In *Ecology and Evolution of Fishes*; Evans, J., Pilastro, A., Eds.; Chicago Press: Chicago, IL, USA, 2011; pp. 18–22.
103. Marsh-Matthews, E.; Brooks, M.; Deaton, R.; Tan, H. Effects of maternal and embryo characteristics on post-fertilization provisioning in fishes of the genus *Gambusia*. *Oecologia* **2005**, *144*, 12–24. [CrossRef] [PubMed]
104. Ripley, J.L.; Foran, C.M. Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). *J. Comp. Physiol. B* **2008**, *179*, 325–333. [CrossRef]
105. Kim, J.; Vallet, J. Secreted and Placental Membrane Forms of Folate-Binding Protein Occur Sequentially During Pregnancy in Swine1. *Biol. Reprod.* **2004**, *71*, 1214–1219. [CrossRef]

Supplementary Material

Text S1: Inclusion criteria for Table 1

For viviparous species, only dry mass or lean mass was included, with wet mass and ash-free dry mass measurements excluded. Parentotrophy indices were not presented if stages of embryonic comparison were not consistent within a study (e.g., Pires *et al.,* 2010 real data). Studies looking at hybridisation across species were excluded. Studies were excluded if they only graphically displayed dry mass data. For studies where raw data were not available, MI/PI calculations were not checked. Not all papers provided an MI/PI but provided dry mass data that could be used to calculate one. Both freeze-dried and oven-dried measures were included. Unfertilised egg comparisons were not used unless specified as mature or ovulated eggs. Due to the limited reference material of oviparous loss in dry mass, all available % loss in dry or lean dry mass in oviparous species was included. All calculations were performed with the number of decimal places provided in the data but were always rounded to 2 decimal places for the final MI value presented here. Calculations were checked if possible and corrected if necessary. All provisioning classifications presented are as stated or suggested by the assigned threshold values in the original reference. We grouped all types of parentotrophy including incipient, moderate, substantial and their synonyms, into "parentotrophy". All species that presented an MI or PI between and including 0.6 and 1.1, were tabulated here, including species name(s), MI value, estimated or real MI/PI calculation, the threshold value in which parentotrophy is classified, any MI/PI specific statistical analysis, the classification given by the study and the source. We chose this range because most lecithotrophic or incipiently parentotrophic species fall within this range and it allows us to focus on the differences in distinguishing between the two across the literature.

Chapter 3: Paternal protein provisioning to embryos during male seahorse pregnancy

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RESEARCH

Paternal protein provisioning to embryos during male seahorse pregnancy

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Abstract

In brief: Seahorses exhibit male pregnancy and are thus valuable comparative models for the study of the physiology and evolution of pregnancy. This study shows that protein is transported from fathers to developing embryos during gestation, and provides new knowledge about paternal contributions to embryonic development.

Abstract: Syngnathid embryos (seahorses, pipefishes and seadragons) develop on or in the male in a specialised brooding structure (brood pouch). Seahorse brood pouches supply nutrients, including lipids, to developing embryos (patrotrophy). We tested the hypothesis that proteins, vital for gene regulation and tissue growth during embryogenesis, are also transported from father to embryos, using the Australian pot-bellied seahorse, *Hippocampus abdominalis.* We used dry masses and total nitrogen content to estimate the total protein content of newly fertilised egg and neonate *H. abdominalis*. Neonates contained significantly greater protein mass than newly fertilised eggs. This result indicates that paternal protein transport to developing embryos occurs during *H. abdominalis* pregnancy. This study is the first to show paternal protein transport during pregnancy in seahorses, and furthers our understanding of paternal influence on embryonic development in male pregnant vertebrates.

Introduction

Teleosts exhibit a diverse range of parental care and reproductive strategies across two parity modes: oviparity (egg-laying) and viviparity (live birth) (Blackburn 2015*a*, Sutton & Wilson 2018). Viviparity is limited to live-bearing species that fertilise and incubate developing embryos within the female reproductive tract (Blackburn 2000). Some oviparous species incubate their embryos on or inside the parental body until the release of live young, which is termed 'brooding' (Ostrovsky *et al.* 2016, Whittington & Friesen 2020). Brooding is analogous to viviparity, in that fertilisation and gestation occur in or on parental tissues other than the female reproductive tract (Ostrovsky *et al.* 2016, Whittington & Friesen 2020). Brooding and viviparity may allow greater parental or embryonic control over the timing of parental resource allocation, compared to oviparity (Trexler & DeAngelis 2003, Van Dyke *et al.* 2014).

In all species, the nutrients for embryonic development can be provided utilising two general strategies: either supplied entirely by the maternal yolk of the egg (lecithotrophy) or supplemented post fertilisation (parentotrophy). Parentotrophy can come from the mother (matrotrophy) or the father (patrotrophy) (Blackburn 1992). Lecithotrophy and parentotrophy represent two extremes on a continuum of embryonic provisioning, with varying degrees of reliance on parentotrophy (Blackburn 1992, Trexler 1997, Frazer *et al.* 2012, Skalkos *et al.* 2023). Parentotrophy has evolved in mammals, coelacanths (Wourms *et al.* 1991), various squamate reptiles (lizards and snakes) (Blackburn 2015*b*),

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amphibians (Wake 2015), chondrichthyans (Dulvy & Reynolds 1997, Frazer *et al.* 2012, Buddle *et al.* 2019) and teleost fishes (e.g. Cohen *et al.* 2015, Skalkos *et al.* 2023), via various mechanisms, including placentation (e.g. Blackburn, 2015*a*, Whittington *et al.* 2022).

Syngnathidae is a teleost family comprised of pipefish, seadragons and seahorses, with 328 described species (Fricke *et al.* 2023). Syngnathids exhibit a rare trait among vertebrates, whereby males incubate developing embryos on or in a structure known as the brood pouch and give birth to live young (oviparity with brooding). Here we refer to syngnathid embryonic brooding as 'gestation' or 'male pregnancy'. Syngnathid brooding structures are located on the ventral surface of the male and vary in complexity, ranging from an unprotected area for egg attachment in some pipefishes to a fully enclosed brood pouch in seahorses (*Hippocampus* spp.) (reviewed in Whittington & Friesen 2020). Thus, seahorses, with the most complex pouch type, undergo the most substantial physiological and morphological changes during pregnancy. The separation of the external and internal environment in the closed brood pouch is adaptively significant due to the physiological challenges placed on the male, and the potential for increased paternal control over embryonic development. A closed brood pouch allows for paternal control of osmoregulation and nutrient provisioning of developing embryos, which potentially increases the size and survival of neonates in unstable environments (Watanabe *et al.* 1999, McCoy *et al.* 2001). However, physiological challenges are placed on the male, including the requirement to facilitate gas exchange, remove wastes and immunologically protect developing embryos during development (Stölting & Wilson 2007, Whittington & Friesen 2020, Dudley *et al.* 2021). Syngnathid pregnancy places maintenance and growth constraints on gestating males, which can reduce their reproductive fitness for future clutches, representing a trade-off against current and future reproduction (reviewed in Whittington & Friesen 2020).

Hippocampus abdominalis seahorses are patrotrophic, transporting lipids to developing embryos during gestation (Skalkos *et al.* 2020) and *Hippocampus erectus* embryos can take up environmental calcium (Linton & Soloff 1964). The extent to which the seahorse pouch provides other nutrients to developing embryos is not well understood, despite knowledge of brood pouch physiology being an important prerequisite for understanding the evolution of male pregnancy (Whittington & Friesen 2020). Here, we focused on measuring the transport of proteins, which are essential during embryogenesis for gene regulation, maintenance, structural growth and development and energy through catabolism (Finn *et al.* 1995, Heming & Buddington 1988, Gilbert 2001, Van Dyke & Griffith 2018, Rayon *et al.* 2020). Due to the importance of protein during embryogenesis, the amount of protein a developing embryo receives either from the yolk or in the form of parentotrophy can have significant effects on offspring survival and development, with increased protein concentrations likely to improve embryonic development (Brooks *et al.* 1997). Paternal protein provisioning to embryos is suspected to occur in *H. abdominalis*, because amino acid transporter genes (e.g. *Solute Carrier Family 38A5* and *38A10*) are upregulated during pregnancy compared to non-pregnant pouch (Whittington *et al.* 2015). Furthermore, paternal amino acids are transported to developing embryos in other syngnathid species including *Syngnathus typhle* (Kvarnemo *et al.* 2011), *Syngnathus fuscus* (Haresign & Shumway 1981) and *Syngnathus floridae* (Ripley & Foran 2009). Since seahorses and *Syngnathus* spp. are closely related (Hamilton *et al.* 2017, He *et al.* 2022) and diverged ~36 million years ago (Stiller *et al.* 2022), pipefish and seahorses may share common adaptations. Here, we aimed to determine whether protein is paternally transported to developing embryos during embryogenesis in *H. abdominalis*. We tested the hypothesis that *H. abdominalis* neonate protein mass is equal to or greater than newly fertilised egg (NFE) protein mass, using mass spectrometry.

Materials and methods

Housing and breeding

Reproductively mature *H. abdominalis* were acquired from a captive-bred population (Seahorse Australia, Tasmania, Australia), and housed under previously described aquarium conditions, as outlined in Whittington *et al.* (2013; University of Sydney Animal Ethics Committee approval number: 2021/1995). Twenty-five male and 15 female *H. abdominalis* were used for this study. Male and female *H. abdominalis* were rotated through a 750 L breeding tank for 72 h per cycle, to enable breeding (Woods 2000), and to allow adequate time for gamete production in between. Males were tagged with non-invasive small glass bead 'necklaces', as described in Whittington *et al.* (2013). Four males and five females were housed in the breeding tank each rotation. Afterwards, they were separated into single-sex tanks. Male reproductive status was determined by behavioural assays over three consecutive testing days to determine whether males exhibited behavioural signs of courtship, the absence of which indicates putative pregnancy (Whittington *et al.* 2013). Briefly, the behaviours that indicate that a male is non-pregnant are pumping water into the pouch, courting head tilts, rapid fin fluttering and dancing with females. This method identifies 94% of pregnant males when used over three consecutive testing days (Whittington *et al.* 2013). All adult fish were fed the same amount (approximately 1.5 g per individual, per day) and type of *Mysis* spp. Shrimp (thawed Ocean Nutrition frozen mysis, Newark) for food throughout the study. Individual consumption of food was not measured. After breeding, pregnant males were housed

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in single-sex tanks until euthanasia for NFE collection, or parturition, for neonate collection. NFE was between 3 and 6 days post fertilisation, as described in Skalkos *et al.* (2020). Neonates were euthanised within 18 h of birth.

Sample collection

Adult male seahorses (individually) and neonates (on a per-clutch basis) were euthanised by an overdose of anaesthetic (ethanol). The ethanol was gradually poured into an opaque container to a final dose rate of 30 mL of 95% ethanol/L, over a 45–60-min period. Adult euthanasia was followed by immediate decapitation and pithing. Euthanasia followed the guidelines of Leary *et al.* (2020) and was approved by the University of Sydney Animal Ethics Committee (protocol 2021/1995). NFEs were fixed in 95% ethanol/ 5% salt water at 30 ppt for 30–45 min, to separate NFE from paternal pouch tissue, as described in Skalkos *et al.* (2020). Neonates were blotted with Kim wipes to remove excess water and salt. NFEs and neonates were placed in pre-weighed 1.5 mL certified protein-free microcentrifuge tubes (Eppendorf). The clutch size varies greatly in *H. abdominalis* (5-1116) (Foster & Vincent 2004). Our neonate clutch sizes varied from 30 to 200. As the individual NFE and neonates have a small dry mass, five NFE/neonates from the same clutch were pooled and treated as one sample to estimate their total masses more accurately. If the total number of NFE/neonates collected for a clutch was not divisible by 5, the remaining two to four NFE/neonates were placed into a 1.5 mL microcentrifuge tube and treated as a single sample. Three samples were chosen at random from each clutch for dry mass and mass spectrometry analysis. We collected and measured a total of 12 neonate clutches and 11 NFE clutches for dry mass and protein content.

Dry mass

All samples in pre-weighed 1.5 mL microcentrifuge tubes were dried at 60°C until a stable dry mass was reached (at least 48 h), following the protocol of Skalkos *et al.* (2020). A total of 59 euthanised NFE samples and 207 euthanised neonate samples, from 11 and 12 clutches respectively, were measured for dry mass. Any clearly underdeveloped/aborted embryos were excluded from the neonate clutches. Samples were allowed to return to room temperature in a desiccation jar and then weighed using a 4-decimal point scale. A patrotrophy index (PI) was calculated using $\frac{neonate\,dry\,mass}{NFE\,dry\,mass}$. Neonates and NFE were stored in two separate desiccation chambers prior to mass spectrometry analysis. Tools were used sparingly and cleaned with methanol between each sample.

Mass spectrometry

Neonate and NFE samples used for mass spectrometry were homogenised, individually covered with parafilm, then stored in separate desiccation chambers. A total of 25 and 32 samples (containing up to five pooled NFE/ neonates each) were analysed for NFE and neonates, from 11 and 12 clutches, respectively. An additional 18 positive controls of glutamic acid with known carbon/ nitrogen isotope ratios were included as standards. Total protein mass was estimated by measuring total nitrogen content using a Thermo Delta V EA-IRMS elemental analyser at the University of New South Wales Bioanalytical Mass Spectrometry Facility. Nitrogen content (%) was multiplied by 6.25 to estimate protein content (%) in each sample, following the Dumas method (Jung *et al.* 2003). Gnaiger and Bitterlich (1984) proposed a conversion of 5.8 for aquatic animals because the nitrogen fraction in protein, as calculated from amino acid compositions, is significantly higher than terrestrial animals. However, most fish embryo energetics literature uses a conversion factor of 6.25 (e.g. Gómez-Requeni *et al.* 2012, Hachicho *et al.* 2015, Benini *et al.* 2022). Thus, to make this study comparable to previous fish literature, we applied the Dumas estimation to both NFE and neonates. Percent protein was then multiplied by the total dry mass of each sample to determine the total protein mass for embryos and neonates. The total protein mass per sample was then divided by the number of NFE/neonates within each sample to provide an average NFE/neonate total protein mass per clutch.

Statistical analysis

Pooling NFE/neonates per sample allowed multiple replicates within a clutch to be measured for clutch mean dry mass. For mass spectrometry, the minimum number of samples per clutch was 1, the maximum was 3 (mean = 2.73, median = 3). A Shapiro–Wilk test was used to ensure that all data met assumptions of normality and heteroscedasticity of variance. Dry mass data were analysed using one-way ANOVA to determine whether the mean values of groups were significantly different at an α-level of 0.05. All statistical analyses were performed using the lme4 package in RStudio 4.2.2. A one-way ANOVA with generalised linear mixed model was also used to analyse protein mass, where the clutch was considered as a random effect, for NFE (n = 11 clutches) and neonates (n = 12 clutches).

Results

Dry mass

There was no significant difference in clutch means between NFE and neonate dry mass ($F_{1,21}$ = 2.78, P = 0.11) (Fig. 1). NFE had greater intra-clutch variability than neonates, with standard deviations of 0.171 and 0.140 mg, respectively.

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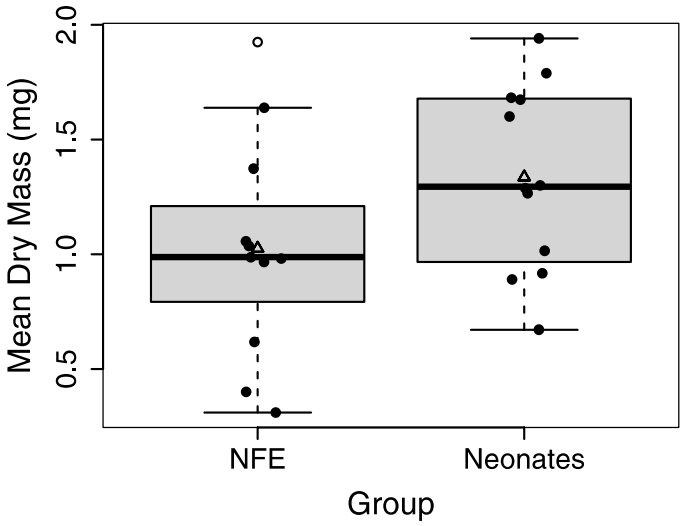

Figure 1

Boxplot of dry mass comparison of *Hippocampus abdominalis* newly fertilised eggs and neonates. A one-way ANOVA revealed no significant difference ($P > 0.05$). The bold lines indicate the median for each group, and the boxes contain 50% of the data, while whiskers extend to the highest/lowest data points within 1.5× interquartile range. Solid data points represent average dry mass values per individual embryo/neonate per clutch. The means are plotted as open triangles. Outliers are plotted as open circles.

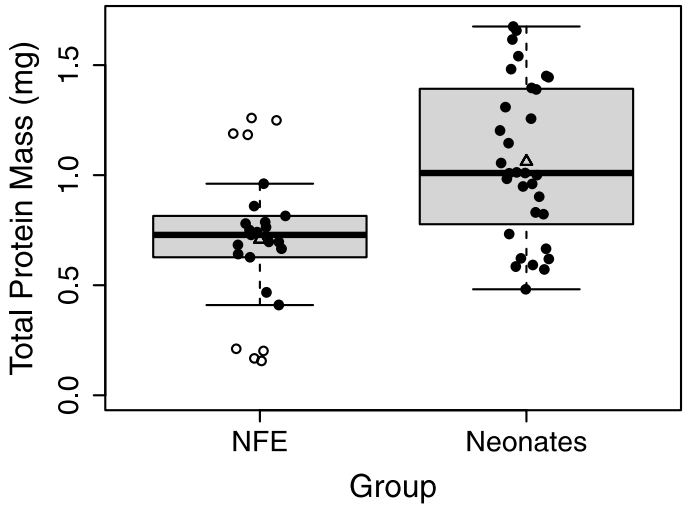

Figure 2

Boxplot of total protein mass comparison of *Hippocampus abdominalis* newly fertilised eggs and neonates. A one-way ANOVA revealed significant increase in neonate total protein mass from newly fertilised egg ($P < 0.001$). The bold lines indicate the median for each group, and the boxes contain 50% of the data, while whiskers extend to the highest/lowest data points within 1.5× interquartile range. Data points represent average individual embryo/neonate total protein mass per sample. The means are plotted as open triangles. Outliers are plotted as open circles.

Mass spectrometry

NFE clutches had an average %nitrogen of 11.10 and %protein of 69.41, while neonate clutches had an average %nitrogen of 12.55 and %protein of 78.44, respectively. Average total protein mass for individual NFE and neonates was 0.707 and 1.069 mg, respectively. Neonates had significantly greater total protein mass than NFE ($F_{1,21} = 6.051$, $P = 0.0227$) (Fig. 2).

Discussion

Here we provide evidence that male *H. abdominalis* transport protein to their developing embryos during gestation. We observed a significant increase in total protein mass in neonates compared to NFE, indicating that some of the maternal yolk-protein reserves being catabolised by developing embryos are replaced and provided in excess by fathers during pregnancy. There is no significant change in dry mass, indicating that embryo composition changes, with the proportion of total protein increasing during embryonic development. Our results illustrate that protein is a major nutrient class that fathers provide to embryos during pregnancy in *H. abdominalis*, in addition to lipids (Skalkos *et al.* 2020).

Total protein content in teleost eggs and newborns is variable across teleosts, but protein generally makes up a large proportion of the dry mass (Kamler 1992). For example, crude protein makes up 34.7%, 24.95%, 23.83% and 19.82% of mature egg dry mass in the oviparous *Cyprinus carpio* (Oroian *et al.* 2013), *Salmo trutta*, *Oncorhynchus mykiss* and *Acipenser baerii*, respectively (Kowalska-Góralska *et al.* 2020). In contrast, the total protein content in *Hippocampus guttulatus* and *Hippocampus hippocampus* is 56.2% and 53.3% of mature egg dry mass (Álvarez *et al.* 2009). Here, total protein contributes an average of 70.21% of NFE dry mass in *H. abdominalis*, which is much higher than values for other teleost eggs. This result may mean that some protein had already been transferred from males to developing embryos early in pregnancy before the NFE was collected (3–6 days post fertilisation). Alternatively, the eggs were provided with very protein-rich yolks by the mother, which is possible in mothers receiving ample dietary protein during oogenesis (Bobe & Labbé 2010, Reading *et al.* 2018); in this study, the thawed frozen mysis shrimps (Ocean Nutrition) fed to the seahorses are high in protein (Segade *et al.* 2015). Protein content in oviparous teleost neonates is also variable. For example, the total protein content in *Scophthalmus maximus* and *Rachycentron canadum* larvae represents 34–41% and 17.81% of dry mass, respectively (Weltzien *et al.* 1999, Huang *et al.* 2021). In contrast, *H. abdominalis* neonates contained an average of 78.06% total protein, which is also a greater proportion than the ~65.5% protein of *Hippocampus reidi* seahorse neonates (Planas *et al.* 2021). In both seahorse studies, pregnant males were fed similar diets. The possibility of nitrogen contamination was reduced by using certified protein-free materials and appropriate air-tight storage. The high protein content of seahorse neonates in this study may be explained by the fact that

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the *H. reidi* were kept at a much higher water temperature (~26°C), compared to this study (18–19°C), which likely increased their rate of protein catabolism, as seen in grass carp, *Ctenopharyngodon idella* (Liang *et al.* 2022). The significant increase in protein in *H. abdominalis* neonates compared to NFE observed here and compared to neonates of other teleost species suggests that protein may be important *for H. abdominalis* survival after birth, either structurally or as a nutrient reserve prior to first feed if prey is limited or of low-quality (Ofelio *et al.* 2018, Planas *et al.* 2021). Further studies on the proportions of protein, lipid and carbohydrates in *H. abdominalis* are required to understand intra- and inter-clutch variability in embryonic and neonate nutrient contents.

The main source of energy in fish embryogenesis is debated (Finn *et al.* 1995, Rosa *et al.* 2005, Kamler 2008, Hölttä-Vuori *et al.* 2010), and the relative importance of protein and lipids for maintenance, growth and development varies interspecifically (Rønnestad & Fyhn 1993, Kamler 2008, Finn & Finn 2010). Across *H. abdominalis* embryonic development, there is no significant increase in lipid content, showing no excess lipid supplementation from the father above what has been catabolised by the embryos (Skalkos *et al.* 2020). Importantly though, neither Skalkos *et al.* (2020) nor this study examined the catabolism of lipids and protein throughout development, so it is not possible to determine the main source of nutrients for *H. abdominalis* embryogenesis. Further research is required to determine the specific protein and lipid requirements of *H. abdominalis* development, and what quantities of each nutrient are catabolised across different stages of development. We recommend quantifying protein, lipid and carbohydrate contents across embryonic development simultaneously.

The level of nutrient supplementation provided by the father may vary both throughout embryonic development, as well as intraspecifically. Lipids and proteins could be catabolised at different rates throughout embryonic development (Kamler 2008), as seen in *D. rerio* (Hachicho *et al.* 2015). In some teleosts, protein catabolism is vital for survival just after hatching (Heming & Buddington 1988). In *Syngnathus* species, paternal nutrients are taken up by embryos at different rates throughout embryonic development (Ripley & Foran 2009). For example, paternal protein transport appears to take place in later stages of embryogenesis in pipefishes *S. fuscus* and *S. floridae*, as embryonic uptake of paternally transported stable isotope-labelled essential amino acid lysine is significantly increased in later stages of pregnancy, once embryos have consumed most of their yolk (Ripley & Foran 2009). Further research investigating when and to what degree different nutrients are paternally transported to *H. abdominalis* embryos throughout gestation is required to better understand the relative contribution of paternal vs maternal nutrients to seahorse embryogenesis, and how that may be influenced by different environmental conditions. We recommend the use of specifically labelled nutrients tracked between parent and offspring (Haresign & Shumway 1981, Marsh-Matthews *et al.* 2005, Marsh-Matthews & Deaton 2006, Kvarnemo *et al.* 2011).

Pregnancy in syngnathids provides a rare opportunity to examine the biology of internal embryo incubation independent of the female reproductive tract. This study is the first to our knowledge to provide evidence of paternal protein provisioning to embryos during gestation in seahorses, which have the most complex form of vertebrate male pregnancy. This work contributes to our knowledge of the fundamental reproductive biology of seahorses and provides further clarity on the complex physiology of the seahorse brood pouch. Future studies should consider measuring the nutritional requirements of *H. abdominalis* embryogenesis, and determine whether reliance on and paternal supply of, different nutrients changes throughout development. A direct measure of embryonic uptake of parentotrophic nutrients is recommended, for example, tracking specifically labelled nutrients from father to embryos during different stages of pregnancy (Haresign & Shumway 1981, Marsh-Matthews & Deaton 2006, Kvarnemo *et al.* 2011). To determine the plasticity of relative parental nutritional investment in male pregnancy, it is important to understand how parentotrophy varies with individual variation and environmental fluctuation. Functional studies to examine the mechanisms by which nutrients are transported across the paternal–embryonic interface are also recommended.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the study reported.

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References

Álvarez JP, Blanco A, Silva C & Planas M 2009 *Proximate biochemical composition and enzymatic activities in eggs of farmed seahorse hippocampus guttulatus (Project hippocampus)*. Veracruz, Mexico: World Acquaculture Society.

Benini E, Politis SN, Nielsen A, Sørensen SR, Tomkiewicz J & Engrola S 2022 Type of hormonal treatment administered to induce vitellogenesis in European eel influences biochemical composition of eggs and yolk-sac larvae. *Fish Physiology and Biochemistry* **48** 185–200. (https://doi.org/10.1007/s10695-021-01042-4)

Blackburn DG 1992 Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *American Zoologist* **32** 313–321. (https://doi.org/10.1093/icb/32.2.313)

Blackburn DG 2000 Classification of the reproductive patterns of Amniotes. *Herpetological Monographs* **14** 371–377. (https://doi.org/10.2307/1467051)

Blackburn DG 2015*a* Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *Journal of Morphology* **276** 961–990. (https://doi.org/10.1002/jmor.20272)

Blackburn DG 2015*b* Viviparous placentotrophy in reptiles and the parent–offspring conflict. *Journal of Experimental Zoology* **324** 532–548. (https://doi.org/10.1002/jez.b.22624)

Bobe J & Labbé C 2010 Egg and sperm quality in fish. *General and Comparative Endocrinology* **165** 535–548. (https://doi.org/10.1016/j.ygcen.2009.02.011)

Brooks S, Tyler CR & Sumpter JP 1997 Egg quality in fish: what makes a good egg? *Reviews in Fish Biology and Fisheries* **7** 387–416. (https://doi.org/10.1023/A:1018400130692)

Buddle AL, Van Dyke JU, Thompson MB, Simpfendorfer CA & Whittington CM 2019 Evolution of placentotrophy: using viviparous sharks as a model to understand vertebrate placental evolution. *Marine and Freshwater Research* **70** 908. (https://doi.org/10.1071/MF18076)

Cohen SN, Regus JU, Reynoso Y, Mastro T & Reznick DN 2015 Comparative life histories of fishes in the subgenus *Limia* (Pisces: Poeciliidae). *Journal of Fish Biology* **87** 100–114. (https://doi.org/10.1111/jfb.12695)

Dudley JS, Hannaford P, Dowland SN, Lindsay LA, Thompson MB, Murphy CR, Van Dyke JU & Whittington CM 2021 Structural changes to the brood pouch of male pregnant seahorses (*Hippocampus abdominalis*) facilitate exchange between father and embryos. *Placenta* **114** 115–123. (https://doi.org/10.1016/j.placenta.2021.09.002)

Dulvy NK & Reynolds JD 1997 Evolutionary transitions among egg–laying, live–bearing and maternal inputs in sharks and rays. *Proceedings of the Royal Society of London* **264** 1309–1315. (https://doi.org/10.1098/rspb.1997.0181)

Finn RN, Henderson JR & Fyhn HJ 1995 Physiological energetics of developing embryos and yolk-sac larvae of Atlantic cod (*Gadus morhua*). II: lipid metabolism and enthalpy balance. *Marine Biology* **124** 371–379. (https://doi.org/10.1007/BF00363910)

Finn RN, Fyhn HJ & Fyhn HJ 2010 Requirement for amino acids in ontogeny of fish. *Aquaculture Research* **41** 684–716. (https://doi.org/10.1111/j.1365-2109.2009.02220.x)

Foster SJ & Vincent ACJ 2004 Life history and ecology of seahorses: implications for conservation and management. *Journal of Fish Biology* **65** 1–61. (https://doi.org/10.1111/j.0022-1112.2004.00429.x)

Frazer HA, Ellis M & Huveneers C 2012 Can a threshold value be used to classify chondrichthyan reproductive modes: systematic review and validation using an oviparous species. *PLoS One* **7** e50196. (https://doi.org/10.1371/journal.pone.0050196)

Fricke R, Eschmeyer WN & van der Laan R 2023 *Eschmeyer's Catalog of Fishes: Genera, Species, References*. Available at: https://researcharchive.calacademy.org/research/ichthyology/catalog/fishcatmain.asp (Accessed July 12th 2023).

Gilbert SF 2001 Developmental Biology. Sixth Edition. By Scott F. Gilbert. *American Journal of Medical Genetics*, 6th ed. **99** 170–171. (https://doi.org/10.1002/1096-8628(2000)9999:999<00::AID-AJMG1133>3.0.CO;2-G)

Gnaiger E & Bitterlich G 1984 Proximate biochemical composition and caloric content calculated from elemental CHN analysis: a stoichiometric concept. *Oecologia* **62** 289–298. (https://doi.org/10.1007/BF00384259)

Gómez-Requeni P, Kraemer MN & Canosa LF 2012 Regulation of somatic growth and gene expression of the GH–IGF system and PRP-PACAP by dietary lipid level in early juveniles of a teleost fish, the pejerrey (*Odontesthes bonariensis*). *Journal of Comparative Physiology* **182** 517–530. (https://doi.org/10.1007/s00360-011-0640-9)

Hachicho N, Reithel S, Miltner A, Heipieper HJ, Küster E & Luckenbach T 2015 Body mass parameters, lipid profiles and protein contents of zebrafish embryos and effects of 2,4-dinitrophenol exposure. *PLoS One* **10** e0134755. (https://doi.org/10.1371/journal.pone.0134755)

Hamilton H, Saarman N, Short G, Sellas AB, Moore B, Hoang T, Grace CL, Gomon M, Crow K & Brian Simison W 2017 Molecular phylogeny and patterns of diversification in syngnathid fishes. *Molecular Phylogenetics and Evolution* **107** 388–403. (https://doi.org/10.1016/j.ympev.2016.10.003)

Haresign TW & Shumway SE 1981 Permeability of the marsupium of the pipefish *Syngnathus fuscus* to [14C]-alpha amino isobutyric acid. *Comparative Biochemistry and Physiology Part A* **69** 603–604. (https://doi.org/10.1016/0300-9629(81)93030-9)

He L, Long X, Qi J, Wang Z, Huang Z, Wu S, Zhang X, Luo H, Chen X, Lin J, *et al.* 2022 Genome and gene evolution of seahorse species revealed by the chromosome-level genome of *Hippocampus abdominalis*. *Molecular Ecology Resources* **22** 1465–1477. (https://doi.org/10.1111/1755-0998.13541)

Heming TA & Buddington RK 1988 Yolk absorption in embryonic and larval fishes. In *Fish Physiology*. Amsterdam: Elsevier Science & Technology, pp. 407–446.

Hölttä-Vuori M, Salo VTV, Nyberg L, Brackmann C, Enejder A, Panula P & Ikonen E 2010 Zebrafish: gaining popularity in lipid research. *Biochemical Journal* **429** 235–242. (https://doi.org/10.1042/BJ20100293)

Huang J, Amenyogbe E, Chen G & Wang W 2021 Biochemical composition and activities of digestive and antioxidant enzymes during the egg and yolk-sac larval development of the cobia (*Rachycentron canadum*). *Aquaculture Research* **52** 1643–1656. (https://doi.org/10.1111/are.15017)

Jung S, Rickert DA, Deak NA, Aldin ED, Recknor J, Johnson LA & Murphy PA 2003 Comparison of Kjeldahl and Dumas methods for determining protein contents of soybean products. *Journal of the American Oil Chemists' Society* **80** 1169–1173. (https://doi.org/10.1007/s11746-003-0837-3)

Kamler E 1992 *Early Life History of Fish: an Energetics Approach*, 1st ed. Dordrecht: Springer Science+Business Media, B.V.

Kamler E 2008 Resource allocation in yolk-feeding fish. *Reviews in Fish Biology and Fisheries* **18** 143–200. (https://doi.org/10.1007/s11160-007-9070-x)

Kowalska-Góralska M, Formicki K, Dobrzański Z, Wondołowska-Grabowska A, Skrzyńska E, Korzelecka-Orkisz A, Nędzarek A & Tański A 2020 Nutritional composition of Salmonidae and Acipenseridae fish

eggs. *Annals of Animal Science* **20** 629–645. (https://doi.org/10.2478/aoas-2019-0072)

Kvarnemo C, Mobley KB, Partridge C, Jones AG & Ahnesjö I 2011 Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. *Journal of Fish Biology* **78** 1725–1737. (https://doi.org/10.1111/j.1095-8649.2011.02989.x)

Leary SL, Underwood W, Anthony R, Carter S, Grandin T, Greenacre C, Gwaltney-Brant S, McCrackin MA, Meyer R, Miller D, *et al.* 2020 *AVMA Guidelines for the Euthanasia of Animals*. Schaumburg: American Veterinary Medical Association, pp. 84–85.

Liang H, Xu H, Ge X, Zhu J, Ren M & Mi H 2022 Water temperature affecrts the protein requirements, growth performance and nutritional metabolism of grass carp (*Ctenopharyngodon idella*) juveniles. *Aquaculture Reports* **25**. (https://doi.org/10.1016/j.aqrep.2022.101267)

Linton JR & Soloff BL 1964 The physiology of the brood pouch of the male sea horse *Hippocampus erectus*. *Bulletin of Marine Science* **14** 45–61.

Marsh-Matthews E & Deaton R 2006 Resources and offspring provisioning: a test of the Trexler-DeAngelis model for matrotrophy evolution. *Ecology* **87** 3014–3020. (https://doi.org/10.1890/0012-9658(2006)87[3014:raopat]2.0.co;2)

Marsh-Matthews E, Brooks M, Deaton R & Tan H 2005 Effects of maternal and embryo characteristics on post-fertilization provisioning in fishes of the genus *Gambusia*. *Oecologia* **144** 12–24. (https://doi.org/10.1007/s00442-005-0030-7)

McCoy EE, Jones AG & Avise JC 2001 The genetic mating system and tests for cuckoldry in a pipefish species in which males fertilize eggs and brood offspring externally. *Molecular Ecology* **10** 1793–1800. (https://doi.org/10.1046/j.0962-1083.2001.01320.x)

Ofelio C, Díaz AO, Radaelli G & Planas M 2018 Histological development of the long-snouted seahorse *hippocampus guttulatus* during ontogeny. *Journal of Fish Biology* **93** 72–87. (https://doi.org/10.1111/jfb.13668)

Oroian TE, Cighi V, Oroian RG & Gavrilă V 2013 Chemical composition of spawns and milt in *Cyprinus carpio* populations from Arinış fishery complex, Maramureş area. *Aquaculture, Aquarium, Conservation & Legislation* **6** 614–617.

Ostrovsky AN, Lidgard S, Gordon DP, Schwaha T, Genikhovich G & Ereskovsky AV 2016 Matrotrophy and placentation in invertebrates: a new paradigm. *Biological Reviews of the Cambridge Philosophical Society* **91** 673–711. (https://doi.org/10.1111/brv.12189)

Planas M, Olivotto I, González MJ, Laurà R, Angeletti C, Amici A & Zarantoniello M 2021 Pre-breeding diets in the seahorse *Hippocampus reidi*: how do they affect fatty acid profiles, energetic status and histological features in newborn? *Frontiers in Marine Science* **8**. (https://doi.org/10.3389/fmars.2021.688058)

Rayon T, Stamataki D, Perez-Carrasco R, Garcia-Perez L, Barrington C, Melchionda M, Exelby K, Lazaro J, Tybulewicz VLJ, Fisher EMC, *et al.* 2020 Species-specific pace of development is associated with differences in protein stability. *Science* **369** eaba7667. (https://doi.org/10.1126/science.aba7667)

Reading BJ, Andersen LK, Ryu Y-W, Mushirobira Y, Todo T & Hiramatsu N 2018 Oogenesis and egg quality in finfish: yolk formation and other factors influencing female fertility. *Fishes* **3**. (https://doi.org/10.3390/fishes3040045)

Ripley JL & Foran CM 2009 Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). *Journal of Comparative Physiology. Part B* **179** 325–333. (https://doi.org/10.1007/s00360-008-0316-2)

Rønnestad I & Fyhn HJ 1993 Metabolic aspects of free amino acids in developing marine fish eggs and larvae. *Reviews in Fisheries Science* **1** 239–259. (https://doi.org/10.1080/10641269309388544)

Rosa R, Calado R, Andrade AM, Narciso L & Nunes ML 2005 Changes in amino acids and lipids during embryogenesis of European lobster, *Homarus gammarus* (Crustacea: Decapoda). *Comparative Biochemistry and Physiology* **140** 241–249. (https://doi.org/10.1016/j.cbpc.2004.10.009)

Segade Á, Robaina L, Otero-Ferrer F, Garciá Romero J & Molina Domínguez L 2015 Effects of the diet on seahorse (*hippocampus hippocampus*) growth, body colour, and biochemical composition. *Aquaculture Nutrition* **21** 807–813. (https://doi.org/10.1111/anu.12202)

Skalkos ZMG, Van Dyke JU & Whittington CM 2020 Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus abdominalis*. *Journal of Comparative Physiology* **190** 547–556. (https://doi.org/10.1007/s00360-020-01289-y)

Skalkos ZMG, Van Dyke JU & Whittington CM 2023 Distinguishing between embryonic provisioning strategies in Teleost fishes using a threshold value for parentotrophy. *Biomolecules* **13** 166–180. (https://doi.org/10.3390/biom13010166)

Stiller J, Short G, Hamilton H, Saarman N, Longo S, Wainwright P, Rouse GW & Simison WB 2022 Phylogenomic analysis of Syngnathidae reveals novel relationships, origins of endemic diversity and variable diversification rates. *BMC Biology* **20** 75–75. (https://doi.org/10.1186/s12915-022-01271-w)

Stölting KN & Wilson AB 2007 Male pregnancy in seahorses and pipefish: beyond the mammalian model. *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology* **29** 884–896. (https://doi.org/10.1002/bies.20626)

Sutton FB & Wilson AB 2018 Parental behavior in fish. In *Encyclopedia of Reproduction*. Skinner MK, ed. Amsterdam: Elsevier Science & Technology.

Trexler JC 1997 Resource availability and plasticity in offspring provisioning: embryo nourishment in Sailfin mollies. *Ecology* **78** 1370–1381. (https://doi.org/10.1890/0012-9658(1997)078[1370:RAAPIO]2.0.CO;2)

Trexler JC & DeAngelis DL 2003 Resource allocation in offspring provisioning: an evaluation of the conditions favoring the evolution of matrotrophy. *American Naturalist* **162** 574–585. (https://doi.org/10.1086/378822)

Van Dyke JU & Griffith OW 2018 Mechanisms of reproductive allocation as drivers of developmental plasticity in reptiles. *Journal of Experimental Zoology* **329** 275–286. (https://doi.org/10.1002/jez.2165)

Van Dyke JU, Griffith OW & Thompson MB 2014 High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. *American Naturalist* **184** 198–210. (https://doi.org/10.1086/677138)

Wake MH 2015 Fetal adaptations for viviparity in amphibians. *Journal of Morphology* **276** 941–960. (https://doi.org/10.1002/jmor.20271)

Watanabe S, Kaneko T & Watanabe Y 1999 Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, *Syngnathus schlegeli*: structural comparison with mitochondria-rich cells in the gills and larval epidermis. *Cell and Tissue Research* **295** 141–149. (https://doi.org/10.1007/s004410051220)

Weltzien F-A, Planas M, Cunha I, Evjen MS & Fyhn HJ 1999 Free amino acid and protein contents of start-feeding larvae of turbot (*Scophthalmus maximus*) at three temperatures. *Marine Biology* **133** 327–336. (https://doi.org/10.1007/s002270050471)

Whittington CM, Musolf K, Sommer S & Wilson AB 2013 Behavioural cues of reproductive status in seahorses *Hippocampus abdominalis*. *Journal of Fish Biology* **83** 220–226. (https://doi.org/10.1111/jfb.12156)

Whittington CM & Friesen CR 2020 The evolution and physiology of male pregnancy in syngnathid fishes. *Biological Reviews of the Cambridge Philosophical Society* **95** 1252–1272. (https://doi.org/10.1111/brv.12607)

Whittington CM, Griffith OW, Qi W, Thompson MB & Wilson AB 2015 Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution* **32** 3114–3131. (https://doi.org/10.1093/molbev/msv177)

Whittington CM, Buddle AL, Griffith OW & Carter AM 2022 Embryonic specializations for vertebrate placentation. *Philosophical Transactions of the Royal Society of London Series B* **377** 20210261. (https://doi.org/10.1098/rstb.2021.0261)

Woods CMC 2000 Preliminary observations on breeding and rearing the seahorse *Hippocampus abdominalis* (Teleostei: Syngnathidae) in captivity. *New Zealand Journal of Marine and Freshwater Research* **34** 475–485. (https://doi.org/10.1080/00288330.2000.9516950)

Wourms JP, Atz JW & Stribling MD 1991 Viviparity and the maternal-embryonic relationship in the coelacanth *Latimeria chalumnae*. *Environmental Biology of Fishes* **32** 225–248. (https://doi.org/10.1007/BF00007456)

Chapter 4: Paternal transport of inorganic molecules involved in skeletal development during seahorse pregnancy

The data from this chapter will be incorporated into a manuscript alongside other data including a histological and morphological approach to quantifying mineral demand during development and identifying the potential mechanisms of their transport, before submission to a peer-reviewed journal.

Abstract

Patrotrophic nutrient transport of organic molecules, including lipid and protein, to developing embryos has been confirmed in seahorses. The ability of fathers to transport inorganic molecules such as minerals has not yet been measured in syngnathids. We tested the hypothesis that the inorganic nutrients calcium, magnesium and phosphorus are paternally transported to developing embryos in the male brood pouch. We measured the dry masses and total mineral isotope contents of newly fertilised eggs (NFE) and neonates of *H. abdominalis* using ICP-MS to test whether Ca, Mg, and P had been transported and at what quantities. Neonates contained significantly greater amounts of all measured minerals compared to NFE, indicating paternal transport of the three minerals occurs during *H. abdominalis* pregnancy. Although significant phosphorus transport occurs during embryogenesis, most neonate phosphorus is already present in NFE, indicating maternal contribution during oogenesis is the primary supply. In contrast, neonate calcium and magnesium are primarily provided by the gestating father. This chapter provides the first experimental evidence for paternal mineral transport to developing embryos in syngnathids and deepens our understanding of the relative parental nutritional contribution to the success of embryogenesis in seahorses.

Introduction

Nutrient transport from pregnant parents to developing embryos is a vital form of parental care in eutherian mammals (Carter, 2012), some viviparous reptiles (Thompson, 1999), sharks (Buddle *et al.*, 2019), and teleost fishes, as well as some brooding teleost fishes, including the seahorses examined in my thesis (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4, 5**). Parentotrophy is performed by the gestating parent and can occur either maternally (matrotrophy) or paternally (patrotrophy) (discussed in **Chapter 2**: Skalkos *et al.*, 2023). Nutrient classes include organic (carbohydrates, proteins, lipids, and vitamins) or inorganic (minerals, water, and gases) molecules. The uptake of inorganic molecules, such as single ion minerals, requires relatively simpler mechanisms compared to the transport of organic molecules, which are generally much larger macromolecules (Silverthorn, 2018). This chapter examines the transport of inorganic minerals in *Hippocampus abdominalis*, with a focus on bone formation during embryogenesis.

Paternal transport of inorganic molecules has been hypothesised to occur in *H. abdominalis* based on transcriptome data (Whittington *et al.*, 2015), and in two other seahorse (*Hippocampus*) spp. In *Hippocampus erectus* and *H. barbouri*, calcium (Ca) in the brood pouch fluid is absorbed by embryos (Linton and Soloff, 1964; Oconer *et al.*, 2006). However, whether these minerals are paternally supplied is unknown, thus patrotrophy of inorganic molecules in syngnathids has not yet been experimentally confirmed. In *H. erectus*, the concentration of thirteen minerals including Ca, magnesium (Mg), and phosphorus (P) inside the brood pouch is significantly higher than in the external sea water, suggesting fathers release minerals into the brood pouch (Linton and Soloff, 1964). However, the pouch fluid likely contains nutrients from eggs (both viable and unfertilised), that male *H. erectus* actively absorb (Lin *et al.*, 2024), which could contribute to the differences observed. Thus, a comparison of pouch fluid nutrient composition to the external seawater alone is not necessarily indicative of patrotrophy. Interestingly though, late-stage embryos of *H. erectus* contain Ca deposits in the gut epithelium, indicating a likely site for inorganic mineral absorption from surrounding pouch fluid. The hypothesis that paternal inorganic nutrient transport occurs during pregnancy in syngnathids is not currently supported by experimental evidence.

In *H. abdominalis*, the Ca ion transporter, ATPase calcium–transporting type 2C member 1 (ATP2C1), Ca binding gene, Calreticulin, and Mg ion transporter, SLC41A1, are significantly upregulated in the pregnant brood pouch compared to the non-pregnant brood pouch (Whittington *et al.*, 2015). The ATP2C1 gene encodes the human secretory pathway Ca^{2+}/Mn^{2+} -ATPase protein 1 (hSPCA1), an ATP-driven pump that transports Ca ions across cell membranes and stores them in the Golgi apparatus of cells in vertebrates and invertebrates (Dode *et al.*, 2005; Vanoevelen *et al.*, 2005; Micaroni *et al.*, 2016) and is important for embryonic survival (Okunade *et al.*, 2007). Calreticulin is a Ca^{2+} binding protein primarily involved in Ca^{2+} storage and buffering (Llewelyn *et al.*, 1998). It is a highly conserved protein among mammals (Michalak *et al.*, 1992), that is highly expressed in the human placenta during pregnancy (Højrup *et al.*, 2001; Crawford *et al.*, 2013), suggests importance for placentation and pregnancy maintenance (Iwahashi *et al.*, 2021). SLC41A1 mediates Mg^{2+} uptake and extrusion in fishes and is required to maintain a Mg ion balance in zebrafish larvae (Arjona *et al.*, 2019). The significant upregulation of these mineral transport genes in the pregnant brood pouch of *H. abdominalis* (Whittington *et al.*, 2015), provide testable hypotheses about how paternal nutrient transport of Ca and Mg during embryogenesis is achieved.

Seahorses undergo greater bone ossification compared to other bony fishes (Shi *et al.*, 2023), and thus likely require a relatively large parental allocation of minerals to support skeletogenesis. Seahorses, in contrast to most teleosts, have a modified bone structure that undergoes increased and delayed ossification (Shi *et al.*, 2023). Seahorse skeletons contain internal bony plates, arranged in articulating square ring-like segments from the base of the cranium until the end of the tail (Lees *et al.*, 2012; Praet *et al.*, 2012; Porter *et al.*, 2013) (Figure 1). Like many teleost fish, seahorse bony plates are acellular (Porter *et al.*, 2013), thus contain no osteocytes (Cohen *et al.*, 2012), a major distinguishing characteristic from mammalian bone (Moss, 1961; Witten *et al.*, 2004; Witten and Huysseune, 2009). In teleosts, acellular bones are associated with significantly higher mineral content than cellular bones (Cohen *et al.*, 2012), but their metabolism and regulation remain an understudied area, despite its importance in the understanding of skeletal development and growth in fishes (Lall and Lewis-McCrea, 2007). Osteogenesis of the seahorse skeleton occurs primarily by the development of a cartilage skeleton, followed by endochondral ossification, the formation of bone derived from cartilage (Weigele and Franz-Odendaal, 2016; Novelli *et al.*, 2017; Shi *et al.*, 2023), as is standard in vertebrate embryogenesis (Cancedda *et al.*, 2000). The

ossification of bony plates of seahorse neonates varies between endochondral or intramembranous (no cartilage precursor) ossification (Franz-Odendaal and Adriaens, 2014; Novelli *et al.* 2017; Shi *et al.,* 2023). In most vertebrates, bone ossification starts during embryogenesis, and continues as a component of health well after parturition (Cancedda *et al.*, 2000; Breeland *et al.*, 2023). However, in *Hippocampus reidi* and *H. subelongatus* vertebral and dermal bony plate bone ossification starts soon after birth (Franz-Odendaal and Adriaens, 2014). The skeletal tissues in neonate *H. hippocampus, H. subelongatus,* and *H. reidi* are mainly cartilaginous, with evidence of ossification within 1 day after birth (DAB) (Van Wassenbergh *et al.*, 2009; Franz-Odendaal and Adriaens, 2014; Novelli *et al.*, 2017). Interestingly, neonate *H. abdominalis* (Figure 2), start vertebral and bony plate bone ossification 7 days after birth (DAB) (Shi *et al.*, 2023). Notably though, in Shi *et al.* (2023) the whole-mount staining with Alizardin Red, which stains ossified tissues, and Alcian Blue, which stains cartilage, likely demineralises the tissue due to the acidity of the Alcian Blue and the study conducted no Alizardin Red only controls. For example, Alzarin Red stained *Nerophis ophidion, Syngnathus typhle*, and *H. erectus* revealed calcification begins mid-late stage embryogenesis (Schneider *et al.*, 2023). Furthermore, preliminary results using Micro-CT and histology on embryonic *H. abdominalis* indicates that mineralisation of the skeleton begins at mid-pregnancy (Poda *et al.*, unpublished data). Similarly, *H. zosterae* begins vertebral bone ossification during embryogenesis, when embryos are still contained in the pouch (Azzarello, 1990). We hypothesised that the main mineral constituents of teleost bone are likely paternally supplied to developing embryos during gestation to support skeletogenesis during embryogenesis and after birth.

In teleosts, the main components of the mineral fraction of bone are P, Ca, and Mg (Meunier, 2002; Toppe *et al.*, 2007; Lall and Kaushik, 2021), making these minerals essential for skeletal development during embryogenesis. The majority of the body's P, Ca, and Mg are stored in skeletal tissues: 85-88 % (Volkoff and London, 2018; Taylor, 2021), ~ 99% (Taylor, 2021), and 50-70 % (Bijvelds *et al.*, 1998; Lall, 2002) respectively. Calcium and P play a major role in the development and maintenance of the skeletal tissues in fishes (Lall and Lewis-McCrea, 2007), and are the two main constituents of hydroxyapatite, the bone mineral that strengthens the mechanical resistance of the organic matrix (Bonjour, 2011; Lall and Kaushik, 2021). For example, the bony plates of *H. kuda* are composed of approximately 40 +/- 5 wet wt.% mineral (calcium phosphate) (Porter *et al.*, 2013). Thus, Ca plays a major role in the development of fish skeletal tissue (Flik *et al.*, 1986; Flik and Verbost, 1995; Flik

et al., 1995), including the development of the bony plate endoskeleton during embryogenesis in seahorses (Linton and Soloff, 1964). Although skeletogenesis in terrestrial vertebrates relies mainly on Ca, in fish, bone mineralisation primarily relies on P (Cruz and C., 1992; Lall and Lewis-McCrea, 2007; Volkoff and London, 2018; Taylor, 2021). The pouch transcriptomic data of *H. abdominalis* shows no significant upregulation of the phosphate transporters SLC20A1, SLC20A2, and SLC34A2 (Whittington *et al*., 2015). However, other unannotated phosphate transporters such as SLC34A1 and ALPL could be involved in phosphorus transport during pregnancy. Given the importance of P for bone mineralisation, we hypothesise it may be paternally provided during embryogenesis. Lastly, Mg is also required for the formation and growth of the teleost skeleton (Bijvelds *et al.*, 1998; Lall, 2002). Embryonic utilisation of Ca, Mg, and P is essential for the growth and development of the skeletal system during embryogenesis (Lall and Lewis-McCrea, 2007), and thus must be sufficiently supplied for development.

The large mineral requirement for the high level of ossification required during seahorse skeletal development, in combination with the significant upregulation of mineral transporters in the pregnant brood pouch of *H. abdominalis*, suggest minerals are likely paternally transported to developing embryos during pregnancy. Here we aimed to determine if the three minerals most abundant and important for bone development in vertebrates are transported from fathers to developing embryos during seahorse pregnancy. We tested the hypothesis that the Ca, Mg, and P content of *H. abdominalis* neonates is greater than the Ca, Mg, and P content of newly fertilised eggs (NFE), using mass spectrometry.

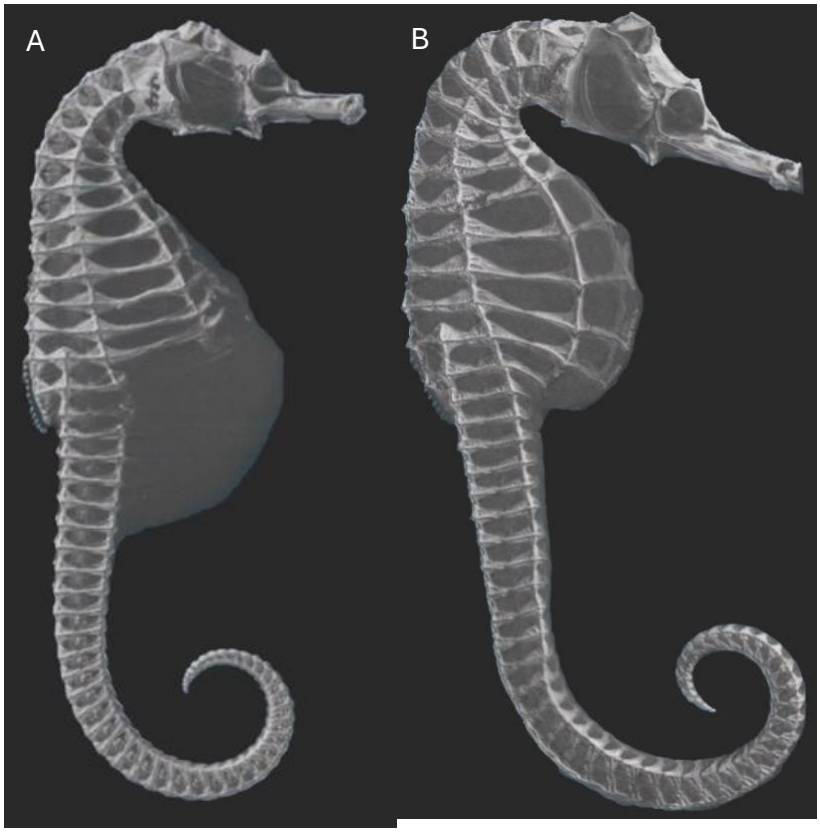

Figure 1. Micro-CT scans of adult Hippocampus abdominalis *male (A) and female (B). Figures modified with permission from Dudley et al. 2022 supplementary material).*

Figure 2. Hippocampus abdominalis *neonates (< 18 hours post-parturition) of variable sizes exhibiting the structural development of the skeletal tissues including the cranium, spine, and prehensile tail.*

Methods

Housing and breeding

Reproductively mature *H. abdominalis* were acquired from a captive-bred population (Seahorse Australia, Tasmania, Australia) and housed as described in Whittington et al. (2013; University of Sydney Animal Ethics Committee approval number: 2021/1995). Twenty-five male and 15 female *H. abdominalis* were used for this study and bred as described in Skalkos *et al.* (2024). Male reproductive status was determined by behavioural assays over three consecutive testing days following Whittington *et al.* (2013), whereby the absence of behavioural signs of courtship indicates putative pregnancy. All adult fish were fed the same amount (approximately 1.5 g per individual, per day) and type of *Mysis* spp. shrimp (thawed Ocean Nutrition frozen mysis, Newark), supplemented with marine frozen food enrichment (Vitalis Aquatic Nutrition, Doncaster) throughout the study. Individual consumption of food was not measured. Following breeding and behavioural testing, pregnant males were housed in single-sex tanks until euthanasia for NFE collection, or parturition for neonate collection. Newly-fertilised embryos were between 3- and 6-days post fertilisation, as described in Skalkos *et al.* (2020), and neonates were euthanised within 18 hours of birth.

Sample collection

Adult male seahorses (individually) and neonates (as a clutch) were euthanised via gradual overdose of anaesthetic (ethanol) as described in Skalkos *et al.* (2024), adhering to the guidelines established by Leary *et al.* (2020) and approved by the University of Sydney Animal Ethics Committee (protocol 2021/1995). Embedded NFEs were fixed in 95 % ethanol/5 % saline solution at 30 ppt for 30–45 minutes to separate them from paternal pouch tissue, as described in Skalkos *et al.* (2020). Neonates were blotted dry with lint-free tissue to reduce excess moisture and salt. The neonate clutch sizes ranged from 20 to 220 individuals, within the broad range for *H. abdominalis* (Foster and Vincent, 2004). Neonates and NFEs were placed in pre-weighed 1.5 mL certified protein-free microcentrifuge tubes (Eppendorf). Due to the small dry mass of individual neonates and NFEs, to improve total mass accuracy, each 1.5 mL microcentrifuge tube contained five pooled NFEs/neonates that were treated as a single sample. All sample measurements were calculated per sample and then divided by the

number of NFE/neonates per sample, to more accurately estimate per NFE/neonate values. When the total number of collected neonates/NFEs for a clutch was not divisible by 5, the remaining two to four specimens were grouped together and treated as a single sample in a 1.5 mL microcentrifuge tube. In total, we collected and analysed 12 neonate clutches (164 total individuals) and 7 NFE clutches (88 total individuals) to measure mineral content. Three samples were randomly selected from each clutch for mass spectrometry analysis and considered as pseudoreplicates for clutch. For clutches where less than 3 samples were available, two (n=4) or one (n=1) psuedoreplicate/s were used to represent the clutch.

ICP Mass Spectrometry

All pooled samples were dried and weighed following the protocol of Skalkos *et al.* (2020) and **Chapter 3**; Skalkos *et al.* (2024). Neonates and NFE were stored in two separate desiccation chambers prior to homogenisation. Neonate and NFE samples were individually homogenised using a TissueLyser LT (QIAGEN, Hilden), individually covered with parafilm, then stored in separate desiccation chambers until analysis. A total of 34 neonate and 18 NFE samples containing up to five pooled NFE/neonates each, were sent to The University of Sydney Mass Spectrometry Facility Service Lab for analysis of P (P^{31}), Ca (Ca^{43}), and Mg (Mg^{24}) content using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Hydrogen peroxide (0.5ml/sample) followed by 70 % trace metal free grade nitric acid (1mL/sample) was added to all NFE and neonate samples, and each was vortexed for 30 seconds, twice. Samples were left at room temperature for 96 hours with the tube lids partially open. Samples were vortexed, centrifuged, and diluted in ultra-pure water using a Hamilton autodiluter to a 100 ppt – 1 ppm concentration range. ICP-MS measurements were performed on a PerkinElmer Nexion 300 x mass spectrometer operating with kinetic energy discrimination using helium as a collision gas at 4L/min, ICP power 1.5 kW. All elements were measured at an integration time of 1000 s. A 10 ppb Rh/Ir internal standard solution was added into the flow system to correct for any variation in kinetic energy. Samples were injected with a cetac ASX520 autosampler. Instrument precision was determined by the relative standard deviation (RSD) for each individual sample. Calcium was corrected for strontium. All elemental isotopes were measured and automatically calculated to represent total elemental composition based on relative isotope abundance per element.

Two samples had to be excluded during the mass spectrometry process due to overflow which would have resulted in inaccurate results. One sample was excluded because the recorded dry mass per neonate was almost three times that of any other sample, which we regarded as a measurement or recording error. These excluded samples are not included in the sample sizes recorded for this study.

Statistical analysis

Data did not meet normality assumptions for multivariate or univariate analysis of variance, including when log and square root transformed. Thus, values were averaged for each clutch to calculate mean mineral content per individual NFE/neonate per clutch. A Mardia's test was then performed to ensure that all data met assumptions of multivariate normality. A multivariate analysis of variance (MANOVA) was used to detect overall differences in mean clutch mineral content between developmental groups, revealing a significant effect ($p < 0.001$). For each mineral, a Shapiro-Wilk test and a Levene's test was used to test whether average total mineral mass met assumptions of normality and heteroscedasticity between developmental groups. The data were log transformed to meet assumptions of heteroscedasticity of variance. Follow-up univariate analysis of variances (ANOVAs) were performed for each log-transformed mineral, to examine differences in means between developmental groups for Ca, Mg, and P at an α-level of 0.05.

Results

The MANOVA provided a significant result ($F_{1,17} = 34.921$, $p < 0.001$), illustrating that mineral concentrations differed between NFE and neonates. Subsequent individual univariate ANOVAs for log-transformed mineral data for each mineral showed significant increases in Ca ($F_{1,17} = 83.40$, $p < 0.001$), Mg ($F_{1,17} = 58.71$, $p < 0.001$), and P ($F_{1,17} = 5.365$, $p = 0.037$) across embryonic development (Figure 3). In both NFE and neonates, P is the most abundant mineral of the three measured, followed by Ca and Mg (Figure 4). Calcium, Mg, and P displayed a 339 %, 212 % and 46 % increase in average mass across development, respectively (Figure 4). The correlations between mineral concentrations were assessed using Pearson's correlation. To account for multiple comparisons, p-values were adjusted using the Holm correction. After applying the Holm-Bonferroni correction, a strong positive correlation was observed between Ca and Mg ($p < 0.001$, $\alpha = 0.016$)), and Ca and P ($p <$

0.001, α = 0.025). A weaker correlation was observed between Mg and P (p = 0.025, α = 0.05) (Figure 5). The Ca:P ratio in neonates is 1:1.194, falling within the normal range of 1:1 and 1:1.7 for commercial whole adult fishes (Chavez-Sanchez *et al*., 2000; Ye *et al.,* 2006).

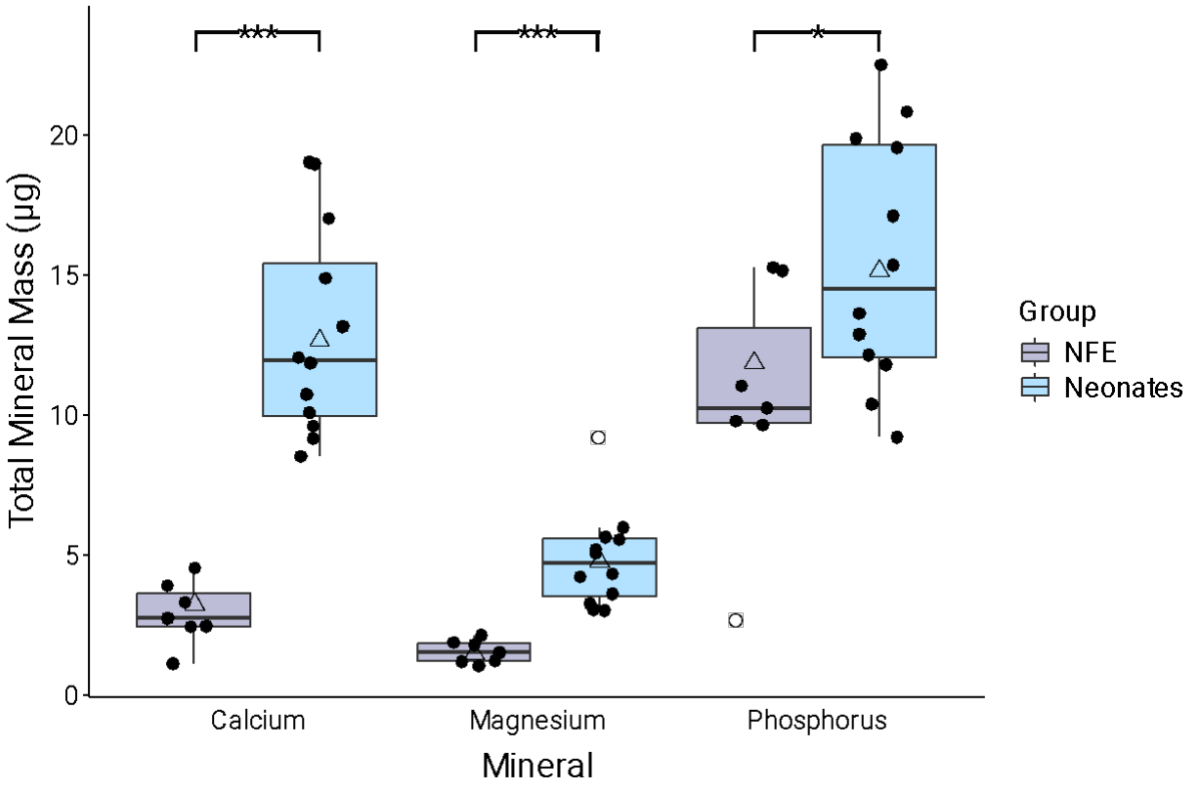

Figure 3. Boxplot of average total calcium, magnesium, and phosphorus content of individual newly fertilised Hippocampus abdominalis *eggs (NFE) and individual neonates. A one-way ANOVA revealed significant increase in total calcium (*p < *0.001), magnesium (*p < *0.001), and phosphorus mass (*p = *0.018) per neonate, compared to NFE. The bold lines indicate the median for each group, and the boxes contain 50 % of the data, while whiskers extended to the highest/lowest data points within 1.5x interquartile range. Data points represent average individual NFE/neonate total mineral mass per sample. The means are plotted as open triangles. Outliers are plotted as open circles.*

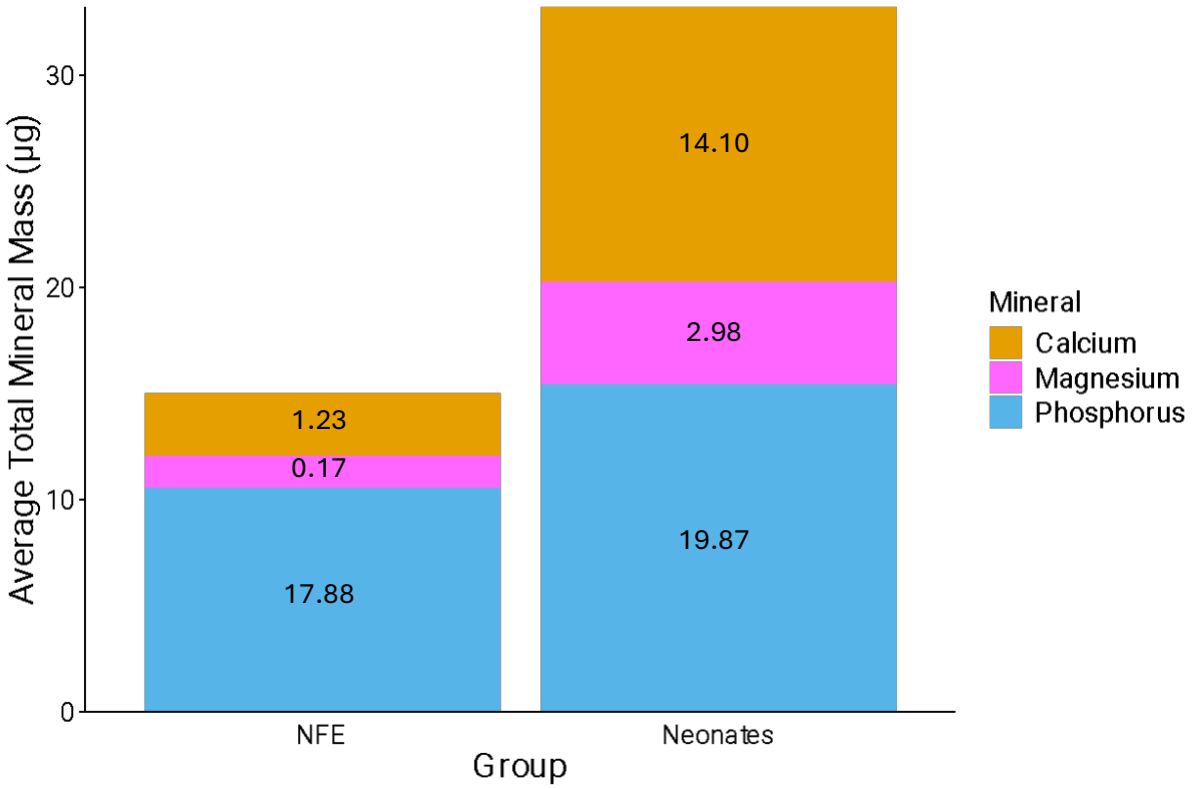

Figure 4. Stacked bar graph of average total calcium, magnesium, and phosphorus mass per newly fertilised Hippocampus abdominalis egg or neonate. The variance for each mineral is given.

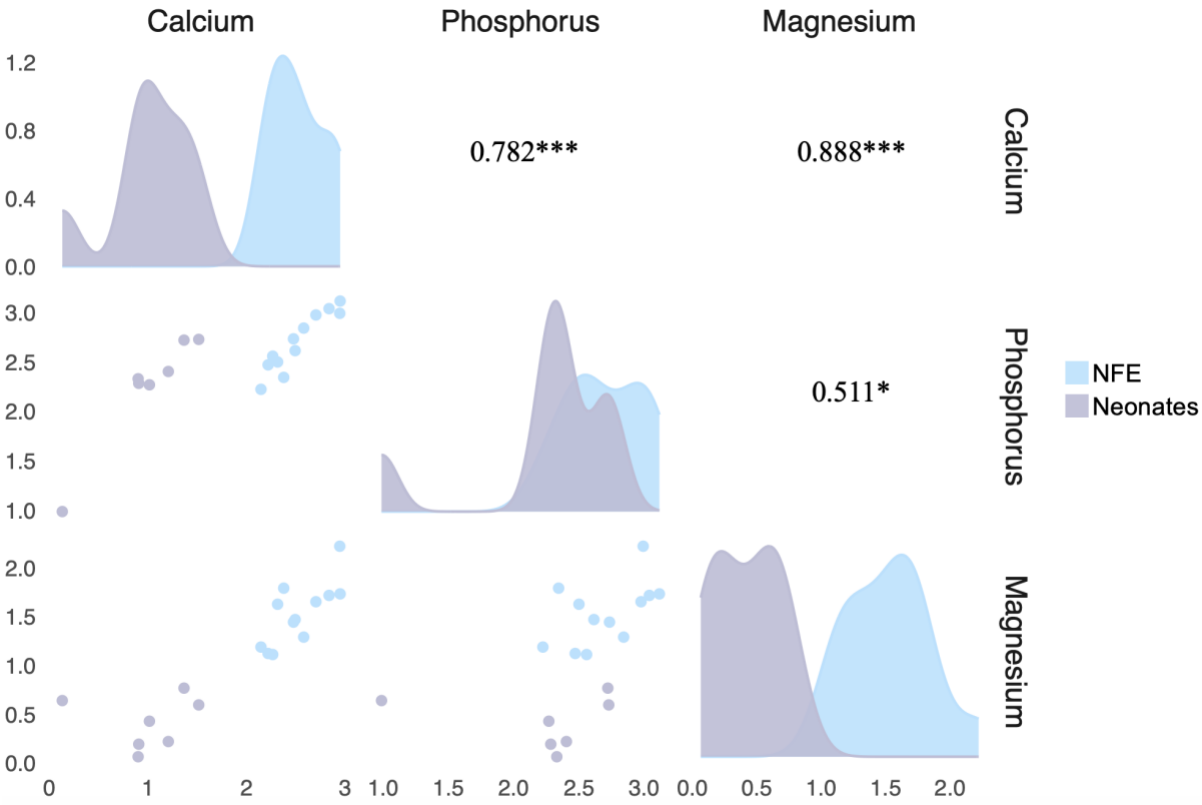

Figure 5. A pairwise scatter plot matrix showing the correlation between log-transformed concentrations of calcium, phosphorus, and magnesium for NFE and Neonates. The numbers in the upper panels represent the correlation coefficients,

*and asterisks indicate the statistical significance of these correlations (*** $p < 0.001$, * $p < 0.05$). The diagonal shows the density distributions for each mineral.*

Discussion

In this chapter, I provide evidence for paternal transport of Ca, Mg, and P to developing embryos during embryogenesis in *H. abdominalis*, as all minerals significantly increased in mass over embryonic development. We observed an average neonatal Ca content of 10.161 mg/g dry mass, P content of 12.015 mg/g dry mass, and Mg content of 3.871 mg/g dry mass. The mineral values observed here, particularly Ca and Mg, are relatively high compared to that of many commercial species, which range between 0.021-1.030 mg/g, 0.170 – 1.090 mg/g and 1.180 – 12.870 mg/g for Ca, Mg, and P respectively (El-Faer *et al.*, 1992; Shehawy *et al.*, 2016; Kiczorowska *et al.*, 2019). This difference may be explained by the significantly higher mineral content present in acellular bones compared to cellular boned teleosts (Cohen *et al.*, 2012). Furthermore, seahorse neonates require mineral stores for continued rapid skeletal formation, tissue growth, and energy metabolism (Cruz and C., 1992; Ye *et al.*, 2006; Nathanailides *et al.*, 2023), which may also explain the increased Ca and Mg in *H. abdominalis* neonates, compared to other adult fishes. In seahorses, bone ossification can continue past 25 days post-birth (Franz-Odendaal and Adriaens, 2014). Seahorse neonates would require substantial quantities of minerals during this period, either through reserves received during oogenesis and embryogenesis, their aquatic environment, or via consumption, to meet their growth demands following embryogenesis. It is unknown how syngnathids, which lack scales, store, and mobilise Ca, Mg, and P or to what extent their acellular bony plates are involved. The mechanisms by which the functions of osteocytes including bone mineralisation are carried out in acellular boned teleosts are unknown (Reznick *et al.*, 2002; Witten and Huysseune, 2009; Cohen *et al.*, 2012; Shahar and Dean, 2013). Male *H. abdominalis* may provision minerals to developing embryos to support mineralisation during embryogenesis (Poda *et al.*, unpublished data from using Micro CT and histological staining) and in excess prior to birth, to ensure sufficient supply for the continuation of skeletogenesis.

In contrast to terrestrial vertebrates, minerals can be absorbed by fish from surrounding water either via their gills, drinking, or via their skin (Lall, 2002). Thus, we cannot exclude the possibility that embryos receive some minerals from the seawater that enters the brood pouch

during mating. The mineral content for the saltwater (Red Sea, Tel Aviv) in which the animals were housed at ~ 32 ppm was ≤ 381 mg/L, ≤ 1131 mg/L and ≤ 0.02 mg/L for Ca, Mg, and P respectively (Supplementary Material Table 1). The available liquid in the brood pouch of *H. abdominalis* during gestation in this study was < 150 µl (Whittington lab, unpublished data). For example, the < 0.381 µg/µl of Ca present in the salt water would equate to < 57.15 µg of Ca in the brood pouch. Our results indicate individual neonates uptake ~ 10 µg of Ca throughout embryogenesis. Our clutch sizes in this study varied from 20 to 220 with an average of ~ 100 NFE/neonates per clutch. Therefore, if Ca were provided exclusively from seawater, ~ 1000 µg of Ca per clutch would be required, which equates to up to 2,625 µl of liquid present in the pouch (> 17 times the maximum measured pouch volume). Therefore, based on the concentration of minerals in the seawater of the housed aquariums, the mineral content available in the liquid in the brood pouch during gestation would be negligible compared to the initial amounts present in the yolk reserves of NFE, and the quantity gained during embryogenesis. Notably though, seahorses may perform "pouch flushing" towards the end of pregnancy to acclimate pouch osmolality to the external environment (Whittington and Friesen, 2020), during which embryos have well-developed gills (Sommer et al., 2012), and could uptake minerals more readily. However, pouch flushing has not yet been confirmed in seahorses. In *S. schlegeli*, pregnant males allow some water to seep into the brood pouch during late pregnancy to acclimate embryos to the external osmolality (Watanabe, 1999), due to the "loosening" of the brood pouch opening before parturition. In *H. abdominalis*, it is unlikely that any regular flushing at the end of gestation exclusively meets embryonic mineral requirements because bone mineralisation in *H. abdominalis* begins mid-pregnancy (Whittington lab, unpublished data). Lastly, the "pouch flushing" required to meet embryonic mineral requirements would have to occur daily or several times per day throughout gestation to meet the mineral increase across embryogenesis observed here. This flushing should result in consistent salinity in the brood pouch that matches the external seawater. However, in *H. erectus* (Linton and Soloff, 1964), *Hippocampus guttulatus*, and *Hippocampus brevirostris* (Leiner, 1936) brood pouch osmolarity is isosmotic to paternal blood during early gestation and increases to match seawater in late pregnancy. Therefore, it is highly unlikely that pouch flushing occurs consistently throughout gestation. Consequently, our results indicate that Ca, Mg, and P were mainly paternally supplied to developing embryos in *H. abdominalis*, although marginal amounts may be environmentally sourced towards late embryogenesis.

The magnitude of increase of Ca, Mg, and P differs, suggesting patrotrophy of each mineral occurs at different amounts. In contrast to organic molecules, minerals cannot be catabolised, therefore embryonic mineral content is expected to stay the same or decrease marginally over development, unless supplemented by the environment or gestating parent. The observed differences in patrotrophy between minerals may be due to what is already present by means of vitellogenin in the yolk and the discrepancy between this quantity and the embryonic requirement throughout embryogenesis, which likely differs for each mineral. My results show that more Ca and Mg are absorbed by embryos post-fertilisation than are provided in the yolk of NFE. In contrast, while some P transport appears to take place, most of this mineral is already present in the yolk of NFE. In *H. abdominalis*, the mother supplies most of the protein and lipids required for embryonic development (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024). This is the first study showing nutrients that are primarily provided by the father, not the mother, to support embryonic development. The combined contribution of minerals by male and female *H. abdominalis* reflects evolutionary trade-offs in optimal resource allocation for embryonic development and survival and prompts interesting discussion on how the relative parental allocation of resources and division of roles evolved. Syngnathids thus represent an unusual comparative group for parental resource allocation as both the mother, by means of vitellogenesis, and the father by patrotrophy, can provide nutrients to developing embryos prior to birth. For example, female *N. ophidion*, a lecithotrophic pipefish, invest more energy into a single zygote than males, however in *S. typhle,* a patrotrophic pipefish, both males and females invest similar amounts (Berglund *et al.*, 1986). In contrast to viviparous and most brooding vertebrates, including pipefishes and seadragons, male seahorses carry eggs from fertilisation to birth, in a fully enclosed brood pouch (Stölting and Wilson, 2007), providing additional opportunities for patrotrophy (Whittington and Friesen, 2020). Therefore, patrotrophic seahorses are likely capable of providing more resources to developing embryos and having greater contribution to meeting the nutritional requirements of embryogenesis. The overall maternal vs paternal resource allocation and investment in *H. abdominalis* embryogenesis is currently unknown, however the results provided from this study, Skalkos *et al.* (2020), and **Chapter 3**: Skalkos *et al.* (2024), provide greater insight into the relative parental mineral contribution in the most complex form of male pregnancy in vertebrates. Furthermore, these results broaden our understanding of the fundamental biology of male brooding, and the paternal contribution to the success of reproduction.

Moreover, *H. abdominalis* may exhibit patrotrophic plasticity, as observed in many matrotrophic teleosts (Thibault and Schultz, 1978; **Chapter 2:** Skalkos *et al.*, 2023**)**, and squamates (Stewart, 1989; Itonaga *et al.*, 2012; Van Dyke *et al.*, 2014). Patrotrophic plasticity in pregnant *H. abdominalis* when faced with inadequate nutrition, would likely fluctuate paternal nutrient provisioning. Given that P is the largest mineral component of fish skeletons (Cruz and C., 1992; Lall and Lewis-McCrea, 2007; Volkoff and London, 2018; Taylor, 2021), is primarily obtained through diet (Ohata *et al.*, 2016; Hernando and Wagner, 2018; Shaker and Deftos, 2023), and is the most abundant mineral in both *H. abdominalis* NFE and neonates (**Chapter 4**), patrotrophic plasticity may have led to selection for egg clutches with greater P supply in the yolk. I recommend studying the effects of food abundance or inconsistent food supply on paternal and embryonic growth and nutrient composition whilst brooding, to determine whether fathers prioritise provisioning to the clutch over their physical and potentially future reproductive fitness, as in *P. entrecasteauxii* (Van Dyke *et al.*, 2014).

The mechanisms that underpin embryonic uptake of paternal nutrients including lipid (Skalkos *et al.*, 2020), protein (**Chapter 3**: Skalkos *et al.*, 2024) and minerals, in *Hippocampus* spp. are currently unknown. One possibility is the placental transfer of nutrients through direct contact of paternal and embryonic tissues. Embryos of *H. abdominalis* are deeply embedded in paternal pouch epithelium (Boisseau, 1965; Wetzel and Wourms, 1991; Carcupino *et al.*, 1997; Skalkos *et al.*, 2020; Harada *et al.*, 2022), and thus could be receiving nutrients directly at the paternal-embryonic interface. The diffusion distance between the capillaries and pouch lumen of *H. abdominalis* decreases during pregnancy, and the density of the vascular bed increases, compared to the non-pregnant pouch (Dudley et al. 2021). These changes during pregnancy likely facilitate gas and nutrient exchange between paternal and embryonic tissues (Ripley *et al.*, 2010; Dudley *et al.*, 2021). Another possible route of nutrient uptake could be via non-placental forms of parentotrophy, including absorption (histotrophy) or digestion (histophagy) of paternal pouch secretions (Wourms *et al.*, 1988; Wake, 2014; Ostrovsky *et al.*, 2016) after release into the brood pouch. Placentotrophic and non-placentotrophic means of nutrient uptake are not mutually exclusive (Blackburn, 2015; Ostrovsky *et al.*, 2016), and we hypothesise that a combination of both may occur in *H. abdominalis*. Whittington *et al.* (2015) hypothesised that nutrient transport in *H. abdominalis* occurs via histotrophy, as in some viviparous sharks and rays (Wourms, 1981; Buddle *et al.*, 2021). This would enable embryonic P, Ca, and Mg uptake via the skin

before the development of the gills and GIT, further enabling the absorption of histotrophe. The most likely route of Ca absorption in embryonic *H. abdominalis* is via skin ionocytes, specialised ion-transporting, mitochondria-rich cells (McCormick *et al.*, 1992; Flik and Verbost, 1995; Flik *et al.*, 1995; Evans *et al.*, 2005; Guh *et al.*, 2015). Extrabranchial ionocytes on the skin and yolk sac membranes of embryos and larval stages have been reported in several teleosts (e.g. Hwang *et al.*, 1999; reviewed in Varsamos *et al.*, 2005) and are the major sites of embryonic environmental Ca^{2+} absorption (Hwang *et al.*, 1999; Evans *et al.*, 2005; Hwang and Perry, 2010; Hwang *et al.*, 2011). As skin ionocytes contain high amounts of Na^{+}/K^{+}ATPase (Lin *et al.*, 2006), a pump involved in Mg transport in fresh-water fishes (Kayne and Lee, 1993; Bijvelds *et al.*, 1996), we hypothesise Mg may also be absorbed via the ionocytes of embryonic skin in *H. abdominalis*. We hypothesise that *H. abdominalis* embryonic Ca and Mg absorption likely occurs through the yolk sac membrane and skin of the embryo via ionocytes, either indirectly from the pouch fluid or through direct contact with paternal pouch tissue. Moreover, ionocytes are exclusively present in the pregnant pouches of *S. abaster, S. schlegeli* and *S. scovelli* (Carcupino *et al.*, 1997; Watanabe *et al.*, 1999; Carcupino *et al.*, 2002; Partridge *et al.*, 2007), and may be involved in pouch osmoregulation (Whittington and Friesen, 2020) and paternal mineral transport. Embryonic skin of syngnathids likely also facilitates absorption of macromolecules (Healey *et al.,* 2024). The epidermal cells of *Syngnathus scovelli* embryos express an abundance of epidermal-specific C-type lectin genes, carbohydrate binding proteins (Healey *et al.,* 2024). This recent finding supports previous hypotheses that maternally derived nutrients may be epidermally absorbed in viviparous fishes (Tengfei *et al*., 2021; Wourms, 1981). Lastly, in adult teleosts, P is primarily absorbed in the GIT by diet but the mechanisms by which this occurs are currently unknown (Ohata *et al.*, 2016; Hernando and Wagner, 2018; Shaker and Deftos, 2023) but complimentary to passive transport, uptake in the GIT may occur via enterocyte sodium-phosphate co-transporters, as in humans (Hernando and Wagner, 2018). We further hypothesise that the paternal provisioning of P is released into the pouch lumen and digested by developing embryos via histophagy.

In summary, Ca, Mg, and P are significantly increased in neonates of *H. abdominalis* compared to NFE, demonstrating paternal transport of inorganic nutrients during pregnancy. This expands our current understanding of the extent of patrotrophy in seahorses from organic nutrients alone (lipids and proteins) (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024), to also include inorganic nutrients including minerals. Thus in *H. abdominalis,*

patrotrophy is comprehensive with evidence of paternal transport of all the major organic and inorganic nutrients (except for carbohydrates), as occurs in matrotrophic viviparous vertebrates. The P supply in neonates is primarily supplied in the yolk, while Ca and Mg are primarily supplied by the gestating father, advancing our understanding of the division of parental resource allocation in male brooding vertebrates. The mechanisms by which minerals are transported and taken up by developing embryos in *H. abdominalis* are hypothesised. We suggest localisation of conserved genes associated with nutrient transport in the brood pouch during pregnancy to deepen our understanding of the mechanisms that underpin organic and inorganic nutrient transport from gestating fathers to developing embryos during pregnancy in *H. abdominalis*.

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Supplementary material

Table S1: Red Sea Fish pharm Ltd. Red Sea Salt Analysis 2022 of synthetic seawater with Red Sea Salt given in mg/L (ppm) for sea water at a salinity of 35 ppt. Received 22nd March 2023.

Mineral	**Red Sea mg/L**		**Analysis Method**
	Min	**Max**	
Calcium	415	445	ICP-OES
Magnesium	1240	1320	ICP-OES
Phosphorus	0	0.025	Spectrophotometric

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References

Arjona, F. J., Latta, F., Mohammed, S. G., Thomassen, M., Van Wijk, E., Bindels, R. J. M., Hoenderop, J. G. J. & de Baaij, J. H. F. (2019). SLC41A1 is essential for magnesium homeostasis in vivo. *Pflügers Archiv: European Journal of Physiology* **471**, 845-860.

Azzarello, M. Y. (1990). A comparative study of the developmental osteology of *Syngnathus scovelli* and *Hippocampus zosterae* (Pisces: Syngnathidae) and its phylogenetic implications. *Evolutionary Monographs* **12**, 1-19.

Berglund, A., Rosenqvist, G. & Svensson, I. (1986). Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. *Marine Ecology* **29,** 209-215.

Bijvelds, M. J., Velden, J. A., Kolar, Z. I. & Flik, G. (1998). Magnesium transport in freshwater teleosts. *Journal of Experimental Biology* **201** 1981-1990.

Bijvelds, M. J. C., Kolar, Z. I., Wendelaar Bonga, S. E. & Flik, G. (1996). Magnesium transport across the basolateral plasma membrane of the fish enterocyte. *The Journal of Membrane Biology* **154**, 217-225.

Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *Journal of Morphology* **276**, 961-990.

Boisseau, J. P. (1965). Action de quelques hormones sur l'incubation d'hippocampes males normaux, ou castres, ou hypophysectomises. *Comptes Rendus de l'Académie des Sciences* **260**, 313-314.

Bonjour, J.-P. (2011). Calcium and phosphate: A duet of ions playing for bone health. *Journal of the American College of Nutrition* **30**, 438S-448S.

Breeland, G., Sinkler, M. A. & Menezes, R. G. (2023). Embryology, bone ossification. In *StatPearls*. StatPearls Publishing.

Buddle, A. L., Van Dyke, J. U., Thompson, M. B., Simpfendorfer, C. A., Murphy, C. R., Dowland, S. N. & Whittington, C. M. (2021). Structure of the paraplacenta and the yolk sac placenta of the viviparous Australian sharpnose shark, *Rhizoprionodon taylori*. *Placenta* **108**, 11-22.

Buddle, A. L., Van Dyke, J. U., Thompson, M. B., Simpfendorfer, C. A. & Whittington, C. M. (2019). Evolution of placentotrophy: using viviparous sharks as a model to understand vertebrate placental evolution. *Marine and Freshwater Research* **70**, 908.

Cancedda, R., Castagnola, P., Cancedda, F. D., Dozin, B. & Quarto, R. (2000). Developmental control of chondrogenesis and osteogenesis. *International Journal of Developmental Biology* **44**, 707-714.

Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. (1997). Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue & Cell* **29**, 21-30.

Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. (2002). Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *Journal of Fish Biology* **61**, 1465-1480.

Carter, A. M. (2012). Evolution of placental function in mammals: the molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. *Physiological Reviews* **92**, 1543-1576.

Chavez-Sanchez, C., Martinez-Palacios, C. A., Martinez-Perez, G. & Ross, L. G. (2000). Phosphorus and calcium requirements in the diet of the American cichlid *Cichlasoma urophthalmus* (Günther). *Aquaculture Nutrition* **6**, 1–9.

Cohen, L., Dean, M., Shipov, A., Atkins, A., Monsonego-Ornan, E. & Shahar, R. (2012). Comparison of structural, architectural and mechanical aspects of cellular and acellular bone in two teleost fish. *Journal of Experimental Biology* **215**, 1983-1993.

Crawford, K. E., Stevenson, J. L., Wlodek, M. E. & Gude, N. M. (2013). No change in calreticulin with fetal growth restriction in human and rat pregnancies. *Placenta* **34**, 1066-1071.

Cruz, M. L. A. & Tsang, R. C. (1992). Introduction to infant mineral metabolism In *Calcium Nutriture for Mothers and Children.* New York Raven Press.

Dode, L., Andersen, J. P., Raeymaekers, L., Missiaen, L., Vilsen, B. & Wuytack, F. (2005). Functional comparison between secretory pathway Ca2+/Mn2+-ATPase (SPCA) 1 and sarcoplasmic reticulum Ca2+-ATPase (SERCA) 1 isoforms by steady-state and transient kinetic analyses. *Journal of Biological Chemistry* **280**, 39124-39134.

Dudley, J. S., Hannaford, P., Dowland, S. N., Lindsay, L. A., Thompson, M. B., Murphy, C. R., Van Dyke, J. U. & Whittington, C. M. (2021). Structural changes to the brood pouch of male pregnant seahorses (*Hippocampus abdominalis*) facilitate exchange between father and embryos. *Placenta* **114**, 115-123.

El-Faer, M. Z., Rawdah, T. N., Attar, K. M. & Arab, M. (1992). Mineral and proximate composition of some commercially important fish of the Arabian Gulf. *Food Chemistry* **45**, 95-98.

Evans, D. H., Piermarini, P. M. & Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews* **85**, 97-177.

Flik, G., Fenwick, J. C., Kolar, Z., Mayer-Gostan, N. & Bonga, S. E. W. (1986). Effects of low ambient calcium levels on whole-body Ca2+ flux rates and internal calcium pools in the freshwater Cichlid Teleost, *Oreochromis Mossambicus*. *Journal of Experimental Biology* **120**, 249-264.

Flik, G. & Verbost, P. M. (1995). Cellular mechanisms in calcium transport and homeostasis in fish. In: *Biochemistry and Molecular Biology of Fishes*, pp. 251-263: Elsevier Science & Technology.

Flik, G., Verbost, P. M. & Bonga, S. E. W. (1995). Calcium transport processes in fishes. In: *Fish Physiology*, pp. 317-342: Academic Press.

Foster, S. J. & Vincent, A. C. J. (2004). Life history and ecology of seahorses: implications for conservation and management. *Journal of Fish Biology* **65**, 1-61.

Franz-Odendaal, T. A. & Adriaens, D. (2014). Comparative developmental osteology of the seahorse skeleton reveals heterochrony amongst *Hippocampus* sp. and progressive caudal fin loss. *EvoDevo* **5**, 45-45.

Guh, Y. J., Lin, C. H. & Hwang, P. P. (2015). Osmoregulation in zebrafish: ion transport mechanisms and functional regulation. *EXCLI Journal* **14**, 627-659.

Harada, A., Shiota, R., Okubo, R., Yorifuji, M., Sogabe, A., Motomura, H., Hiroi, J., Yasumasu, S. & Kawaguchi, M. (2022). Brood pouch evolution in pipefish and seahorse based on histological observation. *Placenta* **120**, 88-96.

Healey, M. H., Penn, H. B., Small, C. M., Bassham, S., Goyal, V., Woods, M. A. & Cresko, W. A. (2024). Single cell sequencing provides clues about the developmental genetic basis of evolutionary adaptations in syngnathid fishes. *eLife* **13**, RP97764.

Hernando, N. & Wagner, C. A. (2018). Mechanisms and regulation of intestinal phosphate absorption. *Comprehensive Physiology* **8**, 1065-1090.

Højrup, P., Roepstorff, P. & Houen, G. (2001). Human placental calreticulin characterization of domain structure and post-translational modifications. *European Journal of Biochemistry* **268**, 2558-2565.

Hwang, P.-P. & Perry, S. F. (2010). *Ionic and acid–base regulation*. pp. 311-344: Elsevier Science & Technology.

Hwang, P. P., Lee, T. H. & Lin, L. Y. (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology* **301**, R28-47.
Hwang, P. P., Lee, T. H., Weng, C. F., Fang, M. J. & Cho, G. Y. (1999). Presence of Na-K-ATPase in mitochondria-rich cells in the yolk-sac epithelium of larvae of the Teleost *Oreochromis mossambicus*. *Physiological and Biochemical Zoology* **72**, 138-144.
Itonaga, K., Jones, S. M. & Wapstra, E. (2012). Effects of maternal basking and food quantity during gestation provide evidence for the selective advantage of matrotrophy in a viviparous lizard. *PloS one* **7**, e41835-e41835.
Iwahashi, N., Ikezaki, M., Nishitsuji, K., Yamamoto, M., Matsuzaki, I., Kato, N., Takaoka, N., Taniguchi, M., Murata, S. I., Ino, K. & Ihara, Y. (2021). Extracellularly released Calreticulin induced by endoplasmic reticulum stress impairs syncytialization of cytotrophoblast model BeWo cells. *Cells* **10**.
Kayne, L. H. & Lee, D. B. (1993). Intestinal magnesium absorption. *Mineral and Electrolyte Metabolism* **19**, 210-217.
Kiczorowska, B., Samolińska, W., Grela, E. R. & Bik-Małodzińska, M. (2019). Nutrient and mineral profile of chosen fresh and smoked fish. *Nutrients* **11**, 1448.
Lall, S. P. (2002). The minerals. In: *Fish Nutrition,* pp. 259-308: Academic Press.
Lall, S. P. & Kaushik, S. J. (2021). Nutrition and metabolism of minerals in fish. *Animals* **11**, 2711.
Lall, S. P. & Lewis-McCrea, L. M. (2007). Role of nutrients in skeletal metabolism and pathology in fish — An overview. *Aquaculture* **267**, 3-19.
Leary, S., Underwood, W., Athony, R., Cartner, S., Grandin, T., Greenace, C., Gwaltney-Brant, S., McCrackin, M. A., Meyer, R., Miller, D., Shearer, J., Turner, T. & Yanong, R. (2020). *AVMA Guidelines for the Euthanasia of Animals (2020 Edition)*. Schaumburg: American Veterinary Medical Association.
Lees, J., Märss, T., Wilson, M. V. H., Saat, T. & Špilev, H. (2012). The sculpture and morphology of postcranial dermal armor plates and associated bones in gasterosteiforms and syngnathiforms inhabiting Estonian coastal waters. *Acta Zoologica* **93**, 422-435.
Leiner, M. (1936). Die physiologischen grundlagen der nachkommenfürsorge bei den seenadeln (Syngnathidae). *Zeitschrift für Vergleichende Physiologie* **23**, 147-207.
Lin, L.-Y., Horng, J.-L., Kunkel, J. G. & Hwang, P.-P. (2006). Proton pump-rich cell secretes acid in skin of zebrafish larvae. *American Journal of Physiology-Cell Physiology* **290**, C371-C378.

Lin, T., Liu, X., Li, S., Zhang, D. & Shen, F. (2024). Brooding fathers actively consume viable embryos to take up nutrients in a monogamous syngnathid fish. *Frontiers in Marine Science* **11**.
Linton, J. R. & Soloff, B. L. (1964). The physiology of the brood pouch of the male sea horse *Hippocampus erectus*. *Bulletin of Marine Science* **14**, 45-61.
Llewelyn, R. H., Llewellyn, D. H., Campbell, A. K. & Kendall, J. M. (1998). Role of calreticulin in regulating intracellular Ca2+ storage and capacitative Ca2+ entry in HeLa cells. *Cell Calcium* **24**, 253-262.
McCormick, S. D., Hasegawa, S. & Hirano, T. (1992). Calcium uptake in the skin of a freshwater teleost. *Proceedings of the National Academy of Sciences* **89**, 3635-3638.
Meunier, F. (2002). Skeleton. In: *Manual of Fish Sclerochronology*, pp. 65-87: Ifremer.
Micaroni, M., Giacchetti, G., Plebani, R., Xiao, G. G. & Federici, L. (2016). ATP2C1 gene mutations in Hailey-Hailey disease and possible roles of SPCA1 isoforms in membrane trafficking. *Cell Death & Disease* **7**, e2259-e2259.
Michalak, M., Milner, R. E., Burns, K. & Opas, M. (1992). Calreticulin. *Biochemical Journal* **285**, 681-692.
Moss, M. L. (1961). Osteogenesis of acellular teleost fish bone. *American Journal of Anatomy* **108**, 99-109.
Nathanailides, C., Kolygas, M., Tsoumani, M., Gouva, E., Mavraganis, T. & Karayanni, H. (2023). Addressing phosphorus waste in open flow freshwater fish farms: Challenges and solutions. *Fishes* **8**, 442.
Novelli, B., Otero-Ferrer, F., Socorro, J. A., Caballero, M. J., Segade-Botella, A. & Molina Domínguez, L. (2017). Development of short-snouted seahorse (*Hippocampus hippocampus,* L. 1758): osteological and morphological aspects. *Fish Physiology and Biochemistry* **43**, 833-848.
Oconer, E., Pascual, C., Herrera, A., Sakamoto, T. & Wetzel, J. (2006). Chemical components of the pouch fluid in the seahorse, *Hippocampus barbouri* Jordan and Richardson 1908 and their uptake in embryos. *Asia Life Sciences* **15**, 73-84.
Ohata, Y., Ozono, K. & Michigami, T. (2016). Current concepts in perinatal mineral metabolism. *Clinical Pediatric Endocrinology* **25**, 9-17.
Okunade, G. W., Miller, M. L., Azhar, M., Andringa, A., Sanford, L. P., Doetschman, T., Prasad, V. & Shull, G. E. (2007). Loss of the Atp2c1 secretory pathway Ca(2+)-ATPase (SPCA1) in mice causes Golgi stress, apoptosis, and midgestational death in homozygous

embryos and squamous cell tumors in adult heterozygotes. *Journal of Biological Chemistry* **282**, 26517-26527.

Ostrovsky, A. N., Lidgard, S., Gordon, D. P., Schwaha, T., Genikhovich, G. & Ereskovsky, A. V. (2016). Matrotrophy and placentation in invertebrates: a new paradigm. *Biological Reviews of the Cambridge Philosophical Society* **91**, 673-711.

Partridge, C., Shardo, J. & Boettcher, A. (2007). Osmoregulatory role of the brood pouch in the euryhaline Gulf pipefish, *Syngnathus scovelli*. *Comparative Biochemistry and Physiology, Part A* **147**, 556-561.

Porter, M. M., Novitskaya, E., Castro-Ceseña, A. B., Meyers, M. A. & McKittrick, J. (2013). Highly deformable bones: Unusual deformation mechanisms of seahorse armor. *Acta Biomaterialia* **9**, 6763-6770.

Praet, T., Adriaens, D., Cauter, S. V., Masschaele, B., Beule, M. D. & Verhegghe, B. (2012). Inspiration from nature: dynamic modelling of the musculoskeletal structure of the seahorse tail. *International Journal for Numerical Methods in Biomedical Engineering* **28**, 1028-1042.

Reznick, D. N., Mateos, M. & Springer, M. S. (2002). Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. *Science* **298**, 1018-1020.

Ripley, J., Cantrell, P. & Foran, C. (2010). Morphological and quantitative changes in paternal brood-pouch vasculature during embryonic development in two Syngnathus pipefishes. *Journal of Fish Biology* **77**, 67-79.

Schneider, R. F., Woltering, J. M., Adriaens, D. & Roth, O. (2023). A comparative analysis of the ontogeny of syngnathids (pipefishes and seahorses) reveals how heterochrony contributed to their diversification. *Developmental Dynamics* **252**, 553-588.

Shahar, R. & Dean, M. N. (2013). The enigmas of bone without osteocytes. *BoneKEy Reports* **2**, 343-343.

Shaker, J. L. & Deftos, L. (2023). Calcium and phosphate homeostasis. In *Endotext*. MDText.

Shehawy, S. M. E., Gab-alla, A. & Mutwally, H. M. (2016). Proximate and elemental composition of important fish species in Makkah Central fish market, Saudi Arabia. *Food and Nutrition Sciences* **7**, 429-439.

Shi, X., Tang, X., Zhang, Y., Wang, W., Qin, S., Liu, Q. & Mei, J. (2023). The early allometric growth and osteological ontogeny of Pot-Bellied Seahorse (*Hippocampus abdominalis*, L. 1827) under mass-scale captive breeding conditions in North China. *Fishes* **8**, 604.

Skalkos, Z., Van Dyke, J., Dowland, S. & Whittington, C. (2024). Paternal protein provisioning to embryos during male seahorse pregnancy. *Reproduction* **167**, e230420.
Skalkos, Z. M. G., Van Dyke, J. U. & Whittington, C. M. (2020). Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus abdominalis*. *Journal of Comparative Physiology, Part B* **190**, 547-556.
Skalkos, Z. M. G., Van Dyke, J. U. & Whittington, C. M. (2023). Distinguishing between embryonic provisioning strategies in Teleost fishes using a threshold value for parentotrophy. *Biomolecules* **13**, 166-180.
Sommer, S., Whittington, C. M. & Wilson, A. B. (2012). Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family Syngnathidae). *Developmental Biology* **12**, 39-39.
Stewart, J. R. (1989). Facultative placentotrophy and the evolution of squamate placentation: quality of eggs and neonates in *Virginia striatula*. *The American Naturalist* **133**, 111-137.
Stölting, K. N. & Wilson, A. B. (2007). Male pregnancy in seahorses and pipefish: beyond the mammalian model. *BioEssays* **29**, 884-896.
Taylor, S. N. (2021). Calcium, magnesium, phosphorus, and vitamin D. *Nutritional Care of Preterm Infants* **122**, 122-139.
Tengfei, D, Yongshuang, X., Haixia, Z., Li, Z., Qinghua, L., Xueying, W., Jun, L., Shihong, X., Ynafeng, W., Jiachen, Y., Lele, W., Yunong, W. & Guang, G. (2021). Multiple fetal nutritional patterns before parturition in viviparous fish *Sebastes schlegelii* (Hilgendorf, 1880). *Frontiers in Marine Science* **7**, 571946.
Thibault, R. E. & Schultz, R. J. (1978). Reproductive adaptations among viviparous fishes (Cyprinodontiformes: Poeciliidae). *Evolution* **32**, 320-333.
Thompson, M. B. (1999). Comparison of nutrient transport across the placenta of lizards differing in placental complexity. *Comparative Biochemistry and Physiology. Part A* **124**, S113-S113.
Toppe, J., Albrektsen, S., Hope, B. & Aksnes, A. (2007). Chemical composition, mineral content and amino acid and lipid profiles in bones from various fish species. *Comparative Biochemistry and Physiology. Part B* **146**, 395-401.
Van Dyke, J., U. , Griffith, O., W. & Thompson, M., B. (2014). High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. *The American Naturalist* **184**, 198-210.

Van Wassenbergh, S., Roos, G., Genbrugge, A., Leysen, H., Aerts, P., Adriaens, D. & Herrel, A. (2009). Suction is kid's play: extremely fast suction in newborn seahorses. *Biology Letters* **5**, 200-203.

Vanoevelen, J., Dode, L., Van Baelen, K., Fairclough, R. J., Missiaen, L., Raeymaekers, L. & Wuytack, F. (2005). The secretory pathway Ca2+/Mn2+-ATPase 2 is a golgi-localized pump with high affinity for Ca2+ ions. *Journal of Biological Chemistry* **280**, 22800-22808.

Varsamos, S., Nebel, C. & Charmantier, G. (2005). Ontogeny of osmoregulation in postembryonic fish: A review. *Comparative Biochemistry and Physiology, Part A* **141**, 401-429.

Volkoff, H. & London, S. (2018). Nutrition and Reproduction in Fish. In: *Encyclopedia of Reproduction*, pp. 743-748: Elsevier Science & Technology.

Wake, M. H. (2014). Fetal adaptations for viviparity in amphibians: amphibian fetal adaptations. *Journal of Morphology* **276**, 941-960.

Watanabe, S. (1999). The role of male brood pouch in the reproduction of the seaweed pipefish, *Syngnathus schlegeli*. Univeristy of Tokyo.

Watanabe, S., Kaneko, T. & Watanabe, Y. (1999). Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, *Syngnathus schlegeli*: Structural comparison with mitochondria-rich cells in the gills and larval epidermis. *Cell and Tissue Research* **295**, 141-149.

Weigele, J. & Franz-Odendaal, T. A. (2016). Functional bone histology of zebrafish reveals two types of endochondral ossification, different types of osteoblast clusters and a new bone type. *Journal of Anatomy* **229**, 92-103.

Wetzel, J. & Wourms, J. P. (1991). Paternal-embryonic relationships in pipefishes and seahorses (Syngnathidae). *American Zoologist* **31**, 83A-83A.

Whittington, C. M. & Friesen, C. R. (2020). The evolution and physiology of male pregnancy in syngnathid fishes. *Biological Reviews of the Cambridge Philosophical Society* **95**, 1252-1272.

Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B. & Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution* **32**, 3114-3131.

Whittington, C. M., Musolf, K., Sommer, S. & Wilson, A. B. (2013). Behavioural cues of reproductive status in seahorses *Hippocampus abdominalis*. *Journal of Fish Biology* **83**, 220-226.

Witten, P., Huysseune, A., Franz-Odendaal, T., Fedak, T., Vickaryous, M., Cole, A. & Hall, B. K. (2004). Acellular teleost bone: primitive or derived, dead or alive? *The Palaeontology Newsletter* **55**, 37-41.

Witten, P. E. & Huysseune, A. (2009). A comparative view on mechanisms and functions of skeletal remodelling in teleost fish, with special emphasis on osteoclasts and their function. *Biological Reviews of the Cambridge Philosophical Society* **84**, 315-346.

Wourms, J. P. (1981). Viviparity: the maternal-fetal relationship in fishes. *American Zoologist* **21**, 473-515.

Wourms, J. P., Grove, B. D. & Lombardi, J. (1988). The maternal-embryonic relationship in viviparous fishes. In: *Fish Physiology*, pp. 1-134: Elsevier Science & Technology.

Ye, C.-X., Liu, Y.-J., Tian, L.-X., Mai, K.-S., Du, Z.-Y., Yang, H.-J. & Niu, J. (2006). Effect of dietary calcium and phosphorus on growth, feed efficiency, mineral content and body composition of juvenile grouper, *Epinephelus coioides*. *Aquaculture* **255**, 263-271.

Chapter 5: Acid mucin acellular layer and oviductal glycoprotein 1 in the brood pouch of the male-pregnant pot-bellied seahorse, *Hippocampus abdominalis*

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Title: Acid mucin acellular layer and oviductal glycoprotein 1 in the brood pouch of the male-pregnant pot-bellied seahorse, *Hippocampus abdominalis*

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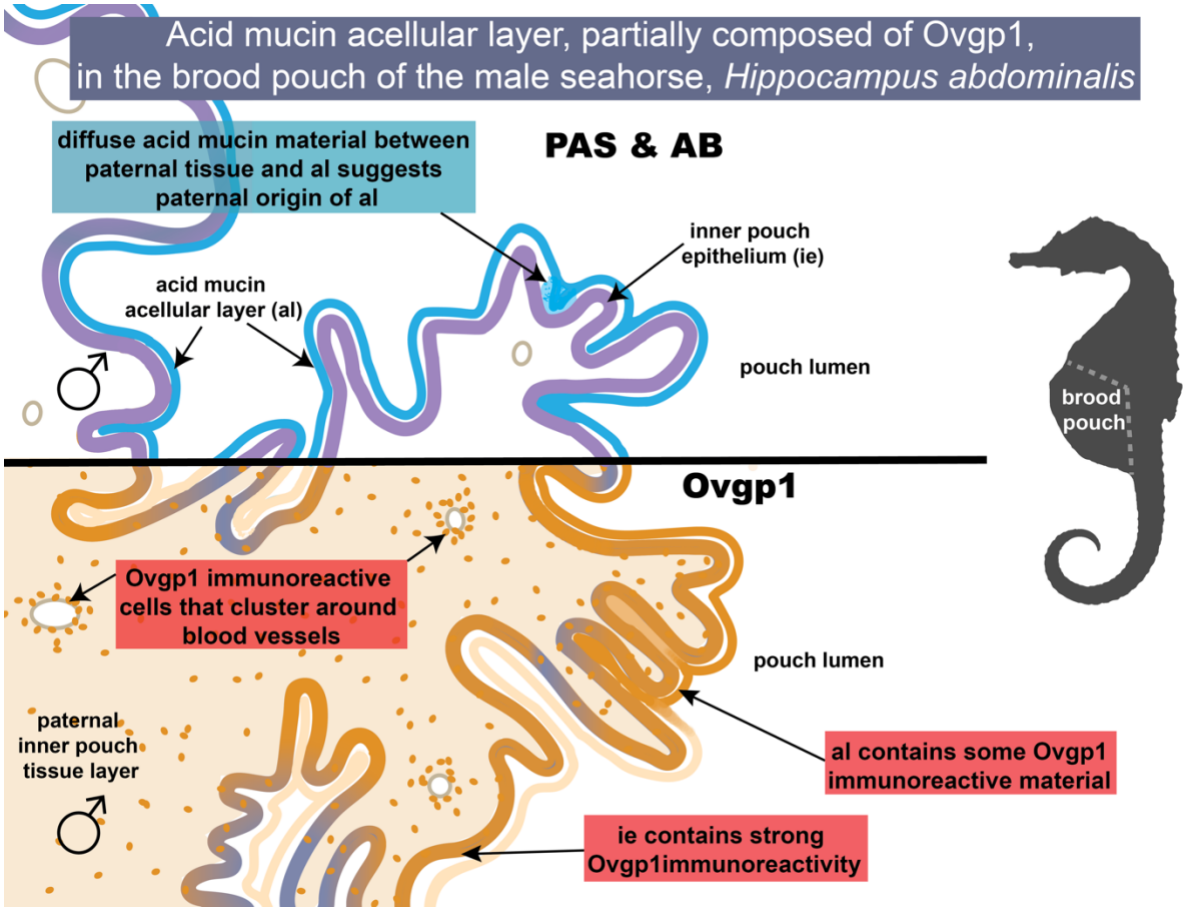
Abstract

Seahorses display a rare form of reproduction whereby fertilisation and incubation occurs inside the male, in a specialised structure, the brood pouch. We characterise acid mucins in an acellular layer identified in the brood pouch of the male-pregnant pot-bellied seahorse, *Hippocampus abdominalis*. The layer closely apposes the brood pouch epithelial lining, in pregnant animals only. Oviductal glycoprotein 1 (Ovgp1) is a minor constituent of the acellular mucin layer and is also present in both the external pouch epithelium and brood pouch inner tissue layer across all reproductive stages, although it is more localised to the inner epithelium in pregnant stages compared to non-pregnant. We speculate that the acellular layer is likely to be paternally-derived, but the presence of acid mucins and Ovgp1 in the ovaries and oocytes of the female *Hippocampus abdominalis* cannot exclude the possibility that the layer may be partially maternally-derived. The acellular layer may function as physical protection for paternal tissue, whilst also facilitating fertilisation, embedment, nutrient transport, and immunological protection of the embryos throughout pregnancy.

Key words: Ovgp1, syngnathid, embryogenesis, immunohistochemistry

Graphical Abstract



Introduction

Fishes are the most reproductively diverse group of vertebrates, with a wide range of maternal and paternal care strategies, making them crucial comparative models in studying the evolution of parental care and investment [1-5]. The Syngnathidae (seahorses, pipefish, and seadragons) are one of the few vertebrate taxa that display male pregnancy, whereby fertilisation and gestation occur on or inside the male in a specialised structure called the brood pouch [5, 6]. Male pregnancy in syngnathids is analogous to viviparity (the retention of embryos in the female reproductive tract followed by birth of live young) [1, 7, 8]. Complex morphological and physiological adaptations for the protection and provisioning of embryos are present in both viviparity and male seahorse pregnancy.

The anatomy of the brooding structure in syngnathids varies greatly across syngnathid species [9-11]. Seahorses (genus *Hippocampus*) have the most complex brooding structures, with a fully enclosed brood pouch that undergoes extensive physiological changes during gestation [reviewed in 6, 12]. These physiological changes in the seahorse brood pouch allow for nutrient transport and both physical and immunological protection of offspring during embryogenesis [reviewed in 6, 12]. Additional putative functions of the seahorse brood pouch include waste removal and osmoregulation [13]. Following female egg transfer into the male's brood pouch, embryos embed non-invasively in pits of the pouch wall, and many are completely enveloped in paternal epithelial tissue [11, 14-17]. The inner layer of the brood pouch closely apposes the embryonic egg coat and facilitates molecular exchange, and thus is analogous to the maternal portion of the mammalian placenta [12, 18-20]. In fact, the inner layer forms a functional placenta, which is defined as 'any intimate apposition or fusion of the fetal organs to the maternal [or paternal] tissues for physiological exchange' [21, p156]. During seahorse pregnancy, nutrients, including lipid and protein, are transported from the gestating father to the embryos across this structure [17, 22]. Expansion of the brood pouch vascular bed during pregnancy also allows for exchange of respiratory gases and other molecules, to meet the demands of embryonic development [23].

The black-striped pipefish, *Syngnathus abaster*, has a less complex brood pouch than seahorses, described here. In this species, embryos are also embedded into the brood pouch, into pits which fill with acellular mucous material that surrounds the embryos during gestation [15]. Following dissection and fixation, the mucous between the brood pouch and

embedded embryos in *S. abaster* appears as a hard thick layer, closely associated with the egg coat on the luminal side, and with the brood pouch epithelium on the other [15]. An acellular layer has also been observed in the pregnant seahorse, *Hippocampus abdominalis,* closely apposed to the inner brood pouch epithelium and embryos, and is hypothesised to be comprised of mucins [23]. However, the role and origins of the layer are unknown.

Here, we aimed to confirm the presence of the acellular layer in the brood pouch epithelium of the Australian pot-bellied seahorse, *H. abdominalis*, characterise its composition by determining the presence of any mucins in the layer, and determine the likely origin of the acellular layer. We subsequently aimed to characterise a reproductive-specific mucin that may contribute to the acellular layer, by localising oviductal glycoprotein 1 (Ovgp1) to brood pouch tissues using immunohistochemistry. This gene is significantly upregulated during *H. abdominalis* pregnancy compared to the non-pregnant pouch [13], and is speculated to contribute to an increase in PAS positive staining seen in pregnant and post-parturition pouch tissues of *Hippocampus erectus* [24].

Methodology

1. Husbandry

Reproductively mature *H. abdominalis* were acquired from a captive-bred population (Seahorse Australia, Tasmania, Australia) and housed under previously described aquarium conditions [25]. To facilitate breeding, animals were housed under a summer light cycle, with 15.5 hr photoperiods, including simulated dawn and dusk periods of 1 hr each. Male and female *H. abdominalis* were rotated through a 750 L breeding tank as described in Skalkos, et al. [17]. Males were tagged with non-invasive small glass bead 'necklaces', as in Whittington, et al. [25]. Reproductive status was determined by behavioural assays described by Whittington, et al. [25], which identifies 94 % of pregnant males when used over three consecutive testing days. All adult fish were fed equal amounts and type of *Mysis* spp. shrimp (thawed ocean Nutrition frozen Mysis, Newark) throughout the study. After breeding, pregnant males were housed until euthanasia before brood pouch and other tissues were collected.

2. Sample collection

Adult male and female seahorses were euthanised by overdose of anaesthetic (ethanol), followed by decapitation and immediate pithing, or immediate decapitation and pithing [26, 27] (AVMA 2013, University of Sydney Animal Ethics Committee approval 2018/1302; AVMA 2020, University of Sydney Animal Ethics Committee approval AEC 2021/1995). The numbers of individuals at each male reproductive stage examined for mucin characterisation were non-pregnant (n = 7), early pregnancy (≤ 25 % of the way through embryonic development, i.e. eye development) (n = 2), mid-pregnancy (~ 65 % of the way through embryonic development, i.e. frontal jaw development) (n = 3), late pregnancy (embryos with protruding snouts in the last third of development) (n = 5) and post-parturition (< 24 hrs post parturition) (n = 1) [embryonic stages as per 28]. The number of individuals at each male reproductive stage examined via immunohistochemistry of Ovgp1 were non-pregnant (n = 5), early pregnant (3 – 6 days post-fertilisation) (n = 6), mid-pregnant (14 – 17 days post-fertilisation) (n = 5), late-pregnant (21-24 days post-fertilisation) (n= 4) and post parturition (<18 hours post parturition) (n = 5). Brood pouches were excised whole from the body followed by a sagittal cut along the midline from the opening of the pouch to the caudal extremity. Each sagittal half was cut approximately in half in transverse orientation, yielding 4 quadrants. Small pieces (~1 cm3) of pouch tissue were also excised for western blot analysis. Ovaries from two females were also collected, to determine the potential presence of mucins in ovarian tissue. Ovaries were excised from females and either kept whole, or transversely cut in half. Ovary and brood pouch tissue were fixed in 10 % neutral buffered formalin (NBF) for ~ 24 hours, then rinsed and stored in 70 % ethanol. During fixation and tissue processing, almost all pouch-embedded embryos became naturally detached from the samples.

3. Mucin characterisation of the acellular layer and ovaries

Brood pouch and ovarian tissues were dehydrated with ethanol and cleared with xylene using a Thermo Scientific Excelsior ES Tissue Processor, then embedded in paraffin using a Tissue-Tek TEC embedding station (both ThermoFisher Scientific, Waltam). Transverse sections of 7 μm thickness were cut from each paraffin block using a HM 325 Rotary Microtome (ThermoFisher Scientific). Sections were hydrated (modified from Kiernan (2015)) and stained with Alcian blue (AB) at pH 2.5, Periodic acid-Schiff (PAS), or

combined. Sections were stained with AB for 45 minutes and then washed before being immersed in 1% periodic acid for 10 minutes, followed by Schiff's reagent for 6 minutes, then washed. All sections were counterstained with haematoxylin. All images were taken using a Leica ICC50 W (Leica, Sydney). Archived seahorse and pre-existing human gastrointestinal tract tissue blocks were used as positive controls for PAS and AB staining.

4. Western Blot

Western blotting was performed to validate the Ovgp1 antibody in *H. abdominalis*. A qualitative western blot was performed to confirm the presence of Ovgp1 at the correct molecular weight, in the brood pouch of *H. abdominalis*.

4.1 Tissue homogenisation and protein quantification

One early-stage and one late-stage sample of male pouch tissue (~ 3 mm cube) was homogenised with homogenising beads in lysis buffer (50 mM Tris-HCl, 1mM EDTA, 150 mM NaCl, 1 % Igepal, MilliQ H_20; Sigma-Aldrich, Victoria Australia) with protease inhibitor cocktail (Sigma-Aldrich). Homogenised tissue was centrifuged 12,000 rcf at 4 ^oC for 10 minutes. The supernatant was then removed, aliquoted, snap frozen in liquid nitrogen, and stored at -80 ^oC. A BCA assay was used to determine protein concentration of each resulting sample. (ThermoFisher Scientific, CN 23235). Protein content was calculated from absorbance readings taken by a CLARIOstar Plus microplate reader (BMG Labtech, Mornington).

4.2 Western blot analysis

Protein samples (20 ⌈g) were combined with sample loading buffer (8% glycerol, 50 mM Tris-HCl pH 6.8, 1.6 % SDS, bromophenol blue and 4 % ®-mercaptoethanol) and water (7.08:4:8.92 ⌈L) and heated at 95 ^oC for 5 minutes. The protein samples and molecular weight marker (Thermo Fisher Scientific, CN 26616) were loaded into 10 % TGX FastCast acrylamide gels (Bio-Rad Laboratories, San Diego). Samples were separated by SDS-PAGE gel electrophoresis at 200 V for 35 minutes and then transferred to an immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Danvers) at 100 V for

1.5 hours. Membranes were blocked with 5 % powdered skim milk diluted in Tris-buffered saline containing 0.05 % Tween 20 (TBST) for 1 hour at room temperature. Membranes were then incubated with rabbit Ovgp1 polyclonal IgG, unconjugated primary antibody (ABclonal, Woburn, PC A13036, LN 0063540101) in 1 % powdered skim milk in TBST at 0.26 ⌈g/mL overnight at 4 ºC. Membranes were washed three times in TBST, 10 minutes each time. All subsequent TBST washes were identical. Membranes were then incubated in secondary HRP-conjugated goat anti-rabbit secondary antibody (Dako, Denmark) diluted to 0.15 ⌈g/mL in 1 % skim milk in TBST for 2 hours at room temperature. Membranes were again washed in TBST, then visualised using the Bio-Rad ChemiDoc MP System (Bio-Rad Laboratories, Hercules) and associated Image Lab software (version 6.1.0, Bio-Rad Laboratories).

5. Immunohistochemistry of Oviductal glycoprotein 1

Transverse brood pouch and ovary sections were cut at 7 ⌈m from each block using a HM 325 Rotary microtome (ThermoFisher Scientific) at room temperature and mounted onto glass slides. Sections were dried at 38 ºC overnight. Four slides with two to eight sections on each were collected per animal. Archival mouse oviduct tissue was used as a positive control for each run. Two slides were randomly selected as experimental slides and the remaining two were used as negative and non-immune IgG control slides. Sections were cleared with xylene, rehydrated through a series of decreasing ethanol concentrations, and rinsed with water. Antigens were unmasked by immersing the slides in sodium citrate buffer (10 mM Sodium Citrate, 0.05 % Tween 20, pH 6.0) which was first heated to ~ 95 ºC or until opaque, then cooled to a translucent ~ 80 – 85 ºC before exposure to the slides. Slides were immersed at this temperature for 20 minutes. Slides were cooled for 20 minutes, then washed in phosphate-buffered saline with 0.05 % Tween 20 (PBS-T), three times for five minutes each wash. All subsequent PBS-T washes were identical. Slides were blocked with 1 % bovine serum albumin (BSA) in PBS-T solution for 1 hour. Rabbit Ovgp1 antibody was diluted to 5.2 ⌈g/mL in 1 % BSA/PBS-T solution (1 ⌈g /mL). The experimental sections were covered with the diluted primary antibodies. The negative control section was covered with 1 % BSA/PBS-T solution only. The non-immune IgG control section was covered with a solution of non-immune IgG control antibodies (Jackson ImmunoResearch, Pennsylvania) in BSA at a concentration of 5.2 ⌈g/ml to match the concentration of the primary antibody. All sections were incubated at 4 ºC overnight. Sections were washed with PBS-T, blocked in 0.3 %

hydrogen peroxide in PBS-T solution for 15 minutes and washed with PBS-T again. Secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Dako, Glostrup, Denmark) was diluted in 1 % BSA/PBS-T solution (0.02 ⌈g /mL). All sections were covered with the diluted secondary antibody and incubated at room temperature for 2 hours. All sections were washed with PBS-T and then incubated with 3,3' -diaminobenzidine-tetrahydrochloride (DAB) substrate (Sigma-Aldrich; St Louis, MO) for 20 minutes. Nuclei were counterstained with hematoxylin. Sections were dehydrated to 100 % ethanol, cleared in xylene and cover slipped with DPX mounting medium (Sigma-Aldrich, Melbourne). An Olympus BX53 microscope (Olympus, Tokyo, Japan) was used to view and image the slides. All images were processed and labelled using Adobe Photoshop (2024, 25.0.0).

Results

1. Mucin characterisation of the acellular layer and ovaries

The acellular layer is closely apposed to the inner epithelial layer of the paternal gestational tissues (Figure 1A-E.), and is present in early-, mid-, and late-pregnant brood pouch. It is not present in the brood pouch of non-pregnant or post-parturition animals [see Figure 2B-E in 23]. The layer consistently stained positively for PAS throughout pregnancy (Figure 1B,C). Positive PAS staining suggests the presence of polysaccharides and/or mucosubstances (e.g. neutral and/or acid mucins) [29, 30]. The acellular layer also stained positively for AB throughout pregnancy. Positive AB staining specifically confirms the presence of acid mucin [29, 31]. When both PAS and AB were used simultaneously, the AB stain overwhelms the PAS stain, and the acellular layer appears blue (Figure 1D,E). The haematoxylin counterstain confirms that the layer is acellular, as no nuclei are present. In female seahorses, the oocyte yolk within the ovaries stained positively for PAS, and the ovarian stroma and cortical alveoli in oocyte cytoplasm stained positively for AB (Figure 1F).

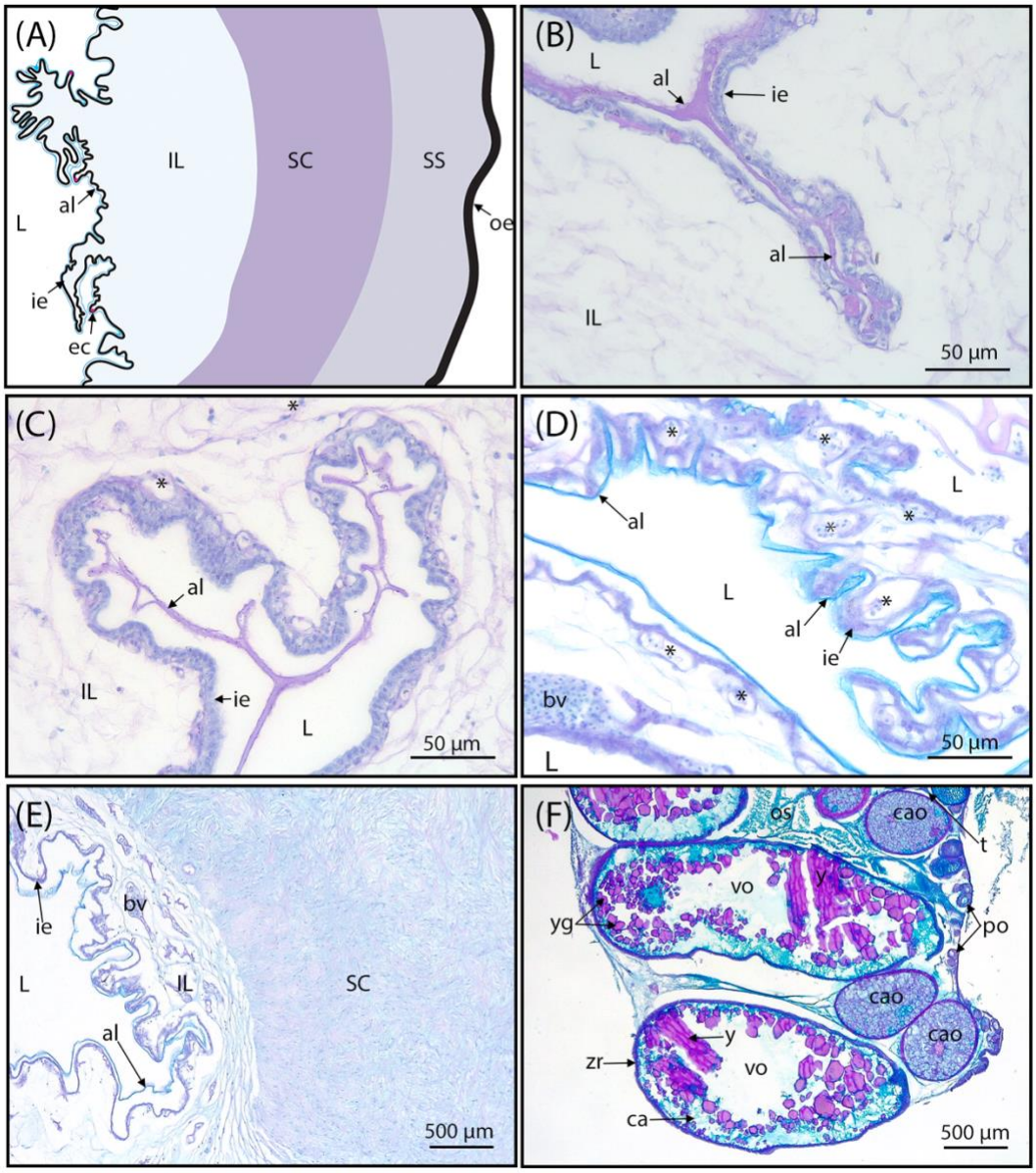


Figure 1. *Representative transverse sections of pregnant* Hippocampus abdominalis *brood pouch showing the acellular layer lining the interior of the brood pouch. During fixation and tissue processing, pouch-embedded embryos became naturally detached from the samples so are absent from the images. The acellular layer encompasses and closely apposes the inner epithelium of the brood pouch in pregnant animals. A diagram of the layers of the brood pouch in transverse orientation, including eosinophilic cells observed in Dudley, et al. [23] and the inner tissue layer comprised of diffuse connective tissue with extensive vasculature, that represents the paternal side of a functional placenta. The brood pouch exhibits PAS + AB + staining in the lumen and across the pouch tissue layers. (**A**). Periodic acid-Schiff (PAS) staining in early pregnancy (**B**) and late pregnancy (**C**). Alcian Blue (AB) and PAS staining in late pregnancy (**D,E**). All are counterstained with Haematoxylin. The ovary (**F**) of*

H. abdominalis*, stained with PAS and AB and counterstained with Haematoxylin. Tissue stained pink/purple is positive for PAS, whilst tissue stained light blue is positive for AB. The ovary displays oocytes in differing developmental stages within their developing follicles. Oocytes at each stage exhibit PAS + AB + staining. Abbreviations: Acellular layer (al), Inner pouch epithelium (ie), Inner tissue layer (IL), Lumen (L), Outer pouch epithelium (oe), Stratum compactum (SC), Stratum spongiosum (SS). Symbols: Capillaries (*). Cortical alveoli (ca), Cortical alveoli oocyte (cao), Ovarian stroma (os), immature oocytes up to and including the primary growth phase (po), Theca (t), Vitellogenic oocyte (vo), Yolk (y), Yolk globules (yg), Zona radiata (zr).*

2. Localisation of Ovgp1 to the brood pouch and ovaries

2.1. Pregnant H. abdominalis

In all pregnancy stages, the inner tissue layer and inner pouch epithelium exhibit Ovgp1 immunoreactivity (Figure 2). There is variable positive staining at the apical and basal surfaces of the inner epithelium (Figure 2A-D). The acellular layer inconsistently stains positively for Ovgp1 (Figure 2C,D). The acellular layer in pregnant samples for immunohistochemistry was not always as clearly visible in comparison to our histology results, likely due to the additional treatment of tissue, or because the layer is more difficult to distinguish from paternal or embryonic tissues when unstained (Figure 2D). Positively staining amorphous globules consistently line the base of the inner pouch epithelium (Figure 2A-D). Positive staining cells (psc) are abundantly scattered throughout the inner tissue layer and form clusters surrounding the borders of capillaries and blood vessels (Figure 2A). All non-immune and negative controls exhibited no staining (Figure 2E), signifying there is no non-specific binding of the primary or secondary antibodies, and exogenous peroxidases were successfully blocked.

Western blotting identified Ovgp1 protein at ~79 kDa (Figure 2F), which is within the expected range, thereby validating the primary antibody. Mature and fully glycosylated Ovgp1 varies in molecular weight (90–95 kDa in domestic animals; 110–150 kDa in primates; 160–350 kDa in rodents) [32]. However, the size of the protein core of several species studied to date has a molecular weight of approximately 70 kDa [e.g. 32, discussed in 33], with the observed molecular weight of the protein core this Ovgp1 antibody targets to be

75 kDa. The variability in molecular weight is attributed to differences in glycosylation patterns [34, 35].

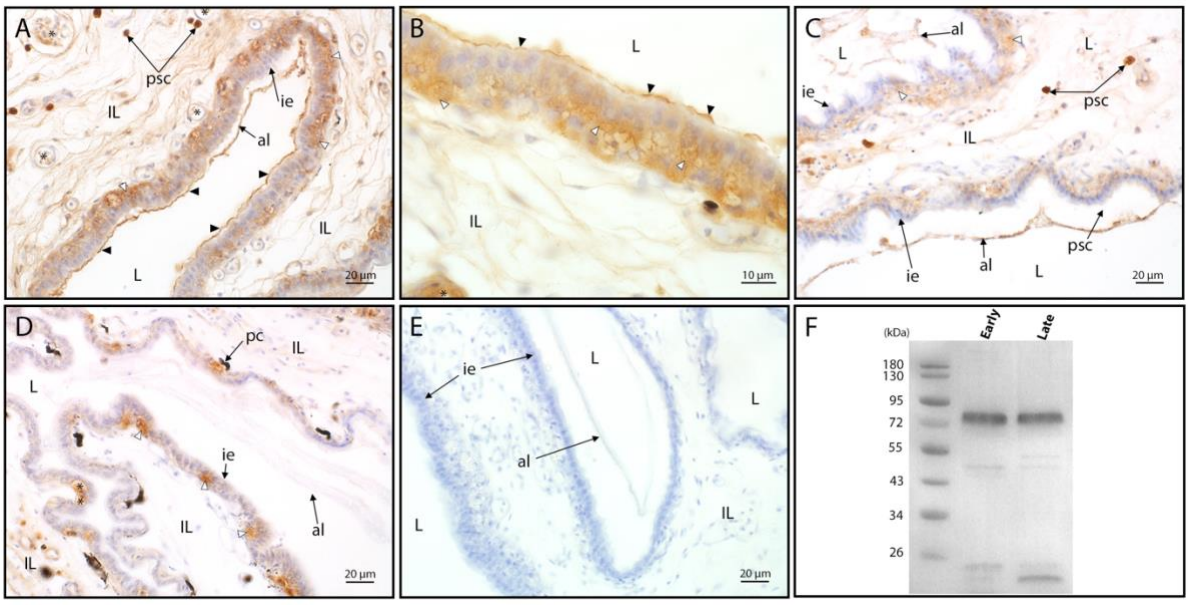


Figure 2. *Immunolocalisation of* Ovgp1 *in the brood pouch of pregnant (early, mid and late-stage)* Hippocampus abdominalis. *The brown precipitate (DAB) represents positive staining for* Ovgp1. ***A****: Immunoreactivity for* Ovgp1 *at the basal and apical surfaces of the inner epithelium and in the acellular layer.* ***B****: Immunoreactivity for* Ovgp1 *in the inner epithelium of the brood pouch, with strong staining at the apical surface.* ***C****: Immunoreactivity for* Ovgp1 *at the base of the inner epithelium and in the acellular layer.* ***D****: Immunoreactivity for* Ovgp1 *at the base of the inner epithelium and not in the acellular layer.* ***E****: Non-immune control of the brood pouch inner epithelium and acellular layer.* ***F****: Western blot of* Ovgp1 *protein in early and late-stage pregnancy brood pouch, with a molecular weight of ~79 kDa. Abbreviations: Acellular layer (al), Inner pouch epithelium (ie), Inner tissue layer (IL), Lumen (L), Pigment cells (pc), Positive staining cells (psc), Symbols: Capillaries (*),* Ovgp1 *staining amorphous globules (white arrowheads),* Ovgp1 *staining apical surface of inner pouch epithelium (black arrowheads).*

In the few early pouch sections where embedded or hatched embryos remained intact and in contact with the brood pouch throughout fixation and tissue processing, embedded embryos are very closely apposed to the paternal inner epithelium (Figure 3), with a faint or absent acellular layer (Figure 3A,B). All non-immune and negative controls exhibited no staining (Figure 3C).

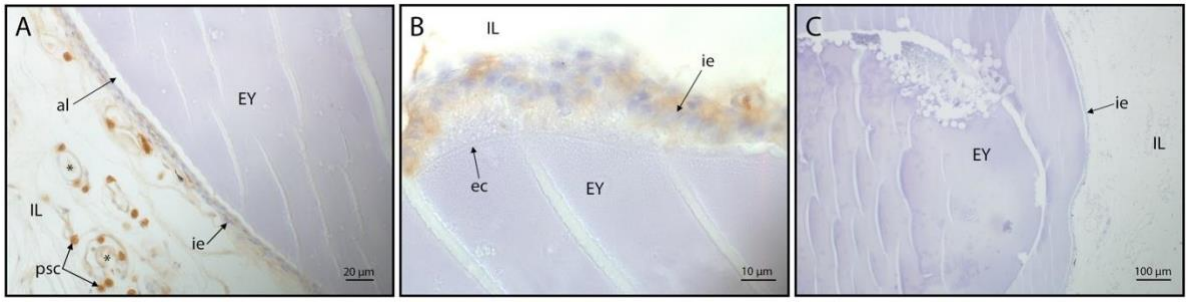


Figure 3. *Immunolocalisation of* Ovgp1 *protein at the early embryo-paternal tissue interface of* Hippocampus abdominalis. *The brown precipitate (DAB) represents positive staining for* Ovgp1. ***A****: Early-stage embryos (3 – 6 days post-fertilisation) embedded into the paternal brood pouch with a faint unstained acellular layer in between embryonic and paternal tissue.* ***B****: Early-stage embryos (3 – 6 days post fertilisation) embedded into the paternal brood pouch with no acellular layer visible. No positive staining on the outside of the embryonic yolk. Egg coat visible.* ***C****: Non-immune control of early-stage embryo embedded into the paternal brood pouch. Abbreviations: Acellular layer (al), Embryonic coat (ec), Embryonic yolk (EY), Inner tissue layer (IL), Acellular layer (al), Inner epithelium of the brood pouch (ie), Positive staining cells (psc). Symbols: Capillaries (*).*

2.2. Post-parturition and non-pregnant H. abdominalis

In post-parturition (PP) pouch tissue the apical surface of the epithelium appears to have less staining than the pregnant pouch (Figure 4A-C), although quantification would be required to confirm. There is no acellular layer present. There are psc present in the inner tissue layer of the pouch and stained amorphous globules in the inner epithelium (Figure 4A-C). All non-immune and negative controls exhibited no staining. All non-pregnant (NP) tissues exhibited minimal staining in the inner epithelium, with some psc throughout the inner tissue layer (Figure 4D-F). There is no acellular layer present. Ovgp1 was immunolocalised to positive control mouse tissue for validation (Figure 5).

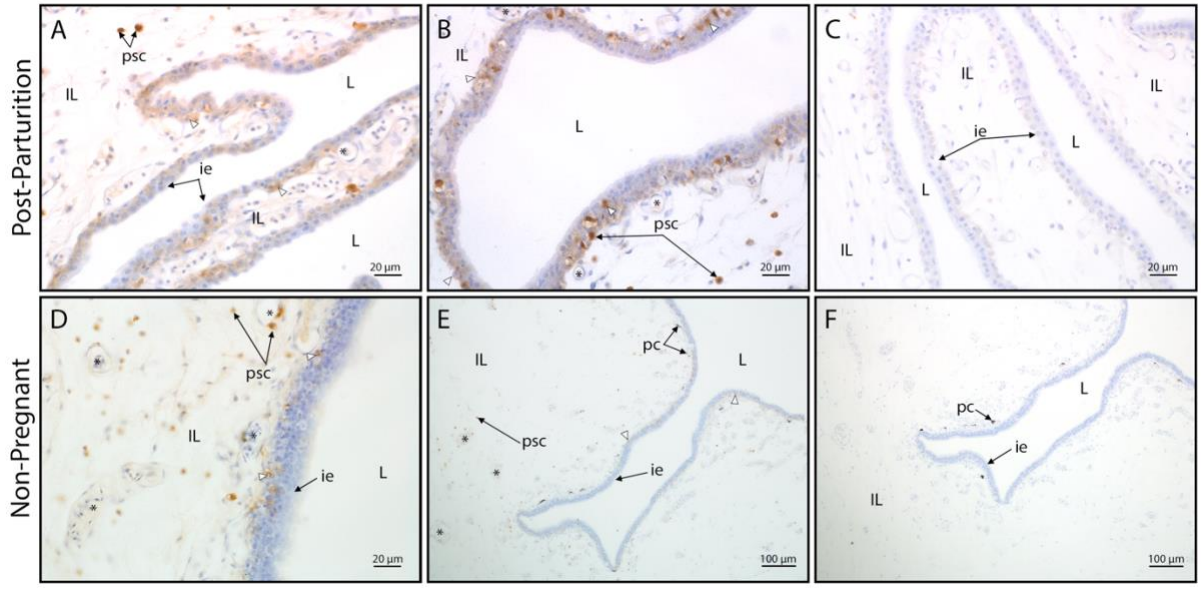


Figure 4. *Immunolocalisation of* Ovgp1 *to the brood pouch tissue of post parturition and non-pregnant* Hippocampus abdominalis. *The brown precipitate (DAB) represents positive staining for* Ovgp1. ***A,B****: Post-parturition pouch tissue, displaying positive staining cells in the inner tissue layer and substantial amorphous globular positive staining at the base of the inner epithelium. No acellular layer present.* ***C****: Post-parturition* H. abdominalis *non-immune control.* ***D,E****: Non-pregnant pouch tissue, displaying positive staining cells in the inner tissue layer and minimal amorphous globular positive staining at the base of the inner epithelium. No acellular layer present.* ***F****: Non-pregnant* H. abdominalis *non-immune control. Abbreviations: Inner pouch epithelium (ie), Inner tissue layer (IL), Lumen (L), Pigment cells (pc), Positive staining cells (psc), Symbols: Capillaries (*),* Ovgp1 *staining amorphous globules (white arrowheads).*

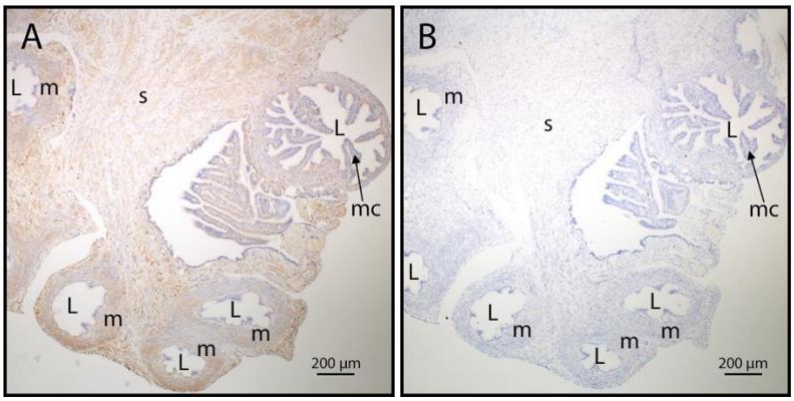


Figure 5. A: *Positive control mouse oviduct immunolocalisation of* Ovgp1. ***B****: Mouse oviduct non-immune control. Abbreviations: Lumen (L), Tunica mucosa (mc), Tunica muscularis (m), Tunica serosa (s).*

2.3. Ovary of H. abdominalis

The ovary stains positively for Ovgp1 (Figure 6). Vitellogenic oocytes and cortical alveoli oocytes contain no positive staining except at the follicle/egg-coat interface (Figure 6A,B). Conversely, there is strong staining within less mature oocytes up to and including the primary growth stages at the germinal ridge (Figure 6B,C). The luminal and external germinal epithelium stains positively for Ovgp1 (Figure 6A,B). No staining is exhibited in non-immune and negative controls (Figure 6D-F).

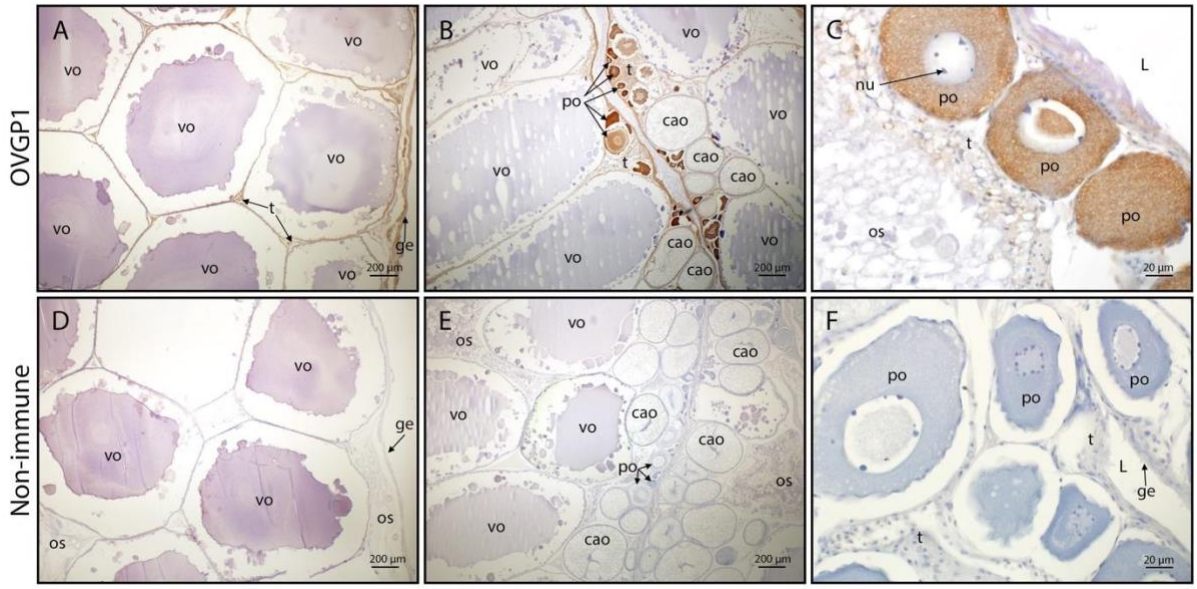


Figure 6. *Immunolocalisation of* Ovgp1 *to the ovarian tissue of reproductively active* Hippocampus abdominalis. *The brown precipitate (DAB) represents positive staining for* Ovgp1. ***A****: Ovarian theca and germinal epithelium stain positively for* Ovgp1. *Vitellogenic oocytes stain lightly along the follicle cells and egg coat, but not inside the oocytes.* ***B:*** Ovgp1 *is highly concentrated at the ovarian germinal ridge, in which the theca and immature (up to and including primary growth stage) oocytes stain very strongly.* ***C****: Immature oocytes staining strongly for* Ovgp1. ***D-F****: Respective non-immune controls. Abbreviations: Cortical alveoli oocyte (cao), Ovarian stroma (os), Germinal epithelium (ge), Immature oocytes up to and including the primary growth phase (po), Vitellogenic oocyte (vo), Yolk (y), Yolk globules (yg), Theca (t), Ovarian lumen (L). Nuclei are counterstained by hematoxylin.*

2.4. Male skin of H. abdominalis

The outer pouch epithelium (skin) of *H. abdominalis* stains positively for Ovgp1 (Figure 7) across all reproductive stages. The outer pouch epithelium contains positively staining amorphous globules, dispersed psc, and mucous globule contents (Figure 7A-C). The microridges of flame cone cells and their mucous caps exhibit positive staining for Ovgp1 (Figure 7A-C). Non-immune and negative controls exhibit no staining (Figure 7D).

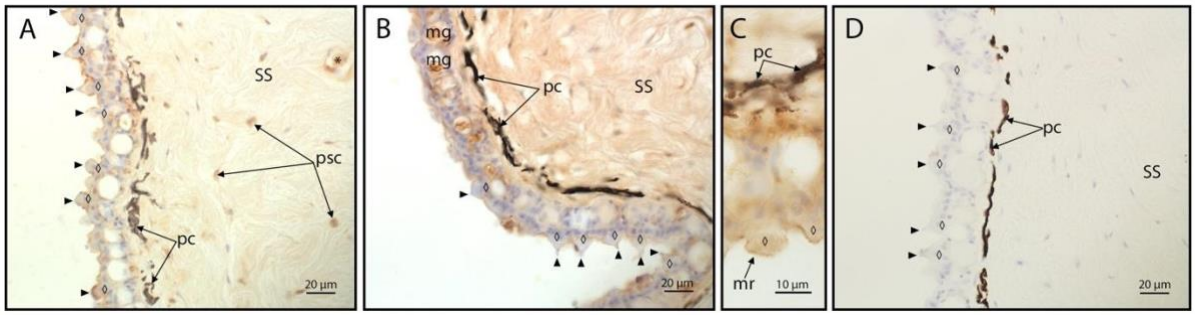


Figure 7. *Immunolocalisation of* Ovgp1 *to the outer pouch epithelium (skin) of male* Hippocampus abdominalis *pouch tissue. The brown precipitate (DAB) represents positive staining for* Ovgp1. ***A****: Outer pouch epithelium where flame cone cells contain positive staining in the mucous cap and the stratum spongiosum contains some positive staining cells.* ***B****: Outer pouch epithelium with positive staining amorphous material within mucous granules and throughout the epithelium, with some staining in the flame cone cells and their mucous caps.* ***C****: An outer pouch epithelium flame cone cell with positive staining micro ridges visible.* ***D****: Non-immune control of outer pouch epithelium. Abbreviations: Micro ridges of flame cone cells (mr), Mucous granules (mg), Pigment cells (pc), Positive staining cells (psc), Stratum spongiosum (SS). Symbols: Capillaries (*), Flame cone cells (◊), Mucous caps of flame cone cells (black arrowheads).*

Discussion

Here, we identify that an acellular layer previously identified at the paternal-embryo interface in *H. abdominalis* [23] closely interdigitates with the brood pouch epithelium, is exclusive to pregnancy, and is not present in post-parturition or non-pregnant tissues. We aimed to characterise the mucin content of this acellular layer and examine whether Ovgp1, a reproductive-specific mucin, contributes to this layer in the brood pouch.

1. The acellular layer and mucins in the brood pouch

1.1. *Acid mucins in the brood pouch and acellular layer*

In the pregnant *H. abdominalis* brood pouch, the acellular layer consistently stains positively with PAS and AB-positive mucins, indicating that mucins are a substantial component of the layer, specifically acid mucins. Interestingly though, the pregnant brood pouch of *H. erectus* [24], and *S. abaster* [15], contain only PAS-positive material and neither PAS nor AB-positive material, respectively, indicating that mucin composition may vary between species. Mucins are a family of glycoproteins that are abundant in non-sterile parts of the body including skin, digestive, and reproductive tracts of marine vertebrates [36, 37]. Seahorse brood pouches are derived from skin [19], and during pregnancy, are non-sterile environments [12], where mucins are likely to be present [23, 37, 38]. Fish skin is covered with a mucous epidermis, which is responsible for immunological protection against microbial adherence, colonisation, and attack [39-42], physical protection [36, 43, 44], osmoregulation [44, 45], and gas exchange [45]. Other roles of mucin lubrication on epithelial surfaces includes protection against dehydration [46-50], modulating cell attachment [51-54], and inhibiting immune cell function [48, 55, 56].

1.2. *Ovgp1 in the brood pouch and acellular layer*

Oviductal glycoprotein 1, also known as oviductin, estrogen-dependent oviduct protein, oviduct-specific glycoprotein, or MUC9, is the major glycosylated protein secreted by mammalian oviductal epithelium [32]. Oviductal glycoprotein 1 is classified as a member of the mucin family [57], plays important roles in gametogenesis, fertilisation, gamete transport and early embryogenesis including facilitating trophoblast adhesion [discussed in 32, 58] and has been characterised in the oviductal epithelium of various mammals such as humans [59], monkeys [60, 61], canines [62], and livestock [e.g. 63, 64]. Oviductal glycoprotein 1 has also been detected in other female reproductive tissues [61, 65-68], and is expressed in mice oviducts during fertilisation, endometrium during implantation, and testis and epididymis of males [69,70], suggesting specific roles in both female and male gonadal physiology. However, Ovgp1 has not yet been localised or characterised in non-mammalian species. Oviductal glycoprotein 1 is prevalent in the inner tissue layer and inner epithelium in the pregnant brood pouch of *H. abdominalis*. The inconsistent Ovgp1 immunoreactivity of the

acellular layer that we identified here suggests Ovgp1 is present in the layer but is not the primary component. The patchy staining of OVGP1 may indicate the protein controls the spacing of embryos, facilitating where they embed. Brood pouch transcriptome data of *H. abdominalis* [13] identified multiple other mucin genes that are highly upregulated during pregnancy that may contribute more to the acellular layer including *muc2* (secretory), *muc5*, and *muc13*. The presence of the acellular mucous layer and its constituents raise important questions for its function in the pregnant brood pouch.

In mammals, Ovgp1 plays a key role in early pregnancy, with uterine OVGP1 expression decreasing after early pregnancy [e.g. 34, 70]. In contrast, the stage with the greatest upregulation of Ovgp1 expression in the pouch transcriptome of *H. abdominalis*, is late pregnancy [13]. *Hippocampus abdominalis* is the first species in which Ovgp1 expression is consistently present in the tissue where both fertilisation and gestation occur, regardless of reproductive stage. This consistency implies Ovgp1 functions are not exclusive to reproduction, and that it may be constitutively expressed in skin (from which the brood pouch is derived, [19]), and/or function in pouch maintenance and immunological protection of the father during non-pregnancy. For example, the psc are present in pregnant, post-parturition and non-pregnant tissues and thus, we suspect are unlikely to have a reproductive role. These psc dispersed throughout the inner tissue layer of the pouch tissue, particularly around blood vessels have not been described in mammalian reproductive tissues and have only been characterised in *H. abdominalis*. In contrast, the Ovgp1 immunolocalisation in the epithelium of *H. abdominalis* brood pouch during pregnancy, both at the apical surface and the amorphous globules within epithelial cells, appears similar to the reproduction-specific Ovgp1 staining in the oviducts of the olive baboon (*Papio anubis*) [61], domestic dog (*Canis lupus familiaris*) [62], and bovine (*Bos taurus*) [71]. These similarities in localisation to mammalian ovarian tissues suggest some functional parallels. The reduced Ovgp1 immunoreactive material in post-parturition and minimal staining in non-pregnant at the inner epithelium implies that Ovgp1 is important during pregnancy. We hypothesise that Ovgp1 plays a functional role in seahorse pregnancy but is not exclusive to reproduction as seen in mammals.

1.3. *Ovgp1 in the outer pouch epithelium*

In *H. abdominalis*, the skin is immunoreactive for Ovgp1, which is unsurprising given the pouch's origin, but interesting given the reproduction-specific role of Ovgp1 in mammals. The Ovgp1 immunoreactive flame-cone cells and their associated mucous caps in *H. abdominalis* suggest Ovgp1 plays a part in their hypothesised epiphyte adhesive role [72]. Interestingly, these flame-cone cells are not present in *Urocampus nanus* or *Syngnathus schlegeli* [73], suggesting these cells may be exclusive to seahorses. Moreover, the brood pouch epithelium of *S. schlegelii* and *S. abaster* lacks typical dermal mucal cells [15], indicating the pouch evolved from the skin to also perform more specialised, reproduction-specific functions in the syngnathid family. The acellular layer and presence of Ovgp1 in pregnant *H. abdominalis* likely evolved from the skin mucous layer to provide many of the same protective and adhesive functions mentioned above.

1.4. *Functions of the acellular layer and future directions*

We speculate that the acellular mucin layer protects both the father and embryos from pathogens, and shields embryos from the father's immune response. Bacteriophages, viruses that infect bacteria, bind to mucin glycoproteins [74] in epithelial mucous layers in fishes [75], mammals [74, 75], and the mucous layer of some coral [76], acting as active non-host immunity against bacterial infections [75]. Furthermore, exposure to mucous makes bacteria more susceptible to bacteriophage infections [74-76]. The acellular layer in *H. abdominalis* likely facilitates the adhesion of bacteriophages as an antimicrobial defence. Due to the adhesion, cell attachment modulation and immune inhibiting functions of epithelial mucins, including Ovgp1, we also speculate that the acellular layer may aid in the embedding of embryos into the epithelial lining of the brood pouch [discussed in 32, 77]. While embryos contain no Ovgp1 immunoreactive material, the acellular layer could possibly have a nutritive function if it were broken down to smaller components throughout pregnancy that could be absorbed through the embryonic coat prior to hatching or absorbed/ingested after hatching. Mucoid histotrophe, secreted from the uterus, is a common form of matrotrophy in viviparous sharks [78-81], and the acellular layer may contribute to this in *H. abdominalis*. The mechanisms underlying paternal nutrient transport in seahorses are not yet known and further research is required. To deepen our understanding of paternal nutrient transport in seahorses, we recommend the immunohistochemical localisation of proteins putatively

involved in nutrient transport, to the brood pouch of pregnant seahorses. Tracking labelled nutrients in pregnant seahorses, combined with histology of the pregnant brood pouch and embryos in situ [82] can determine how patrotrophic nutrients are provided across the acellular layer and taken up by embryos. We also recommend scanning electron microscopy of the paternal-embryo interface of embedded embryos to confirm the consistent presence of the mucous acellular layer in between, as observed in *S. abaster* [15]. Transmission electron microscopy may identify cellular structures likely involved in patrotrophy such as vacuoles and vesicles within the inner tissue layer and epithelium of the brood pouch [72], and provide hypotheses for how the acellular layer is involved.

2. Origin of the acellular layer

The origin of the acellular layer present in pregnant *H. abdominalis brood pouch* is currently unknown and could be maternal, paternal or both. Our results show that the acellular layer forms a clearly defined, compact barrier between paternal tissue and the pouch lumen. There is diffuse AB-positive and PAS-positive staining in the paternal epithelium and/or between the acellular layer and the paternal epithelium, which is suggestive of accumulation of a secretory product, making the acellular layer likely paternally derived. Immunohistochemistry also shows Ovgp1 immunoreactive and non-reactive diffuse material at the apical surface of brood pouch inner epithelial cells, which further suggests that the acellular layer is paternally secreted and that Ovgp1 contributes to its formation. The strong Ovgp1 immunoreactivity at the apical surface of the pregnant pouch, compared to the non-pregnant, suggests the secretions contributing to the acellular layer sometimes contain Ovgp1.

However, a maternal contribution to the acellular layer cannot be excluded. Oocytes at all stages of oogenesis in the ovary exhibit positive staining for AB and PAS and thus, this material could be transferred into the brood pouch at oviposition. While immature oocytes stain strongly for Ovgp1, more mature oocytes contain no positive staining inside the egg, but are still surrounded by lightly staining egg coats, follicles, and ovarian theca. This change in immunolocalisation across oogenesis indicates that Ovgp1 may be important during early oogenesis but becomes less so as oocytes mature. If there is maternal contribution to the formation of the acellular layer, there are three possible sources: 1. the egg coat contributing to the formation of the acellular layer after hatching, 2. embryonic secretions, or 3. the

medium in which eggs are transferred to the male's brood pouch during mating. Given seahorse embryos do not hatch out of their egg coats until after the acellular layer is present, the likelihood of the acellular layer deriving from the egg coat itself (option 1) is unlikely. Moreover, anything the embryos secrete pre-hatching must move through the egg coat. For teleosts such as zebrafish (*Danio rerio*), the egg coat is permeable to molecules no larger than 4 kDa [83]. Mucins vary between 200-10,000 kDa [84], making embryonic contribution to the acellular layer (option 2) also unlikely. Furthermore, we found no Ovgp1 staining within embryos, so any Ovgp1 contribution to the acellular layer is unlikely to originate from the embryo. The medium in which eggs are transported into the pouch (option 3) has not yet been characterised, nor is its origin known and thus it is possible that it may contribute to the formation of the acellular layer. We recommend determining the origin of the transport medium and characterising its composition using techniques such as ELISA assays of mucin epitopes [85] and compositional analysis using mass spectrometry [86], to determine if acid mucins are transported into the brood pouch with the eggs.

3. Conclusion

This is the first study to characterise the presence of an acellular layer throughout the reproductive cycle of male pregnant *H. abdominalis*. Our results indicate that Oviductal glycoprotein 1 (Ovgp1) is localised to this layer as well as to the inner epithelial lining of pregnant seahorses. Localisation patterns of Ovgp1 and acid mucins provide evidence for the acellular layer being at least partially paternally secreted. The layer forms a barrier between paternal tissue and the pouch lumen where embryonic development occurs, putatively playing a role in nutrient transfer, and immune protection of embryos throughout pregnancy.

References

1. Wourms JP, Lombardi J. Reflections on the evolution of piscine viviparity. *Am Zool* 1992; **32**:276-293.
2. Woodroffe R, Vincent A. Mother's little helpers: patterns of male care in mammals. *Trends Ecol Evol* 1994; **9**:294-297.
3. Ah-King M, Kvarnemo C, Tullberg BS. The influence of territoriality and mating system on the evolution of male care: a phylogenetic study on fish. *J Evol Biol* 2005; **18**:371-382.
4. Mank JE, Promislow DEL, Avise JC. Phylogenetic perspectives in the evolution of parental care in ray-finned fishes. *Evol* 2005; **59**:1570-1578.
5. Sutton FB, Wilson AB. Parental behavior in fish. In: *Encyclopedia of Reproduction*, 2nd ed. Amsterdam: Elsevier Science & Technology; 2018: 106-114.
6. Stölting KN, Wilson AB. Male pregnancy in seahorses and pipefish: beyond the mammalian model. *BioEssays* 2007; **29**:884-896.
7. Blackburn DG. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *J Morph* 2015; **276**:961-990.
8. Wourms JP, Grove BD, Lombardi J. The maternal-embryonic relationship in viviparous fishes. In: *Fish Physiology*, vol. 11. Amsterdam: Elsevier Science & Technology; 1988: 1-134.
9. Wilson AB, Ahnesjö I, Vincent A, Meyer A. The dynamics of male brooding, mating patterns and sex roles in pipefishes and seahorses (Family Syngnathidae) *Evol* 2003; **57**:1374-1386.
10. Wilson AB, Orr JW. The evolutionary origins of Syngnathidae: pipefishes and seahorses. *J Fish Biol* 2011; **78**:1603-1623.
11. Harada A, Shiota R, Okubo R, Yorifuji M, Sogabe A, Motomura H, Hiroi J, Yasumasu S, Kawaguchi M. Brood pouch evolution in pipefish and seahorse based on histological observation. *Placenta* 2022; **120**:88-96.
12. Whittington CM, Friesen CR. The evolution and physiology of male pregnancy in syngnathid fishes. *Biol Rev Cambridge Philos Soc* 2020; **95**:1252-1272.
13. Whittington CM, Griffith OW, Qi W, Thompson MB, Wilson AB. Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Mol Biol Evol* 2015; **32**:3114-3131.

14. Boisseau JP. Action de quelques hormones sur l'incubation d'hip- pocampes males normaux, ou castres, ou hypophysectomises. *C R Acad Sci* 1965; **260**:313-314.
15. Carcupino M, Baldacci A, Mazzini M, Franzoi P. Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue Cell* 1997; **29**:21-30.
16. Wetzel J, Wourms JP. Paternal-embryonic relationships in pipefishes and seahorses (Syngnathidae). *Am Zool* 1991; **31**:83A-83A.
17. Skalkos ZMG, Van Dyke JU, Whittington CM. Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus abdominalis*. *J Comp Physiol [B]* 2020; **190**:547-556.
18. Laksanawimol P, Damrongphol P, Kruatrachue M. Alteration of the brood pouch morphology during gestation of male seahorses, *Hippocampus kuda*. *Mar Freshw Res* 2006; **57**:497-502.
19. Kawaguchi M, Okubo R, Harada A, Miyasaka K, Takada K, Hiroi J, Yasumasu S. Morphology of brood pouch formation in the pot-bellied seahorse *Hippocampus abdominalis*. *Zool Lett* 2017; **3**:19-19.
20. Whittington CM, Buddle AL, Griffith OW, Carter AM. Embryonic specializations for vertebrate placentation. *Phil Trans R Soc Lon B* 2022; **377**:20210261-20210261.
21. Mossman HW. Comparative morphogenesis of the fetal membranes and accessory uterine structures. *Placenta* 1937; **12**:1-5.
22. Skalkos Z, Van Dyke J, Dowland S, Whittington C. Paternal protein provisioning to embryos during male seahorse pregnancy. *Reproduction* 2024; 167.
23. Dudley JS, Hannaford P, Dowland SN, Lindsay LA, Thompson MB, Murphy CR, Van Dyke JU, Whittington CM. Structural changes to the brood pouch of male pregnant seahorses (*Hippocampus abdominalis*) facilitate exchange between father and embryos. *Placenta* 2021; **114**:115-123.
24. Linton JR, Soloff BL. The physiology of the brood pouch of the male sea horse *Hippocampus erectus*. *Bull Mar Sci* 1964; **14**:45-61.
25. Whittington CM, Musolf K, Sommer S, Wilson AB. Behavioural cues of reproductive status in seahorses *Hippocampus abdominalis*. *J Fish Biol* 2013; **83**:220-226.
26. Leary S, Underwood W, Athony R, Cartner S, GCorey D, Grandin T, Greenace C, Gwaltney-Brant S, McCrackin MA, Meyer R, Miller D, Shearer J, et al. AVMA Guidelines for the Euthanasia of Animals: 2013 Edition. Schaumburg: American Veterinary Medical Association; 2013: 38-42.

27. Leary S, Underwood W, Athony R, Cartner S, Grandin T, Greenace C, Gwaltney-Brant S, McCrackin MA, Meyer R, Miller D, Shearer J, Turner T, et al. AVMA Guidelines for the Euthanasia of Animals: 2020 Edition. Schaumburg: American Veterinary Medical Association; 2020: 44-48.
28. Sommer S, Whittington CM, Wilson AB. Standardised classification of pre-release development in male-brooding pipefish, seahorses, and seadragons (Family Syngnathidae). *Dev Biol* 2012; **12**:39-39.
29. Mowry RW. The special value of methods that colour both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of alcian blue G8X and their combinations with the periodic acid-schiff reaction. *Ann N Y Acad Sci* 1963; **106**:402-423.
30. Kiernan JA. *Histological and histochemical methods: theory and practice*. London: Arnold; 2015.
31. Lai M, Lü B. Tissue preparation for microscopy and histology. In: *Comprehensive Sampling and Sample Preparation*. Oxford: Academic Press; 2012: 53-93.
32. Zhao Y, Vanderkooi S, Kan FWK. The role of oviduct-specific glycoprotein (OVGP1) in modulating biological functions of gametes and embryos. *Histochem Cell Biol* 2022; **157**:371-388.
33. O'Day-Bowman MB, Mavrogianis PA, Fazleabas AT, Verhage HG. A human oviduct-specific glycoprotein: synthesis, secretion, and localization during the menstrual cycle. *Microsc Res Tech* 1995; **32**:57-69.
34. Roux E, Bleau G, Kan FW. Fate of hamster oviductin in the oviduct and uterus during early gestation. *Mol Reprod Dev* 1997; **46**:306-317.
35. Verhage HG, Mavrogianis PA, Boomsma RA, Schmidt A, Brenner RM, Slayden OV, Jaffe RC. Immunologic and molecular characterization of an estrogen-dependent glycoprotein in the rhesus (*Macaca mulatta*) oviduct. *Biol Reprod* 1997; **57**:525-531.
36. Brown RB, Hollingsworth MA. Mucin family of glycoproteins. In: *Encyclopedia of Biological Chemistry,* vol 3, 2nd Edition: Amsterdam Elsevier Science and Technology; 2013:200-204.
37. Esteban MÁ. An overview of the immunological defenses in fish skin. *ISRN Immunol* 2012; **2012**:853470.
38. Dash S, Das SK, Samal J, Thatoi HN. Epidermal mucus, a major determinant in fish health: a review. *Iranian J Vet Res* 2018; **19**:72-81.

39. Ingram GA. Substances involved in the natural resistance of fish to infection-a review. *J Fish Biol* 1980; **16**:23-60.
40. Daniel TL. Fish mucus: In situ measurements of polymer drag reduction. *Biol Bull* 1981; **160**:376-382.
41. Lamblin G, Roussel P. Airway mucins and their role in defence against micro-organisms. *Respir Med* 1993; **87**:421-426.
42. Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. *Mucosal Immunol* 2008; **1**:183-197.
43. Rosen MW, Cornford NE. Fluid friction of fish slimes. *Nature* 1971; **234**:49-51.
44. Oosten JV. The skin and the scales. In: *The Physiology of Fishes*. New York: Academic Press; 1957: 207-244.
45. Shephard KL. Functions for fish mucus. *Rev Fish Biol Fish* 1994; **4**:401-429.
46. Jentoft N. Why are proteins O-glycosylated. *Trends Biochem Sci* 1990; **15**:291-294.
47. Devine PL, McKenzie IFC. Mucins: Structure, function, and associations with malignancy. *BioEssays* 1992; **14**:619-625.
48. Pelaseyed T, Bergström JH, Gustafsson JK, Ermund A, Birchenough GMH, Schütte A, Van der Post S, Svensson F, Rodríguez-Piñeiro AM, Nyström EEL, Wising C, Johansson MEV, et al. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. *Immunol Rev* 2014; **260**:8-20.
49. Crouzier T, Boettcher K, Geonnotti AR, Kavanaugh NL, Hirsch JB, Ribbeck K, Lieleg O. Modulating mucin hydration and lubrication by deglycosylation and polyethylene glycol binding. *Adv Mater Interfaces* 2015; **2**:1500308-n/a.
50. Albèr C, Engblom J, Falkman P, Kocherbitov V. Hydration of hyaluronan: effects on structural and thermodynamic properties. *J Physical Chem B* 2015; **119**:4211-4219.
51. Hilkens J, Ligtenberg MJL, Vos HL, Litvinov SV. Cell membrane-associated mucins and their adhesion-modulating property. *Trends Biochem Sci* 1992; **17**:359-363.
52. Ligtenberg MJL, Buijs F, Vos HL, Hilkens J. Suppression of cellular aggregation by high levels of episialin. *Cancer Res* 1992; **52**:2318-2324.
53. Hey NA, Aplin JD. Sialyl-Lewis x and Sialyl-Lewis a are associated with MUC1 in human endometrium. *Glycoconj J* 1996; **13**:769-779.
54. Zhang K, Baeckström D, Brevinge H, Hansson GC. Secreted MUC1 mucins lacking their cytoplasmic part and carrying sialyl-Lewis a and x epitopes from a tumor cell

line and sera of colon carcinoma patients can inhibit HL-60 leukocyte adhesion to E-selectin-expressing endothelial cells. *J Cell Biochem* 1996; **60**:538-549.

55. Fung PYS, Longenecker BM. Specific immunosuppressive activity of epiglycanin, a mucin-like glycoprotein secreted by a murine mammary adenocarcinoma (TA3-HA). *Cancer Res* 1991; **51**:1170-1176.
56. Bhatia R, Gautam SK, Cannon A, Thompson C, Hall BR, Aithal A, Banerjee K, Jain M, Solheim JC, Kumar S, Batra SK. Cancer-associated mucins: role in immune modulation and metastasis. *Cancer Metastasi Rev* 2019; **38**:223-236.
57. Lapensée L, Paquette Y, Bleau G. Allelic polymorphism and chromosomal localization of the human oviductin gene (MUC9). *Fertil Steril* 1997; **68**:702-708.
58. Buhi WC. Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. *Reproduction* 2002; **123**:355-362.
59. Verhage HG, Fazleabas AT, Donnelly K. The in vitro synthesis and release of proteins by the human oviduct. *Endocrinology* 1988; **122**:1639-1645.
60. Fazleabas AT, Verhage HG. The detection of oviduct-specific proteins in the baboon (*Papio anubis*). *Biol Reprod* 1986; **35**:455-462.
61. Wang C, Mavrogianis PA, Fazleabas AT. Endometriosis is associated with progesterone resistance in the Baboon (*Papio anubis*) oviduct: evidence based on the localization of oviductal glycoprotein 1 (OVGP1). *Biol Reprod* 2009; **80**:272-278.
62. Saint-Dizier M, Marnier C, Tahir MZ, Grimard B, Thoumire S, Chastant-Maillard S, Reynaud K. OVGP1 is expressed in the canine oviduct at the time and place of oocyte maturation and fertilization. *Mol Reprod Dev* 2014; **81**:972-982.
63. Buhi WC, Alvarez IM, Sudhipong V, Dones-Smith MM. Identification and characterization of de novo-synthesized porcine oviductal secretory proteins1. *Biol Reprod* 1990; **43**:929-938.
64. Pradeep MA, Jagadeesh J, De AK, Kaushik JK, Malakar D, Kumar S, Dang AK, Das SK, Mohanty AK. Purification, sequence characterization and effect of goat oviduct-specific glycoprotein on in vitro embryo development. *Theriogenology* 2011; **75**:1005-1015.
65. Woo MMM, Gilks CB, Verhage HG, Longacre TA, Leung PCK, Auersperg N. Oviductal glycoprotein, a new differentiation-based indicator present in early ovarian epithelial neoplasia and cortical inclusion cysts. *Gynecol Oncol* 2004; **93**:315-319.
66. Maines-Bandiera S, Woo MMM, Borugian M, Molday LL, Hii T, Gilks B, Leung PCK, Molday RS, Auersperg N. Oviductal glycoprotein (OVGP1, MUC9): a

differentiation-based mucin rresent in serum of women with ovarian cancer. *Int J Gynecol Cancer* 2010; **20**:16-22-22.

67. Hendrix E, Hewetson A, Mansharamani M, Chilton BS. Oviductin (Muc9) is expressed in rabbit endocervix. *Endocrinology* 2001; **142**:2151-2154.
68. Woo MMM, Alkushi A, Verhage HG, Magliocco AM, Leung PCK, Gilks CB, Auersperg N. Gain of OGP, an estrogen-regulated ovidcut-specific glycoprotein, is associated with the development of endometrial hyperplasia and endometrial cancer. *Clin Cancer Res* 2004; **10**:7958-7964
69. Laheri S, Modi D, Bhatt P. Extra-oviductal expression of oviductal glycoprotein 1 in mouse: detection in testis, epididymis and ovary. *J Biosci* 2017; **42**:69-80.
70. Laheri S, Ashary N, Bhatt P, Modi D. Oviductal glycoprotein 1 (OVGP1) is expressed by endometrial epithelium that regulates receptivity and trophoblast adhesion. *J Assist Reprod Genet* 2018; 35:1419-1429.
71. Kürüm A, Kocamiş H, Karahan S, Türk M, Özkabadayi Y. Ovgp1 expression in boec and oviduct: An immunohistochemical and immunocytochemical study. Vet Fak Derg 2021; 27:151-158.
72. Bereiter-Hahn J, Richards KS, Elsner L, Voth M. Composition and formation of flame cell caps: A substratum for the attachment of micro-organisms to sea horse epidermis. *Proc R Soc Edinb*. *Section B, Biological Sciences* 1980; **79**:105-112.
73. Kawaguchi M, Chang W-S, Tsuchiya H, Kinoshita N, Miyaji A, Kawahara-Miki R, Tomita K, Sogabe A, Yorifuji M, Kono T, Kaneko T, Yasumasu S. Orphan gene expressed in flame cone cells uniquely found in seahorse epithelium. *Cell Tissue Res* 2023; **393**:47-62.
74. Barr JJ, Auro R, Furlan M, Whiteson KL, Erb ML, Pogliano J, Stotland A, Wolkowicz R, Cutting AS, Doran KS, Salamon P, Youle M, et al. Bacteriophage adhering to mucus provide a non-host-derived immunity. *Proc Natl Acad Sci* 2013; **110**:10771-10776.
75. Almeida GMF, Laanto E, Ashrafi R, Sundberg LR. Bacteriophage adherence to mucus mediates preventive protection against pathogenic bacteria. *MBio* 2019; **10**.
76. Rubio-Portillo E, Robertson S, Antón J. Coral mucus as a reservoir of bacteriophages targeting Vibrio pathogens. *ISME J* 2024; **18**.
77. Van Klinken BJW, Dekker J, Buller HA, Einerhand AWC. Mucin gene structure and expression: protection vs. adhesion. *Am J Physiol Gastrointest Liver Physiol* 1995; **269**:G613-G627.

78. Hamlett WC, Koob T. Female reproductive system. In: *Sharks, Skates, and Rays: the Biology of Elasmobranch Fishes*. Baltimore: Johns Hopkins University Press; 1999: 398-443.
79. Storrie M. Microscopic modifications of the female reproductive tissues of *Mustelus antarcticus*. Deakin University; 2004.
80. Hamlett WC. Chondrichthyan Parity, Lecithotrophy and Matrotrophy. In: *Reproductive Biology and Phylogeny of Chondrichthyes:* CRC Press; 2005: 405-421.
81. Conrath CL, Musick JA. Reproductive Biology of Elasmobranchs. In: *Biology of Sharks and their Relatives 2*: CRC Press; 2012: 307-328.
82. Goicoechea O, Garrido O, Jorquera B. Evidence for a trophic paternal-larval relationship in the frog *Rhinoderma darwinii*. *J Herpetol* 1986; **20**:168-178
83. Pelka KE, Henn K, Keck A, Sapel B, Braunbeck T. Size does matter – determination of the critical molecular size for the uptake of chemicals across the chorion of zebrafish (*Danio rerio*) embryos. *Aquat Toxicol* 2017; **185**:1-10.
84. Behera SK, Praharaj AB, Dehury B, Negi S. Exploring the role and diversity of mucins in health and disease with special insight into non-communicable diseases. *Glycoconj J* 2015; **32**:575-613.
85. Munster DJ, Quin RJ, Bansal AS, Ward BG, McGuckin MA. Peritoneal fluid from ovarian cancer patients stimulates MUC1 epithelial mucin expression in ovarian cancer cell lines. *Int. J. Cancer Res.* 1998; **76**:393-398.
86. Vitiazeva V, Kattla JJ, Flowers SA, Lindén SK, Premaratne P, Weijdegård B, Sundfeldt K, Karlsson NG. The O-linked glycome and blood group antigens ABO on mucin-type glycoproteins in mucinous and serous epithelial ovarian tumors. *PLoS One* 2015; **10**.

Chapter 6: General discussion

Summary of results

In this thesis, I explored the extent of patrotrophy in the oviparous male brooding seahorse, *H. abdominalis*, as a comparative model for the evolution of live birth and embryonic provisioning in vertebrates. Seahorses are patrotrophic, by means of paternal lipid supplementation of developing embryos during pregnancy (Skalkos *et al.*, 2020), providing the first experimental evidence that the inner tissue layer of the seahorse brood pouch is the paternal side of a functional placenta. In this thesis, I expand on our current understanding of what nutrients male brooding species are capable of transporting to their developing embryos during pregnancy. I focus on the *what,* as an important prerequisite for the *how* in the most complex form of male pregnancy known in vertebrates. With this work, I broaden our understanding of the evolution of parentotrophy and the diverse mechanisms underlying it. The main findings/outcomes of this thesis include:

a) Protein is paternally transported to developing embryos during male pregnancy to supplement maternal yolk supply. This is the first time a range of organic molecules has been demonstrated to contribute to patrotrophy in an oviparous brooding vertebrate.
b) Inorganic molecules including calcium (Ca), magnesium (Mg), and phosphorus (P) are paternally transported to developing embryos during male pregnancy in *H. abdominalis.*
    a. In terms of overall proportional parental contributions of these molecules, Ca and Mg are primarily supplied by the father whilst P is primarily supplied by the mother before fertilisation.
c) The characterisation of an acid mucin acellular layer, partially composed of oviductal glycoprotein 1 (Ovgp1), which closely apposes paternal and embryonic tissue during male pregnancy in *H. abdominalis.*
    a. Given the functions of mucins and Ovgp1 in the reproductive tracts of female vertebrates, the acellular layer likely functions as physical protection for paternal tissue and immunological protection of embryos, whilst also facilitating fertilisation and embedment.
    b. The acellular layer is most likely paternally derived, as there is also acid mucin staining in the paternal inner epithelium and sometimes between the epithelium and the acellular layer.

c. The role the acellular layer plays in parentotrophy is unknown. Examining the brood pouch for proteins putatively involved in nutrient transport using immunohistochemistry, in addition to tracking labelled nutrients combined with histology of the brood pouch and embryos *in situ*, would provide valuable insight into how patrotrophic nutrients are provided to embryos across the acellular layer.

d) The data presented in this thesis supports the hypothesis that viviparity or the close apposition of parental and embryonic tissues with the birth of live young, has convergently co-evolved across vertebrate groups and sexes.

Convergent co-evolution of viviparity/complex brooding and parentotrophy in oviparous brooders

Despite the diverse ways in which male brooding occurs in vertebrates, research on the provisioning strategies and supporting mechanisms male brooders have evolved is extremely limited. Nonetheless, oviparous brooding species offer considerable opportunity as comparative models for studying the convergent evolution of live birth and parentotrophy across vertebrate lineages and sexes. In this thesis, I 1our current understanding of parentotrophy in vertebrates with internal gestation and live birth, by demonstrating that complex male oviparous brooding (male pregnancy) is functionally comparable to viviparity, and patrotrophy via the male placenta in *H. abdominalis* is functionally equivalent to matrotrophy in incipient matrotrophic viviparous species (**Chapter 3, 4**). I build on knowledge on the complexity of male pregnancy and patrotrophy in the syngnathid lineage, as an extreme model for convergent evolution of reproductive traits. This expanded understanding of *H. abdominalis* reproductive biology provides a new perspective, supporting the hypothesis that parentotrophy has co-evolved with viviparity or complex brooding (defined here as the internal incubation of embryos closely associated with parental tissues with subsequent release of live young) irrespective of vertebrate taxa or sex. My thesis contributes to the growing body of evidence supporting this theory in squamates and mammals (Blackburn, 1992; Stewart and Thompson, 2000; Thompson *et al.*, 2004), some fishes (Hamlett, 2005b; Cotton *et al.*, 2015; Morrison *et al.*, 2017) and various caecilians (Wake, 1977; 1993).

Convergent evolution of patrotrophy in syngnathids

My thesis expands on the current understanding of patrotrophy in the most complex form of male pregnancy known in vertebrates: seahorse pregnancy. Modes of embryonic provisioning strategies, specifically lecithotrophy and matrotrophy, have been reviewed extensively in vertebrate literature, with a particular focus on squamates (e.g. Blackburn, 1992; Blackburn, 2015), and fishes (Wourms *et al.*, 1988; Frazer *et al.*, 2012; **Chapter 2**: Skalkos *et al.*, 2023). However, patrotrophy in vertebrates is a relatively recently defined concept, and within teleosts, has only been confirmed in the syngnathid family: in one seahorse species, *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**), and in three pipefish species with less complex closed brooding structures (*Syngnathus fuscus, Syngnathus floridae* and *Syngnathus typhle*) (Haresign and Shumway, 1981; Stölting and Wilson, 2007; Ripley and Foran, 2009; Kvarnemo *et al.*, 2011). Patrotrophy is putative but not confirmed in some other syngnathid species including *Syngnathus schlegeli* (Watanabe, 1999), *Hippocampus barbourin* (Oconer *et al.*, 2003), *Hippocampus erectus* (Linton and Soloff, 1964), and *Nerophis ophidion* (Berglund *et al.*, 1986), all but the last of which have closed brood pouches. Syngnathids exhibit an array of brooding structures (Whittington and Friesen, 2020), and thus offer a plethora of opportunities to study how male pregnancy, parentotrophy, and their supporting mechanisms evolve. The diversity of brooding structures and complexities allow several hypotheses to be tested, including: is patrotrophy more substantial in species with more complex brood pouches, and where there is closer association of paternal and embryonic tissues (Berglund *et al.*, 1986)? Or, at what "point" of brood pouch complexity and embryonic embedment has patrotrophy of organic and/or inorganic nutrients evolved alongside brooding? My work on *H. abdominalis* provides an essential point of reference when answering these questions and is a pivotal stepping stone for determining the adaptations that support male pregnancy and patrotrophy across a male placenta.

Seahorses exhibit paternal transport of organic molecules including lipids (Skalkos *et al.*, 2020) and proteins (**Chapter 3**: Skalkos *et al.*, 2024), and inorganic molecules such as Ca, Mg, and P (**Chapter 4**) across a non-invasive placenta, to developing embryos during pregnancy. My research demonstrates that despite fundamental biological differences

between male and female pregnancy, the male placenta in *H. abdominalis* is functionally analogous to the female non-invasive placenta in other taxa (Blackburn and Starck, 2015; Buddle *et al.*, 2019; Whittington and Friesen, 2020), and that parentotrophy in male vertebrates has convergently evolved to be as wide-ranging as (at least incipient) matrotrophy in female viviparous vertebrates. The convergent evolution of parentotrophy is widespread and has multiple independent origins in squamates (Thompson *et al.*, 2000; Adams *et al.*, 2005), chondrichthyans (Lombardi *et al.*, 1993; Hamlett, 2005a; Sato *et al.*, 2016; Buddle *et al.*, 2020), amphibians (Wake, 1993; Sandberger-Loua *et al.*, 2017), and teleosts (Olivera-Tlahuel *et al.*, 2019; **Chapter 3:** Skalkos *et al.*, 2024). For example, the viviparous lizard, *Pseudemoia entrecasteauxii*, has a non-invasive highly vascularised placenta that secretes histotrophe containing lipids and proteins (Thompson *et al.*, 2000; Adams *et al.*, 2005; Van Dyke *et al.*, 2014). Comparably, in the only known matrotrophic viviparous anuran, the non-placental *Nimbaphrynoides occidentalis* (previously *Nectophrynoides occidentalis*), histotrophe includes mucopolysaccharides and amino acids (Vilter and Lugand, 1959; Xavier, 1971; Xavier, 1973; Sandberger-Loua *et al.*, 2017). The parentotrophic transport of organic molecules across non-invasive placentae, like in *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024), is prevalent across matrotrophic viviparous vertebrates.

Traditionally, the evolution of viviparity was viewed as preceding the evolution of matrotrophy (Blackburn 1992). However, more recent hypotheses include the transition from oviparity [lecithotrophy] to viviparity with incipient matrotrophy as simultaneous in taxa such as squamates, rather than as successive stages of evolution (Blackburn 1992). All studied squamates (oviparous and viviparous), provide at least some post-fertilisation provisioning to developing embryos (Blackburn, 1992; Stewart and Thompson, 2000; Blackburn, 2015). For example, all oviparous squamates uptake minerals from their external environment, particularly Ca mobilised from the eggshell (Florian, 1990; Stewart and Thompson, 2000; Thompson *et al.*, 2001; Thompson and Speake, 2006). Thus, the basic physiology for Ca provisioning for the egg-shelling process is already present in the uterus of oviparous lizards (Thompson *et al.*, 2004). Similarly, "lecithotrophic" viviparous sharks have uteri with secretory tissues, implying matrotrophy occurs in these species (Hamlett, 2005b; Cotton *et al.*, 2015). Moreover, in teleosts, active transport mechanisms for small organic molecules are present in oviparous fish embryos (Morrison *et al.*, 2017), suggesting the mechanisms supporting embryonic uptake precede the evolution of viviparity. Likewise, in

many oviparous caecilians, hatched embryos exhibit histophagy adaptations like those in larval matrotrophic viviparous caecilians (Wake, 1977; 1993; Kupfer *et al.*, 2006; Wilkinson *et al.*, 2008), suggesting the evolution of embryonic uptake likely evolved at least concurrently with viviparity. Thus, the female reproductive tract, eggs and embryos of some oviparous vertebrates including squamates, caecilians, and teleosts, are pre-adapted for the evolution of live birth and parentotrophy. Using *H. abdominalis* as a model, I provide strong circumstantial evidence that when complex brooding/viviparity evolves, regardless of taxa, sex or analogous organ, parentotrophy has co-evolved concomitantly (Figure 1.).

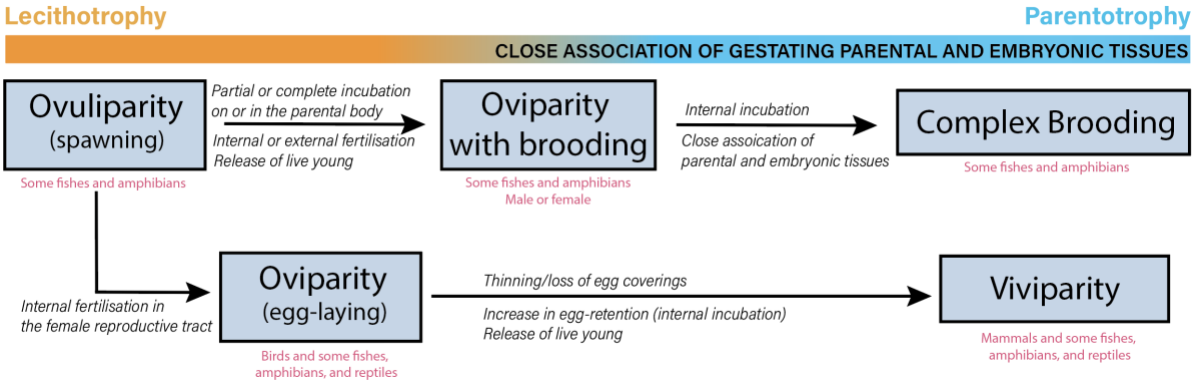


Figure 1. An adapted co-evolution hypothesis for the simultaneous convergent evolution of viviparity/complex brooding and parentotrophy to include oviparity with brooding.

Convergent evolution of patrotrophy in other male brooding vertebrates

Patrotrophy in the complex brooding *H. abdominalis* includes both organic *and* inorganic nutrient transport (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**). These findings suggest that patrotrophy of inorganic nutrients has potentially convergently co-evolved in other male brooding vertebrates, and the transfer of organic nutrients is likely to occur where there are opportunities for close association of embryonic and paternal tissues, as often occurs in female pregnancy in other taxa (Schindler and Hamlett, 1993; Thompson *et al.*, 1999; Thompson and Speake, 2006; Buddle, 2021; Whittington *et al.*, 2022). In the only male brooding anuran, *Rhinoderma darwinii*, a vocal sac brooding frog, paternally injected labelled horseradish peroxidase enzyme, as well as valine and leucine (essential amino acids), are detected in the vocal sac in the form of histotrophe, and in larvae, indicating patrotrophy of protein (Goicoechea *et al.*, 1986). Oviparous anuran embryos and larvae can absorb environmental compounds and minerals including Ca (McDonald *et al.*, 1984; Stiffler, 1993; Fort *et al.*, 1998; Qin and Xu, 2006; Carotenuto *et al.*, 2023). If oviparous anuran embryos are predisposed for parentotrophy like teleost (Morrison *et al.*, 2017) and squamate (Florian,

1990; Thompson and Speake, 2006) embryos, it is highly likely that minerals are paternally transported in *R. darwinii* to developing embryos, and possible that other organic nutrients (e.g. lipids) are likewise transported. To test this, I suggest comparing total lipid, as well as Ca, Mg, and P contents, which are important in anuran embryogenesis (Packard *et al.*, 1977), in embryos at the beginning and end of male incubation.

Two important distinctions exist between Syngnathidae and *R. darwinii*: 1. fertilisation occurs on or in the male in syngnathids whereas *R. darwinii* have external fertilisation, and 2. the entirety of embryogenesis occurs on or in the male in syngnathids, whereas only larval development and metamorphosis occurs inside the vocal sac of *R. darwinii*, following ~ 20 days of embryonic development in the external environment (Goicoechea *et al.*, 1986). Therefore, although *R. darwinii* fits the current definition for oviparity with brooding in males, syngnathid male-pregnancy is much more complex and covers the entire duration of embryogenesis. Notably though, *R. darwinii* is patrotrophic, thus providing evidence that internal fertilisation and embryonic incubation for the entirety of embryogenesis are not prerequisites for parentotrophy (Figure 1.). This is interesting given the prerequisites of viviparity include internal fertilisation and egg retention (Blackburn, 1992). Thus, the drivers of parentotrophy are unlikely to be internal fertilisation/extended egg retention and may instead be the close association of tissues is all you probably need. As such, other common features likely associated with parentotrophy include at least some internal incubation of embryos and the close apposition between parental and embryonic tissues (Figure 1.). Considering male *R. darwinii* transport large molecules and likely minerals, this oviparous frog can provide unique insight into how patrotrophy and the mechanisms underpinning it have convergently evolved with complex male brooding, without internal fertilisation.

Convergent evolution of matrotrophy in female brooding vertebrates

Matrotrophy has evolved at least once in female brooding vertebrates and can be an important point of comparison for the convergent co-evolution of internal incubation, live birth and parentotrophy outside of the female reproductive tract. For example, in the skin-brooding anuran, *Gastrotheca excubitor*, there is an increase in dry mass across embryonic development, providing the first evidence for matrotrophy in an oviparous female brooding anuran (Warne and Catenazzi, 2016; **Chapter 2**: Skalkos *et al.*, 2023). Furthermore, labelled fatty acids and amino acids injected into the gestating female are localised to pouch tissues

and within developing embryos, specifically showing provisioning of lipid and protein to developing embryos (Warne and Catenazzi, 2016), as occurs in *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024). The evolution of diverse parentotrophy of organic nutrients in two convergent homoplastic brooding structures across sexes strongly suggests that complex brooding and viviparity have co-evolved with the ability to provide developing embryos with nutritional supplementation. Thus, studying the physiology and trophic abilities of other oviparous brooding vertebrates may provide more comparative data to test the convergent co-evolution hypothesis of viviparity/complex brooding, and parentotrophy.

Other likely convergent co-evolution oviparous brooding models

Research into the provisioning strategies and gestating parental role in the success of reproduction of oviparous brooding vertebrates remains preliminary. Parentotrophy has convergently evolved in the male brooding vertebrates, *H. abdominalis* and *R. darwinii*, and female brooding *G. excubitor,* providing several examples of evidence of the convergent co-evolution hypothesis. The data presented in this thesis supports the hypothesis that with close apposition of parental and embryonic tissues in an internally incubating structure, wide-ranging parentotrophy convergently co-evolves (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**). Consequently, my work predicts that complex brooding vertebrates may also be parentotrophic. Future research on the fundamental biology and physiology of oviparous brooding teleosts and anurans will provide valuable comparative information on how male brooding, female brooding, and viviparity, in addition to parentotrophy convergently evolved across taxa. Defining provisioning strategies and the mechanisms that support parentotrophy in a broad range of comparative brooding models will help test whether the hypothesis that parentotrophy and viviparity/its equivalents consistently and predictably convergently co-evolve.

Male brooding vertebrates

Other than syngnathids and *R. darwinii*, currently no other male brooding anurans or teleosts have been studied to determine provisioning strategy or the physiological role of the father on embryonic development. Future studies on other male brooding vertebrates would provide comparative data that examines the range and degree of patrotrophic provisioning, and

mechanisms underpinning it, across different lineages. I hypothesise that the oviparous male skin-brooding teleost, *Kurus gulliveri*, is likely patrotrophic. The forehead skin of *K. gulliveri* is folded into crypts that extend deeply into the dermis and contain embedded embryos (Berra and Humphrey, 2002), like in the non-invasive placenta of *H. abdominalis* (Skalkos *et al.*, 2020). These crypts in *K. gulliveri* are probable specialisations that evolved to facilitate adhesion of the sticky egg mass. The crypts in *H. abdominalis* in which embryos are embedded, in addition to the mucin acellular layer present at the paternal-embryo interface of these crypts (**Chapter 5**), likely also facilitate embryo adhesion. Moreover, the tissue immediately associated with the crypts is highly vascularised in *K. gulliveri* and *H. abdominalis*, likely facilitating gas exchange and nutrient supplementation of embryos in both (Berra and Humphrey, 2002; Whittington and Friesen, 2020; Dudley *et al.*, 2021), and may also contribute to keeping embryos embedded (Berra and Humphrey, 2002). Comparatively in the viviparous gummy shark, *Mustelus antarcticus*, uterine mucosa create individual embryo compartments that increase the surface area for fetomaternal exchange of water and likely inorganic nutrients (Storrie *et al.*, 2009). The patrotrophic capabilities of *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**), the similar "crypts", increased vascularisation, and close proximity of embryos to paternal tissue observed in *K. gulliveri* brooding tissue, suggest that male transport of organic and inorganic molecules may similarly occur in this species. Therefore, I suspect that *K. gulliveri* could offer another teleost model in which male brooding has convergently co-evolved with patrotrophy and can contribute valuable information on how male pregnancy and the physiological role the father has on its success, has evolved.

Additionally, the male vocal sac brooding frog, *Rhinoderma rufum* is like *R. darwinii* in that fertilisation is external and after eight days of larval development, embryos are taken into the vocal sac, then released into the aquatic environment, but *R. rufum* embryos are released before the end of metamorphosis (Jorquera *et al.*, 1974; Jorquera *et al.*, 1982). Current knowledge on the morphology and physiology of male *R. rufum* are very limited, and thus there is little that suggests *R. rufum* may be patrotrophic. Nonetheless, the similar patrotrophic abilities and brooding morphology of *R. darwinii* and *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**), and the very close relation and reproductive behaviour of *R. darwinii* to *R. rufum*, it is likely that *R. rufum* is patrotrophic. However, I suggest further histological examination of embryos *in situ* and nutrient content

comparisons across incubational development, as *R. rufum* may represent another comparative model for how male incubation has evolved, and whether it co-evolved with patrotrophic abilities, without internal fertilisation and with the release of metamorphosing young.

Female brooding vertebrates

Female brooding vertebrates can also provide valuable morphological and physiological comparisons for the convergent co-evolution of internal incubation, the birth of live young, and parentotrophy, outside of the traditional definition of viviparity. By comparing the range and degree of matrotrophy in female brooders with that of viviparous and male brooding vertebrates we can better understand how viviparity/complex brooding and parentotrophy evolved across different sexes and gestating structures. This data can also help determine whether the mechanisms driving parentotrophy evolve consistently across sexes *and* gestating organs, or whether novel pathways evolve each time. In the female brooding frog, *Gastrotheca riobambe*, embryos develop highly vascularised gill placentae that are intimately apposed to maternal tissues, like in the matrotrophic brooding frog, *G. excubitor* (Jones *et al.*, 1973; del Pino *et al.*, 1975; Greven, 2011; Wake, 2015; del Pino, 2018). During embryonic incubation in *G. riobambae*, the pouch walls become enlarged, highly vascularised, and envelop individual embryos (del Pino *et al.*, 1975), similar to the crypts observed in patrotrophic *K. gulliveri* and *H. abdominalis* (Berra and Humphrey, 2002; Skalkos *et al.*, 2020; Dudley *et al.*, 2021) and matrotrophic *G. excubitor* (Jones *et al.*, 1973; del Pino, 2018). Moreover, calculating a PI can help distinguish between parentotrophy and lecithotrophy (**Chapter 2**: Skalkos *et al.*, 2023). Like *H. abdominalis*, in *G. riobambae* the dry mass of embryos does not decrease across development (del Pino and Escobar, 1981), suggesting parentotrophy (Skalkos *et al.*, 2020; **Chapter 2**: Skalkos *et al.*, 2023). However, calculating PI alone to distinguish between lecithotrophy and incipient patrotrophy is not sufficient (**Chapter 2**: Skalkos *et al.*, 2023), and it remains unconfirmed in anuran literature whether *G. riobambae* mothers are matrotrophic (del Pino, 2018). Given the PI of 1 and morphological similarities of *G. riobambae* to patrotrophic *H. abdominalis*, *K. gulliveri* and matrotrophic *G. excubitor*, I strongly encourage research into the trophic and other physiological capabilities involved in supporting embryogenesis in this female brooding frog.

Additionally, I suggest defining the provisioning strategies of the female skin-brooding teleost genuses *Platystacus* (Apredinidae family), and *Solenostomus* (Solenostomidae

family), as potential comparative models for the convergent evolution of embryonic incubation in or on the female body, outside of the female reproductive tract. Both genera brood embryos on maternal cotylephores that contain extensive connective tissue and heavy vascularisation where the egg envelope adheres to the maternal tissue (Wetzel *et al.*, 1997), similar to the close apposition of paternal-embryonic tissues in *H. abdominalis* (Carcupino *et al.*, 1997; Skalkos *et al.*, 2020; Dudley *et al.*, 2021; Harada *et al.*, 2022). Syngnathidae and Solenostomidae are sister lineages (Wilson and Orr, 2011; Longo *et al.*, 2017), but it is unlikely that male pregnancy evolved from female skin-brooding (Whittington and Friesen, 2020), and thus the brooding structures of both and that of *Platystacus*, in structure and probable parentotrophic function, represent convergent evolution of similar embryonic incubation structures across sexes (Wetzel and Wourms, 1995). Comparing distantly related skin brooders can tell us whether the same nutrients that are transported in *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**), and other brooders, are widespread, and whether all skin-brooding vertebrates have evolved similar functions and underlying mechanisms, or whether different mechanisms developed with each independent evolution.

Mucins in complex brooding structures

In contrast to the oviparous brooders mentioned and all viviparous species currently known, in *H. abdominalis*, an acid mucin acellular layer containing OVGP1 borders the paternal pouch tissue and developing embryos. A similar layer has been identified within syngnathids in *S. abaster* (Carcupino *et al.*, 1997), but not in any other family of vertebrates (**Chapter 5**). How the brooding specific mucous layer in both *S. abaster* and *H. abdominalis* is involved in male pregnancy is currently unknown, but likely acts as a facilitator of patrotrophic provisioning (discussed in **Chapter 5**). However, some valuable comparisons of mucous in the gestational tissues of brooding and viviparous species can be drawn. Mucous cells are absent in the brood pouch luminal epithelium of *H. abdominalis* (Kawaguchi *et al.*, 2017), despite mucous cells being present in the external epithelium (**Chapter 5**). Similarly, the forehead skin of the non-brooding male *K. gulliveri* contains no secretory mucous cells, which differs from normal fish skin (Berra and Humphrey, 2002). Conversely, in the female skin-brooding *G. riobambae*, pouches contain mucous glands (Jones *et al.*, 1973), that secrete a PAS+ mucous which helps attach embryos to the maternal brood pouch tissue (del Pino and Escobar, 1981; del Pino, 2018). Similarly, in the incipiently matrotrophic shark,

Carcharodon carcharias, PAS+ material is present on the surface and in the cytoplasm of the epithelial cells of the uterine wall (Sato *et al.*, 2016), like what is observed in *H. abdominalis* (**Chapter 5**). The PAS+ material in *C. carcharias* is also present in the uterine fluid, likely secreted from the uterine epithelium along with lipid histotrophe (Hamlett, 2005a; Conrath and Musick, 2012; Sato *et al.*, 2016). The mucous material in the uterine epithelium and fluid is likely evidence of "mucoid histotrophe", the simplest form of matrotrophy in viviparous sharks (Hamlett and Koob, 1999; Storrie, 2004; Conrath and Musick, 2012). However mucous cells in the uterine epithelium have not been confirmed in *C. carcharias*. Therefore, it is likely that mucous cells are not required for mucoid histotrophe across internally gestating vertebrates, and that mucoid histotrophe may have convergently evolved in viviparous chondrichthyans and brooding teleosts. Furthermore, the acellular layer in *H. abdominalis* is PAS+, appears to be paternally derived and thus may contribute some patrotrophy (**Chapter 5**). However, the mechanisms behind production of the mucin, nor its role in paternal internal incubation and patrotrophy in *H. abdominalis*, are currently unconfirmed. To better understand the role of mucins and the acellular layer in *H. abdominalis* pregnancy, additional localisation of other mucins to the brood pouch epithelium would be optimal. More broadly, deeper research into the fundamental biology and morphology of egg attachment sites in brooding and incipiently matrotrophic viviparous vertebrates is required to test if the acellular layer in *H. abdominalis* is an adaptation unique to syngnathids or if it has convergently evolved in other viviparous/complex brooding parentotrophic vertebrates.

Although not a brooding species, the oviparous *Symphpysodon aequifasciata* is a species of discus fish in which parents feed fry from secreted paternal skin mucous after hatching (Blüm and Fiedler, 1965; Schradin and Anzenberger, 1999; Wei *et al.*, 2021), a strategy similar to some skin-feeding caecillians (Kupfer *et al.*, 2006; Wilkinson *et al.*, 2008). This implies that the typical mucous function of fish epithelium has evolved transport mechanisms to supply nutrition in non-brooding lecithotrophic oviparous vertebrates, displaying a likely predisposition to mucoid histotrophy, particularly in skin brooders. Moreover, the transport of minerals and organic molecules across a skin-derived placenta in *H. abdominalis* (Skalkos *et al.*, 2020; **Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**), suggests it is possible that the mechanisms underpinning mucous secretion may have been co-opted for use in wide-spread parentotrophy. *Symphpysodon aequifasciata*, like *H. abdominalis*, is an oviparous species

that provides circumstantial evidence that the evolution of internal gestation does not precede the evolution of parentotrophic mechanisms.

Mechanisms of patrotrophy in syngnathids

Potential mechanisms of paternal nutrient transport

The mechanisms underpinning the transport of nutrients across viviparous/complex brooding vertebrates are understudied, with studies primarily focussed on net embryonic uptake/maternal transport of minerals in squamates (Thompson and Speake, 2006), teleosts (**Chapter 2**: Skalkos *et al.*, 2023) and chondrichthyans (Frazer *et al.*, 2012). My thesis identifies the range of nutrients that are transported in the most complex form of male brooding. The mechanisms by which syngnathids transport nutrients to developing embryos during pregnancy and how embryos uptake them are currently unknown, and likely diverse given the various molecule sizes involved (**Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**). Nutrients may be transported from the paternal blood to the pouch by transcytosis across the brood pouch epithelium or may be synthesised or modified in the epithelial cells themselves (Whittington and Friesen, 2020). Modified flame-cone cells (MFCC) in the brood pouch epithelium of *H. hippocampus* may be involved in extraembryonic nutrition, including protein synthesis (Carcupino *et al.*, 2002) but are not present in the brood pouch luminal epithelium of *H. abdominalis* (Kawaguchi *et al.*, 2023; **Chapter 5**), or suspected patrotrophic *S. schlegeli* (Watanabe, 1999; Watanabe *et al.*, 1999; Kawaguchi *et al.*, 2023), implying MFCC are not necessary for patrotrophy across the Syngnathid family. In pregnant *S. abaster*, intra-cellular spaces develop at the basal surface of the pouch epithelial cells that may facilitate patrotrophy and transport of other substances into or out of the pouch lumen as histotrophe (Carcupino *et al.*, 1997). I hypothesise that paternal nutrients are transported to embryos across the male placenta of patrotrophic syngnathids via histotrophe released into the brood pouch, as occurs in the male brooding *R. darwinii* (Goicoechea *et al.*, 1986), matrotrophic viviparous *N. occidentalis* (Vilter and Lugand, 1959; Xavier, 1971; Xavier, 1973; Sandberger-Loua *et al.*, 2017), many viviparous chondrichthyans with no or non-invasive placentae (Hamlett, 1989; Buddle *et al.*, 2019), and putatively in the female brooding *G. excubitor* (Warne and Catenazzi, 2016). Histotrophe can contribute to substantial parentotrophy and in the histotrophic gummy shark, *M. antarcticus*, contributes to a PI of ~ 9

(Storrie *et al.*, 2009; **Chapter 2**: Skalkos *et al.*, 2023). I also hypothesise that histotrophe may be supplemented by simple parentotrophy (**Chapter 4**). I recommend testing this hypothesis by tracking labelled nutrients to the brood pouch fluid of *H. abdominalis* using enzyme- or radio-immunoassay techniques (Sánchez-Vizcaíno Rodríguez and Cambra, 1981) as in *R. darwinii* (Goicoechea *et al.*, 1986), to establish whether paternal nutrients are supplied to embryos in the form of histotrophe.

Lipid droplets may be involved in lipid patrotrophy in some syngnathids. Lipid droplets are abundant in the pouch tissues of *H. erectus* (Linton and Soloff, 1964) and *H. barbouri* (Oconer et al., 2003), which may support fatty acid release into the pouch, although neither species are confirmed patrotrophic. In the viviparous incipiently matrotrophic shark, *C. carcharias,* lipid histotrophe occurs via lipid droplets that are present on the surface and in the cytoplasm of the epithelial cells of the uterine wall (Sato *et al.*, 2016), and released via a mucous. Lipid droplets in *H. erectus, H. barbouri* and *C. carcharias* may represent convergent evolution of mechanisms that support parentotrophy of lipids in varying gestating organs. Furthermore, the mucin acellular layer in *H. abdominalis* (**Chapter 5**), may be involved in lipid patrotrophy, in the form of mucoid histotrophe like *C. carcharias* (Hamlett, 2005a; Conrath and Musick, 2012; Sato *et al.*, 2016). Future studies should test the hypothesis that lipid droplets/secretions are present in *H. abdominalis* pouch tissue by histological staining similar to those used in *H. erectus* (Linton and Soloff, 1964) and *C. carcharias* (Sato *et al.*, 2016), or by using SEM as demonstrated in *H. barbouri* (Oconer *et al.*, 2003), or tracking labelled fatty acids e.g. ^{13}C as in *G. excubitor* (Warne and Catenazzi, 2016).

Several other nutrient transporter genes are hypothesised to be involved in patrotrophy in *H. abdominalis* including amino acid solute carriers SLC38A10 and SLC38A5, and Ca transporter ATP2C1 (Whittington *et al.*, 2015). Solute carriers are a large family of membrane bound transporters that transport amino acids and minerals across placentae in mammals (Carter, 2012), and putatively in the viviparous skink *Pseudemoia entrecasteauxii* (Griffith *et al.*, 2016), and shark *Rhizoprionodon taylori* (Buddle *et al.*, 2021). Furthermore, in lizards, Ca^{2+} ATPase channels in shell glands and/or the uterine epithelium are hypothesised to be important for Ca provisioning (Thompson *et al.*, 2004; Thompson and Speake, 2006). The localisation of these proteins using immunohistochemistry or correlative light and electron microscopy (Moore *et al.*, 2016) in the brood pouch of *H. abdominalis* will

improve our understanding of the mechanisms supporting patrotrophy in the most complex form of male brooding known to vertebrates.

My thesis primarily focuses on identifying the macronutrients paternally transported across the male placenta to developing embryos during *H. abdominalis* pregnancy (**Chapter 3**: Skalkos *et al.*, 2024; **Chapter 4**), to provide a foundation for understanding the mechanisms of patrotrophy and embryonic uptake (**Chapter 5**), as a comparative model for the evolution of viviparity/complex brooding and parentotrophy. Transcriptomic comparisons of viviparous vertebrates with independent origins of live birth, show that different genes evolved to support the same underlying morphological and physiological functions of viviparity (Foster *et al.*, 2022). Studying the morphology, physiology, and molecular biology of brooding vertebrates as comparative models for the evolution of parentotrophy, will allow determination of whether the mechanisms underlying parentotrophy in homoplastic brooding organs repeatedly evolve across taxa or if it evolved through recruitment of different genes, like viviparity (Foster *et al.*, 2022). Lastly, fundamental knowledge on the mechanisms supporting parentotrophy and live birth/its equivalents in a range of vertebrates where these traits have convergently evolved, allows us to test whether these two traits consistently co-evolve.

Potential mechanisms of embryonic nutrient uptake

In **Chapter 4** I delve into the potential mechanisms for paternal nutrient transport and embryonic uptake of inorganic molecules Ca, Mg, and P. Inorganic nutrient transport alone does not constitute parentotrophy (**Chapter 2**: Skalkos *et al.*, 2023), as embryonic uptake occurs in oviparous [lecithotrophic] vertebrates (Thompson *et al.*, 2000; Thompson *et al.*, 2001; Oftedal, 2002). However, the mechanisms by which they occur can provide insight into the initial step for the evolution of parentotrophy and the complexity of provisioning during internal incubation in oviparous male brooders. The ability of parentotrophic embryos to absorb nutrients from their environment was likely retained from an egg-laying ancestor and is a prerequisite for parentotrophy (Wourms *et al.*, 1988; Blackburn, 2015). Studies on parentotrophy in vertebrates including fishes, anurans, and squamates have focussed on defining provisioning strategy, identifying what is transported, measuring net transport of nutrients, and deriving hypotheses on how they are transported (**Chapter 2**: Skalkos *et al.*, 2023), as fundamental stepping stones to ascertaining the mechanisms underpinning their

transport. However, research on embryonic uptake of the nutrients that the parents transport is relatively under emphasised yet constitutes a crucial aspect of the functionality of parentotrophy. Deriving an embryonic transcriptome at various stages of embryogenesis in complex brooding parentotrophic vertebrates like *H. abdominalis*, as recently done in *Syngnathus scovelli* (Healey *et al.,* 2024), could identify genes associated with embryonic uptake of nutrients in other vertebrates, and thus provide testable hypotheses for mode of nutrient uptake and the mechanisms that support it/them. In *S. scovelli*, the embryonic skin expresses an abundance of carbohydrate binding proteins, supporting the hypothesis that absorption of nutrients including macromolecules likely occurs via the skin of embryos (Healey *et al.,* 2024). This molecular research opens new avenues for testing evolutionary hypotheses on the other side of the same process; do embryos always convergently evolve the same mechanisms for the same nutrient uptake modes, or do novel mechanisms evolve with each independent origin?

Histotrophy and histophagy are modes of embryonic uptake of nutrients that have been hypothesised for male and female brooding vertebrates and are common in matrotrophic viviparous vertebrates. *Rhinoderma darwinii* embryos are hypothesised to utilise both histophagy and histotrophy for organic nutrient uptake (Jorquera *et al.*, 1982; Goicoechea *et al.*, 1986), with extracytoplasmic vacuoles in the larval skin likely involved in the latter (Jorquera *et al.*, 1982). Similarly, *N. occidentalis* embryos uptake mucopolysaccharides by histotrophy but amino acids by histophagy (Vilter and Lugand, 1959; Xavier, 1971; Xavier, 1973; Sandberger-Loua *et al.*, 2017). Moreover, the embryos of *G. excubitor* likely uptake organic nutrients via their bell gills (histotrophy) (Warne and Catenazzi, 2016). As histotrophy and histophagy are embryonic uptake modes present in parentotrophic Syngnathids and both male and female brooding amphibians, I hypothesise that embryos of *H. abdominalis* uptake patrotrophic nutrients through a combination of both. Specifically, I hypothesise that embryos absorb minerals primarily through ionocytes in the skin (histotrophy) during early embryogenesis, which is later combined with absorption via the gills and gastrointestinal tract (histophagy) (**Chapter 4**). Calcium, in contrast to Mg and P, is primarily absorbed from the environment rather than from digestion (**Chapter 5**). The ability to absorb Ca from the environment via skin ionocytes before the development of the gills and GIT may contribute to the large increase in paternal Ca contribution, compared to Mg and P, across development. In *S. abaster,* mitochondria-rich cells (ionocytes) are scattered in the

epidermis of the brood pouch that apoptose after the reproductive period (Carcupino *et al.*, 2002), highlighting a brooding-specific role. Provisioning mode has not yet been examined in *S. abaster,* a pipefish with a closed brood pouch (Carcupino *et al.*, 2002). However, since I have demonstrated ionic transport in a closely related species (**Chapter 4**), ionocytes may be involved in the release of minerals into the brood pouch lumen. I hypothesise that ionocytes are involved in both the release of minerals into the brood pouch lumen, and the absorption by embryos in patrotrophic syngnathids. Future studies using transition and scanning electron microscopy on *H. abdominalis* embryos and inner tissue layer of the brood pouch in situ should test the hypothesis that ionocytes are present in embryonic skin which will provide further testable hypotheses for how they are involved in paternal secretion of minerals and/or embryonic uptake (Carcupino *et al.*, 1997). Utilising these techniques to examine the interface of paternal-embryonic tissues will also provide greater insight into the role of the acellular layer in patrotrophy and offer greater understanding of how complex male brooding evolved.

Beyond the pouch: Evolutionary implications

This thesis provides pioneering evidence that *H. abdominalis* males transport a broad range of nutrients to their developing offspring in the pouch during pregnancy. This is the first time a brooding vertebrate has been demonstrated to have such a wide-ranging parentotrophic ability. Thus, this thesis provides strong evidence supporting the hypothesis for the co-evolution of parentotrophy and viviparity/complex brooding across various taxa, tissue structures, and sexes. The work in this thesis advances our knowledge on the fundamental biology of male pregnancy, which provides greater insight into how pregnancy and its supporting mechanisms evolve across taxa. The morphology and physiology of viviparity and the placenta have convergently evolved highly similar functions across diverse taxa (Blackburn, 2015), despite a lack of convergence in supporting gene recruitment (Foster *et al.*, 2022). This extreme convergent co-evolution of reproductive traits suggests that the evolution of viviparity/its equivalents is predisposed to co-evolve with the mechanisms underpinning parentotrophy, performing similar functions. The close apposition of parental and embryonic tissues observed in a diverse range of viviparous and some brooding species is repeatedly concomitant with parentotrophy. This body of work demonstrates that gestating organs that closely appose embryonic tissues during pregnancy/brooding across different taxa and sexes have evolved to transport similar molecules. Future work should expand on this

foundation to examine whether nutrient transport is widespread amongst complex brooding vertebrates and whether transport is achieved via similar or unique mechanisms. Lastly, the male placenta of *H. abdominalis* can be used as a model for how complex organs in general are predisposed to convergently evolve the same physiological functions with each independent evolution, even if gene expression and underlying mechanisms differ.

References

Adams, S. M., Biazik, J. M., Thompson, M. B. & Murphy, C. R. (2005). Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: A new placental morphotype. *Journal of Morphology* **264**, 264-276.

Berglund, A., Rosenqvist, G. & Svensson, I. (1986). Reversed sex roles and parental energy investment in zygotes of two pipefish (Syngnathidae) species. *Marine Ecology* **29,** 209-215.

Berra, T. M. & Humphrey, J. D. (2002). Gross anatomy and histology of the hook and skin of forehead brooding male Nurseryfish, *Kurtus gulliveri,* from Northern Australia. *Environmental Biology of Fishes* **65**, 263-270.

Blackburn, D. G. (1992). Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. *American Zoologist* **32**, 313-321.

Blackburn, D. G. (2015). Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. *Journal of Morphology* **276**, 961-990.

Blackburn, D. G. & Starck, J. M. (2015). Morphological specializations for fetal maintenance in viviparous vertebrates: An introduction and historical retrospective. *Journal of Morphology* **276**, 1-16.

Blüm, V. & Fiedler, K. (1965). Hormonal control of reproductive behavior in some Cichlid fish. *General and Comparative Endocrinology* **5**, 186-196.

Buddle, A. (2021). The evolution of viviparity and placentae in sharks. The University of Sydney.

Buddle, A. L., Otway, N. M., Van Dyke, J. U., Thompson, M. B., Murphy, C. R., Dowland, S. N., Simpfendorfer, C. A., Ellis, M. T. & Whittington, C. M. (2020). Structural changes to the uterus of the dwarf ornate wobbegong shark (*Orectolobus ornatus*) during pregnancy. *Journal of Morphology* **281**, 428-437.

Buddle, A. L., Van Dyke, J. U., Thompson, M. B., Simpfendorfer, C. A., Murphy, C. R., Dowland, S. N. & Whittington, C. M. (2021). Structure of the paraplacenta and the yolk sac placenta of the viviparous Australian sharpnose shark, *Rhizoprionodon taylori*. *Placenta* **108**, 11-22.

Buddle, A. L., Van Dyke, J. U., Thompson, M. B., Simpfendorfer, C. A. & Whittington, C. M. (2019). Evolution of placentotrophy: using viviparous sharks as a model to understand vertebrate placental evolution. *Marine and Freshwater Research* **70**, 908-924.

Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. (1997). Morphological organization of the male brood pouch epithelium of *Syngnathus abaster* Risso (Teleostea, Syngnathidae) before, during, and after egg incubation. *Tissue & Cell* **29**, 21-30.

Carcupino, M., Baldacci, A., Mazzini, M. & Franzoi, P. (2002). Functional significance of the male brood pouch in the reproductive strategies of pipefishes and seahorses: a morphological and ultrastructural comparative study on three anatomically different pouches. *Journal of Fish Biology* **61**, 1465-1480.

Carotenuto, R., Pallotta, M. M., Tussellino, M. & Fogliano, C. (2023). *Xenopus laevis* (Daudin, 1802) as a model organism for bioscience: A historic review and perspective. *Biology* **12**, 890.

Conrath, C. L. & Musick, J. A. (2012). *Reproductive biology of Elasmobranchs*. pp. 307-328: CRC Press.

Cotton, C. F., Dean Grubbs, R., Dyb, J. E., Fossen, I. & Musick, J. A. (2015). Reproduction and embryonic development in two species of squaliform sharks, *Centrophorus granulosus* and *Etmopterus princeps*: Evidence of matrotrophy? *Deep-sea Research, Part II* **115**, 41-54.

del Pino, E. M. (2018). The extraordinary biology and development of marsupial frogs (Hemiphractidae) in comparison with fish, mammals, birds, amphibians and other animals. *Mechanisms of Development* **154**, 2-11.

del Pino, E. M. & Escobar, B. (1981). Embryonic stages of *Gastrotheca riobambae* (fowler) during maternal incubation and comparison of development with that of other egg-brooding hylid frogs. *Journal of Morphology* **167**, 277-295.

del Pino, E. M., Galarza, M. L., de Albuja, C. M. & Humphries, A. A. (1975). The maternal pouch and development in the marsupial frog *Gastrotheca riobambae* (Fowler). *The Biological Bulletin* **149**, 480-491.

Dudley, J. S., Hannaford, P., Dowland, S. N., Lindsay, L. A., Thompson, M. B., Murphy, C. R., Van Dyke, J. U. & Whittington, C. M. (2021). Structural changes to the brood pouch of male pregnant seahorses (*Hippocampus abdominalis*) facilitate exchange between father and embryos. *Placenta* **114**, 115-123.

Florian, J. D. (1990). Development of extraembryonic membranes and chemical composition of eggs and hatchlings of the oviparous lizard, *Eumeces fasciatus* (reptilla, Squamata). University of Tulsa.

Fort, D. J., Propst, T. L., Stover, E. L., Strong, P. L. & Murray, F. J. (1998). Adverse reproductive and developmental effects in *Xenopus* from insufficient boron. *Biological Trace Element Research* **66**, 237-259.

Foster, C. S. P., Van Dyke, J. U., Thompson, M. B., Smith, N. M. A., Simpfendorfer, C. A., Murphy, C. R. & Whittington, C. M. (2022). Different genes are recruited during convergent evolution of pregnancy and the placenta. *Molecular Biology and Evolution* **39**.

Frazer, H. A., Ellis, M. & Huveneers, C. (2012). Can a threshold value be used to classify chondrichthyan reproductive modes: systematic review and validation using an oviparous species. *PloS one* **7**, e50196-e50196.

Goicoechea, O., Garrido, O. & Jorquera, B. (1986). Evidence for a trophic paternal-larval relationship in the frog *Rhinoderma darwinii*. *Journal of Herpetology* **20**, 168-178.

Greven, H. (2011). *Maternal adaptations to reproductive modes in Amphibians.* pp. 117-141.

Hamlett, W. C. (1989). Evolution and morphogenesis of the placenta in sharks. *Journal of Experimental Zoology, Part B* **252**, 35-52.

Hamlett, W. C. (2005a). Chondrichthyan parity, lecithotrophy and matrotrophy. In: *Reproductive Biology and Phylogeny of Chondrichthyes*, pp. 405-421: CRC Press.

Hamlett, W. C. (2005). *Reproductive Biology and Phylogeny of Chondrichthyes: Sharks, Batoids, and Chimaeras,* vol 3: Taylor & Francis Group.

Hamlett, W. C. & Koob, T. (1999). Female reproductive system. In: *Sharks, skates, and rays: the biology of elasmobranch fishes*, pp. 398-443: Johns Hopkins University Press.

Harada, A., Shiota, R., Okubo, R., Yorifuji, M., Sogabe, A., Motomura, H., Hiroi, J., Yasumasu, S. & Kawaguchi, M. (2022). Brood pouch evolution in pipefish and seahorse based on histological observation. *Placenta* **120**, 88-96.

Haresign, T. W. & Shumway, S. E. (1981). Permeability of the marsupium of the pipefish *Syngnathus fuscus* to [14C]-alpha amino isobutyric acid. *Comparative Biochemistry and Physiology, Part A* **69**, 603-604.

Healey, M. H., Penn, H. B., Small, C. M., Bassham, S., Goyal, V., Woods, M. A. & Cresko, W. A. (2024). Single cell sequencing provides clues about the developmental genetic basis of evolutionary adaptations in syngnathid fishes. *eLife* **13**, RP97764.

Jones, R. E., Gerrard, A. M. & Roth, J. J. (1973). Estrogen and brood pouch formation in the marsupial frog, *Gastrotheca riobambae*. *The Journal of Experimental Zoology* **184**, 177-183.

Jorquera, B., Garrido, O. & Pugin, E. (1982). Comparative studies of the digestive tract development between *Rhinoderma darwinii* and *R. rufum*. *Journal of Herpetology* **16**, 204-214.

Jorquera, B., Pugin, E. & Goicoechea, Ó. (1974). Tabla de desarrollo normal de Rhinoderma darwini (Concepción).

Kawaguchi, M., Chang, W.-S., Tsuchiya, H., Kinoshita, N., Miyaji, A., Kawahara-Miki, R., Tomita, K., Sogabe, A., Yorifuji, M., Kono, T., Kaneko, T. & Yasumasu, S. (2023). Orphan gene expressed in flame cone cells uniquely found in seahorse epithelium. *Cell and Tissue Research* **393**, 47-62.

Kawaguchi, M., Okubo, R., Harada, A., Miyasaka, K., Takada, K., Hiroi, J. & Yasumasu, S. (2017). Morphology of brood pouch formation in the pot-bellied seahorse *Hippocampus abdominalis*. *Zoological Letters* **3**, 19-19.

Kupfer, A., Müller, H., Antoniazzi, M. M., Jared, C., Greven, H., Nussbaum, R. A. & Wilkinson, M. (2006). Parental investment by skin feeding in a caecilian amphibian. *Nature* **440**, 926-929.

Kvarnemo, C., Mobley, K. B., Partridge, C., Jones, A. G. & Ahnesjö, I. (2011). Evidence of paternal nutrient provisioning to embryos in broad-nosed pipefish *Syngnathus typhle*. *Journal of Fish Biology* **78**, 1725-1737.

Linton, J. R. & Soloff, B. L. (1964). The physiology of the brood pouch of the male sea horse *Hippocampus erectus*. *Bulletin of Marine Science* **14**, 45-61.

Lombardi, J., Jones, K. B., Garrity, C. A. & Files, T. (1993). Chemical composition of uterine fluid in four species of viviparous sharks (*Squalus acanthias, carcharhinus plumbeus, mustelus canis* and *rhizoprionodon terraenovae*). *Comparative Biochemistry and Physiology, Part A* **105**, 91-102.

Longo, S. J., Faircloth, B. C., Meyer, A., Westneat, M. W., Alfaro, M. E. & Wainwright, P. C. (2017). Phylogenomic analysis of a rapid radiation of misfit fishes (Syngnathiformes) using ultraconserved elements. *Molecular Phylogenetics and Evolution* **113**, 33-48.

McDonald, D. G., Ozog, J. L. & Simons, B. P. (1984). The influence of low pH environments on ion regulation in the larval stages of the anuran amphibian, *Rana clamitans*. *Canadian Journal of Zoology* **62**, 2171-2177.

Moore, C. L., Cheng, D., Shami, G. J. & Murphy, C. R. (2016). Correlated light and electron microscopy observations of the uterine epithelial cell actin cytoskeleton using fluorescently labeled resin-embedded sections. *Micron* **84**, 61-66.

Morrison, K. R., Ngo, V., Cardullo, R. A. & Reznick, D. N. (2017). How fish eggs are preadapted for the evolution of matrotrophy. *Proceedings of the Royal Society, Part B* **284**, 20171342-20171342.

Oconer, E. P., Herrera, A. A., Amparado, E. A., Wetzel, J. T. & De La Paz, R. M. (2003). Immunolocalization of hormones involved in male gestation in the seahorse, *Hippocampus barbouri* Jordan and Richardson 1908. *Phillipine Agricultural Scientist* **86**, 84-91.

Oftedal, O. T. (2002). The origin of lactation as a water source for parchment-shelled eggs. *Journal of Mammary Gland Biology and Neoplasia* **7**, 253-266.
Olivera-Tlahuel, C., Moreno-Mendoza, N. A., Villagrán-Santa Cruz, M. & Zúñiga-Vega, J. J. (2019). Placental structures and their association with matrotrophy and superfetation in poeciliid fishes. *Acta Zoologica* **100**, 167-181.
Packard, G. C., Tracy, C. R. & Roth, J. J. (1977). The physiological ecology of reptilian eggs and embryos and the evolution of viviparity within the class reptilia. *Biological Reviews of the Cambridge Philosophical Society* **52**, 71-105.
Qin, Z. & Xu, X. (2006). Application of *Xenopus laevis* in ecotoxicology (I) - Introduction and quality control of laboratory animal. *Chinese Science Bulletin* **51**, 1273-1280.
Ripley, J. L. & Foran, C. M. (2009). Direct evidence for embryonic uptake of paternally-derived nutrients in two pipefishes (Syngnathidae: *Syngnathus* spp.). *Journal of Comparative Physiology, Part B* **179**, 325-333.
Sánchez-Vizcaíno Rodríguez, J. M. & Cambra, M. (1981). *Técnicas inmunoenzimáticas en patología animal y vegetal*: Instituto Nacional de Investigaciones Agrarias.
Sandberger-Loua, L., Müller, H. & Rödel, M. O. (2017). A review of the reproductive biology of the only known matrotrophic viviparous anuran, the West African Nimba toad, *Nimbaphrynoides occidentalis*. *Zoosystematics and Evolution* **93**, 105-133.
Sato, K., Nakamura, M., Tomita, T., Toda, M., Miyamoto, K. & Nozu, R. (2016). How great white sharks nourish their embryos to a large size: evidence of lipid histotrophy in lamnoid shark reproduction. *Biology Open* **5**, 1211-1215.
Schindler, J. F. & Hamlett, W. C. (1993). Maternal–embryonic relations in viviparous teleosts. *The Journal of Experimental Zoology* **266**, 378-393.
Schradin, C. & Anzenberger, G. (1999). Prolactin, the hormone of paternity. *News in Physiological Sciences* **14**, 223-231.
Skalkos, Z., Van Dyke, J., Dowland, S. & Whittington, C. (2024). Paternal protein provisioning to embryos during male seahorse pregnancy. *Reproduction* **167**, e230420.
Skalkos, Z. M. G., Van Dyke, J. U. & Whittington, C. M. (2020). Paternal nutrient provisioning during male pregnancy in the seahorse *Hippocampus abdominalis*. *Journal of Comparative Physiology, Part B* **190**, 547-556.
Skalkos, Z. M. G., Van Dyke, J. U. & Whittington, C. M. (2023). Distinguishing between embryonic provisioning strategies in Teleost fishes using a threshold value for parentotrophy. *Biomolecules* **13**, 166-180.

Stewart, J. R. & Thompson, M. B. (2000). Evolution of placentation among squamate reptiles: recent research and future directions. *Comparative Biochemistry and Physiology, Part A* **127**, 411-431.
Stiffler, D. F. (1993). Amphibian calcium metabolism. *Journal of Experimental Biology* **184**, 47-61.
Stölting, K. N. & Wilson, A. B. (2007). Male pregnancy in seahorses and pipefish: beyond the mammalian model. *BioEssays* **29**, 884-896.
Storrie, M. (2004). Microscopic modifications of the female reproductive tissues of *Mustelus antarcticus*. Deakin University.
Storrie, M. T., Walker, T. I., Laurenson, L. J. & Hamlett, W. C. (2009). Gestational morphogenesis of the uterine epithelium of the gummy shark (*Mustelus antarcticus*). *Journal of Morphology* **270**, 319-336.
Thompson, M. B., Adams, S. M., Herbert, J. F., Biazik, J. M. & Murphy, C. R. (2004). Placental function in lizards. *International Congress Series* **1275**, 218-225.
Thompson, M. B. & Speake, B. K. (2006). A review of the evolution of viviparity in lizards: structure, function and physiology of the placenta. *Journal of Comparative Physiology, Part B* **176**, 179-189.
Thompson, M. B., Speake, B. K., Russell, K. J. & McCartney, R. J. (2001). Utilisation of lipids, protein, ions and energy during embryonic development of Australian oviparous skinks in the genus *Lampropholis*. *Comparative Biochemistry and Physiology, Part A* **129**, 313-326.
Thompson, M. B., Stewart, J. R. & Speake, B. K. (1999). Comparison of nutrient transport across the placenta of lizards differing in placental complexity. *Comparative Biochemistry and Physiology, Part A* **127**, 469-479.
Thompson, M. B., Stewart, J. R. & Speake, B. K. (2000). Comparison of nutrient transport across the placenta of lizards differing in placental complexity. *Comparative Biochemistry and Physiology, Part A* **127**, 469-479.
Van Dyke, J., U. , Griffith, O., W. & Thompson, M., B. (2014). High food abundance permits the evolution of placentotrophy: evidence from a placental lizard,*Pseudemoia entrecasteauxii*. *The American Naturalist* **184**, 198-210.
Vilter, V. & Lugand, A. (1959). Trophisme intra-utérin et croissance embryonnaire chez le *Nectophrynoides occidentalis* Ang., crapaud totalement vivipare du Mont Nimba (Haute Guinée). *Comptes Rendus des Séances de la Société de Biologie et de ses Filiales* **153**, 29-32.

Wake, M. H. (1977). Fetal maintenance and its evolutionary significance in the Amphibia: Gymnophiona. *Journal of Herpetology* **11**, 379-386.
Wake, M. H. (1993). Evolution of oviductal gestation in amphibians. *The Journal of Experimental Zoology* **266**, 394-413.
Wake, M. H. (2014). Fetal adaptations for viviparity in amphibians: amphibian fetal adaptations. *Journal of Morphology* **276**, 941-960.
Warne, R. W. & Catenazzi, A. (2016). Pouch brooding marsupial frogs transfer nutrients to developing embryos. *Biology Letters* **12**, 20160673.
Watanabe, S. (1999). The role of male brood pouch in the reproduction of the seaweed pipefish, *Syngnathus schlegeli*. Univeristy of Tokyo.
Watanabe, S., Kaneko, T. & Watanabe, Y. (1999). Immunocytochemical detection of mitochondria-rich cells in the brood pouch epithelium of the pipefish, *Syngnathus schlegeli*: Structural comparison with mitochondria-rich cells in the gills and larval epidermis. *Cell and Tissue Research* **295**, 141-149.
Wei, Y.-L., Wen, B., Gao, J.-Z. & Chen, Z.-Z. (2021). Brain transcriptome analysis reveals genes involved in parental care behaviour in discus fish (*Symphysodon haraldi*). *General and Comparative Endocrinology* **309**, 113793.
Wetzel, J. & Wourms, J. P. (1995). Adaptations for reproduction and development in the skin-brooding ghost pipefishes, *Solenostomus*. *Environmental Biology of Fishes* **44**, 363-384.
Wetzel, J., Wourms, J. P. & Friel, J. (1997). Comparative morphology of cotylephores in *Platystacus* and *Solenostomus*: Modifications of the integument for egg attachment in skin-brooding fishes. *Environmental Biology of Fishes* **50**, 13-25.
Whittington, C. M., Buddle, A. L., Griffith, O. W. & Carter, A. M. (2022). Embryonic specializations for vertebrate placentation. *Philosophical Transactions of the Royal Society of London, Series B* **377**, 20210261-20210261.
Whittington, C. M. & Friesen, C. R. (2020). The evolution and physiology of male pregnancy in syngnathid fishes. *Biological Reviews of the Cambridge Philosophical Society* **95**, 1252-1272.
Whittington, C. M., Griffith, O. W., Qi, W., Thompson, M. B. & Wilson, A. B. (2015). Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Molecular Biology and Evolution* **32**, 3114-3131.
Wilkinson, M., Kupfer, A., Marques-Porto, R., Jeffkins, H., Antoniazzi, M. M. & Jared, C. (2008). One hundred million years of skin feeding? Extended parental care in a Neotropical caecilian (Amphibia: Gymnophiona). *Biology Letters* **4**, 358-361.

Wilson, A. B. & Orr, J. W. (2011). The evolutionary origins of Syngnathidae: pipefishes and seahorses. *Journal of Fish Biology* **78**, 1603-1623.

Wourms, J. P., Grove, B. D. & Lombardi, J. (1988). The maternal-embryonic relationship in viviparous fishes. In: *Fish Physiology*, pp. 1-134: Elsevier Science & Technology.

Xavier, F. (1971). Recherches sur l'endocrinologie sexuelle de la femelle de *Nectophrynoides occidentalis* Angel (Amphibien Anoure vivipare).

Xavier, F. (1973). Le cycle des voies génitales femelles de *Nectophrynoides occidentalis* Angel, Amphibien Anoure vivipare. *Cell and Tissue Research* **140**, 509-534.