Stress and sex: does cortisol mediate sex change in fish?

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Abstract

Cortisol is the main glucocorticoid (GC) in fish and the hormone most directly associated with stress. Recent research suggests that this hormone may act as a key factor linking social environmental stimuli and the onset of sex change by initiating a shift in steroidogenesis from estrogens to androgens. For many teleost fish, sex change occurs as a usual part of the life cycle. Changing sex is known to enhance the lifetime reproductive success of these fish and the modifications involved (behavioral, gonadal and morphological) are well studied. However, the exact mechanism behind the transduction of the environmental signals into the molecular cascade that underlies this singular process remains largely unknown. We here synthesize current knowledge regarding the role of cortisol in teleost sex change with a focus on two well-described transformations: temperature-induced masculinization and socially regulated sex change. Three non-mutually exclusive pathways are considered when describing the potential role of cortisol in mediating teleost sex change: cross-talk between GC and androgen pathways, inhibition of aromatase expression and upregulation of *amh* (the gene encoding anti-Müllerian hormone). We anticipate that understanding the role of cortisol in the initial stages of sex change will further improve our understanding of sex determination and differentiation across vertebrates, and may lead to new tools to control fish sex ratios in aquaculture.

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Introduction

Teleost fish as models for understanding vertebrate sexual development

Glucocorticoids (GCs) are an essential component of the hormonal cascade underlying human reproduction (Whirledge & Cidlowski 2010). Precise levels of GCs are necessary for a balanced gonadal function; and increased cortisol (the most important GC) levels due to stress can cause loss of pregnancy and/or have a negative effect on fertility (Nepomnaschy *et al.* 2006, Whirledge & Cidlowski 2010). In recent years, cortisol has been proposed as a key factor linking external environmental stimuli with internal physiological responses during sexual development, whereby environmental stress may initiate a steroidogenic shift at the onset of sex change (Solomon-Lane *et al.* 2013, Nozu & Nakamura 2015). However, the exact role of cortisol or stress in transducing the external signals to elicit physiological responses during sexual development and sex change remains a mystery.

Studies in the last few decades have revealed that sexual development is more plastic in non-mammals, especially teleost fish (reviewed by Devlin & Nagahama 2002, Avise & Mank 2009). It has been shown in many teleost species that environmental factors can interact and sometimes override genetic factors to control sexual

development. More dramatically, in many marine fish, individuals can change sex as an adaptive response to environmental changes even during adulthood (Kobayashi *et al.* 2013). Such sensitivity to environmental stimuli may explain why teleost fishes display such highly diverse sex determination and developmental systems, which make them good models for understanding vertebrate sexual development (reviewed by Wilhelm & Koopman 2006, Matson & Zarkower 2012, Herpin & Scharlt 2015).

From mounting data in mammalian systems, we now comprehend that there is a direct competition between antagonistic male and female signaling networks. Following activation of one sex-specific network in early development, sexual fate must be actively maintained through ongoing suppression of the opposing network throughout adulthood (Matson *et al.* 2011, Windley & Wilhelm 2015). Disruption of this process likely causes disorders of sex development (DSD) (Todd *et al.* 2016) involving pathologies causing ambiguous genitalia that affect 1 in 4500 live births (Sax 2002). Our understanding of the dysregulation of sexual development that results in various human DSD may be greatly enhanced by elucidating how a usually committed sexual fate remains plastic in sex-changing fish. Here, we review the latest advances in our understanding of the process of sex change in

teleost fish, highlighting recent research suggesting a key role for stress in mediating temperature-induced masculinization (TIM) and socially regulated sex change. We present a synthesis of previous research regarding the mechanistic role of cortisol in mediating teleost sex change, emphasizing the importance of three key pathways through which cortisol may induce maleness, as originally proposed by [Fernandino et al. \(2013\)](#). We build on previous reviews in this area by incorporating critical recent studies that lend further support to the cortisol hypothesis.

Sex change in teleosts

Teleost fishes display a diversity of sex determination and developmental systems found in no other vertebrate lineage. They may exhibit gonochorism (fixed separate sexes), simultaneous (or synchronous) or sequential hermaphroditism ([Devlin & Nagahama 2002](#)). Teleost species are the only known vertebrates with sequentially hermaphroditic representatives, which undergo sex change as an adaptive part of the life cycle ([Robertson & Choat 1974](#), [Shapiro 1979](#)). Sequential hermaphroditism has multiple evolutionary origins in fishes, having been reported in 27 taxonomic families spread across nine orders ([Munday et al. 2006](#), [Avisé & Mank 2009](#), [Godwin 2009](#)). In such species, the reproductive value is typically strongly size dependent, and sex change enables an individual to maximize the lifetime reproductive fitness by reproducing as one sex while small, and another sex when older and larger (the size advantage model or SAM; [Warner 1988](#)). Three strategies are observed: protogynous (female to male, common among coral reef fishes such as groupers, parrotfishes and wrasses), protandrous (male to female, as in anemone fishes) and sequentially bidirectional sex change (sex change in either direction, common in gobies). All three approaches entail modifications to behavior, gonadal restructuring and alterations in external morphology ([Warner 1984](#), [Nakamura & Kobayashi 2005](#), [Godwin 2009](#)). Sex change in teleost fish illustrates a dramatic example of environmentally cued phenotypic plasticity unprecedented in any other group of vertebrates ([Frisch 2004](#), [Moczek 2015](#)).

Many commercially valuable fish species naturally change sex (e.g. grouper). In aquaculture settings, artificial control of sex ratios using sex-change technology has become essential for the accomplishment of successful operations ([Frisch 2004](#), [Budd et al. 2015](#)). For example, artificial achievement of monosex populations can be valuable for increasing growth rates or to ensure availability of broodstock of a particular sex ([Frisch 2004](#)). A molecular-level understanding of sex change, and especially the upstream factors that initiate this process, would enable the development of efficient, low-cost genetic tools for controlling sex ratios,

thereby avoiding the use of steroid treatments that pose significant environmental contamination risk.

Sex determination, sex change and the environment

Sex change involves switching the sexual characteristics associated with one of the sexes (male or female) into those of the opposite one. To fully comprehend this diversity, we need to backtrack to the point at which sexual fate is first decided, sex determination. Sex determination is the regulatory system that determines whether the undifferentiated gonads will form either ovaries or testes, while sex differentiation constitutes the process of gonad development thereafter ([Bull 1983](#), [Devlin & Nagahama 2002](#)). Sex determination encompasses a series of mechanisms that have classically been divided into those having either a genetic or environmental basis. Genetic sex determination (GSD) takes place when the individual's primary sex is determined at fertilization by inherited genetic elements, which are different between males and females (e.g. XX/XY chromosomal sex determination in mammals). Environmental sex determination (ESD) involves sexual fate being determined by external environmental conditions experienced during early development (e.g. temperature) ([Bull 1983](#)). Nevertheless, increasing evidence challenges this strict GSD–ESD dichotomy. Numerous examples exist where organisms are influenced by both strategies; their phenotypic sex being defined by the interaction between GSD and ESD ([Sarre et al. 2004, 2011](#), [Stelkens & Wedekind 2010](#), [Holleley et al. 2016](#)). Moreover, there are instances where specific external conditions can induce functional sex reversal in otherwise GSD species, an event referred to as environmental sex reversal (ESR) ([Shao et al. 2014](#)). Examples of ESR can be found across both invertebrates (as reported in the oviparous oyster *Crassostrea virginica*; [Coe 1943](#)) and vertebrates such as fishes (as seen, under both laboratory and wild conditions, in the pejerrey, *Odontesthes bonariensis*; [Hattori et al. 2009](#), [Yamamoto et al. 2014](#)), reptiles (occurring naturally in the Australian bearded dragon, *Pogona vitticeps*; [Holleley et al. 2015](#)) or amphibians (as observed in wild populations of the frog *Rana temporaria*; [Alho et al. 2010](#)). Herein, the term 'sex change' is used to refer exclusively to the male-to-female or female-to-male transformation that sequentially hermaphroditic teleost fishes undergo as a usual part of the life cycle. We use the term 'sex reversal', as it is common in the literature, to refer to the process whereby environmental conditions override genetic factors during early stages of development and induce the phenomenon of ESR, as it occurs in fishes, reptiles and amphibians. A range of biotic and abiotic factors, e.g. temperature, social changes, pH, hypoxia, relative density, pathogens, etc., have been reported to act as environmental cues for ESR (reviewed by [Devlin & Nagahama 2002](#), [Stelkens & Wedekind 2010](#)). Of these, water temperature and

social factors have been the most extensively studied (Warner 1984, Ospina-Alvarez & Piferrer 2008, Godwin 2009, Kato *et al.* 2011, Ribas *et al.* 2017). Studies on temperature sex determination (a type of ESD) in fish constitute a great example of the interaction and blurred lines between the false GSD–ESD dichotomy, showing that the production of cortisol as a result of thermal stress can lead to masculinization of female fish under artificial conditions, in a transformation known as TIM. Recent research suggests that in addition to possibly mediating TIM, cortisol may similarly act as a key physiological signal underlying sex change in sequential hermaphroditic fish, which most often occurs in response to a change in social circumstances (Nozu & Nakamura 2015).

The role of cortisol

Cortisol as a transducer of thermal and social cues

Elevated temperatures have been shown to cause gonadal masculinization by inhibiting ovarian development in several gonochoristic species under artificial conditions (Hattori *et al.* 2009, Hayashi *et al.* 2010, Yamaguchi *et al.* 2010, Fernandino *et al.* 2012, Yamaguchi & Kitano 2012, reviewed by Fernandino *et al.* 2013). Stressful conditions can affect many steroidogenic enzymes during maturation of the gametes, and several authors have emphasized the role of GCs in influencing gonadal fate during TIM (Hattori *et al.* 2009, Hayashi *et al.* 2010,

Yamaguchi *et al.* 2010). Furthermore, recent studies have also reinforced the hypothesis of stress (i.e. plasma levels of cortisol) being associated with sex change in sequential hermaphrodites induced by social interactions (Table 1) (Godwin & Thomas 1993, Perry & Grober 2003, Solomon-Lane *et al.* 2013, Nozu & Nakamura 2015). Protogynous, protandrous and bidirectional sex changes in sequential hermaphrodites are thought to be mediated by factors that control steroidogenic gene expression and regulate agonistic behavior and social status. Socially induced sex change depends both on the physical status of the fish and on the appropriate social conditions to occur (Munday *et al.* 2006, Lamm *et al.* 2015). In protogynous species, for example, this involves loss of a dominant male from a social group of females leading to the sex change of the dominant alpha female (Robertson & Choat 1974, Jones & Thompson 1980, Warner & Swearer 1991). It remains unanswered whether differential stress responsiveness is a cause or a consequence of the individual's social rank (Øverli *et al.* 1999). However, it has been shown that the hypothalamic–pituitary–interrenal or HPI axis (homologous to the hypothalamic–pituitary–adrenal or HPA axis) response and subsequent cortisol production cascade are indeed influenced by social status (Øverli *et al.* 1999) and could affect sexual fate (Iwata *et al.* 2012).

The exact ways in which cortisol may act as a mediator in the masculinization process remain unclear, although several hypotheses exist (reviewed by Baroiller & D'Cotta 2016, Todd *et al.* 2016, Liu *et al.* 2017). One

Table 1 Summary of current knowledge regarding the involvement of cortisol in the processes of temperature-induced masculinization and socially regulated sex change.

Species	Reproductive strategy	Induction of sex change	Effects observed	References
<i>Amphiprion melanopus</i> (cinnamon clownfish)	Protandrous	Removal of dominant female	Gonadal sex change accompanied by increase in plasma cortisol levels, reaching a peak on day 20 post female removal	Godwin and Thomas (1993)
<i>Halichoeres trimaculatus</i> (three-spot wrasse)	Protogynous	Prolonged administration of cortisol	Gonadal sex change accompanied by reduction in E2 levels	Nozu and Nakamura (2015)
<i>Lythrypnus dalli</i> (bluebanded goby)	Bidirectional	Removal of dominant male CRF administration	Female-to-male sex change accompanied by increase in cortisol levels during early sex change stages. Single i.c.v. injection did not cause sex change	Solomon-Lane <i>et al.</i> (2013)
<i>Odontesthes bonariensis</i> (pejerrey)	Gonochoristic	High temperature and cortisol administration	Temperature-induced masculinization. Downregulation of gonadal aromatase and increased 11KT synthesis	Hattori <i>et al.</i> (2009), Blázquez and Somoza (2010), Shinoda <i>et al.</i> (2010), Fernandino <i>et al.</i> (2011)
<i>Oryzias latipes</i> (medaka)	Gonochoristic	High temperature and cortisol administration	Temperature-induced masculinization. Downregulation of gonadal aromatase. Suppression of female-type PGC proliferation	Hattori <i>et al.</i> (2007), Selim <i>et al.</i> (2009), Hayashi <i>et al.</i> (2010)
<i>Paralichthys olivaceus</i> (Japanese flounder)	Gonochoristic	High temperature and cortisol administration	Temperature-induced masculinization. Downregulation of gonadal aromatase. Delay in start of germ cell proliferation	Kitano <i>et al.</i> (1999), Yamaguchi <i>et al.</i> (2010), Yamaguchi & Kitano (2012)
<i>Paraperis cylindrica</i> (sandperch)	Protogynous	Cortisol administration and removal of dominant male	Cortisol-treated fish changed sex, as did all sham-treated and control fish	Frisch <i>et al.</i> (2007)

Modified from Fernandino *et al.* (2013).

11KT, 11-ketotestosterone; CRF, corticotropin-releasing factor; E2, 17 β -estradiol; i.c.v., intracerebroventricular; PGC, primordial germ cell.

of the first published reports of the association between stress and sequential hermaphroditism in a teleost fish was for socially induced sex change in the protandrous cinnamon clownfish (*Amphiprion melanopus*) in which serum cortisol levels were observed to peak in the later sex change stages (Godwin & Thomas 1993). Perry and Grober (2003) suggested that environmental stimuli affect cortisol levels which, in turn, modulate brain activity, effectively providing a mechanism for communication between the brain and the gonads (Perry & Grober 2003, Blázquez & Somoza 2010). According to Perry and Grober (2003), dominant males in social groups of protogynous species were responsible for inhibiting socially induced sex change of subordinate females through their aggressive behavior, and the release from this high stress (i.e. drop in cortisol) would trigger the sex change of the alpha female, which would, in turn, keep the cortisol levels of the rest of the females elevated.

Furthermore, Perry and Grober (2003) also proposed another hypothesis stating that GCs could act as modulators of transcription as well as regulators of steroid production directing the function of 11 β -hydroxylase (Cyp11b) and 11 β -hydroxysteroid dehydrogenase (Hsd11b) enzymes through substrate competition with androgens testosterone (T) and 11-ketotestosterone (11KT, the most powerful androgen in teleosts). In teleost fish, T acts as a prohormone, producing either 17 β -estradiol (E2, the most potent estrogen in both teleosts and mammals) through the gonadal or brain aromatase (Cyp19a1a and Cyp19a1b respectively) or 11KT by Cyp11b and Hsd11b enzymes (Fig. 1) (Frisch 2004). Cyp11b and Hsd11b are critical not only for the synthesis of 11-oxygenated androgens, but also for the metabolism of GCs (Perry & Grober 2003, Alderman & Vijayan 2012, Fernandino et al. 2012).

However, both the Release from Social Stress and the Substrate Competition for Enzymes hypotheses were refuted by work on the protogynous *Paraperca cylindrica* (sandperch) in which cortisol implants inserted into the dominant female of a social group neither showed detectable effects on the pattern of steroidogenesis nor inhibited sex change under permissive conditions (i.e. removal of dominant male) (Frisch et al. 2007). Research on gonochoristic pejerrey has also come into conflict with the Substrate Competition for Enzymes hypothesis, since results on pejerrey larvae showed that the cortisol treatment was associated with significant increases in 11KT, but not T, during the first 4 weeks (Hattori et al. 2009). In combination with data from Frisch et al. (2007), an increase in cortisol levels was proposed as part of the trigger initiating sex change in sequential hermaphrodites by Solomon-Lane et al. (2013), who argued that this elevation would increase corticotropin-releasing factor (CRF) signaling and activity of HPI axis. Their work on the bidirectional hermaphrodite *Lythrypnus dalli* (bluebanded goby) showed that cortisol peaked in early stages of sex change during which

differences in HPI axis activity were also shown to reflect social status (Solomon-Lane et al. 2013). Most recently, long-term cortisol administration was shown to induce sex change in protogynous three-spot wrasse (*Halichoeres trimaculatus*; Nozu & Nakamura 2015). These recent studies suggest that elevated cortisol levels should promote, rather than inhibit, protogynous sex change, which is worthy of future investigation.

Cross-talk between hypothalamic–pituitary–gonadal (HPG) and HPI axes

In all vertebrates, the HPG axis plays a principal role in development and reproduction and is considered the primary signaling pathway by which environmental

Figure 1 Simplified illustration of steroidogenic pathways relevant to triggering and maintaining sex change in teleost fishes. Contrarily to most vertebrates, higher levels of T (testosterone) are not directly associated with maleness, and equal levels (or even higher) of T concentrations can be found in female individuals compared to other males (Borg 1994). The balance between 11KT (11-ketotestosterone) and E2 (17 β -estradiol) influences the differentiation of germ cells into ovaries or testes, but it is still not clear whether androgens and estrogens can redirect the fate of existing germ cells, or if these are replaced by newly proliferated ones (Lo Nostro et al. 2003, Liu et al. 2017). Dashed boxes represent their predominance in male (δ) and female (ϕ) fishes, respectively. 11BOHA4, 11 β -hydroxyandrostenedione; 11BOHT, 11 β -hydroxytestosterone; 11KA4, adrenosterone; A4, androstenedione; Cyp11b, 11 β -hydroxylase; E1, estrone; Hsd11b, 11 β -hydroxysteroid dehydrogenase. Adapted from Baroiller et al. (1999) and Liu et al. (2017).

cues are transduced into behavioral and gonadal sex changes in teleosts (Godwin 2010). The HPI axis, also commonly referred to as the stress axis, affects sexual development and reproduction via its cross-talk with the HPG axis (Fig. 2). It has been speculated that these two axes interact with each other to mediate sex change, but the detailed mechanism remains poorly understood.

The cross-talk between the two axes is exemplified by the effects of signaling molecules associated with neural activity on circulating cortisol, which has been proposed to indirectly regulate gonadal sex change (Liu *et al.* 2017). These neurotransmitters include norepinephrine (NE), arginine vasotocin (AVT), serotonin (5-HT), dopamine (DA), kisspeptin and melatonin (reviewed by Godwin 2010, Lamm *et al.* 2015, Liu *et al.* 2017). NE is known to influence both the liberation of gonadotropin-releasing hormone (GnRH) and the production of gonadotropins (GtHs) in gonochoristic fish (Chang & Peter 1984, Yu *et al.* 1991). Moreover, in mammals, NE has been demonstrated to control CRF

neurons (Itoi *et al.* 1999), and evidence in rainbow trout (*Oncorhynchus mykiss*) suggests that NE stimulates HPI axis activity (Øverli *et al.* 2000). Neuropeptide AVT is homologous to mammalian arginine vasopressin and has been the focus of the study of behavioral sex change in fish (Donaldson & Young 2008). Interestingly, AVT has been shown to increase plasma cortisol levels in rainbow trout (Baker *et al.* 1996, Gesto *et al.* 2014), which links cortisol with the transduction of social information. Furthermore, serotonin, which promotes the liberation of GnRH and mediates the increase of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) levels in gonochoristic fish (Yu *et al.* 1991, Senthilkumaran *et al.* 2001), can stimulate or inhibit the HPI axis through different neuronal projections (Lepage *et al.* 2002). Therefore, the interaction among AVT, NE and 5-HT seems to play an essential role in mediating communication between the brain and the gonads via HPG and HPI axes during sex change (reviewed by Godwin 2010, Lamm *et al.* 2015).

Figure 2 Schematic representation of cross-talk between HPG (Hypothalamic–Pituitary–Gonadal) and HPI (Hypothalamic–Pituitary–Interrenal) axes that regulate steroidogenesis and behavior in teleost fish. HPG axis regulation begins with the Gonadotropin-releasing hormone (GnRH), secreted from the preoptic area (POA) in the hypothalamus by GnRH-expressing neurons, which directly innervate the anterior pituitary (Foran & Bass 1999). When stimulated by GnRH, the pituitary produces the gonadotropins (GtHs) LH and FSH. Once released into the circulatory system, these gonadotropins mediate the release of sex steroids via their receptors in the gonad, including 17 β -estradiol (E2) and 11-ketotestosterone (11KT) in the case of teleost fish (Foran & Bass 1999). Likewise, under a situation of stress, activation of the HPI axis begins in the POA of the hypothalamus, which is stimulated to secrete CRF by CRF neurons, which in turn stimulates corticotrophic cells of the anterior pituitary to release ACTH (also known as corticotropin or adrenocorticotrophic hormone) into the blood stream (Wendelaar Bonga 1997). ACTH circulates to the interrenal cells in the head kidney (homologous to the adrenal gland in mammals) where it binds to the melanocortin receptor 2 (also known as the ACTH or MC2 receptor) to activate cortisol synthesis and release (Wendelaar Bonga 1997, Mommsen *et al.* 1999). Feedback relationships are not illustrated. Solid lines indicate interactions with support from fish models; dashed lines indicate interactions with support from non-teleost systems that are yet to gain supporting evidence in fish. 5-HT, serotonin; AVT, arginine vasotocin; DA, dopamine; FSHR, follicle-stimulating hormone receptor; GR, glucocorticoid receptor; GnIH, gonadotropin-inhibitory hormone; LHR, luteinizing hormone receptor; MEL, melatonin; NE, norepinephrine; T, testosterone. Modified from Frisch (2004), Todd *et al.* (2016), Liu *et al.* (2017).

A recently proposed hypothesis suggests that increased NE activities in the brain can lead to the rapid increase in serum cortisol levels, while a diminished presence of serotonin annuls the inhibitory effects of this neurotransmitter on AVT signaling, enabling the maintenance of high cortisol levels during the first stages of gonadal sex change (Liu *et al.* 2017). In addition, novel findings have revealed possible key roles of melatonin of being involved in the synchronization of circadian rhythms, blood pressure regulation and seasonal reproduction (Altun & Ugur-Altun 2007). Melatonin's response to stressors has been reported in the Mozambique tilapia (*Oreochromis mossambicus*) in which serum melatonin levels were reported to increase after exposure to high temperatures (Relkin 1989). The effects of melatonin on GnRH and gonadotropin signaling are thought to be stage dependent and studies suggest that despite discrepancies, a regulatory mechanism initiated by melatonin and NE transiently stimulates GnRH-induced LH release to start sex change. This is followed by indirect suppression of GnRH and gonadotropin signaling by an increase of cortisol levels and GnIH (gonadotropin-inhibitory hormone) expression (Khan & Thomas 1996, Amano *et al.* 2004, Yuan *et al.* 2012, Alvarado *et al.* 2015). Along with these factors, an increase in circulating cortisol could directly suppress *cyp19a1a* transcription to arrest estrogen production and female-specific gene expression to promote ovarian degeneration (Liu *et al.* 2017).

Potential pathways through which cortisol regulates sex change

Three non-exclusive pathways are considered regarding the role of cortisol in triggering the male pathway: (i) cross-talk between corticosteroid and androgen pathways, (ii) the inhibition of aromatase expression through the binding of cortisol to the glucocorticoid response elements (GRE) in the *cyp19a1a* promoter and (iii) the depletion of primordial germ cells (PGCs) via upregulation of *amh* (the gene encoding anti-Müllerian hormone) (reviewed by Fernandino *et al.* 2013, Todd *et al.* 2016).

Cross-talk between GC and androgen pathways

The stress axis has been suggested to influence plasma levels of androgens in both mammals (Viau 2002, Carré & Mehta 2011) and fish (Godwin & Thomas 1993, Perry & Grober 2003, Ozaki *et al.* 2006, reviewed by Goos & Consten 2002). In teleosts, an example of this cross-talk is visible through key enzymes Cyp11b and Hsd11b (Fig. 3) (Perry & Grober 2003, Bury & Sturm 2007). The physiological roles of these enzymes in the testes are yet to be defined. The metabolism of cortisol to 11-oxygenated androgens *in vitro* has been proposed in various teleost species (Kime 1978), whereby cortisol

would induce spermatogonial mitosis by increasing 11KT production (Ozaki *et al.* 2006). This hypothesis was challenged by Schulz (1986), whose results *in vitro* fitted Kime's concept but questioned its importance *in vivo*, highlighting the possibility that extragonadal steroid metabolism could confound the observed plasma hormone levels.

Only a handful of studies have directly assessed the effect of cortisol on the androgenic pathway in teleost fish (Consten *et al.* 2002, Kusakabe *et al.* 2003, Ozaki *et al.* 2006, Fernandino *et al.* 2012). Higher doses of cortisol were first suggested to act on testicular steroidogenesis by competition with enzymes Cyp11b and Hsd11b in the common carp *Cyprinus carpio* (Consten *et al.* 2002). The dual role of enzymes involved in both GCs and androgen biosynthesis was later addressed in the rainbow trout (Kusakabe *et al.* 2003). Interestingly, low doses of cortisol administration were also demonstrated

Cross-talk between glucocorticoid and androgen pathways

Figure 3 Schematic representing cross-talk between glucocorticoid and androgen pathways. Two types of HSD11B, HSD11B1 and HSD11B2, have been identified and characterized in mammals (Albiston *et al.* 1994). HSD11B1 converts cortisone to cortisol, while HSD11B2 oxidizes active ligand cortisol to cortisone, the inactive form incapable of binding to glucocorticoid receptors (GRs), thus mediating the stress response (Albiston *et al.* 1994, Wendelaar Bonga 1997). In fish, however, only one type, Hsd11b2, has been found, although this discovery is limited to a few species. Hsd11b2 is believed to be the homolog of mammalian HSD11B2 (Baker 2004). Additionally, only one form of Cyp11b has been found in teleosts (Jiang *et al.* 2003, Kusakabe *et al.* 2003, Arterbery *et al.* 2010). 11BOHT, 11 β -hydroxytestosterone; 11KT, 11-ketotestosterone; Cyp11b, 11 β -hydroxylase; Hsd11b, 11 β -hydroxysteroid dehydrogenase; T, testosterone. Adapted from Arterbery *et al.* (2010).

to induce spermatogenesis and enhance spermatogonial proliferation through the synthesis of 11KT *in vitro* in Japanese eel (*Anguilla japonica*) testis explants (Ozaki *et al.* 2006). In pejerrey, cortisol treatment during TIM was observed to promote 11KT synthesis also by modulation of Hsd11b2 enzyme (Fernandino *et al.* 2012). Importantly, levels of T and 11KT were observed to start to elevate prior to the inhibition of gonadal aromatase (*cyp19a1a*), providing clear evidence of the association between the stress axis and the androgens biosynthesis in the masculinization of larval pejerrey (Hattori *et al.* 2009, Fernandino *et al.* 2012). These results suggest that cortisol effects are dose and species specific (reviewed by Fernandino *et al.* 2013).

Inhibition of aromatase expression

Another way in which cortisol may tip the balance in favor of androgen production is by directly inhibiting the expression of the aromatase gene, *cyp19a1a*. Cortisol acts upon the different tissues via its passage through the plasma membrane and subsequent binding to a glucocorticoid receptor (GR), which controls gene expression by binding to GRE within promoter regions of responsive genes (Payvar *et al.* 1983), such as *cyp19a1a*. Aromatase converts androgens to estrogens to maintain ovarian function, and its downregulation is considered a key event initiating female-to-male sex change in fishes (Todd *et al.* 2016, Liu *et al.* 2017).

The effects of cortisol on teleost ovarian steroidogenesis were first evaluated in cultured ovarian follicles of rainbow trout, resulting in the suppression of estrogen synthesis (Carragher & Sumpter 1990). Studies on TIM in Japanese flounder (*Paralichthys olivaceus*), pejerrey and medaka (*Oryzias latipes*) have shown downregulation of gonadal aromatase during high temperature-induced sex reversal (Hattori *et al.* 2009, Yamaguchi *et al.* 2010, Kitano *et al.* 2012, Yamaguchi & Kitano 2012). The masculinizing effects of high temperatures or cortisol can be rescued by 17 β -estradiol treatment, as shown in medaka and Japanese flounder (Kitano *et al.* 2012). Moreover, cortisol was shown to directly suppress transcription of *cyp19a1a* *in vitro* in Japanese flounder by binding to GRE in the gene's promoter region (Yamaguchi *et al.* 2010, Yamaguchi & Kitano 2012).

Upregulation of *amh*, promoting gonadal apoptosis and maleness

Third, cortisol may promote masculinization by upregulating *amh* (Hattori *et al.* 2009, Yamaguchi *et al.* 2010, Yamaguchi & Kitano 2012) to induce germ cell apoptosis and promote maleness (reviewed by Pfennig *et al.* 2015, Todd *et al.* 2016). Although *amh* is expressed in both sexes, high levels of *amh* expression are commonly found in male gonads during fish sex differentiation, which reinforces the involvement of

this gene in the promotion of male sex differentiation (reviewed by Pfennig *et al.* 2015). Cortisol administration has been shown to affect *amh* transcript levels, except for medaka (Hayashi *et al.* 2010, Kitano *et al.* 2012, reviewed by Pfennig *et al.* 2015). Specifically, it is a reduced ratio of PGCs to somatic cells (SC) in the gonad that is considered to have masculinizing effects. Experimental depletion of germ cells in female gonads is associated with male development of the remaining SC, while an increased number of germ cells correlates with the activation and maintenance of the female phenotype by signaling SC to prevent their activation of the male phenotype (Tanaka *et al.* 2008, reviewed by Pfennig *et al.* 2015). Thus, in sex-changing fish, cortisol could link temperature-dependent and social cues to gonadal development while acting on *amh*, which in turn could play a role at the level of the gonads as a downstream effector to inhibit germ cell proliferation and encourage masculinization (reviewed by Pfennig *et al.* 2015). The potential for cortisol to act on germ cells is consistent with the discovery of GRs on germ cells (Goos & Consten 2002). GRs have been identified in Sertoli cells in mammalian testes (Levy *et al.* 1989) but are yet to be demonstrated in fish. Moreover, cortisol has been proposed to play an antagonizing role to FSH, which indeed inhibits the *amh* expression (Yamaguchi *et al.* 2010, reviewed by Pfennig *et al.* 2015).

Upregulation of *amh* expression has been observed during TIM in Japanese flounder (Yoshinaga *et al.* 2004, Kitano *et al.* 2007, Yamaguchi & Kitano 2012), Southern flounder (*Paralichthys lethostigma*; Mankiewicz *et al.* 2013), Nile tilapia (*Oreochromis niloticus*; Poonlaphdechana *et al.* 2013) and pejerrey (Fernandino *et al.* 2008), and likewise during protogynous sex change in ricefield eel (*Monopterus albus*) (Hu *et al.* 2015). Acting as a transcription factor, the Amh protein is also known to directly inhibit the *cyp19a1a* expression in mammals, and an inverse association between *amh* and *cyp19a1a* expression is reported in numerous fishes, including Japanese flounder (Kitano *et al.* 2007), pejerrey (Fernandino *et al.* 2008), rainbow trout (Vizziano *et al.* 2008), southern flounder (Mankiewicz *et al.* 2013) and zebrafish (*Danio rerio*; Rodríguez-Marí *et al.* 2005, Wang & Orban 2007). Recently, a transcriptome-wide study of protogynous sex change in bluehead wrasse (*Thalassoma bifasciatum*) found that the expression of *amh* and its receptor *amh2* increased concurrently with a drop in *cyp19a1a* expression (Liu *et al.* personal communication). Although a causal relationship cannot be established from these data, these results suggest that *amh* may have an important role in initiating protogynous sex change by suppressing gonadal aromatase expression and/or activating a male-specific expression pathway.

The three proposed pathways through which cortisol may induce gonadal masculinization under stressful conditions are not mutually exclusive and may, therefore,

be acting together to promote masculinization, both in TIM and in socially induced sequential hermaphrodites.

Conclusions and future opportunities

Cortisol has emerged as a key physiological mediator of sex change in teleost fish, which may communicate external environmental signals to adaptive reproductive responses. We synthesize mounting evidence reinforcing the involvement of this hormone in two well-described transformations: TIM and, more recently, socially regulated sex change. The mechanistic role of cortisol in sex change involves three non-mutually exclusive pathways: (i) cross-talk between GC and androgen pathways, (ii) inhibition of aromatase expression to arrest estrogen production and (iii) induction of gonadal apoptosis through the upregulation of *amh*, which may also influence sex-specific gene expression. However, these hypotheses only address how cortisol can induce maleness. Serum cortisol levels have been shown to fluctuate across protandrous sex change, for example, in cinnamon clownfish (Godwin & Thomas 1993). We encourage further investigation into the role of cortisol in male-to-female sex change in protandrous, as well as, bidirectional hermaphrodites.

Another fruitful avenue for research will be the potential interaction between cortisol and epigenetic factors influencing sex-specific gene expression during sex change (Deveson *et al.* 2017). Corticosteroid hormones are known to affect methylation of DNA in different mammalian systems. For instance, hydrocortisone (as hormone cortisol is known when supplied as medication) was observed to induce noticeable changes in the 5-MeCyt (5-Methylcytosine) content in the DNA of the rat liver cells, its effect on the level of DNA methylation being both organ and tissue specific (Vanyushin *et al.* 1973). In guinea pigs, prenatal exposure to synthetic GCs led to lifelong and potentially transgenerational effects on organ methylation, such as global hypomethylation and deregulated expression of *mbd2* (methyl-CpG-binding domain protein 2) and *dnmt* (DNA methyltransferase) mRNA, as well as changes in HPA function (Dunn *et al.* 2010, Crudo *et al.* 2012). These critical changes of the epigenome are associated with a higher risk of tumorigenesis (Szyf 2005). In humans, childhood stress (measured in the form of cortisol) has been linked to a genome-wide decrease in DNA methylation (Nätt *et al.* 2015). Cortisol is yet to be associated with epigenetic changes in teleosts. However, TIM-inducing temperatures are shown to induce DNA methylation changes within gene promoters to influence the expression of key sex-pathway genes in several fishes (Navarro-Martín *et al.* 2011, Zhang *et al.* 2013, Shao *et al.* 2014, Sun *et al.* 2016). For example, during high TIM of the European sea bass (*Dicentrarchus labrax*), hyper-methylation of

cyp19a1a led to downregulation of gonadal aromatase expression causing sex reversal (Navarro-Martín *et al.* 2011). Hyper-methylation of *cyp19a1a* gene promoters was similarly observed in testis of the Japanese flounder (Wen *et al.* 2014), medaka (Contractor *et al.* 2004), ricefield eel (Zhang *et al.* 2013) and zebrafish. Genome-wide analysis of methylation and gene expression following TIM in half-smooth tongue sole (*Cynoglossus semilaevis*) revealed that a change in the methylation state was not limited to the gonadal aromatase gene, but occurred across the entire sex development network (Shao *et al.* 2014). Most recently, TIM in Nile tilapia was associated with increased methylation levels on various chromosomes (Sun *et al.* 2016). Given the strong evidence for a mechanistic role for both cortisol and DNA methylation individually in regulating sex-specific gene expression during teleost sex change, and evidence that cortisol can promote epigenetic changes in mammals, their potential interaction during sex change warrants investigation. Special attention might be paid to the possibility that cortisol mediates temperature-induced methylation of the *cyp19a1a* gene.

In teleost fish, one major impediment when measuring cortisol is that being directly related to stress, an absolute control over its quantification and over the handling of fish is essential not to confound its measurement. Current limitations to studying cortisol as a mediator of sex change include the ability to reliably differentiate males and females in dimorphic species, the seasonality of spawning and the individual variation regarding physical fitness. Additionally, it is not an easy task to determine whether differences in cortisol levels observed between social groups (i.e. dominant vs subordinate) may have a role in the triggering of sex change or merely reflect social status. These hurdles can be at least partly overcome by using standardized protocols and consistent laboratory and field conditions and we encourage further investigation. Genomic technologies offer exciting possibilities of testing the hypotheses related to cortisol's influence on the sex-specific gene expression. For example, whole transcriptome sequencing technology (also known as RNA-seq) now enables rapid characterization of global gene expression in any species without the need for prior genomic resources. This approach has already proven successful in identifying candidate triggers of sex change in the bluehead wrasse (Liu *et al.* 2015, Liu 2016) and Red Sea clownfish (Casas *et al.* 2016). Manipulative studies will be a critical next step, and technologies such as nucleofection of cDNAs, siRNA (Ryan *et al.* 2011), vivo morpholinos (Moulton & Jiang 2009) and gene editing techniques (Irion *et al.* 2014) represent promising approaches for targeted induction or suppression of expression of candidate genes implicated in gonadal sex change. Organ explant culture systems could provide a suitable highly controlled *in vitro* system for such

manipulations as demonstrated with testes explants of Japanese eel (Ozaki *et al.* 2006) and in an ovary culture system in the three-spot wrasse (Todo *et al.* 2008).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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