The role of prolactin in fish reproduction

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Abstract:

Prolactin (PRL) has one of the broadest ranges of functions of any vertebrate hormone, and plays a critical role in regulating aspects of reproduction in widely divergent lineages. However, while PRL structure, mode of action and functions have been well-characterised in mammals, studies of other vertebrate lineages remain incomplete. As the most diverse group of vertebrates, fish offer a particularly valuable model system for the study of the evolution of reproductive endocrine function. Here, we review the current state of knowledge on the role of prolactin in fish reproduction, which extends to migration, reproductive development and cycling, brood care behaviour, pregnancy, and nutrient provisioning to young. We also highlight significant gaps in knowledge and advocate a specific bidirectional research methodology including both observational and manipulative experiments. Focusing research efforts towards the thorough characterisation of a restricted number of reproductively diverse fish models will help to provide the foundation necessary for a more explicitly evolutionary analysis of PRL function.

Keywords: prolactin, prolactin receptor, reproduction, parental care, fish, teleost

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1 List of abbreviations: aa (amino acids), GH (growth hormone), GHR (growth hormone receptor), GnRH (gonadotrophin releasing hormone), GPCRs (G-protein coupled receptors), HCG (human chorionic gonadotrophin), PL (placental lactogen), PRL (prolactin), PRLR (prolactin receptor), qPCR (quantitative real-time polymerase chain reaction), RNAi (RNA interference)
Introduction

Prolactin (PRL) is a multifunctional polypeptide hormone present in all vertebrates, with the exception of jawless fish. It has more than three hundred known activities, encompassing the maintenance of water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behaviour, reproduction, and immunoregulation and protection [15]. The exceptional diversity of PRL functions has been reviewed extensively elsewhere; in this review, we focus on its role in reproduction.

PRL is perhaps best known for its function in mammalian reproduction. It has significant effects in the mammary gland, influencing growth and development during pregnancy as well as milk protein synthesis, and it is also involved in regulating testicular, ovary and uterine function [reviewed in 4, 15]. Investigations of PRL function have primarily focused on mammals, but studies of non-mammals indicate that PRL also plays an important role in reproduction in birds, reptiles, amphibians and fish [eg. reviewed in 4, 15, 42, 96, 121]. While these studies have resulted in a greater general appreciation of the importance of this hormone outside the mammalian lineage, they have failed to address the key question of how common evolutionary history has influenced the development of PRL function. At the same time, the wide range of experimental approaches taken, some questionable by today's standards, have complicated efforts at cross-species comparison. Recent studies employing an explicitly phylogenetic approach to the study of endocrine function [eg. 106] demonstrate how an understanding of evolutionary ancestry can provide unique insights into endocrine activity, and allow the identification of common regulatory pathways in divergent lineages. Such an approach is likely to be particularly powerful when studying a multifunctional hormone such as PRL, which has been implicated in a remarkable range of analogous functions across vertebrates.

With more than 25,000 species, fish are the most diverse group of vertebrates and thus offer a valuable comparative system for the study of the association between endocrine activity and
reproductive function. However, despite the potential importance of this model, the endocrine control of fish reproduction remains relatively poorly understood compared to the mammalian system, with research focused on a small number of species. Here, we review the current state of knowledge of the role of prolactin in fish reproduction, identify methodological problems inherent in previous studies of PRL and significant gaps in our present knowledge, and discuss how the application of new methodologies in a comparative context may offer unique insights into the evolution of PRL function.

1. Prolactin: gene structure, evolution, and activities

PRL is a member of a diverse family of proteins that includes growth hormone (GH), placental lactogen (PL), and somatolactin (SL) [reviewed in 15, 64, 85]. The PRL gene is ca. 10kb in length, with five exons and four introns (HUGO Gene Nomenclature Committee ID: HGNC:9445); an additional exon 1a has also been found in humans [reviewed in 4]. PRL is synthesised as a prehormone containing a signal peptide; in humans this prehormone is 277 aa, and includes a 28 aa signal peptide [15].

A study of chum salmon (*Oncorhynchus keta*) was the first to demonstrate that the typical mammalian PRL gene structure is conserved in fish [50]. PRL sequences have since been isolated from a range of species including fugu (*Takifugu rubripes*) [59], rainbow trout (*Oncorhynchus mykiss*) [68], European eel (*Anguilla anguilla*) [87], Atlantic salmon (*Salmo salar*) [65], blue gourami (*Trichopodus trichopterus*) [26], and starry flounder (*Platichthys stellatus* [72]). Although sequence identity differs between species (eg fugu–seabream (*Sparus aurata*) 68%, fugu–goldfish (*Carassius auratus*) 54%), teleost PRLs share a common structure and contain four well-conserved cysteine residues, including one at the C-terminus [59]. The 5’ region of teleost PRL contains a TATA box ca. 20 bp upstream of the transcription initiation site; in several species this region also contains Pit-1 and GHF-1 binding sites involved in regulation of PRL expression (see Section 3) [reviewed in 51].
Fish PRLs are divergent from those of other vertebrates (e.g. the carp PRL amino acid sequence shows <40% identity with mammalian PRL)[120]. Most notably, teleost PRL genes are generally shorter, with a shorter signal peptide (23-24 aa) than mammalian PRLs, and lack 12-14 aa at the N-terminal end of the gene, the location of the first of three disulphide bonds in other vertebrates. In contrast, non-teleost fish (sturgeon, lungfish) PRLs are longer and contain all three disulphide bonds [reviewed in 64, 85]. Figure 1 provides a schematic representation of PRL phylogeny and structure.

PRL is thought to have evolved via duplication of an ancestral gene 400 to 800 million years ago, which gave rise to GH, somatolactin and PRL [reviewed in 15, 64, 85]. The multiple forms of PRL observed in some species, including various fish, have subsequently evolved via lineage-specific gene duplication or polyploidisation [45, 115]. Differences in disulphide bonding between teleost PRL and that of other vertebrates have given rise to a dual-lineage hypothesis of PRL evolution, which suggests that the N-terminal disulphide bond was lost in the common ancestor of teleost fish, but retained in the lineage leading to the lobe-finned fish and tetrapods [reviewed in 64]. It has been suggested that structural differences between teleost PRL and those of other vertebrates reflect differing functions (for example, the loss of the N-terminus resulting in osmoregulatory function), while the four PRL domains conserved across the vertebrate lineage are responsible for its common activities [reviewed in 64]. Repeated bursts of positive selection during the evolutionary history of pituitary PRL have also been proposed to be associated with functional change [115].

Many organisms have two forms of PRL: Whilst PRL1 is common to all vertebrates, an additional copy of PRL (PRL2) has been identified in a number of non-mammalian vertebrates, including fish [45]. The majority of these PRL2 variants contain three disulphide bonds, and are proposed to have evolved via whole genome duplication in vertebrates after their divergence from the jawless fish. PRL2 is potentially able to bind PRLR, is expressed highly in the eye and brain but not pituitary of zebrafish (Danio rerio), and may be involved in teleost retinal development [45]. These PRL2 gene variants cannot be explained by the dual-lineage hypothesis of PRL evolution (see above), and have
apparently been lost from the mammalian lineage, suggesting a complex evolutionary history of
gene duplication which may have contributed to the diverse array of functions commonly attributed
to modern PRL. Two distinct forms of PRL1 encoded by different genes have also been isolated in
several species of fish, including cichlids (tilapia, Oreochromis sp.) and chum salmon [reviewed in
85]. These variants are likely the result of lineage-specific gene duplication [reviewed in 85], and
differ in their activities; for example, Nile tilapia (Oreochromis niloticus) PRL variants tiPRL177 and
tiPRL188 differ in their effects on ion retention at varying water salinities [reviewed in 64].
Throughout this paper, PRL1 is generally referred to as PRL, with any references to PRL2 or long or
short variants of PRL1 specifically indicated.

PRL is considered to be a pituitary hormone, and is typically produced at high levels in pituitary
tissues. However, extrapituitary production of PRL is also common [7]. In fish, extrapituitary PRL
expression has been observed in the seabream (liver, intestine, gonads [94]), European seabass
(Dicentrarchus labrax gill, intestine [17]) and goldfish (gonads, testis, liver, kidney, spleen, gill,
muscle and brain [48]). Boutet et al. [17] suggest that extrapituitary PRL in fish may play an
important role in osmoregulation. Research in mammalian models suggests that extrapituitary PRL
expression may be regulated in a different manner to that produced by the pituitary [94], although
this is not a general pattern: extrapituitary PRL expression is controlled by an alternative promoter in
some species (eg. humans), whilst in others (goats, sheep) the promoter remains the same
[reviewed in 94]; non-coding intronic regions may also influence expression regulation and
alternative splicing.

Most extrapituitary PRL research in fish has involved the identification of tissue-specific expression
patterns. Several studies have discovered tissue-specific promoter binding sites and regulatory
regions in individual species [eg. 2, 3, 17, 84], but in situ analyses and functional assays will be
necessary in order to determine whether alternative regulatory mechanisms act upon fish PRL
synthesised in different tissues. Of particular interest is PRL expression in tissues that also express
prolactin receptor (PRLR), raising the possibility that PRL may be able to act in an autocrine or paracrine manner, in addition to its known endocrine effects [reviewed in 15].

2. Prolactin receptor

PRLR is a member of the class 1 cytokine receptor superfamily, and forms a transmembrane chain that is embedded in the cell membrane, through which PRL exerts its effects (see Section 3) [15]. PRLR is encoded by a gene with 11 exons and a gDNA length of >200kb in humans (HUGO Gene Nomenclature Committee ID: HGNC:9446); in mammals, multiple PRLR isoforms have been detected, differing in lengths due to alternative splicing and promoters [reviewed in 15, 64]. PRLR sequences have been characterised for several species of fish, including goldfish [113], seabream [95], blue discus (Symphysodon aequifasciatus) [52], Japanese flounder (Paralichthys olivaceus) [41], starry flounder [72], fugu [59], and Nile tilapia [92]. These genes encode mature peptides of ca. 600 aa that are similar in structure to the long isoform of mammalian PRLR, although their sequence is highly divergent (26−37% similarity to mammalian PRLR) [reviewed in 85]. A schematic diagram of PRLR structures and phylogenetic relationships is shown in Figure 2.

Key features of mammalian PRLRs include two disulphide bonds and a WS motif in the extracellular portion of the protein, both of which are required for correct processing but do not appear to play a role in the binding of the PRL ligand [reviewed in 51]. Intracellular Box 1 (a conserved proline-rich motif close to the membrane required for correct folding) and Box 2 domains (which are less conserved) are also typical of mammalian PRLR [reviewed in 51, 64, 85]. All of these features are also present in fish PRLRs, although the WS motif is slightly modified and fish PRLRs do not contain an N-glycosylation site between C2 and C3 (Fig. 2) [reviewed in 64, 85].

Similar to the pattern observed for PRL, several teleost species have been found to have two forms of PRLR (PRLR1 and PRLR2), which are thought to have evolved from a fish-specific gene duplication (but see [73]) and exhibit different expression domains [46]. Although both forms resemble the long
variant of mammalian PRLR, they have only about 30% similarity to one another [46]. The Box 2 domain of PRLR2 may be present or absent, depending on species [33, 46], and functional studies indicate that PRLR1 and PRLR2 mediate different downstream signalling events, suggesting differing biological functions [22].

Although PRLR levels in fishes tend to be highest in tissues with osmoregulatory function such as gill, intestine and kidney [eg. 52], transcripts of both PRLR forms are also found in a range of other tissues including brain, gonad, liver, muscle, skin, spleen, head kidney, fertilised eggs, bone, and lymphocytes, similar to the expression pattern found in mammals [reviewed in 15]; tissue-specific expression profiles differ among species [reviewed in 51, 64].

3. Regulation of PRL release

PRL is predominantly produced in specialized cells of the anterior pituitary, known as lactotrophs. Much is known about the regulation of PRL release in mammals, particularly in rodents. Aspects of particular importance to rodent reproduction include the stimulation of PRL synthesis and release by ovarian steroids; contact with young, suckling and copulation (via a stimulus-secretion reflex); and auditory and olfactory cues [for a review, see 36]. Although there have been fewer studies of PRL regulatory mechanisms in fish, there are several known stimulators that are obvious candidates for the regulation of PRL during reproduction. It is known that regulation of fish pituitary PRL synthesis and secretion is influenced by both stimulatory and inhibitory substances including neurohormones from the hypothalamus, sex steroids, plasma factors secreted from other tissues, and osmolality [reviewed in 51]. Many of these regulatory substances appear to be directly related to the non-reproductive functions of PRL (eg. osmolality and osmoregulation). PRL regulatory mechanisms known to be related to reproduction in fish are discussed here.

Gonadotrophin releasing hormone (GnRH) is a neurohormone with diverse roles in reproduction. GnRH is known to stimulate PRL release in Mozambique tilapia (Oreochromis mossambicus) [117], and GnRH neurones have been found to innervate the pituitary [119]. GnRH acts by interacting with
G-protein coupled receptors (GPCRs) on cell membranes, activating G-proteins and producing a cellular response. GPCRs for GnRH have been found in fish lactotrophs [eg. 81]. In Mozambique tilapia cells, GnRH has been shown to increase signalling by phospholipase C and inositol triphosphate, mobilising intracellular calcium (required for exocytosis) and triggering the release of PRL from lactotrophs [112]. In masu salmon (Oncorhynchus masou masou) pituitary cells, GnRH treatment has also been found to increase mRNA levels of pituitary-specific transcription factor (Pit-1) [76], which interacts with Pit-1 binding sites that have been found in PRL gene promoters in various fish [eg. 2, 3, 17, 84]. This Pit-1 mRNA increase was found to coincide with an increase in PRL mRNA, suggesting that Pit-1 is indeed important for regulation of PRL gene expression. The same study found that sex steroids (oestradiol-17 beta, testosterone, and 11-ketotestosterone) also induced a modification in Pit-1 and PRL mRNA levels; the direction and magnitude of these changes varied with the stage in the reproductive cycle [76]. Judging by the range of PRL regulatory mechanisms known in mammalian reproduction, our understanding of the reproductive regulation of fish PRL is undoubtedly incomplete, and further molecular work will be needed to systematically characterise PRL regulation in this group.

Once the mature PRL protein moves to the site of action, its activity is exercised by the binding of a single PRL molecule to PRLRs via two binding sites. After PRL binding, dimerised PRLRs activate a JAK kinase molecule, which in turn phosphorylates STAT transcription factors; STAT then dimerises, and migrates to the nucleus, where it activates PRL-responsive genes by binding to specific promoters, thus causing biological effects. PRL may also activate the MAP kinase pathway, which is involved in cell proliferation [15, 51]. A detailed discussion of the signal transduction pathways of PRL is beyond the scope of this review; for an in-depth discussion, readers are directed to the reviews of Bole-Feyssot et al. [15] and Kawauchi et al. [51]. The mechanism of action of PRL is far better described in mammals than in fish, and further research is required to determine to what extent the processes differ between these two groups.
4. **Prolactin and signalling in fish reproduction**

The importance of PRL in fish osmoregulation has been known for some time [eg. reviewed in 64, 90], but the hormone has a wide array of additional functions in this group [reviewed in 52], including effects on the immune system, pigmentation, seasonal acclimatisation, growth, and reproduction; the effects of PRL specific to reproduction will be discussed here.

Many of the reproductive functions of PRL in mammals and birds have analogues in fish reproduction, making this group an important comparative system for investigating the evolution of endocrine regulation of reproductive activity. In this section, we explore the parallels between the reproductive effects of PRL in different lineages, and discuss current evidence for its role in fish reproduction. This field has a long history, and includes research carried out between the late 1930s and the present day. While this body of research includes a wide diversity of organisms and aspects of reproduction, early studies of PRL function typically employed methodologies which are considered questionable by today's standards. Although the results themselves are biologically interesting, many of these studies will require replication using modern methodologies and controls. Methodological limitations are briefly discussed throughout this section; a detailed discussion of the implications of these limitations can be found in Section 5.2. A systematic survey of experimental methods and results is presented in Table 1.

4.1 **Migration**

Migration plays an important role in the reproductive cycle of many vertebrates, although it is not associated with reproduction in every species. PRL injections induce migratory behaviour in the white-crowned sparrow [66] and migration from land to water in the salamander [69]. The mechanisms by which PRL acts in these examples are unclear, but both of these behaviours are necessary prerequisites for reproduction. Migration is also of reproductive significance in many fish species; however, studies of the relationship between PRL and migration have thus far been largely restricted to salmonids.
Expression of PRL mRNA, GH and SL increase with the start of anadromy in chum salmon, which is part of the maturation process [78]. Presumably part of this effect is due to osmoregulatory functions of PRL facilitating the transition from salt water to fresh water, but experiments have shown that pre-spawning animals held in salt water also experience increased PRL mRNA expression during the final stages of maturation [77]. There also appears to be a sex-specific difference in chum salmon PRL secretion during transfer to freshwater [74]. These facts, and the fact that GnRH and sex steroids have been found to regulate PRL expression in maturing salmon [78], point to a potential role of PRL in the onset of reproductive migratory behaviour in these species. Similarly, Japanese eel (*Anguilla japonica*) exhibit reduced PRL mRNA expression after ‘silvering’, when the fish mature and migrate downstream [40], and sticklebacks (*Gasterosteus aculeatus*) show increases in PRL hormone levels when moving from saltwater to freshwater [57]. These studies highlight the fact that PRL expression is associated with migration in many fish species; however whether PRL actually induces migratory behaviour or whether it is simply part of a pre-adaptive process remains unknown.

4.2 Reproductive development and cycling

In mammals and birds, PRL has been shown to play a role in reproductive development (including attainment of sexual maturity) and cycling, including mammary growth and oocyte maturation (mammals) and crop sac growth (birds). PRL also regulates a variety of reproductive activities and influences hormone receptor abundance and gonadal functions [reviewed in 15] (for example it plays an important role in promoting survival and steroidogenesis of the corpus luteum [reviewed in 16]). Although there has been less research in this area in fish, there is evidence that PRL has a similar function in this group (see below).

PRL has been implicated in fish reproductive development and attainment of sexual maturity. Singh and Singh [101] found that PRL increases along with gonadotrophin during gonadal development in freshwater catfish *Clarius batrachus*, and reaches a maximum during spawning. In contrast, Ozaki *et al.* found no differences in PRL production during sexual maturation of Japanese eels [79]; however,
it is worth noting that these authors measured only the number of PRL-producing cells, which may not reflect the actual levels of circulating PRL. Treatment with oestradiol-17-beta in live seabream produced an increase in PRL mRNA depending on the maturity of the animal [20], an effect similar to the results of Degani et al. [26], who found lower levels of PRL mRNA in juvenile male blue gouramis relative to adults, suggesting that PRL may play a role in gonadal development.

A number of studies have shown that PRL also influences fish reproductive cycling. Firstly, PRLR mRNA and mature protein have been found in the gonads of a number of fish species, including Mozambique tilapia [31], Nile tilapia [91], seabream [20, 95], Japanese flounder [41], goldfish [113], fugu (Takifugu rubripes) [59], and starry flounder [72], suggesting that PRL may be involved in spermatogenesis, vitellogenesis, and/or ovulation. Further studies involving immunohistochemical analyses of fish gonads across the full reproductive cycle are required to clarify these functions.

Secondly, PRL levels affect or are affected by sex hormones, as mentioned in Section 3: oestradiol 17-beta increases PRL protein synthesis in vitro in Mozambique tilapia cells [118]; GnRH and oestrogen stimulate PRL release [70, 117]; and PRL suppresses progesterone as well as oestradiol expression when it is at its peak after vitellogenesis and before ovulation [38]. Thirdly, PRL levels change during breeding cycles: for example, Nile tilapia plasma PRL levels are highest in females after spawning, during vitellogenesis [110]. Similarly, PRL serum levels in Mozambique tilapia also change across the reproductive cycle, although this effect was found to be inconsistent between experimental treatments [116].

PRL is involved in steroidogenesis and gonadogenesis in both testes and ovaries: it directly stimulates testosterone production in courting male Mozambique tilapia testicular tissue [89]; stimulates oestradiol 17-beta secretion in guppy (Poecilia reticulata) oocytes during their development [111]; and has been found to have a gonadotrophic and steroidogenic action in experiments with hypophysectomised Fundulus heteroclitus [100]. In a series of experiments with intact, hypophysectomised, and castrated catfish Heteropneustes fossilis, Sundaraj and Goswami
showed that while hypophysectomy causes rapid regression of seminal vesicles, PRL acts in a synergistic manner with HCG and androgens to stimulate seminal vesicle growth and secretion.

### 4.3 Brood care behaviour

PRL has an important role in parental behaviour in mammals and birds [eg. reviewed in 4, 15, 121], mediated by PRLRs that are expressed widely throughout the brain. PRL is able to enter the brain to affect neuronal function; sensitivity to PRL varies during the reproductive cycle; and the hormone has neurogenic activity during pregnancy [reviewed in 58]. In mammals, PRL induces maternal care after parturition, enhancing rodent pup retrieval [eg. 19] and grooming behaviour [eg. 29], and paternal care behaviours such as pup licking in rodents and in offspring care in primates [reviewed in 121]. In birds, PRL affects aspects of parental behaviour including nesting, incubation, and feeding [reviewed in 15]. It should be noted that while PRL can induce reproductive behaviours, the reverse is also true, that is, the behaviours themselves can influence neuronal activity and PRL synthesis and release; this effect has been particularly well explored in rodents [eg. reviewed in 36], but has yet to be investigated in fish. Specific examples of parental care behaviours in fish that are influenced or induced by PRL are discussed below.

#### 4.3.1 Mouth brooding

Several lineages of fish incubate eggs and larvae in their oral cavity (mouth brooding). Research in cichlids suggests that a combination of PRL and oestrogen promotes oral egg carrying behaviours [5, as cited in 10]. More recent work indicates that pituitary tiPRL177 concentrations increase in female Mozambique tilapia brooding eggs or early stage embryos, with serum concentrations of tiPRL177 also increasing in females brooding late-stage larvae [116], although the changes in PRL concentration were found to be relatively small compared to those induced by changing water salinities or fasting. The fact that the expression of tiPRL177 increased in fasting fish raises the possibility that the hormone may play an indirect role in metabolic regulation during reproduction,
when feeding activity of mouth brooding species is reduced [116]. In Nile tilapia, increased plasma tiPRL177 variance was found in brooding females [110], although the functional implications of this increased variance remain unknown. Summers and Zhu [108] found evidence of positive selection along the tiPRL177 lineage in Oreochromis cichlids (including tilapia), and have speculated that selection may have contributed to the evolution of complex parental behaviour exhibited in this genus. Clearly, detailed functional analyses of the two PRL isoforms will be essential to test this hypothesis.

4.3.2 Nest building

Many species of fish display nest building behaviour, in which they create floating nests of bubbles and mucous to protect, aerate and provide nutrients to hatchlings [reviewed in 49]. There is evidence to suggest that mucous production associated with nest construction is under the control of PRL in some species: experimental administration of PRL increases mucous production, and stimulates increased numbers of mucous cells and nest building behaviour in paradisefish (Macropodus opercularis) [61, 62]. Somewhat surprisingly, these two early studies are the only investigations of hormonal control of nest building behaviour in fish, making it difficult to determine whether qualitatively similar behaviours in other species may also be under the control of PRL.

4.3.3 Fanning

Nest fanning behaviour provides a flow of oxygenated water to ventilate developing eggs in the nest, and parental fanning activity has been shown to be under PRL control in several species [80]. An early experiment showed increased fanning behaviour in male and female angelfish (Pterophyllum scalare) and brown discus (Symphysodon haraldi) following PRL injection, even in the absence of eggs [11, 12]. This increased activity was found to be dose-dependent, and higher doses of PRL inhibited fanning behaviour. PRL also induces fanning in other species of fish, including wrasse males (Symphodus ocellatus) [reviewed in 12] and stickleback males [80]. Experimental treatment of
convict cichlid (*Amatitlania nigrofasciata*) females and male bluegills (*Lepomis macrochirus*) with PRL inhibitors results in decreased fanning behaviour [reviewed in 43, 55].

Observational data also support the role of PRL in mediating fanning behaviour: PRL secretory cell activity increases during the fanning period in untreated male sticklebacks [102]; and PRL sensitive neurons are present in the forebrains of fish species that display parental fanning behaviour. Interestingly, PRL has little effect on forebrain activity in mouthbrooding fish or those providing no parental care [reviewed in 43].

4.3.4 Other parental care behaviours

PRL influences a range of other behaviours related to brooding, including pit-digging behaviour (promoted by PRL in blue acara *Andinoacara pulcher*, which sweep pits in the substrate to corral and protect their young [9]); nest guarding and defence (male bluegills treated with a PRL inhibitor display decreased aggression against model predators [55]); guarding of schooling fry (parental jewel fish (*Hemichromis bimaculatus*) make marked fin ‘calling movements’ to their fry, which can be induced by PRL in non-brooding adults with previous spawning experience [71]); and feeding inhibition (PRL reduces feeding behaviour in jewel fish [71], angelfish, platinum acara (*Andinoacara latifrons*), and brown discus [11, 12], similar to the pattern observed in some mouth brooding species (see Section 4.3.1), a behavioural change suggested to prevent cannibalism of young.

While the majority of studies show a positive relationship between PRL expression and brood care, there is one notable exception to this pattern - the alloparental daffodil cichlid (*Neolamprologus pulcher*). In this species, PRL does not appear to be important for nest maintenance, and females providing brood care have lower PRL mRNA levels than non-brooding females [8]. As this study used both observational and manipulative experiments and a highly sensitive quantitative real-time PCR (qPCR) assay (see Section 5.3), its counterintuitive results appear to be robust, and may be explained by the fact that *N. pulcher* is a cooperatively brooding species with a complex social hierarchy. Studies of other alloparental systems would be particularly useful to test the generality of these observations.

4.4 Pregnancy
Peptides in the same family as PRL, and with PRL-like activity, are expressed in rodent placenta, and are thought to be the result of species-specific gene expansions [reviewed in 103]. Similarly, PRL-like molecules are expressed in the human placenta, decidua and uterus during pregnancy [reviewed in 36]. PRL has a range of important activities consistent with physiological changes during pregnancy, sustaining the function of the hormone-secreting corpus luteum that is necessary to maintain pregnancy in its early stages [reviewed in 104], and acting to regulate angiogenesis [reviewed in 25].

As the highly developed forms of reproduction exhibited by many viviparous fish species show remarkable convergence with mammalian reproduction [107], the location of PRL expression and its mode of action in such systems is of particular interest.

A study of the viviparous poeciliid mosquitofish (Gambusia sp.) showed that hypophysectomy of pregnant females adversely affected embryonic survival, particularly during early development, where almost all offspring died; hypophysectomy close to parturition produced no discernible effect [21]. Similarly, experiments by Ishii [reviewed in 32] showed that oestrogen-induced abortion in mosquitofish was reduced by PRL administration. Although PRL has not been specifically examined in the viviparous guppy, females exhibit pituitary secretory cycles related to gestation [105].

The best evidence for a role for PRL in fish pregnancy, stemming from a combination of traditional and modern methodologies [14, 82], comes from an extensive study of seahorses (Hippocampus sp.), which exhibit a highly derived form of paternal care. The complex pouches of male seahorses provide protection, aeration, osmoregulation and trace nutrients to developing embryos [reviewed in 107]. In Boisseau’s seminal study [14], methods for hypophysectomy and castration of seahorses were developed and the progress of pregnancy was followed, and surgical manipulations were applied with experimental administration of various steroid and peptide hormones.

In H. guttulatus and H. hippocampus, PRL-producing cells of the pituitary vary in activity during the reproductive cycle. Hypophysectomy adversely affected pregnant males in the early and particularly the mid stages, resulting in pouch regression, an excess of abnormal embryos, atypical pouch fluid,
extended parturition, and preterm births [13, 14], suggesting that male pregnancy is under pituitary control. In a result similar to that observed in mosquitofish, late-term hypophysectomy had no effect on offspring survival. The effects of hypophysectomy on seahorse pregnancy were not reversed by testosterone or oestradiol, but administration of exogenous PRL and cortisol improved offspring survival to parturition and reduced abnormal births relative to untreated animals, and PRL was found to stimulate the brood pouch epithelium in normal individuals [14].

In a more recent study, Patron et al. [82] detected the presence of PRL and GH proteins in the pouch of seahorses (H. barbouri) during pregnancy, but not outside the brooding period. GH increased during pregnancy, while PRL showed a more complex pattern of activity, present at high levels in early pregnancy, reducing mid-term and increasing again to moderate levels close to parturition. Patron et al. [82] suggest that reductions in free PRL during mid-pregnancy could reflect increased activity of PRLR in brood pouch tissues, though this is yet to be experimentally tested. PRL and GH are hypothesised to act antagonistically in this system to maintain pouch fluid osmolality during embryonic development [82].

While the combined results of these observational and experimental studies strongly suggest that PRL plays an important role in pregnancy in the seahorse, the precise function of the hormone remains unclear, and should be subject to further studies along the lines of those recommended in Section 5.3.

4.5 Nutrient provisioning to young

Finally, PRL also has a well-documented role in nutrient provisioning, an important aspect of the reproductive cycle of both mammals (directing nutrients towards the mammary gland and promoting its growth [reviewed in 6, 15]) and birds (promoting crop sac growth and epithelial proliferation to produce ‘crop milk’ [reviewed in 15, 44]).
Mucous production in fish is also a form of nutrient provisioning, and the production of nutritive mucous is under PRL control, consistent with its role in cell proliferation and nutrient provisioning activities in birds and mammals. Injections of PRL increase the number of epidermal mucous-producing cells in hypophysectomised goldfish [75] and guppy [97], and also influence the size of these cells in goldfish. Mucous production in these species is associated with osmoregulation, but in other species of fish, particularly discus, this mucous is actively fed on by developing fry (note that this mucous is different to the buccal mucous produced by nest building species discussed in Section 4.3.2). The contents of the parental mucous, also known as ‘discus milk’, are currently under investigation, and compounds contained within it have been suggested to be involved in both fry nutrition and parent/offspring immune function [23, 24].

PRL application promotes the production of skin mucous cells in cichlids such as Heros severus and platinum acara, and this effect is particularly strong in discus, where PRL treatment produced an increase of 90 to 140% in the number of mucous-producing cells in brown discus [11, 12]. Exogenous PRL also induced thickened epidermis and mucosal secretions similar to discus milk in juvenile red discus (Symphysodon discus) [32]. Similarly, the upregulation of PRLR mRNA in the skin of both male and female blue discus suggests a role for PRL signalling in mucous production [52]. Consistent results across a range of species and experimental approaches provide clear evidence of the importance of PRL activity in nutrient provisioning in this group.

5. Experimental approaches to PRL research in fish

5.1 Phylogenetic representation

Figure 3 provides a comprehensive phylogenetic overview of studies of the role of PRL in fish reproduction. Research has been heavily taxonomically biased, with particular emphasis on the Percomorpha; of the 45 studies reviewed in Section 4, 36 (80%) are of species within this group (including 24 studies of the Perciformes). The reasons for this lineage bias are twofold. Firstly, Crown Percomorpha contains more than 50% of extant teleosts [93], making it a likely target for any study
of fish diversity. Secondly, this group contains lineages such as the Cichlidae that have undergone
dramatic adaptive radiations and encompass species occupying a remarkable variety of ecological
and reproductive niches [eg. reviewed in 114]. These fish display a range of specialisations, including
complex brood care and nesting behaviour, making them particularly interesting targets for
comparative studies of reproduction. As a result, 19 of the 45 studies reviewed in Section 4 (42%)
are of cichlid species, despite the diversity of other taxa exhibiting brood care and nesting
behaviours (eg Gasterostiformes, Osphronemidae, and Labridae). Economically important groups
such as Salmoniformes and Siluriformes are also somewhat over-represented relative to other
lineages (6/45 references (13%) reviewed in Section 4). This is largely due to the importance of
salmonid fishes in aquaculture, where research has heavily emphasised the role of PRL in
osmoregulation.

The taxonomic bias in this work limits opportunities to draw broader evolutionary conclusions about
the significance of PRL in reproduction. The research outlined in Section 4 strongly suggests that PRL
has a widespread role in fish reproduction, as it does in other animals, but research into other fish
lineages is required before this can be generalised to all teleosts. While such comparative studies
offer great promise for understanding the evolution of endocrine function, cross-species
comparisons are necessarily limited by the quality of the individual studies. This will be discussed
further below.

5.2 Methodological approaches

The methodologies that have been utilised to investigate the role of PRL in fish reproduction are
summarised by effect in Table 1 and by taxonomic group in Figure S1. Experimental approaches can
be divided into two major classes (see below): manipulative experiments, aimed at artificially
increasing or decreasing PRL levels, and observational studies, which have used a variety of methods
to monitor naturally occurring levels of the hormone. Both approaches have their advantages, and
many modern variants of these methods represent possibilities for incorporation into a combined approach to PRL research, discussed in Section 5.3.

5.2.1 Manipulation

Gross surgical manipulations of pituitary tissues (i.e. hypophysectomy to prevent PRL production) dominated early research on PRL in fish reproduction. Such surgical manipulations suffer from a number of limitations, the most significant of which is the fact that the pituitary is responsible for the synthesis of a wide range of substances in addition to PRL. As such, hypophysectomy may induce physiological changes independent of the effects of PRL, which may be incorrectly attributed to the hormone [1]. A more targeted modern alternative is now available: RNA interference (RNAi) offers a method to experimentally knock down the expression of individual genes in target tissues. RNAi is increasing in use in non-model species, and although results in fish have been mixed [reviewed in 98], established protocols are now available [eg. 27]. RNAi would allow the knockdown of PRL or PRLR expression (including the targeting of specific variants), allowing researchers to isolate the effects of reduced PRL on reproduction. The ability to target specific ontogenetic stages (e.g. via injection directly at the site of production in reproductively mature animals; localised administration of RNAi has already been used in other species [eg. 54]) would avoid problems with embryo or fry survival associated with gene knockdown during early development. Importantly, the technique would facilitate targeting of PRL activity across diverse fish lineages. RNAi is thus recommended for studies that aim to experimentally reduce PRL activity.

Administration of exogenous PRL to experimentally increase PRL levels was also common in early research on fish reproduction, and still continues today. This research has been particularly prevalent in studies of behavioural effects of PRL, with researchers using the hormone to elicit specific behavioural responses. Most early studies of fish involved the administration of PRL from an unrelated, typically mammalian, species. Given the nature of the sequence and structural differences between species (see Figures 1 and 2), non-homologous hormones may not bind, or may
bind to related non-target receptors (e.g., GHR). Therefore, such experimental treatments may yield unintended results; for example, ovine PRL interacts with both fish GH and PRL receptors [reviewed in 64], confounding efforts to use ovine PRL to identify tissue-specific PRLR activity in fish. In addition, purified PRL protein may contain trace levels of associated hormones, making it difficult to isolate the effects of PRL. Fortunately, methods for purifying fish PRLs are now available [e.g., 50, 86], and recombinant fish PRL can also be produced in bacterial expression systems [e.g., 88]. The use of pure preparations of homologous PRL is thus recommended in order to maximise binding specificity in future studies.

A further issue with the administration of PRL stems from clear evidence showing that responses to the hormone are dosage-dependent (e.g., PRL has a stimulatory effect on fanning behaviour at low doses but is inhibitory at high levels [11, 12]), illustrating the difficulty in comparing the results of studies administering differing hormone doses. The development of a common set of experimental standards for dosage rates would do much to advance the field, as these would allow researchers to go beyond their individual experimental species to identify results of broader evolutionary relevance.

*In vitro* techniques have been used in more recent studies in order to experimentally manipulate PRL and investigate effects on specific tissues or cell types. Although these have the advantage of allowing an in-depth investigation of the effects of the hormone on a particular organ or tissue, they may not always reflect the *in vivo* response [1]. Fortunately, most of the overarching results from *in vitro* work in PRL reproductive function have been independently confirmed in the same or another species using other methods (Table 1), a result which highlights the value of combining multiple experimental approaches when studying endocrine function.

### 5.5.2 Monitoring

Studies of changes in pituitary morphology and/or histology were common in the early research into fish PRL. However, as mentioned in Section 5.5.1, the limitation of these studies is that changes in
the pituitary as a whole may not necessarily reflect changes in PRL production. The direct measurement of PRL (circulating or otherwise) offers a significant advance over these earlier methods, as it is both more sensitive and more reliable when immunoassays are used [1], as in the majority of studies reviewed in Section 4.

Circulating PRL levels are in most cases correlated with the amount of PRL mRNA present in the pituitary [99], making it possible to use targeted molecular techniques to shed light on PRL and PRLR tissue distribution and function. Most of these approaches have, somewhat surprisingly, not yet been applied to the reproductive effects of fish PRL. Fish genomes, becoming available in increasing numbers, are emerging resources for such studies, and can be used to design targeted molecular probes. There are currently ten complete fish genomes available on Ensembl (release 69, accessed November 2012 [34]; ray-finned fish genomes are indicated in Figure 3), including those of the mouth-brooding Nile tilapia (which exhibits brood care behaviour) and the live-bearing platyfish (*Xiphophorus maculatus*), with more to come as the cost of genome sequencing continues to decline. These are a readily available source of sequence information for use in developing genomic tools to investigate PRL function.

Piggy-backing on the increasing availability of fish genomic data, technologies such as RNA-seq, microarrays and real-time PCR allow sensitive and temporal monitoring of transcript levels between different reproductive states, sexes, and/or treatments, and efforts to standardize these methods are already well-established [eg. 18]. qPCR, a sensitive and relatively inexpensive technique allowing the precise quantification of expression levels of a restricted number of genes of interest, has recently been used to monitor hormonal gene expression profiles (including PRL) in fish [eg. 59, 67]. The fact that each form of PRL is encoded by an independent gene makes it a particularly suitable candidate for this method. In addition, microarray technologies provide a tool for system-wide investigations of gene expression and have been used extensively to investigate various aspects of fish biology [reviewed in 28, eg. 30]. The newer RNA-seq technology, although not yet used
extensively for fish research, also offers great potential [eg. 35, 60], particularly in non-model species for which genomic resources are not yet available. The transition from qPCR approaches to system-wide microarray and/or RNA-seq methods will undoubtedly drive the next phase in the development of reproductive endocrinology as an explicitly systems-based discipline.

5.3 Recommendations

As outlined above, the large number of studies investigating the role of PRL in fish reproduction reinforce the multifunctional nature of this hormone, but the variable quality of this work limits opportunities to identify how shared evolutionary ancestry has influenced the development of its reproductive functions (see Section 5.1). A solid comparative experimental analysis of species showing qualitatively similar behaviours or phenotypes is key to determining to what extent analogous behaviours or phenotypes share commonalities in their hormonal regulation. As closely related fish species often show very different patterns of parental care or modes of reproduction (eg. the cichlid fishes of East Africa [eg. 39, 63]), such systems may offer the best opportunity to determine whether endocrinological changes predate behavioural or phenotypic modifications, occur concurrently, or develop after novel forms of reproduction have become established.

As discussed throughout this review, the study of the reproductive role of PRL in fish is in its infancy relative to major mammalian models. This, combined with a lack of focus on any one species or family of functions, has contributed to confusion concerning PRL activity, and complicated efforts to identify homologous functions and modes of action in divergent evolutionary lineages. While efforts to explore fish diversity will be invaluable in the long term, it is clear that any efforts at a more comprehensive understanding of PRL function and its evolution would benefit tremendously from a solid foundation in a smaller number of model taxa. It is thus suggested that future research efforts should initially be concentrated on a single group of fish before being broadened to include representatives of all teleost lineages. There are a number of fish with ‘model organism’ status, with zebrafish serving as the traditional fish model. However, cichlid fishes arguably represent a more
powerful model for reproductive endocrinology. As outlined in Section 5.1, the overwhelming majority of existing studies on reproductive function of PRL in fishes have been performed in this group, which offers both a wide range of reproductive specialities and a long history of research as both a laboratory and field model for evolutionary biology. The successful application of a broad array of experimental methods (Table 1) and the availability of extensive genomic data for five cichlid species (Broad Institute Cichlid Genome Project) make this group an obvious choice for studies of endocrine function in reproduction. Given these advantages, we advocate the use of a small number of model cichlid species for the establishment of a solid foundation of endocrinological data. Once this groundwork has been laid, the stage is set for broadening the scope of research to include a number of representative groups across the teleost lineage.

As outlined above, the absence of a standardised experimental methodology has confounded efforts to generalise beyond individual studies and species. An integrative experimental approach represents the best candidate for further investigations into the hormonal control of reproduction in fish. For example, Kurata et al. [56] used proteomics in combination with transcriptomics and hormonal manipulations to dissect the effects of GH1 on growth and reproduction in salmon. Using molecular techniques in combination with more traditional methods (e.g. direct manipulations and assays of hormone levels) is a powerful approach which provides the means to detect time lags between cDNA synthesis and the translation and transport of the mature protein, something which could explain counterintuitive patterns observed in earlier studies. This could be done using an approach similar to that taken in several recent studies of various aspects of fish reproduction (e.g. gene expression and hormone levels during sex change [53] and brooding behaviour [47]).

A fully integrated approach to the study of PRL function would involve bidirectional studies involving experiments that monitor as well as manipulate PRL and receptor levels, incorporating modern molecular techniques with traditional methods of hormone administration (using a homologous hormone). This approach was taken by Bender et al. [8] in their study of parental behaviour in the
daffodil cichlid (described in Section 4.3.4). This two-pronged tactic provides an increased degree of sensitivity to identify subtle variation in endocrine function and adds greater confidence to experimental results. Bender et al. found no increase in PRL mRNA in parental fish compared to non-breeders, and PRL administration did not alter brood care behaviours. Although these results differed from expectations based on previous studies, the quality of the experimental data lends credibility to the work, and provides a solid experimental basis for future research in this area.

5.4 Conclusions
PRL has been implicated in a wide array of reproductive traits in fishes, but variation in taxonomic sampling and experimental methodologies have limited efforts to determine how evolutionary ancestry has influenced the evolution of PRL function. The importance of PRL in mammalian and avian reproduction is well established, and in order to put these results in a broader evolutionary context, detailed studies of the endocrine axis in other vertebrate lineages are essential.

We have discussed here a wide range of studies which implicate PRL in fish reproductive function, highlighting the fact that many of these actions are similar to functions observed in mammalian and avian systems. Fish, as a diverse group of species that are relatively easy to experimentally manipulate, represent a particularly suitable comparative experimental system useful in understanding the evolution of endocrine function. Differences in experimental methodologies are likely to prove one of the most significant hurdles to developing a rigorous comparative approach to researching PRL and its relationship to reproductive function in fish, and we suggest that future studies should use a bidirectional approach (both monitoring and manipulating PRL and/or PRLR levels) to provide an additional level of vigour. Concentrating initial research efforts on a more restricted number of fish models will provide a foundation for further comparative studies of a broader range of fishes and the development of a phylogenetic perspective on the evolution of reproductive function in this group. Due to the range of reproductive functions attributed to PRL in
fish, this group is likely to be particularly useful in understanding how changes in patterns of hormone expression influence the evolution of complex reproductive systems.

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The authors declare no conflicts of interest.

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Table 1. Summary of methodologies used to study the role of PRL in fish reproduction, described in detail in Section 4. Source of administrated PRL is labelled as ‘unknown’ where the study did not specify the type of PRL administered. * indicates cichlid species.
<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
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<th>Experimental manipulation</th>
<th>Administration of PRL (source)</th>
<th>Hormone measurement</th>
<th>In vitro studies</th>
<th>Gene Expression</th>
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<td>Likely ovine</td>
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Figure 1. Schematic representation of PRL peptide structures and phylogenetic relationships. Peptides are shown approximately to scale. Branch lengths are not drawn to scale. Linked arrows indicate the binding pattern of cysteine amino acids. Dashed arrows show regions of PRL which are absent in the teleost PRL1 lineage. The sequences and NCBI accession numbers from top to bottom of the figure are as follows: *Mus musculus* PRL1 (AAH61141), *Gallus gallus* PRL1 (BAB18728), *Oreochromis niloticus* PRL1 (AAA53281), *O. niloticus* PRL1 short variant (B32477), *Acipenser gueldenstaedtii* PRL1 (AAB28396), *G. gallus* PRL1 (NP_001159384), *O. niloticus* PRL2 (ACQ73170), *A. gueldenstaedtii* PRL2 (ACQ73168), *M. musculus* growth hormone (AAH61157). Signal peptides were predicted using SignalP [83]. Phylogenetic relationships follow the phylogeny of Ocampo Daza et al. [73]. aa, amino acids.
Figure 2. Schematic representation of PRLR peptide structures and phylogenetic relationships after [64]. Branch lengths are not drawn to scale.

Linked arrows on the mammalian PRLR long isoform indicate the typical disulphide binding pattern; the binding pattern in avian PRLR is also indicated. The sources of the sequences or the NCBI accession numbers from top to bottom of the figure are as follows: *Rattus rattus* PRLR [64], *Gallus gallus* PRLR (AAS93635), *Oreochromis niloticus* PRLR1 (Q91513), *O. niloticus* PRLR2 (EF429094), *O. niloticus* GHR (XP_003446130). Signal peptides were predicted using SignalP [83]. Phylogenetic relationships follow the phylogeny of Ocampo Daza *et al.* [73]; the phylogeny of Huang *et al.* [46] differs in its placement of teleost PRLR2 (represented as a sister group to teleost PRLR1). aa, amino acids.
Figure 3. Phylogenetic tree of ray-finned fish indicating species diversity [after 93], indicating current evidence for the functional role of PRL in fish reproduction. Extant species diversity and species classification taken from FishBase [37] is indicated based on a colour gradient, representative points of which are shown in the legend. Asterisks indicate orders containing a complete genome-sequenced species present in Ensembl [34]. The boxes in the right hand column indicate groups containing species that have been studied with respect to PRL’s role in reproduction; dark grey boxes indicate that PRL has been implicated in this function in at least one species in the group. A complete absence of boxes indicates that no studies of PRL reproductive function have been carried out in this group.

Percopsif.: Percopsiformes; Gadiif.: Gadiiformes
Figure S1. A phylogenetic classification of research methodologies applied to the study of PRL reproductive function. Phylogeny is identical to that shown in Figure 3. Asterisks indicate orders containing a genome-sequenced species present in Ensembl [34]. The dark grey boxes in the right hand column indicate groups in which PRL’s role in reproduction has been studied in one or more species with the respective methodology.

Percopsif.: Percopsiformes; Gadiif.: Gadiiformes