The evolution and physiology of male pregnancy in syngnathid fishes

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1. Abstract

The seahorses, pipefishes and seadragons (Syngnathidae) are among the few vertebrates in which pregnant males incubate developing embryos. Syngnathids are popular in studies of sexual selection, sex-role reversal, and reproductive trade-offs, and are now emerging as valuable comparative models for the study of the biology and evolution of reproductive complexity. These fish offer the opportunity to examine the physiology, behavioural implications, and evolutionary origins of embryo incubation, independent of the female reproductive tract and female hormonal milieu. Such studies allow us to examine flexibility in regulatory systems, by determining whether the pathways underpinning female pregnancy are also co-opted in incubating males, or whether novel pathways have evolved in response to the common challenges imposed by incubating developing embryos and releasing live young. The Syngnathidae are also ideal for studies of the evolution of reproductive complexity, because they exhibit multiple parallel origins of complex reproductive phenotypes. Here we assay the taxonomic distribution of syngnathid parity mode, examine the selective pressures that may have led to the emergence of male pregnancy, describe the biology of syngnathid reproduction, and highlight pressing areas for future research. Experimental tests of a range of hypotheses, including many generated with genomic tools, are required to inform overarching theories about the fitness implications of pregnancy and the evolution of male pregnancy. Such information will be widely applicable to our understanding of fundamental reproductive and evolutionary processes in animals.

Keywords

brood pouch, convergent evolution, embryonic incubation, parent-offspring conflict, pipefish, ~~pseudoviviparity,~~ reproduction, seahorse

2. Contents

3. Introduction

Reproductive success is arguably the critical metric of Darwinian fitness, fundamentally influencing the ability of individuals to contribute genes to the next generation. There are two traditionally recognised parity modes in ~~vertebrate~~ animals, which are major determinants of reproductive success: live-bearing (viviparity) and egg-laying (oviparity) (Blackburn, 2015a). Parity mode is adaptively complex, and embryos in oviparous species may also receive substantial post-parition care by parents, sometimes via incubation on or in the parental body (termed 'brooding') (Ostrovsky *et al.*, 2016). Oviparity is the ancestral state, and the subsequent evolution of embryo incubation by parents has involved selection on a complex set of traits integrating morphology, physiology, and behaviour in support of internally developing embryos (Wourms & Lombardi, 1992). Both brooding (incubation of embryos after they are released externally; post-paritive incubation) and viviparity (incubation of embryos before they are released externally; pre-paritive incubation) can allow parents to protect developing embryos from predators and regulate optimal conditions for embryonic development (Smith & Shine, 1997). Brooding and viviparity can enable parents to manipulate offspring phenotypes to maximise their fitness (e.g. Robert & Thompson, 2001; Shine & Harlow, 1993), and can permit greater parental or embryonic control over the timing of resource allocation (Trexler & DeAngelis, 2003; Van Dyke, Griffith

Oliver & Thompson, 2014b). Furthermore, brooding and viviparity could decrease competition for limited reproductive niches (Wourms & Lombardi, 1992). It~~, and~~ may have facilitated the exploitation of new habitats, such as pelagic regions (Wourms & Lombardi, 1992) and cold climates (Shine, 2004), where oviparity without parental care of eggs would reduce embryonic survival. However, incubating parents also incur costs, including those associated with parent-offspring conflict (Crespi & Semeniuk, 2004), a fecundity and energetic cost of pregnancy, and vulnerability to predation (Shine, 1980; Wourms & Lombardi, 1992).

Studies in a variety of vertebrates show that viviparous and oviparous parents exhibit differences in endocrine activity and metabolism, tissue remodelling and vascularity, nutrient provisioning, immune function, behaviour and performance, and regulation of gene expression (e.g. Whittington *et al.*, 2015b; Griffith *et al.*, 2017; Adams *et al.*, 2007; Qualls & Shine, 1998; Heulin *et al.*, 2002; Heulin *et al.*, 2005; Murphy & Thompson, 2011; Foster *et al.*; Callard *et al.*, 2015). These differences make parity mode a compelling subject for studies of comparative physiology, evolutionary biology, and the genetic basis of novel phenotypes. Furthermore, embryonic incubation has evolved many times independently (Ostrovsky *et al.*, 2016). This includes over 150~~since viviparity has evolved~~ independent~~ly~~ origins of ~~from oviparity over 150 times in~~ vertebrate viviparity~~s~~ (Blackburn, 2015a), and many more ~~times~~ transitions from oviparity to either viviparity or brooding in invertebrates (Ostrovsky *et al.*, 2016). Embryonic incubation~~, it~~ is thus an ideal model for studies of convergent evolution. The evolution of this type of reproductive complexity is currently of broad interest to evolutionary biologists (e.g. Griffith *et al.*, 2015; Whittington *et al.*, 2015b; Cornetti *et al.*, 2018; Pyron, 2015; Van Dyke, Brandley & Thompson, 2014a; Blackburn, 2015b; Buddle *et al.*, 2018; Gao *et al.*, 2019).

a. *Terminology*

After varying interpretations of the term “viviparity” in the early literature, a bipartite model that was originally developed to classify the~~fish~~ reproductive patterns of fish (Wourms, 1981) has been widely adopted by most vertebrate viviparity researchers (Blackburn, 2005; Blackburn, 1982; Blackburn, 2015a). In this model, viviparity (from the Latin *vivus*, live; *pario*, to bear) is defined as reproduction ~~live-bearing~~ with internal fertilisation ~~and~~ followed by embryonic development in the maternal reproductive tract and then release of live young (Blackburn, 2000).~~;~~ ~~o~~Oviparity (*ovum*, egg) is egg-laying (Blackburn, 2000). A second level of classification then refers to the source of embryonic nutrition during development: lecithotrophy (yolk-derived); or matrotrophy (in addition to the yolk). The use of the term “ovoviviparity” is strongly discouraged because its ambiguity generates misconceptions and confusion (Blackburn, 1994; Ostrovsky *et al.*, 2016; Blackburn, 2000).~~:~~ “~~o~~Ovoviviparity” has varyingly been used to refer to the deposition of partially developed embryos; unmetamorphosed anamniote larvae; and fully developed embryos which derive nutrients solely from the yolk, amongst other definitions (Blackburn, 1994).

b. *Unique features of syngnathid reproduction*

There are 316 described species of Syngnathidae (seahorses, pipefish, and seadragons) (Eschmeyer & Fong, 2018). All exhibit some form of male incubation of developing embryos in or on a structure known as a brood pouch (marsupium), after which the young are born live. The unusual nature of syngnathid reproduction and the historical use of different terminologies in different taxa [e.g. see (Blackburn, 2000) for a discussion] makes terminology describing their reproduction a challenging~~difficult~~ issue.

Most vertebrate biologists use the terms oviparity and viviparity in the literal sense: “egg-laying” and “live-bearing”, respectively (Blackburn, 2015a; Blackburn, 2000; Wourms, 1981).

However, the term “viviparity” is widely accepted to apply only to live-bearing species that fertilize and incubate embryos *in the maternal reproductive tract*, which therefore excludes syngnathids. However, male syngnathids have many reproductive characteristics that are analogous to those of viviparous females (Blackburn, 2018). These features include a close association between paternal and embryonic tissues that could facilitate exchange, and the release of offspring that are “are able to feed, locomote, and otherwise interact freely with their environment” as soon as they emerge [the major defining characteristic of viviparity, (Blackburn, 2000)]. To compound the terminological difficulty, some syngnathids (e.g. seahorses) have *physically internal fertilization and embryo incubation*, because their brood pouches are sealed off from the external environment (see Section 4).

“Pseudoviviparity” ~~is~~ has been used to describe a form of oviparous reproduction in which the embryos are incubated on or inside the body of the parent (Blackburn, 2018; Blackburn, 2015a), but the term is not widely used~~, which applies to syngnathids~~. Instead, “~~M~~male pregnancy” ~~also~~ refers to ~~this form of~~ embryonic incubation in syngnathids. Th~~e~~is term has been in widespread use ~~for~~ over decades of syngnathid research (Stolting & Wilson, 2007, and many others; Rosenqvist & Berglund, 2011; Kvarnemo *et al.*, 2011; e.g. Berglund, Rosenqvist & Svensson, 1986b). Therefore, throughout this review, we ~~will~~ use the terms “male pregnancy”~~, “pseudoviviparity”~~ and male “embryo incubation” to describe the complex form of reproduction in syngnathid fishes.

Syngnathids are popular models for examining sexual selection, sex-role reversal and the trade-offs between present and future reproduction (e.g. Berglund, 1991; Berglund & Rosenqvist, 1990; Kvarnemo, 2018; Jones *et al.*, 2000; Hare & Simmons, 2019; Ahnesjö & Craig, 2011). These fish also offer a unique opportunity to examine the evolution of internal embryo incubation, independent of both the female reproductive tract and the female hormonal milieu. Such studies may use syngnathids as models for the flexibility in regulatory systems, by determining whether the hormonal-receptor-cellular response pathways in female pregnancy are co-opted in pregnant males, or whether novel pathways have evolved in response to the common challenges imposed by internal incubation of embryos. Here we comprehensively review the taxonomic distribution and evolution of the brood pouch~~,~~ and the biology of male pregnancy in syngnathid fishes. We examine the selective pressures that may have led to the development of male pregnancy~~,~~ and the evolutionary consequences of these reproductive transitions. We offer a discussion of sex-role reversal and sexual selection in this group only as it pertains to the evolution of pregnancy~~;~~. ~~i~~Interested readers are referred to several excellent reviews examining sex roles in general (Kvarnemo, 2018; Hare & Simmons, 2019; e.g. Berglund & Rosenqvist, 2003). Studies of syngnathid reproduction, which we hope to encourage with our review, have enormous potential to improve our understanding of reproductive physiology, convergence, and the evolution of reproductive complexity. Such information ~~will be~~is widely applicable to our understanding of fundamental reproductive and evolutionary processes in animals.

4. Origins of syngnathid reproductive complexity

a. Variation in and adaptive implications of syngnathid brood pouch morphological complexity

Syngnathid females transfer eggs into or onto a structure on the ventral surface of the male~~,~~ termed the brood pouch. Egg transfer is concomitant with male sperm release, and the fertilised eggs are cared for by males until the release of live young. Brood pouch morphology differs in complexity across the lineage. Building on decades of foundational work (Wilson *et al.*, 2003; Dawson, 1985; Breder, Rosen & History, 1966; Herald, 1959), the brood pouch can be divided into five main morphotypes (Table 1, Figure 1): **Type 1**) gluing of eggs to modified ventral epithelium; **Type 2**) individual open membranous compartments for each egg; **Type 3**) a semi-enclosed brood pouch partially protected by either pouch-plates alone or pouch-folds; **Type 4**) complete enclosure with pouch-folds during embryo incubation, which either meet in everted or inverted fashion; and **Type 5**) an enclosed, sac-like pouch fused along the length and opening via a single anteromesial pore or slit [the pore is inverted in pygmy seahorse pouches, which have a posterior slit (Lourie & Randall, 2003)]. The bony plates characteristic of the body of all syngnathids may be either present (e.g. pipefish *Acentonura* spp., Type 5) or absent (e.g. all seahorses, *Hippocampus* spp., Type 5) in the pouch walls, depending on species (Dawson, 1985). Fertilisation in fully closed brood pouches (Types 4 and 5) is physically internal (Watanabe, 1999; Watanabe, Hara & Watanabe, 2000; Van Look *et al.*, 2007). The pipefish *Syngnathus typhle* (Type 4i in Table 1) does not neatly fit into either the open or closed category, as the pouch closes a few days after egg transfer (Berglund & Rosenqvist, 1990). This shift from external brooding to internal brooding during embryo incubation may represent a transitional form between the open and closed brood pouch.

The closure of the brood pouch is adaptively significant because of the common physiological challenges of embryo incubation experienced by males with an enclosed brood pouch (and the embryos within this structure) and viviparous females in other lineages. For example, given that oxygen is at a much lower concentration in water than in air, respiratory gas supply in an aquatic environment is a ~~key~~ critical limiting factor in fish embryonic development (Little, 1983). An additional challenge of pregnancy in aquatic animals is that respiratory gases diffuse much more slowly through animal tissues than water (Krogh, 1919). Therefore, the need to maintain a supply of respiratory gases, which must diffuse through the closed pouch wall to the embryo, is a major difference between closed and open syngnathid pouches. This situation ~~that~~ may generate selection on pregnant males for mechanisms to facilitate gas exchange (see Section 4). Likewise, in closed pouches, mechanisms to remove or store nitrogenous wastes would be targets of selection (Linton & Soloff, 1964). A closed brood pouch creates additional opportunities that are not afforded by open brood pouches, including for paternal osmoregulation of the brood pouch lumen to provide optimal conditions for embryonic development (Linton & Soloff, 1964; Quast & Howe, 1980), or histotrophic secretion for extraembryonic nutrition, as occurs in some other fish (Wourms, 1981). We therefore expect different adaptations ~~in external versus internally brooding~~across syngnathids in response to the selection imposed on and by the evolution of open versus closed brood pouches~~, respectively~~.

Figure 1. Syngnathid pouch morphology varies in complexity. A. Phyllopteryx taeniolatus- *seadragon pouch, ventral view (Type 2) (Photo: Tom Burd). B.* Syngnathus *sp. pipefish pouch, partially opened along seam to reveal embryos, ventral view (Type 4i) (Photo: © Yuriy Kvach). C.* Hippocampus abdominalis *seahorse pouch (Type 5) (Photo: Jacquie Herbert).*

Table 1. Syngnathid brood pouch classification. All brood pouches are located on the ventral surface of the body (the body cavity is not shown). Diagrams represent transverse sections through the brood pouch, with the dorsal side of the pouch at the top of the image. Embryos are represented by orange circles, with paternal tissues in navy blue. Images are not to scale. Previously, pouch type 3f has been grouped as a sub-type of pouch type 4 (Wilson et al., 2001; Dawson, 1985), but we separate the two due to the differences in incubation environment created by closed (internally-incubating) versus open (externally incubating) brood pouches.

Pouch type	Subtype	Description	Pouch morphology
1		Fully open pouch	
2		Open pouch with invaginated membranous compartments	
3	p	Open pouch partially protected by pouch plates only	
3	f	Open pouch partially protected by pouch folds (plates may also be present)	
4	e	Closed pouch; bilateral pouch folds meet in everted position	
4	i	Closed pouch; bilateral pouch folds meeting in inverted position	

5		Closed pouch; sac-like, opening via a pore or small slit	

b. Phylogeny

Phylogenetic analyses enable us to formulate hypotheses about the origins of brood pouch complexity. The Syngnathidae were historically divided into two major lineages corresponding to the location of the brood pouch on the ventral surface of the male: Nerophinae (Gastrophori; brood pouch located on the abdomen/trunk) and Syngnathinae (Urophori; brood pouch located on the tail) (Herald, 1959; Wilson *et al.*, 2001). Deep nodes in the phylogeny were poorly resolved (Wilson & Orr, 2011), but parallel increases in brood pouch complexity were well-supported in both lineages (Wilson *et al.*, 2003). However, a recent phylogeny (Figure 2) supports a more intricate history of syngnathid reproductive morphology, with multiple independent derivations of brood pouch complexity within the tail and trunk brooders. This analysis is the most complete syngnathid phylogeny to date, and includes almost one third of all syngnathids (91 species; 84 % of described genera) (Hamilton *et al.*, 2017). The tree topology, based on sequences for four nuclear genes, is in strong agreement with morphological data as well as phylogenies constructed using complete mitochondrial genomes of eight species (Wang *et al.*, 2019), and a syngnathiform phylogeny constructed using ultraconserved elements (Longo *et al.*, 2017).

While the abdominal/tail brood pouch location split is generally supported in the newest phylogeny, there are some exceptions. The alligator pipefish *Syngnathoides biaculeatus* is a trunk-brooder nested within a tail-brooding clade (Hamilton *et al.*, 2017; Wilson & Rouse, 2010). The pygmy seahorses (*Hippocampus bargibanti, Hippocampus denise, Hippocampus* *pontohi*) are also trunk-brooders (Lourie & Randall, 2003; Lourie & Kuiter, 2008), nested within the tail-brooding seahorses. We speculate that the pygmy seahorse pouch may have originally been on the tail but was repositioned to the trunk due to correlated selection for both a prehensile tail and diminutive size in these taxa: these fish are 1-2 cm tall, which is far smaller even than pygmy pipehorses and pygmy pipefish (Kuiter, 2009; Lourie & Randall, 2003). Along with morphological data on prehensile tails, fin location, and bony plate arrangement (Hamilton *et al.*, 2017), as well as the large number of transitions between brood pouch types (Figure 2), the phylogeny implies that the syngnathid body plan and morphology was highly labile through evolutionary history.

The evolutionarily plastic body plan of syngnathids is therefore an excellent model to study the development of reproductive complexity. There are multiple origins of more complex brood pouch types, and several possible transitional pouch forms in this group. For example, the pipefish *Maroubra perserrata* has a thin deciduous membrane along the ventral midline separating rows of eggs (Dawson, 1985) that may represent a transitional form from pouch Type 1, in which eggs are glued to the ventral surface of the male, to Type 2, in which eggs are partially enclosed in individual membranous compartments. *Amphelikturus dendriticus* represents a possible transitional pouch form from everted pouch flap closure (Type 4e) to the sac-like pouch (Type 5), as in this species the pouch flaps are completely fused over the posterior two tail rings (Herald, 1959). The phylogeny demonstrates a minimum of three independent origins of the most complex brood pouch type (Type 5), in *Hippocampus* spp., *Acentronura* and *Idiotropiscis* spp., and *Apterygocampus eppinulatus* (Figure 2), from less complex pouch types.

Figure 2. Phylogeny of representative Syngnathidae based on the most recent and comprehensive consensus phylogeny (Hamilton et al., 2017). Pouch morphology is taken from Dawson (1985), with added information from other primary sources: (Herald, 1959) (Anarchopterus criniger; Amphelikturus dendriticus; Bryx *sp.); (Scott, 1961)* (Lissocampus caudalis*); (Dawson, 1981)* (Enneacampus ansorgii*);* Halicampus macrorhynchus *(Kuiter, 2009; Kvarnemo & Simmons, 2004). Star indicates a trunk-brooding species within the tail-brooding lineage. Maximum likelihood support values are shown if <75.*

Brood pouch types are described in Table 1.

c. Multiple origins of the closed brood pouch

Syngnathids with open brood pouches (Types 1-3) brood embryos externally. We describe this complex form of syngnathid parental care as skin-brooding: the incubation of developing embryos on the body surface of an oviparous parent. Skin-brooding encompasses a subset of other ~~widely distributed~~ teleosts, including the external bearers of the ecology-focused reproductive guilds proposed by Balon [(1975) (transfer brooders, forehead brooders, skin-brooders, and some pouch brooders) and Balon (1981) (transfer brooders, auxiliary brooders, and some pouch brooders)]. The internally brooding syngnathids have pouches of Type 4, with sealed folds (e.g. the pipefishes *Hippichthys* spp. and *Syngnathus* spp.), and Type 5, sealed longitudinally apart from a single slit- or pore-like opening [including all 55 *Hippocampus* spp. seahorses, three *Acentronura* spp. pipehorses, three *Idiotropiscis* spp. pygmy pipehorses, and the pipefish *Apterygocampus epinnulatus*, the only member of the genus (species numbers from Froese & Pauly, 2012)].

Ancestral state reconstructions reveal a probable ancestral state of no pouch, followed by repeated origins of different pouch types, including multiple origins of the closed brood pouch (Hamilton *et al.*, 2017). The closed brood pouch has only evolved in the Syngnathinae; all Nerophinae are have open brood pouches (Figure 2). Closed-pouch genera tend to be very speciose, with wide geographic distribution (Dawson, 1985; Wilson *et al.*, 2001). In extant syngnathids, closed pouches are associated with prehensile tails, ecological factors such as low population densities, and monogamous mating systems (Hamilton *et al.*, 2017; Herald, 1959). The potential for correlated evolution of locomotion, dispersal, feeding ecology and the evolution of ~~the~~ brood pouch complexity has yet to be fully explored.

5. The evolution of male pregnancy

a. Teleost parental care

In contrast to most other vertebrates, sole male care is the most common form of parental care in teleosts (Gross, 2005; Gross & Shine, 1981; Gross & Sargent, 1985; Benun Sutton & Wilson, 2019) and has evolved independently at least 22 times in ray-finned fish (Mank, Promislow & Avise, 2005), in a diversity of forms. Examples of paternal care include skin-brooding, mouth brooding, and nest building. Male parental care in fish has evolved most often from external fertilization with no parental care; there have been few to no transitions to male care from bi-parental care or maternal care (Mank *et al.*, 2005; Gross & Sargent, 1985).

Various non-mutually exclusive hypotheses may explain the commonality of male care in teleosts and the striking patterns of its repeated evolution. These include the order of gamete release hypothesis, in which the last parent to deposit gametes (usually the male in external fertilizers) is left in a ‘cruel bind’ to care for the embryos~~,~~ if caring results in increased offspring survival; the association hypothesis, in which externally fertilizing males are more likely to be associated with offspring than internal fertilizers, and therefore have the opportunity to guard embryos; the costs hypothesis, in which caring males experience lower costs to future fecundity than caring females; and the certainty of paternity hypothesis, in which caring males have increased paternity (Kahn, Schwanz & Kokko, 2013; Smith & Wootton, 1995; Kvarnemo, 2006; Gross & Shine, 1981; McNamara & Wolf, 2015; Gross, 2005; Benun Sutton & Wilson, 2019). Many forms of male teleost care still allow males to mate multiply (for example, where females spawn in the home range of a territorial male), which relaxes the usual trade-off between caring and mating. This situation~~,~~ leaves the~~ing~~ potential reproductive rates of caring males unconstrained (Gross & Shine, 1981; Jennions & Fromhage, 2017).

b. *Extreme forms of male care*

Skin-brooding (Figure 3) probably evolved from non-guarding ancestors displaying no parental care (Balon, 1975). Female skin-brooding teleosts include ricefish (e.g. *Oryzias eversi*), which incubate an egg mass in an abdominal concavity and between enlarged pelvic fins (Herder, Hadiaty & Nolte, 2012; Balon, 1975); ghost pipefish (Solenostomidae), which brood eggs on integumentary outgrowths (cotylephores) inside fused pelvic fins (Wetzel & Wourms, 1995); and aspredinid catfish (e.g. *Platystachus*), which incubate developing embryos on cotylephore outgrowths on the abdomen or fin (Wetzel, Wourms & Friel, 1997; Wyman, 1859). Skin-brooding also occurs in other vertebrate mothers, including two lineages of back-brooding frogs (Wake, 2014; Berglund & Rosenqvist, 1990).

Aside from syngnathids, there are only a few examples of such extreme forms of care in males. Male *Rhinoderma darwinii* frogs incubate embryos in the vocal sac (Goicoechea, Garrido & Jorquera, 1986). Male nurseryfish (*Kurtus gulliveri*) have a vascularised forehead hook on which they incubate an egg mass (Berra & Humphrey, 2002). Male dactyloscopids are ‘armpit brooders’, incubating egg masses under each pectoral fin (Balon, 1981); and male anglerfish *Antennarius caudimaculatus* incubate embryos on the dorsal integument (Pietsch & Grobecker, 1980; Crespi & Semeniuk, 2004; Wourms & Lombardi, 1992).

Male incubation of embryos on or in the body is therefore rare, despite the predominance of other forms of male care in teleosts. There are several possible explanations for the scarcity of this extreme form of male care, which are not mutually exclusive. Gamete transfer is potentially risky, which may favour gamete donation from the sex with the fastest gametogenesis (i.e. sperm donation rather than egg donation), resulting in female brooding more often than male brooding (Baylis, 1981). Secondly, there is usually a trade-off between extreme forms of care versus opportunity costs of not being available in the mating pool. Body size limits fecundity in species that incubate embryos, as there is a maximum clutch size that can be accommodated on or in the body of a parent. Since males are usually capable of fertilizing multiple egg clutches, male incubation of embryos could limit the potential reproductive rate of males and thus be selected against (McDiarmid, 1978). Thus, male incubation of embryos would only be selected for if the reduction in male potential reproductive rate is compensated for by increases in paternity assurance or offspring survival, which might occur if female availability or egg clutch size is limiting. It is tempting to speculate that some form of male-biased operational sex ratio (Kokko & Jennions, 2008) has driven the evolution of extreme forms of male care, for example due to the longer reproductive 'time out' of female than male syngnathids (e.g. Masonjones & Lewis, 2000). However, modelling demonstrates that the particular causes of sex ratio differences determine the effect of male-biased sex ratios on the direction of parental care (Fromhage & Jennions, 2016). For example, if maturation sex ratio (ratio of males to females in individuals mating for the first time) is male-biased or male mortality is lower than female mortality, then male care will be selected for (Fromhage & Jennions, 2016; Jennions & Fromhage, 2017). The possibility of sex-biased mortality in syngnathids remains to be examined.

In discussing the possible factors influencing the evolution of male embryo incubation, we must extrapolate from general models of male care, and we are restricted to verbal models, because there are no formal models available. Modelling the evolution of paternal care within syngnathids would aid empirical researchers in defining what ecological variables or traits would be most fruitful to address with experimental manipulation or phylogenetic comparative methods ~~within this group of fish~~. Given the rarity of male pregnancy, even a simple model demonstrating how it *might* have evolved in syngnathids could be useful in understanding other systems where males engage in extreme forms of parental care, and why male pregnancy is so rare.

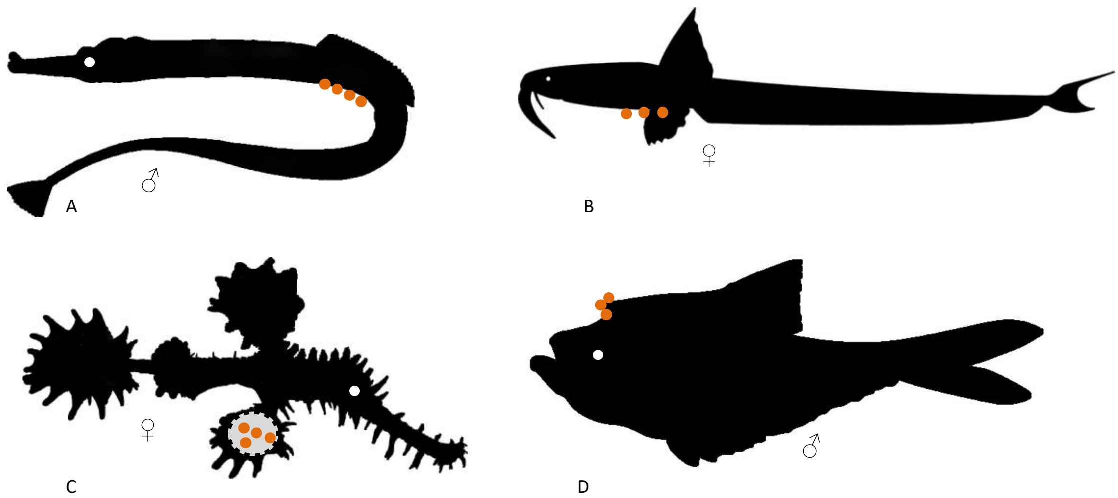

Figure 3. Examples of skin-brooding in teleosts: A) Male pipefish with open brood pouch; B) Female aspredinid catfish; C) Female ghost pipefish; D) Male nurseryfish. Embryos are shown in orange, and images are not to scale.

c. The evolution of syngnathid pregnancy

Phylogenetic analysis repeately recovers Solenostomidae (ghost pipefishes) as the sister lineage to Syngnathidae (Wilson & Orr, 2011; Longo *et al.*, 2017). Solenostomids are female skin-brooders in which developing embryos are incubated inside fused pelvic fins (Figure 3C). Epithelial outgrowths develop on the inside surface of the fins, adjacent to the fin rays, to form cotylephores that attach to the embryonic chorion (Wetzel & Wourms, 1995). However, it is unlikely that the ancestral syngnathid was a female skin-brooder, because teleost transitions from female care to male care are rare to non-existent; male care has most commonly evolved from external fertilisation with no parental care (Mank *et al.*, 2005). It is therefore likely that the ancestral, externally fertilising syngnathid did not have any parental care; this hypothesis is supported by ancestral state reconstruction (Benun Sutton & Wilson, 2019). Pegasids (sea moths and dragon fish) are free-spawning syngnathiform fish related to ghost pipe fish and syngnathids (Longo *et al.*, 2017). These monogamous fish display a stereotyped breeding behaviour in which pairs swim vertically through the water column and spawn at the top (Herold & Clark, 1993), which is strikingly similar to movements preceding egg transfer in some syngnathids (Masonjones & Lewis, 1996; Watanabe, 1999; Woods, 2000; Whittington *et al.*, 2013). Such spawning is common in reef fish, and it is possible that a similar behaviour in ancestral syngnathids might have resulted in a close association between males and fertilised eggs that eventually led to male parental care.

Nest building is an exaptation for male parental care of embryos in some teleosts (Mank *et al.*, 2005), and is common in the sticklebacks (Gasterosteidae) [sticklebacks and syngnathids are related percomorphs (Small *et al.*, 2016)]. Gasterosteids secrete a cement to build nests; a behavioural shift could conceivably result in glue instead being used to attach and transport embryos on the male's body (Baylis, 1981). Ancestral syngnathids could have displayed similar nest gluing behaviour, or even adhesive eggs that could stick to the male integument. Indeed, the eggs of some extant syngnathids are sticky (Watanabe, 1999), and excess eggs can temporarily adhere to the outside of the pouch (Breder *et al.*, 1966; Straughan, 1960). Selection for egg adhesion to the male ~~ventral~~ epithelium could have resulted in a simple open brood pouch (McCoy, Jones & Avise, 2001), followed by the repeated parallel evolution of brood pouch closure, a theory supported by character mapping on the syngnathid phylogeny (Hamilton *et al.*, 2017) (Figure 2). The selective pressures leading to the evolution of syngnathid embryo incubation are likely to be complex and overlapping (Figure 4), and we explore the possibilities here in view of current theories of evolution of male care in teleosts.

d. Potential drivers of syngnathid embryo incubation in open brood pouches

Syngnathid skin-brooding in open brood pouches may have evolved from ancestors in which paternity was maximised by territorial males remaining close to eggs at spawning (Wilson *et al.*, 2001), with this paternity assurance acting as a 'kick start' leading to the evolution of male parental care (Kahn *et al.*, 2013). Even male egg-guarding fishes (e.g. nesting gasterosteids) experience high rates of cuckoldry (DeWoody & Avise, 2001; Jones, Ostlund-Nilsson & Avise, 1998), and so selection in the syngnathid lineage may have favoured adhesion of eggs to the skin of the male to safeguard eggs from sneaker males and increase paternity (Watanabe, 1999; Wilson *et al.*, 2001). Syngnathid gonadosomatic index (GSI) is very low compared to other teleosts, which indicates low levels of sperm competition (Kvarnemo & Simmons, 2004). Initial investment in care by one sex can lead to positive feedback that increases the degree of care by that sex (Fromhage & Jennions, 2016). Therefore, initial selection for male egg-guarding to maximise paternity might produce an association between fathers and offspring, in turn leading to the evolution of extended male care if such care increases offspring survival (Kahn *et al.*, 2013).

Under conditions of high predation, brooding of embryos in an open pouch could have enabled basal syngnathid males to safeguard developing offspring from predators (Watanabe, 1999; Wilson *et al.*, 2001; Smith & Wootton, 1995). Theory predicts that evolution should minimize the time organisms spend in the most dangerous life history stages (Shine, 1978; Williams, 1966). In syngnathids, the exposure of vulnerable developing embryos to the external environment is limited: by the time syngnathid neonates are free-swimming in the external environment, they generally have fully resorbed yolk sacs, cryptic colouration, well-developed vision systems, fully developed snouts, and prehensile tails in species that have them as adults (Sommer, Whittington & Wilson, 2012; Ofelio *et al.*, 2018; Álvarez-Hernán *et al.*, 2019; Wetzel & Wourms, 2004). These precocial neonates are fully independent and can feed, swim and successfully seek cover. In contrast, for example, stickleback hatchlings emerge at a much earlier stage of development, with a heavy yolk sac, poor swimming ability and incomplete jaws and gills (Swarup, 1958). The vulnerable hatchlings are actively guarded by fathers for varying periods until they reach a more advanced developmental stage (e.g. Whoriskey & FitzGerald, 1994). Syngnathid skin-brooding may thus act as an analogous but passive guarding strategy to shield altricial embryonic stages from predators during development.

e. Potential drivers of the closure of the syngnathid brood pouch

Once eggs adhered to the male integument, selection could drive the development of morphological specialisations and closure of the brood pouch. The syngnathid phylogeny indicates multiple independent origins of the closed pouch within the Syngnathinae, likely via ~~successive~~ outgrowth~~s~~ of the integument to eventually fully enclose the developing embryos. There are several potential drivers of pouch closure. The most obvious possibility, paternity assurance, is unlikely, because there is no difference between the GSI of pouch types 3,4,5 and types 1 and 2 (Kvarnemo & Simmons, 2004). Male syngnathids appear to gain full paternity regardless of pouch type (Jones & Avise, 2001; McCoy *et al.*, 2001). As cuckoldry is physically possible in an open pouch (McCoy *et al.*, 2001), the eggs are probably fertilised directly at laying (Kvarnemo & Simmons, 2004; Monteiro, da Natividade Vieira & Almada, 2002). This idea is supported by the fact that pipefish *Nerophis ophidion* spermatozoa (Type 1 pouch) are activated in a mixture of seawater and ovarian fluid, and cannot be activated if they enter seawater first (Ah-King *et al.*, 2006).

We can identify three selective advantages that either alone or in combination may have resulted in brood pouch closure: 1) decreased predation mortality and increased embryonic survival; 2) increased brood size; and 3) increased control over reproduction (Figure 4). Firstly, brooding *Nerophis ophidion* males with open Type 1 pouches are more susceptible to predation than non-reproductive males (Svensson, 1988). Syngnathid eggs and developing embryos are brightly coloured (orange, yellow, pink); camouflaging them by overgrowth of pouch folds may have increased adult and embryo survival rates. Closed brood pouches also offer the opportunity to osmoregulate and provide nutrients to developing embryos, which could increase the rate of embryonic development, the size of resulting fry, and neonate survival in unstable environments (Watanabe, 1999; McCoy *et al.*, 2001). Secondly, closed pouches are correlated with larger brood sizes than open pouches; open pouches have eggs arranged in a single layer, whereas closed pouches allow multiple layers of eggs to be in contact with paternal tissues (Monteiro, Almada & Vieira, 2005). The closed pouch may have facilitated polyandry, in which males have the capacity to accept eggs from more than one female (Monteiro *et al.*, 2005) (although polyandry is also physically possible in open pouches, and many closed-pouch species are monogamous). Finally, there is growing evidence that brood pouches may be an arena of sexual conflict, in which the closed pouch could give males greater reproductive control (Paczolt & Jones, 2010; Da Cunha *et al.*, 2018; Braga Goncalves, Ahnesjö & Kvarnemo, 2016).

f. Male 'control' in the closed brood pouch

Syngnathid pregnancy represents a trade-off ~~between~~ against male survival and future reproduction. Pregnancy increases metabolic rate (Masonjones, 2001) and reduces feeding (Da Cunha *et al.*, 2015) and the somatic growth (Paczolt & Jones, 2015; Svensson, 1988) that would ordinarily increase embryo carrying capacity in future broods, since larger males can carry more embryos (Berglund, Rosenqvist & Svensson, 1986a). Some species experience a trade-off between brood size and offspring size/performance (Ahnesjö, 1992; Faleiro *et al.*, 2016; but see Mobley *et al.*, 2011a). Brood size and resource availability also influence the costs of male care: embryo mortality is inversely proportional to male condition, and male mortality increases with larger brood size (Sagebakken, Ahnesjö & Kvarnemo, 2016). The costs of male pregnancy may be offset by benefits accrued through post-copulatory processes [analogous to benefits suggested to drive the evolution of cryptic female choice ~~(CFC)~~ mechanisms (Eberhard, 1996)]. One post-copulatory mechanism of male choice is via brood reduction, which has only been noted in closed pouches. Brood reduction could be the result of selective egg fertilization, or embryo abortion, thus biasing care toward offspring of preferred mates. In the pipefish *S. typhle*, eggs from more attractive (larger) females have greater survival rates than those from smaller females, although this could be a female-mediated effect via a correlation between female size and the size of her eggs (Partridge *et al.*, 2009). Large eggs have higher survival rates than small eggs in mixed egg-size broods with multiple ~~p~~maternity, and small eggs fare worse in mixed broods than homogeneous broods, suggesting that either: 1) the embryos compete with one another for resources, or 2) the father is able to differentially partition parental care (Ahnesjö, 1996). In *Syngnathus scovelli*, brood reduction increases in broods from less attractive females, and as a function of the characteristics of the prior brood (Paczolt & Jones, 2010). If *Syngnathus abaster* males are exposed to a more attractive female during pregnancy, they have higher rates of brood reduction and produce smaller offspring (Da Cunha *et al.*, 2018).

The closed pouch also makes it possible for the male to capture nutrients from maternal sources. For example, male *S. typhle* can resorb egg nutrients (Sagebakken *et al.*, 2010). Recouping maternally-derived nutrients is more difficult to envisage in an open brood pouch; closed pouch fluid contains yolk from broken or undeveloped eggs (Ripley & Foran, 2006; Boisseau & Lemenn, 1967; Boisseau, 1967) that would be lost to the environment in an open brood pouch. Pouch closure may facilitate a) resource conservation, if eggs that naturally fail to develop are resorbed, or b) direct conflict with females (see Section 7). Conflict with females would occur if either fathers selectively abort and resorb eggs and use the resources for somatic growth/future reproduction, with no benefit to the current brood; or if the aborted egg nutrients are used by half-sibling embryos in ~~polyandrous~~mixed maternity broods, which may also generate parent-offspring conflicts. Such resource partitioning could be particularly adaptive in environments where resources are variable or limiting. However, demonstrating and disentangling mechanisms of selection in cryptic male choice ~~(CMC)~~ and sexual and parent-offspring conflict is fraught with some of the same difficulties as understanding ~~CFC~~ cryptic female choice (Friesen & Olsson, 2016), including female investment into gametes that may obscure male-mediated processes. Investigating the physiological mechanisms that underpin syngnathid pregnancy will help to elucidate variables that may respond to selection under scenarios of sexual and parent-offspring conflict, or as mechanisms of ~~CMC~~cryptic male choice.

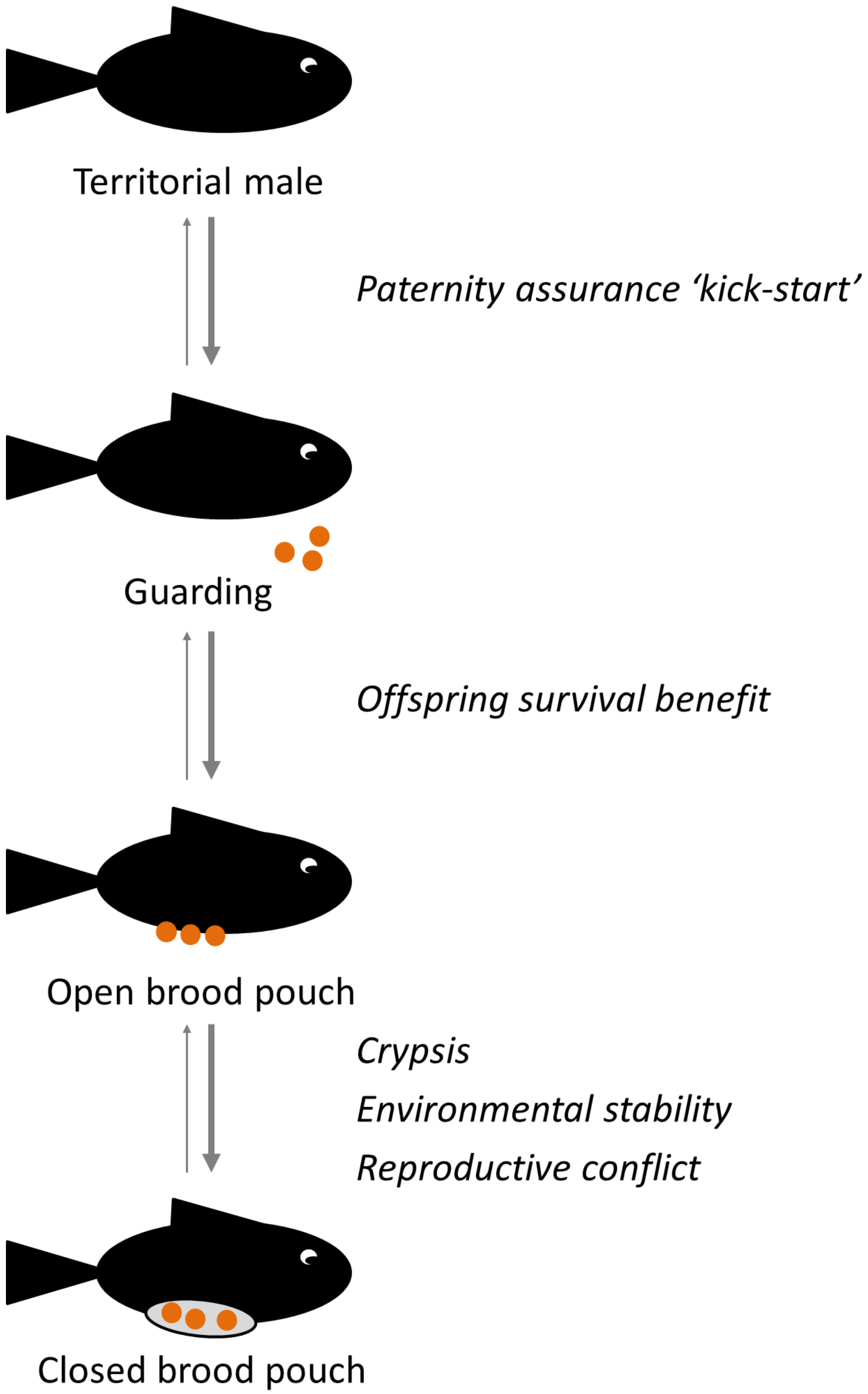

Figure 4. Possible selective forces leading to the evolution of different forms of syngnathid egg incubation. Eggs/embryos are shown in orange.

6. Physiology of syngnathid pregnancy

Despite the utility of syngnathid pregnancy in evolutionary research, much of their reproductive physiology remains poorly understood. This situation is problematic given that evolutionary theory rests on a strong foundation of knowledge of reproductive biology. Here we synthesise the state of the field and highlight pressing areas for future study.

a. Brood pouch morphology

Morphology differs between pouches of differing complexity, possibly reflecting different degrees of male care during embryo incubation. In particular, more complex pouches have increased contact between paternal and embryonic tissues (Carcupino *et al.*, 2002), which may be important for bidirectional exchange of respiratory gases, nutrients, and wastes as in other internally incubating taxa (Ostrovsky *et al.*, 2016). While a vascularised underlying dermis is common to syngnathid pouches, other structures differ across the lineage. The ultrastructure of *Nerophis ophidion* pouch epithelium (Type 1)~~, although it is covered with microridges that may facilitate egg adhesion,~~ is very similar to typical fish epidermis, although it is covered with microridges that may facilitate egg adhesion (Carcupino *et al.*, 2002). In contrast, *Syngnathus* spp. pouches (Type 4i) have a thinner micro-ridged epithelial layer of pavement cells plus unusually mitochondrial-rich cells, which may function in osmoregulation and gas exchange (Carcupino *et al.*, 2002; Carcupino *et al.*, 1997; Watanabe, Kaneko & Watanabe, 1999).

Seahorse (*Hippocampus* spp.) pouches (Type 5) are fully closed apart from a single pore and are very fleshy. The *Hippocampus hippocampus* pouch has folded internal epithelium, plus specialised cell types, including modified secretory flame-cone cells that may be involved in extraembryonic nutrition (Carcupino *et al.*, 2002). Seahorse pouches also consist of multiple layers (Figure 5): an outer epithelium of cuboidal cells, mucous granules, and flame cone cells; a vascularised elastic dermis (stratum spongiosum, made up of loosely associated connective tissue; scattered smooth muscle; and stratum compactum, made up of tightly packed collagen); and an inner layer sometimes referred to as a “pseudoplacenta”. This inner layer, which closely apposes to embryonic membranes, is in fact one half of a functional placenta (Kawaguchi *et al.*, 2017; Laksanawimol, Damrongphol & Kruatrachue, 2006), which is defined as “any intimate apposition or fusion of the fetal organs to the maternal (or paternal) tissues for physiological exchange” (Mossman, 1937). The inner layer consists of crosslinked vascularised connective tissue, bordered by a luminal epithelium without mucous cells (Kawaguchi *et al.*, 2017). The brood pouch is formed developmentally by outgrowths of the male abdominal epithelium on the ventral surface that elongate towards the midline and fuse to form a bag that differentiates into the discrete layers of the mature pouch (Kawaguchi *et al.*, 2017). Rapid morphological change during pregnancy, particularly of the inner layer, suggests precise regulation of the internal environment during embryo development (Laksanawimol *et al.*, 2006).

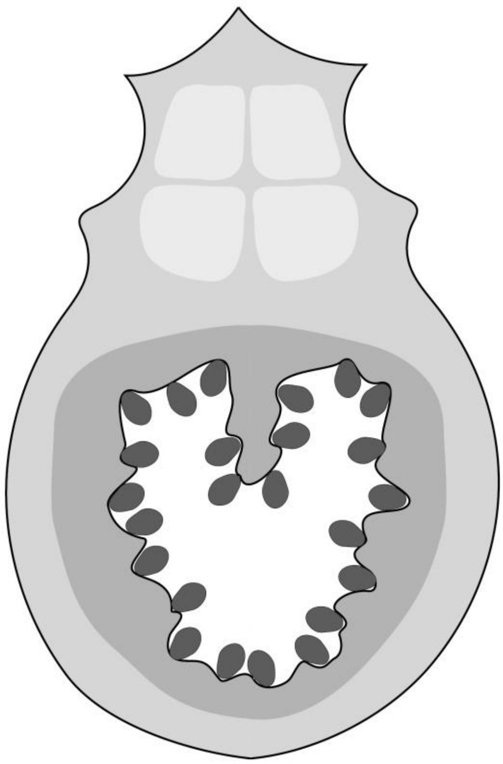

*Figure 5. Transverse section through the tail and brood pouch (Type 5) of a pregnant male seahorse (*Hippocampus *spp.) (top: dorsal, bottom: ventral). Bony plates and vertebra are not shown. Tail skeletal muscle is in palest grey. Dermis is pale grey, inner layer is dark grey, and embryos are darkest grey. Outer and inner epithelium are in black, and the pouch lumen is white. Modified from (Kawaguchi et al., 2017) and (Stolting & Wilson, 2007). Not to scale.*

b. Respiratory gas exchange

One of the major challenges to internal incubation of embryos is meeting the embryonic demand for respiratory gas exchange inside the body of the parent, particularly late in pregnancy~~,~~ when embryonic oxygen demand peaks (Berglund *et al.*, 1986b). Exchange of respiratory gases is critical~~,~~ because hypoxia negatively affects syngnathid embryonic development (Braga Goncalves, Ahnesjo & Kvarnemo, 2015a). In an open brood pouch, embryos are partially exposed to ambient water, whereas respiratory gases must diffuse through the thin pouch flaps or thick pouch walls in species with closed brood pouches. Therefore in species with closed pouches, we expect either: 1) paternal adaptations for the supply of respiratory gases via angiogenesis and vasculogenesis, increased gestational surface area, decreased blood oxygen-~~-~~carrying capacity compared to embryos, or pouch flushing; 2) embryonic development that is robust to low oxygen environments; 3) embryonic adaptations to increase gas exchange, such as highly developed respiratory organs or increased oxygen-~~-~~carrying capacity of blood via modified respiratory pigments; or 4) a combination of adaptations. Such strategies are present in other vertebrates incubating young internally (e.g. Parker *et al.*, 2010; Whittington *et al.*, 2017; Hartvig & Weber, 1984; Skov *et al.*, 2010; Balon, 1975; Ingermann & Terwilliger, 1981; Whittington *et al.*, 2015a; Tomita, Cotton & Toda, 2015).

The eggs of syngnathids with closed brood pouches are larger than those with open pouches (Braga Goncalves, Ahnesjö & Kvarnemo, 2011). Although egg size ~~has no effect on~~does not affect the survival of some syngnathid embryos in hypoxia, possibly because metabolic rate is lower in larger eggs (Braga Goncalves *et al.*, 2015a), egg shape may be important in gas exchange. *Nerophis ophidion* (Type 1) and ~~*Syngnathus*~~ *S. abaster* (Type 4i) eggs are spherical, whilst seahorse (Type 5) eggs are pear-shaped and the narrow end embeds into the vascularised pouch lining (Carcupino *et al.*, 2002). Pear-shaped eggs have~~,~~ ~~with~~ an increased surface area to volume ratio [9 % greater than a sphere of the same volume (Monteiro *et al.*, 2005)], which may result in more efficient gas exchange inside the thicker seahorse pouch. An embryo's position in the pouch may also affect oxygenation (Nygård *et al.*, 2019). Closed pouches are not always well-oxygenated, as *S. typhle* (Type 4i) pouch fluid oxygen saturation decreases during pregnancy in hypoxic waters (Braga Goncalves, Ahnesjö & Kvarnemo, 2015b), and embryonic mortality is higher in closed (*S. typhle, Syngnathus rostellatus*) than open (*Entelurus aequoreus, N. ophidion*) brood pouches (Braga Goncalves *et al.*, 2016). Since closed pouches are a barrier to respiratory gas exchange, this may represent a trade-off against other adaptive advantages (Braga Goncalves *et al.*, 2016):

Blood vessels are present in the inner layer of syngnathid brood pouches (Kawaguchi *et al.*, 2017; Carcupino *et al.*, 2002; Carcupino *et al.*, 1997; Drozdov, Kornienko & Krasnolutsky, 1997; Laksanawimol *et al.*, 2006), possibly as a means of supplying respiratory gases.~~, but have rarely been quantified across pregnancy~~. The high pouch vascularisation in the pouch of *Syngnathus fuscus* and *Syngnathus floridae* (both Type 4i) develops early in pregnancy, remains stable, and then degenerates prior to parturition (Ripley, Williams & Foran, 2010). Angio- and vasculogenesis are therefore likely to be important mediators of gas exchange in closed syngnathid brood pouches. The blood supply is probably most critical to providing gas exchange to developing embryos inside the thick and fleshy seahorse pouch, but the seahorse pouch vascular bed has never been quantified.

c. Osmoregulation

The ability to osmoregulate the lumenal fluids represents another potential selective advantage of the closed brood pouch. Seahorses *Hippocampus erectus* (Linton & Soloff, 1964), *Hippocampus guttulatus*, and *Hippocampus brevirostris* (Leiner, 1934) maintain Type 5 pouch fluid concentrations isosmotic to paternal blood early in gestation, which rise to match the osmotic concentration of seawater by parturition, possibly acclimating young to the external environment. The concentration of Na and Cl in seahorse *Hippocampus barbouri* pouch fluid also changes throughout gestation (Oconer *et al.*, 2006). All seahorses are marine, and thus experience relatively stable osmotic conditions. In contrast, the Type 4 pouches of *S. scovelli* (euryhaline) and *Syngnathus schlegeli* (estuarine) regulate osmolality close to that of paternal blood throughout gestation (Quast & Howe, 1980; Watanabe, 1999). *Syngnathus typhle* maintains consistent pouch osmolality irrespective of salinity of the external environment, and may allow water to seep into the brood pouch late in pregnancy (Partridge, Shardo & Boettcher, 2007), as does *S. schlegeli* (Watanabe *et al.*, 1999). *Syngnathus floridae* and *S.~~yngnathus~~ fuscus* (both marine, Type 4) also regulate pouch osmolality, particularly at mid-stage pregnancy (Ripley, 2009). These species-specific differences in osmoregulation are probably due to the constraints of stable marine versus fluctuating estuarine environments, rather than evolved differences in the osmotic capability of different pouch types (Quast & Howe, 1980).

Syngnathid embryos incubated *in vitro* at low osmolality have a higher survival rate (Linton & Soloff, 1964) and reach a larger size (Azzarello, 1991) than when incubated in sea water. These results suggest that a closed pouch produces optimal conditions for development, particularly until the embryos have developed their own osmoregulatory ability via gills and kidneys (Partridge *et al.*, 2007; Quast & Howe, 1980). The chorion surrounding eggs of species with closed brood pouches (*Hippocampus abdominalis*) is thinner than that of species with open pouches (*Corythoichthys haematopterus, Syngnathoides biaculeatus*)

(Kawaguchi *et al.*, 2016), perhaps because osmoregulation of the closed pouch fluid removes the requirement for a protective chorion, and chorion thinning reduces the barrier to respiratory gas diffusion. In addition, oxygen becomes less soluble at higher salinities, so by reducing pouch salinity, males may enhance oxygen concentration in the pouch (Monteiro *et al.*, 2005).

The mechanism by which closed brood pouch osmoregulation is achieved is unknown. *Syngnathus abaster, S*~~*yngnathus*~~*. schlegeli and S. scovelli* pouches have mitochondrial-rich cells similar to chloride cells, which in fish gills which pump sodium and chloride against a concentration gradient (Carcupino *et al.*, 1997; Watanabe, 1999; Watanabe *et al.*, 1999; Carcupino *et al.*, 2002; Partridge *et al.*, 2007). The pouch mitochondrial-rich cells have apical pits that may facilitate ion transport (Partridge *et al.*, 2009; Watanabe *et al.*, 1999), and contain Na^+,K^+-ATPase, an enzyme involved in ion transport (Watanabe, 1999); their apoptosis at the end of pregnancy underscores their gestation-specific role (Carcupino *et al.*, 1997; Carcupino *et al.*, 2002). Seahorse *H. abdominalis* pouch expresses ion transporter genes including Na^+,K^+-ATPases, but not in a way that explains the salinity increase towards the end of pregnancy (Whittington *et al.*, 2015b). It remains to be seen whether another mechanism such as pouch flushing occurs late in seahorse pregnancy to increase osmolality and acclimate the embryos to the local external environment (which would concurrently increase respiratory gas exchange).

d. Removal of metabolic wastes

The requirement to remove toxic embryonic metabolites from closed pouches (or to sequester these wastes in a non-harmful form) represents a constraint of internal incubation (Monteiro *et al.*, 2005), as in other species (Ostrovsky *et al.*, 2016). Seahorses (Type 5 pouch) excrete both ammonia and urea as a by-product of metabolism (Wilson, Carter & Purser, 2006). Transporters of both molecules are upregulated in seahorse pregnancy, and carbon dioxide transporter genes are similarly upregulated (Whittington *et al.*, 2015b). We do not yet know whether the transporter molecules encoded by these genes are sufficient to remove embryonic wastes from the pouch fluid, or whether another mechanism is involved.

e. Extraembryonic nutrition

Energetic demand during syngnathid development is high (Faleiro & Narciso, 2010). Nutrients transported from father to embryo during syngnathid pregnancy may therefore have significant fitness benefits. Lecithotrophy is the maternal provisioning of nutrients prior to ovulation, whilst species which transport nutrients from the parent to developing embryos are parentotrophic [nutrients provided by the mother (matrotrophic) or father (patrotrophic)] (Ostrovsky *et al.*, 2016; Wourms, 1981; Blackburn, 2000). Lecithotrophy and matrotrophy/patrotrophy represent extremes of a continuum (Ostrovsky *et al.*, 2016; Ostrovsky, 2013). Although syngnathid embryos receive nutrients from mothers via their yolk-rich eggs, patrotrophy may also occur (Ripley & Foran, 2006; Ripley & Foran, 2009).

Matrotrophy indices (MI) have been widely used to determine the source of embryonic nutrients in viviparous vertebrates (Reznick, Mateos & Springer, 2002). The MI is calculated by comparing the dry mass of newborns to the dry mass of ovulated eggs. If the mass of newborns is similar to or larger than that of ovulated eggs, then matrotrophy is indicated. In teleost fish, MI > ~0.65 is often used as the cut-off to indicate that matrotrophy is occurring (Reznick *et al.*, 2002).This threshold was calculated for poecilids (i.e., guppies, mollies, platies and swordtails) and is based on the mass lost during development in oviparous (solely lecithotrophic) species. However, MI thresholds are widely debated, as the amount of nutrients transferred may be small, and the metabolic cost of embryonic development (;

and thus mass lost as metabolic wastes and heat)~~,~~ may differ between species (Frazer, Ellis & Huveneers, 2012; Buddle *et al.*, 2018). The egg size and the degree of patrotrophy ha~~s~~ve been quantified for very few syngnathids (Ripley & Foran, 2009).

Syngnathus schlegeli (Type 4i) has a patrotrophy index of 0.71 (Watanabe & Watanabe, 2002), indicating incipient patrotrophy (minor paternal supplementation); however, its embryos do not survive *in vitro* incubation, suggesting that paternal supplementation may be essential (Drozdov *et al.*, 1997). The *Hippocampus fuscus* patrotrophy index is around 0.72 (Vincent, 1990). The *H. abdominalis* patrotrophy index is approximately 1, indicating potential paternal supplementation during embryonic development (Skalkos, Van Dyke & Whittington, in review). Embryos of other syngnathids can survive solely from their yolk supply *in vitro* (albeit with low survival or only when incubated *in vitro* late in development), so their patrotrophic contribution to embryonic energy demands may be minimal (Azzarello, 1991; Drozdov *et al.*, 1997; Linton & Soloff, 1964). Nutrients from undeveloped embryos may also be absorbed by siblings (Ripley & Foran, 2006), which needs to be taken into account in calculations of patrotrophy indices. Unfortunately, separating paternal supplementation from maternal sources is likely to be technically ~~difficult~~ problematic when based on dry mass alone. We therefore recommend that tracking transport of labelled nutrients ~~injected into~~in pregnant fathers is the ideal method of identifying patrotrophic syngnathids. Transport of specific nutrients from father to embryos has only been tested in a few syngnathids (Table 2).

The mechanisms of potential paternal nutrient transport have not yet been identified. Modified flame-cone cells ~~described above~~(see Section 6a) may be involved in extraembryonic nutrition in *H. hippocampus* (Carcupino *et al.*, 2002). In ~~*Syngnathus*~~ *S. abaster*, intracellular spaces develop at the basal surface of pouch epithelial cells during pregnancy; these spaces may facilitate the transport of nutrients (or other substances) into or out of the lumen (Carcupino *et al.*, 1997). The quantity and source of embryonic nutrition probably differs across species, even with the same pouch type (Ripley & Foran, 2006). Patrotrophy may be more significant in species with more complex pouches (Berglund *et al.*, 1986b), but this hypothesis has not been formally tested. Understanding the differences in paternal investment in pregnancy across syngnathids is essential to informing theories underpinning the evolution of syngnathid pregnancy and brood pouch complexity, as paternal nutrient transport potentially represents a large energetic cost of pregnancy and a source of both sexual and parent-offspring conflict. We therefore recommend that future researchers quantify patrotrophy across syngnathids with different brood pouch morphologies~~ using labelled nutrient transport~~. In other taxa, ultrastructural studies have successfully identified cellular specialisations for nutrient transport (including exo/endocytosis and secretory tissues) (Ostrovsky *et al.*, 2016; Blackburn, 2015a), and further morphological research will provide a complementary approach in identifying the mechanisms underlying patrotrophy in syngnathids.

Table 2. Evidence of paternal nutrient transport in syngnathid fish.

Pouch type	**Species**	**Nutrients transported**	**Method of determination**	**Reference**
1 (open)	*Nerophis ophidion*	Limited transport of energy-yielding molecules	Bomb calorimetry to measure energy content of eggs versus neonates; embryonic respirometry	(Berglund *et al.*, 1986b)
4i (closed)	*Syngnathus floridae*	Amino acids, possibly fatty acids	Stable isotope-labelled nutrient injections (enrichment)	(Ripley & Foran, 2009)

		Proteins, lipids, carbohydrates	Nutrient content of pouch fluid in non-pregnant males, and pregnant males (some nutrients may be maternally derived)	(Ripley & Foran, 2006)
	Syngnathus fuscus	Amino acids, possibly fatty acids	Stable isotope-labelled nutrient injections (enrichment)	(Ripley & Foran, 2009)
		Amino acids	Radioactively labelled nutrient injections	(Haresign & Shumway, 1981)
		Proteins, lipids, carbohydrates	Nutrient content of pouch fluid in non-pregnant males, and pregnant males (some nutrients may be maternally derived)	(Ripley & Foran, 2006)
	Syngnathus schlegeli	Unknown	Embryonic RNA:DNA in starved versus fed parents indicates more protein synthesis in embryos of fed parents Horseradish peroxidase appears in pouch fluid when injected into pregnant males	(Watanabe, 1999)
	Syngnathus typhle	Amino acids, glucose	Radioactively labelled tube-fed nutrients	(Kvarnemo *et al.*, 2011)

		Energy-yielding molecules	Bomb calorimetry to measure energy content of eggs versus neonates; embryonic respirometry	(Berglund *et al.*, 1986b)
5 (fleshy, closed)	*Hippocampus barbouri*	Putatively: lipids or steroids	Morphology: lipid droplets in pouch tissues via scanning electron microscopy; steroid biosynthesis enzyme present in pouch	(Oconer *et al.*, 2003)
	Hippocampus abdominalis	Putatively: lipids, amino acids, Ca^{2+}, $Fe^{2/3+}$, K^{+}, Zn^{2+}, Cu^{2+}, Mg^{2+}	Gene expression analysis	(Whittington *et al.*, 2015b)
	Hippocampus erectus	Putatively: lipids	Morphology: lipid droplets in pouch tissues via light microscopy	(Linton & Soloff, 1964)
		Calcium	Transport of radioactively labelled calcium in external seawater to embryos	(Linton & Soloff, 1964)

f. Immune function

Vertebrate mothers prime the immune system of their offspring with immune defence factors deposited in eggs, via placental transfer, or during lactation (Grindstaff, Brodie &

Ketterson, 2003). Such transgenerational immune priming can enhance offspring survival. The close association between syngnathid embryos and fathers provides ample opportunity for biparental influence on offspring immunity. *Syngnthus typhle* immunity is influenced by paternal (Roth *et al.*, 2012) and grandpaternal (Beemelmanns & Roth, 2017) priming. Paternal transgenerational immune priming affects both innate and adaptive immunity (Roth *et al.*, 2012). Epigenetic regulation may be involved (Beemelmanns & Roth, 2016), and this and other mechanisms underpinning paternal immune priming in pregnant syngnathids are fertile ground for future research.

Immune capabilities of male syngnathids are higher than females (Lin *et al.*, 2016b; Roth *et al.*, 2011). While reproductive activities in *H. erectus* come with an immune cost (Lin *et al.*, 2016b), male plasma concentration of immune factors peaks during pregnancy (Lin *et al.*, 2017b). The mechanisms by which immunity is boosted during pregnancy are unknown. While enhanced immunity in pregnant males could simply be due to a greater paternal resource allocation to immunity during parental care, future studies should examine the possible immune role of maternal factors transferred into the pouch along with eggs.

The syngnathid brood pouch may also function to provide immunological protection to embryos during development. Closed brood pouches provide ideal conditions for bacterial proliferation, because they are filled with non-sterile seawater at the beginning of pregnancy, and the pouch fluid is rich in proteins and lipids and fragmented eggs. The inner epithelial layer of *Hippocampus erectus* pouch is immunologically active during pregnancy (Lin *et al.*, 2017b). During gestation, large quantities of putatively antibacterial C-type lectins are secreted from the inner layer of the pouch into the pouch fluid in *Hippocampus comes* (Melamed *et al.*, 2005) and *Syngnathus* spp. (Small, Harlin-Cognato & Jones, 2013). *Hippocampus kuda* pouch expresses antimicrobial peptides (Sun *et al.*, 2012; Wang *et al.*, 2008), and several genes with putative antibacterial and antifungal activity are highly expressed in pregnant *H. abdominalis* pouch tissue (Whittington *et al.*, 2015b). Innate immune pathways are also differentially regulated in the *S. scovelli* brood pouch during pregnancy (Small *et al.*, 2016). Diverse microbiota are present in the *S. typhle* brood pouch, which changes with pregnancy stage and paternal immune status (Beemelmanns *et al.*, 2019). We hypothesise that the balance of paternal immune molecules and commensal microbiota maintain a pathogen-limited environment in closed syngnathid pouches.

g. Endocrinology of pregnancy

For an extensive discussion of the reproductive endocrinology of male and female syngnathids, readers are directed to the excellent review of Scobell and MacKenzie (2011). Most knowledge of male pregnancy endocrinology comes from a complex series of experiments involving seahorse hypophysectomy, castration, and hormone administration (Boisseau, 1967). Brood pouch development is likely under the control of androgens: castration of non-pregnant males produces abnormal morphology that is rescued by testosterone injection; testosterone also produces a rudimentary brood pouch in females (Boisseau, 1967). A decrease in androgen concentrations may be required for the progression of pregnancy (Boisseau, 1967; Scobell & MacKenzie, 2011; Mayer *et al.*, 1993; Lin *et al.*, 2017b).

Adrenocorticotrophic hormone (ACTH) and prolactin (PRL) seem to be ~~important~~ necessary ~~for the~~to maintai~~e~~n~~ance of~~ syngnathid pregnancy (Whittington & Wilson, 2013; Scobell & MacKenzie, 2011): hypophysectomy during seahorse pregnancy produces abortions, abnormal embryonic development, and pouch abnormalities; some of these effects can be prevented by administering exogenous PRL, and ACTH; oestradiol has no effect (Boisseau, 1967; Boisseau, 1964). Prolactin and growth hormone are both present in pouch fluid at various reproductive stages, although the site of production (embryonic, paternal or both) is unknown (Patron, Herrera & Oconer, 2008). It is possible that extra-pituitary PRL is produced by the brood pouch, and future studies should determine this. Progesterone may also have a role in pregnancy maintenance (Boisseau, 1967) or proliferation of the brood pouch epithelium, but this has not been tested. Both estradiol and progesterone are present in the testis and brood pouch of males, regardless of reproductive status, and the presence of 3-beta-hydroxysteroid-dehydrogenase there suggests that steroidogenesis takes place in these tissues (Oconer *et al.*, 2003). 11-Ketotestosterone may also be involved in pregnancy function (Lin *et al.*, 2017b). Gene expression and immunofluorescence histochemistry identifying tissue-specific receptor expression throughout pregnancy would be invaluable in understanding the evolutionary flexibility of endocrinological pathways in male-female convergence on the pregnancy phenotype.

h. Hatching and release of young from the brood pouch

In syngnathids with closed brood pouches, embryos hatch from their chorions and may become free swimming prior to birth if space in the pouch lumen is available. Studies of hatching in three syngnathids reveal that embryonic chorions are thinner in species with more complex brood pouches, and pseudogenization of hatching enzyme genes may coincide with closure of the brood pouch (Kawaguchi *et al.*, 2016). Seahorse embryos with closed brood pouches and thin chorions may use high choriolytic enzyme to swell the egg envelope and facilitate hatching (Kawaguchi *et al.*, 2016). There is an expansion of this gene family (patristacins) in the *Hippocampus comes* genome (Lin *et al.*, 2016a), and these enzymes may be produced concurrently in the brood pouch as a signal for hatching (Whittington *et al.*, 2015b). At hatching in oviparous fish, choriolytic enzymes are expressed *in the embryo* (Inohaya *et al.*, 1995), illustrating a remarkable evolved change in the ontogeny of gene expression (from hatching embryo to father pre-parturition) in at least some syngnathids − although functional studies are required to confirm gene expression data. A similar expansion of choriolytic enzyme genes is present in the *S. scovelli* genome (Small *et al.*, 2016), and these genes are expressed in the brood pouch of pregnant *Syngnathus* spp. (Harlin-Cognato, Hoffman & Jones, 2006; Small *et al.*, 2016), although their function is debated.

In amniotes, oxytocin and related nonapeptide hormones are key mediators of labour, producing a cascade of effects culminating in contractions of the uterine musculature and birth (Blanks & Thornton, 2003; Guillette, 1979; Paul *et al.*, 2020). These hormones also induce spawning in teleosts (Wilhelmi, Pickford & Sawyer, 1955). Isotocin administration to non-pregnant male seahorses produces the sequence of stereotypical behaviour typical of birth (Fiedler, 1970), and brain concentration of the nonapeptide arginine vasotocin is variable but peaks in some *Syngnathus* spp. individuals during pregnancy (Ripley & Foran, 2010). *In vitro* studies of the effects of isotocin on syngnathid brood pouch would help elucidate whether the mechanisms underpinning labour in male syngnathids are homologous to those involved in female amniote parturition.

7. Where to next for syngnathid reproduction research?

a. Expanding knowledge of physiology and evolution of male pregnancy with molecular tools

An integrated approach spanning comparative genomics, morphological, ecological, and physiological studies (Crespi & Semeniuk, 2004) is likely to lead to the greatest advances in understanding the evolution of male pregnancy. While we have in-depth knowledge of the physiology of some aspects of syngnathid pregnancy, much remains unexplored. Major outstanding questions are the extent of patrotrophy in syngnathids with different brood pouch types, the mechanisms by which the duration of pregnancy and birth are controlled, and the endocrinology of pregnancy and labour. Molecular tools, as well as enabling phylogenetic and conservation genetic studies of syngnathids, offer an unprecedented opportunity to resolve the genetic basis of male pregnancy (Mobley, Small & Jones, 2011b). For example, transcriptomic and other gene expression studies of the brood pouch across pregnancy have identified genes putatively involved in a variety of pouch functions (Melamed *et al.*, 2005; Harlin-Cognato *et al.*, 2006; Small *et al.*, 2013). Given that the physiology of the pouch in some species appears to vary across pregnancy, an approach which has proven particularly powerful is to use RNA-seq at a number of reproductive stages to compare gene expression at a fine scale as embryos develop (Whittington *et al.*, 2015b). These gene expression studies have provided a valuable foundation of testable hypotheses that can now be examined in detail. The next steps to confirming gene function are wet-lab experimental studies using physiological techniques and histological methods to examine pouch ultrastructure and the paternal−embryonic interface.

To this end, we suggest that the research community focus on 1) depth of biological understanding, by continuing to develop key species such as *Syngnathus* and *Hippocampus* spp. (which are already in use by several research groups) as research models; and 2) breadth of study for comparative work, to encompass syngnathids with a variety of brood pouch types including those with open pouches (e.g. *Nerophis* spp.), which have received comparatively less research attention. While a number of syngnathids are threatened (Vincent, Foster & Koldewey, 2011), several abundant species can be caught and held in the laboratory or purchased commercially (sourcing from environmentally sound aquaculture operations is recommended) (Martin-Smith & Vincent, 2006). A Web of Science search (~~November~~ March 20~~19~~20) of topics using the term “~~S~~syngnath*” (and excluding “syngnathia”) returns over ~~1000~~950 peer-reviewed publications, 5~~7~~2 % of which were published in the past decade, since genomic tools started to become tractable for use on non-model species, and syngnathid genomic resources became available. The recent publication of three syngnathid genomes (Lin *et al.*, 2016a; Lin *et al.*, 2017a; Small *et al.*, 2016) promises to further expand the research possibilities in this unusual and useful group of fish.

Syngnathids are ripe for comparative research determining whether the convergent phenotype of pregnancy has evolved in similar ways in independent lineages, or whether there are multiple physiological pathways to reach the same phenotypic end (pregnancy). A number of key areas of gestational physiology are similar in male syngnathids and female amniotes, including tissue remodelling, ion exchange, and immune modulation (Stolting & Wilson, 2007). Similar changes also take place in the uterus of viviparous chondrichthyans (Hamlett & Hysell, 1998) and the ovary (gestational site) of viviparous teleosts, including specialisations to promote the physiological exchange of nutrients and respiratory gases (Wourms & Lombardi, 1992). In line with these observations, the apparent convergent gene use between pregnant amniote uterus, poecilid ovary, and seahorse brood pouch, is particularly striking, because these lineages represent independent origins of internal embryo incubation, with gestational tissue derived from different tissue types (oviduct, ovary, and external epithelium, respectively) (Whittington *et al.*, 2015b). These genetic similarities suggest that there may be a common ‘toolkit’ of genes that has been recruited into gestational tissues each time pregnancy has evolved (Whittington *et al.*, 2015b; Brandley *et al.*, 2012; Van Dyke *et al.*, 2014a; Griffith *et al.*, 2017; Whittington *et al.*, 2018). However, no study has yet approached this idea in a rigorously systematic way. Do we see more genes shared between pregnant syngnathid brood pouch and mammalian uterus than we would expect by chance, for example? It should be possible to systematically compare gene recruitment between male syngnathid pregnancy and female pregnancy in other vertebrates, as well as to determine the genes involved in the evolution of reproductive complexity by comparing the genetic control of brood pouch function in syngnathids with varying pouch morphologies. It is worth noting that the differences found between embryo incubation in male syngnathids and female-pregnant vertebrates will also be informative- because differences would indicate a degree of evolutionary ‘flexibility’ in which pathways are recruited to support internal incubation of embryos.

The phylogenetic replication that is critical for informative comparative analyses (Reznick *et al.*, 2002) is provided in syngnathids by the parallel evolution of complex pouch types. Most molecular work of this sort has focused on *Hippocampus* spp. and *Syngnathus* spp., which have the most complex pouches (Types 5 and 4, respectively). The next step will be to perform comparative genomics across other lineages with less complex brood pouches, and multiple origins of brood pouch closure. Of interest are levels of gene expression, novel genes, gene family expansions, polymorphic sites, and types of selection operating on genes involved in brood pouch function in different species. Such comparisons will inform models aiming to understand the development of parental care and the drivers of its evolution in vertebrates.

b. Signalling and conflict in the brood pouch

Internally fertilising species have ample opportunity for one sex to influence the physiology and behaviour of the other. In viviparous mammals, reptiles and fish, the female reproductive tract is also thought to be a site of conflict over the allocation of resources and duration of pregnancy between mothers and embryos, sibling embryos, and maternal and paternal genomes (Crespi & Semeniuk, 2004; Zeh & Zeh, 2000; Haig, 1996a). Such conflict is physically possible in the male syngnathid brood pouch (Paczolt & Jones, 2010), but surprisingly little research has been done in this area (see Section ~~3~~ 5 for details).

c. Female/male communication and conflict

In many animals, seminal fluid is transferred into the female reproductive tract during mating, along with the sperm (Poiani, 2006; Wolfner, 2009; Ramm *et al.*, 2008; Perry, Sirot & Wigby, 2013). This fluid contains factors that promote both sperm performance and influence female physiology. Such factors include reproductive hormones (oestrogen, testosterone, prostaglandins), cytokines, and growth factors, which induce immune changes in the female and initiate a cascade of pathways producing cellular changes that promote implantation and the correct uterine environment for embryonic development (Robertson, 2005). Seminal fluid components also offer the opportunity for male-male competition within the female reproductive tract, and ~~CFC~~ cryptic female choice in polyandrous species (Perry *et al.*, 2013). These facts raise the question: to what extent can female syngnathid ovarian fluid influence male physiology? Females appear to transfer a viscous material along with the eggs into the brood pouch (Watanabe, 1999). Some species have mucous material surrounding the eggs once they are embedded in the pouch (Carcupino *et al.*, 1997). The source of this material is unknown, but we speculate that it could be maternally derived. We posit that there is substantial opportunity for female syngnathids to influence male physiology in this manner- but this area remains essentially unexamined.

In ~~female pregnant~~ taxa with female pregnancy, there is an expectation that conflict primarily occurs in matrotrophic species (Crespi & Semeniuk, 2004; Pollux *et al.*, 2014). However, syngnathids represent an interesting theoretical exception to this hypothesis, because even lecithotrophic males have the possibility of recouping maternally-derived resources from unfertilised eggs or aborted offspring (see Section 5). These resources could be used to supply half-siblings~~,~~ or ~~to~~ invest in somatic growth and future reproduction. The mechanisms underlying syngnathid brood reduction and the potential conflict between females and males with different pouch types are rich areas for future investigation.

d. Father/embryo and embryo/embryo communication and conflict

Signalling to (and possibly manipulation of) pregnant females by embryos is common in viviparous vertebrates (Crespi & Semeniuk, 2004; Zeh & Zeh, 2000), so the close apposition between the syngnathid brood pouch and embryonic tissues creates a similar arena for embryos to influence the father's physiology, and vice-versa. Outgrowths form from the brood pouch inner lining towards embryos (Drozdov *et al.*, 1997). Very early in seahorse pregnancy, most of the embryos are so firmly embedded into the brood pouch that it is impossible to remove them intact (pers. obs.); there is some suggestion that seahorses may have a limited number of optimal sites for embryonic attachment in the brood pouch (Dzyuba *et al.*, 2006). It would be intriguing to determine the mechanism underlying tissue outgrowth and embryonic embedding in these fish- do embryos secrete factors that stimulate tissue outgrowth? We suggest that this might be the case, because introducing plastic beads approximating the size and density of eggs into the pouch of does not induce the formation of such structures (Boisseau, 1967). Does the ability to embed into the gestational tissues differ between half-sibs in polygynous syngnathids, and how does this affect embryonic survival? In patrotrophic species, can the embryos signal to influence their individual resource allocation from the father? Could embryonic signals be used by the father to differentially partition care? While hormones produced by embryos and entering parental circulation cannot be used to signal information about individual embryos (Haig, 1996b), the fact that some syngnathids appear to have the ability to selectively abort embryos suggests that more localised, paracrine signalling pathways may exist. If this were the case, we might expect offspring size variation to be higher in polygynous than monogynous species (Ala-Honkola, Friman & Lindstrom, 2011), a hypothesis that is easily testable in this group.

In viviparous mammals, parent-specific allelic expression of certain genes (genomic imprinting) may have also evolved because of parent-offspring conflict (Moore & Haig, 1991). Genomic imprinting is present in therian fetal and placentally-expressed genes (Renfree *et al.*, 2009). Imprinted genes are predicted to be paternally expressed when they maximise nutrient allocation from the mother (at a cost to the mother). Imprinted genes should be maternally expressed when they have the opposite function. These differences are predicted due to selection in each sex to optimally balance the trade-offs between present reproduction and future reproductive investment (Renfree *et al.*, 2009). Investigation of some mammalian imprinted genes found no genomic imprinting in a matrotrophic lizard (Griffith *et al.*, 2016); *IGF2*, imprinted in mammals, also shows biallelic expression in a matrotrophic fish (Lawton *et al.*, 2005). However, these studies do not rule out imprinting of genes that are different to those imprinted in mammals. To our knowledge, the possibility of genomic imprinting has never been investigated in syngnathids. We suggest that this would be a fascinating avenue of research in patrotrophic species.

e. Cooperation and conflict in male pregnancy

Syngnathids offer the unique opportunity to compare the situation of female gestation in other fish, amphibians, reptiles and mammals, with the potential for conflict in male gestational tissues. Such comparisons would allow for powerful comparative tests of evolutionary theory and determine whether conflict during gestation is a general feature of pregnancy. The predictions of conflict hypotheses (e.g. Crespi & Semeniuk, 2004; Zeh & Zeh, 2000) lend themselves to testing in syngnathids. Do more complex pouches reflect increased paternal investment and/or increased paternal control over resource allocation? Is the evolution of more complex pouches associated with increased speciation? Is there interspecific variation in the structures involved in resource allocation from fathers to offspring? We know that many placenta-expressed genes involved in resource allocation in mammals have experienced positive selection, possibly as a result of conflict (e.g. Chuong, Tong & Hoekstra, 2010), and several syngnathid brood pouch-expressed genes have evolved under positive selection (Small *et al.*, 2013; Harlin-Cognato *et al.*, 2006). Are syngnathid genes involved in patrotrophy, or embryonic-expressed genes influencing paternal physiology under positive selection, or imprinted? We suggest that answering these questions has enormous potential to expand our knowledge of fundamental evolutionary processes.

8. Conclusions

1. Syngnathid fishes exhibit male pregnancy within a range of gestational structures~~,~~ and are ideal models for evolutionary studies of sexual selection, conflict, the genetic basis of phenotypic variation and evo-devo, the evolution of male pregnancy, and the ecology and evolution of reproductive diversity.
2. Experimental tests of the variety of hypotheses about the physiology of syngnathid embryo incubation that have been generated by genomic studies are now required, including examining the possible convergence in processes supporting pregnancy in male syngnathids and viviparous females in other vertebrate groups.
3. An integrated approach spanning comparative morphology and genomics, ecology, and physiology is likely to lead to the greatest advances.
4. We suggest a focus on depth of biological and ecological understanding of key species with varying pouch types, including *Hippocampus*, *Syngnathus* and *Nerophis* spp., which are already in use as research models and for which several genomes are available. We hope that this review will encourage the research community towards new advances in understanding the biology and evolution of male pregnancy.

9. Figures and Tables

Figure 1. Syngnathid pouch morphology varies in complexity. A. *Phyllopteryx taeniolatus*

seadragon pouch, ventral view (Type 2) (Photo: Tom Burd). B. *Syngnathus* sp. pipefish pouch, partially opened along seam to reveal embryos, ventral view (Type 4i) (Photo: © Yuriy Kvach). C. *Hippocampus abdominalis* seahorse pouch (Type 5) (Photo: Jacquie Herbert).

Figure 2. Phylogeny of representative Syngnathidae based on the consensus phylogeny of (Hamilton *et al.*, 2017). Pouch morphology is taken from Dawson (1985), with added information from Herald (1959) (*Anarchopterus criniger; Amphelikturus dendriticus; Bryx sp.*); Scott (1961) (*Lissocampus caudalis*); Dawson (1981) (*Enneacampus ansorgii*); Kuiter (2009) and Kvarnemo et al. (2004) (Kuiter, 2009; Kvarnemo & Simmons, 2004) *Halicampus macrorhynchus.* Star indicates a trunk-brooding species within the tail-brooding lineage. Maximum likelihood support values shown if <75. Brood pouch types are described in Table 1.

Figure 3. Examples of skin-brooding in teleosts: A) Male pipefish with open brood pouch; B) Female aspredinid catfish; C) Female ghost pipefish; D) Male nurseryfish. Embryos are shown in orange, and images are not to scale.

Figure 4. Possible scenarios of selection that may lead to the evolution of different forms of syngnathid egg incubation. Eggs/embryos are shown in orange.

Figure 5. Transverse section of a pregnant male seahorse (*Hippocampus* spp.) through the brood pouch region (top: dorsal, bottom: ventral). Bony plates and vertebra are not shown. Tail skeletal muscle is in palest grey. Dermis is pale grey, inner layer ~~(pseudoplacenta)~~ is dark grey, and embryos are darkest grey. Outer and inner epithelium are in black, and the pouch lumen is white. Modified from Kawaguchi et al. (2017) and Stolting and Wilson (2007). Diagram is not to scale.

Table 1. Syngnathid brood pouch classification. All brood pouches are located on the ventral

surface of the body (the body cavity is not shown). Diagrams represent transverse sections through the brood pouch, with the dorsal side of the pouch at the top of the image. Embryos are represented by orange circles, with paternal tissues in navy blue. Images are not to scale. Previously, pouch type 3f has been grouped as a sub-type of pouch type 4 (Wilson et al., 2001, Dawson, 1985), but we separate the two due to the differences in incubation environment created by closed versus open brood pouches.

Table 2. Evidence of paternal nutrient transport in syngnathid fish.

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1052 ## 11. References

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