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SYMPOSIUM

The Need for Speed: Neuroendocrine Regulation of Socially-controlled Sex Change

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Synopsis Socially-controlled functional sex change in fishes is a dramatic example of adaptive reproductive plasticity. Functional gonadal sex change can occur within a week while behavioral sex change can begin within minutes. Significant progress has been made in understanding the neuroendocrine bases of this phenomenon at both the gonadal and the neurobiological levels, but a detailed mechanistic understanding remains elusive. We are working with sex-changing wrasses to identify evolutionarily-conserved neuroendocrine pathways underlying this reproductive adaptation. One key model is the bluehead wrasse (*Thalassoma bifasciatum*), in which sex change is well studied at the behavioral, ecological, and neuroendocrine levels. Bluehead wrasses show rapid increases in aggressive and courtship behaviors with sex change that do not depend on the presence of gonads. The display of male-typical behavior is correlated with the expression of arginine vasotocin, and experiments support a role for this neuropeptide. Estrogen synthesis is also critical in the process. Female bluehead wrasses have higher abundance of aromatase mRNA in the brain and gonads, and estrogen implants block behavioral sex change. While established methods have advanced our understanding of sex change, a full understanding will require new approaches and perspectives. First, contributions of other neuroendocrine systems should be better characterized, particularly glucocorticoid and thyroid signaling. Second, advances in genomics for non-traditional model species should allow conserved mechanisms to be identified with a key next-step being manipulative tests of these mechanisms. Finally, advances in genomics now also allow study of the role of epigenetic modifications and other regulatory mechanisms in the dramatic alterations across the sex-change process.

Introduction

The plasticity of the brain of adult vertebrates is much greater than was once appreciated (see Gross 2000; Kaslin et al. 2008). The mechanisms underlying these plastic events are an area of intense interest, particularly in relation to environmental influences. Plasticity of the brain often is most apparent in species in which individuals can undergo dramatic behavioral transformations as adults. Some of the most compelling examples include sex change in many reef fishes (e.g., Fishelson 1970; Robertson 1972; Warner et al. 1975; Fricke and Fricke 1977) and role change in species with morphologically- and behaviorally-distinct alternative reproductive tactics (e.g., Warner et al. 1975; Francis et al. 1993; Oliveira et al. 2001). Some of the same neuroendocrine systems that regulate puberty and reproduction (including gonadotropin-releasing hormone) and dominant male-typical behaviors (including vasotocin/vasopressin) in many vertebrates may also regulate sex change and role change in fishes (reviewed by Foran and Bass 1999; Bass and Grober 2001; Godwin 2009, 2010). Some of these brain systems will be discussed in this review.

In general, switching sex or role to the dominant/territorial phenotype confers reproductive benefits, such as greater access to mates and higher mating success (Ghiselin 1969; Warner 1975; Warner et al. 1975). Socially-induced sex change, whether protogynous (female-to-male), protandrous

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(male-to-female), or bidirectional, has been observed in a wide range of marine fishes and can occur remarkably rapidly: behaviorally, within minutes to hours into the sex-change process for some species, such as the bluehead wrasse (*Thalassoma bifasciatum*; Warner and Swearer 1991) and bluebanded goby (*Lythrypnus dalli*; Reavis and Grober 1999), and as quickly as 1–2 weeks for completion of gonadal sex change in certain species, including the bluehead wrasse (Warner and Swearer 1991) and bluebanded goby (Black et al. 2005a). The initial social trigger typically involves removal of individuals of one phenotype (e.g., dominant/territorial males) from a social group to allow replacement by individuals of another phenotype (e.g., subordinate/non-territorial females). Because individuals often live in highly competitive, complex social groups, it is critical for individuals to rapidly assert and maintain behavioral dominance in order to achieve gonadal/morphological changes that lead to higher reproductive success.

Some species of vertebrates, particularly teleost fishes, also exhibit plastic alternative reproductive tactics among individuals of the same sex (usually male), with or without distinct body morphotypes, in which territorial/bourgeois and non-territorial/parasitic behavioral phenotypes can switch roles depending on the social environment (reviewed by Moore and Thompson 1990; Moore 1991; Moore et al. 1998; Taborsky 2001; Godwin 2010). Role change among members of the same sex appears to occur more rapidly than does change in sex, that is in less than 1 week, since no major gonadal restructuring is required, for example, *Astatotilapia burtoni* (Burmeister et al. 2005; Maruska and Fernald 2010) and the bluehead wrasse (Godwin et al. personal observation).

Functional sex change and role change in adult fishes both offer dramatic examples of how the social environment can rapidly affect behavior and reproduction. This review focuses solely on socially-induced sex change. This phenomenon is valuable for studies exploring the neuroendocrine bases of dominant/subordinate behaviors and reproductive differences, while also allowing exploration of social effects on sex determination and the evolution of socially-sensitive sex-determination systems in teleosts.

In this review, we first discuss behavioral, gonadal, and hormonal aspects of socially-induced sex change in adult fishes, with emphasis on protogynous sex change in the bluehead wrasse. We then discuss known and proposed neuroendocrine pathways mediating rapid events during sex change. We conclude with some promising future directions for the study of sex change in fishes. An exhaustive review of sex change and sex determination in fishes is beyond the scope of this article, but we refer readers to several comprehensive reviews (Devlin and Nagahama 2002; Godwin 2010; Piferrer 2011).

Socially-induced sex change in protogynous fishes

Sexual differentiation and sex change in adult bluehead wrasses

The bluehead wrasse is a small Caribbean reef fish that exhibits socially-controlled protogynous sex change (Warner et al. 1975; Warner and Swearer 1991). Bluehead wrasses display strong sexual dimorphism as well as alternative male phenotypes with divergent reproductive tactics (Feddern 1965; Warner et al. 1975; Semsar and Godwin 2004). Initial sexual differentiation into either a female or a initial phase (IP) male occurs at approximately 30 mm standard length (Shapiro and Rasotto 1993) and is at least partially socially-controlled (Munday et al. 2006); most juveniles that settle on small reefs become females, while up to 50% of those that settle on large reefs become IP males (Warner 1984).

Adults spawn at mid-day year-round, releasing their gametes into the water column (Feddern 1965). Females spawn two out of every 3 days on average (Schultz and Warner 1991), while males can spawn many times per day (Warner et al. 1975). IP males mimic females in morphology and behavior, showing very little aggression, territoriality, or courting of females. IP males either spawn in large groups of 5–20 or more IP males and one female, or obtain fertilizations by ''sneaking'' or ''streaking'' on pair-spawns between females and dominant terminal phase (TP) males (Warner et al. 1975). TP males develop through changes in sex or role in large females and IP males, which involve dramatic alterations in behavior and morphology. TP males can be divided into two social classes: non-territorial (NT-TP) and territorial (T-TP). NT-TP males are generally smaller, less aggressive, not territorial, and display only infrequent courtship (Semsar et al. 2001). NT-TP males can rapidly occupy vacant territories and begin displaying behaviors typical of T-TP males (Table 1).

Development and display of behaviors typical of T-TP males begin minutes to hours after removal of a T-TP male and are not dependent on gonads (Godwin et al. 1996). Ovarian atresia is advanced by Day 3 of sex change, testicular development and initial change in color occur about Day 4–6, and mature sperm are produced as early as Day 8–10

Table 1 Two body morphotypes in the bluehead wrasse, IP and TP, and four behavioral phenotypes, female, IP male, NT-TP male, and T-TP male

	Female	IP male	Female → T-TP male sex changer	NT-TP male	T-TP male
Morph	IP	IP	Intersex	TP	TP
Behavior	Female-typical	IP male-typical	TP male-typical	Less TP male-typical	TP male-typical
Aggression	No	No	Yes	Little	Yes
Spawning	Pair-spawn or group-spawn as female	Group-spawn or sneak/streak-spawn as male	Pair-spawn as male	Opportunistically pair-spawn as male	Pair-spawn as male
Courtship	No	No	Yes	Opportunistic	Yes
Genital papilla	Blunt, with broad genital opening	Pointy, with small genital aperture	Intersex	Pointy, with small genital aperture	Pointy, with small genital aperture
Gonadal status	Ovary	Testis	Intersex	Testis	Testis

(Warner and Swearer 1991; Shapiro and Rasotto 1993; Semsar and Godwin 2003).

Social signals that regulate sex change in protogynous fishes

The earliest clear sign of sex change in female bluehead wrasses is the display of behaviors typical of T-TP males (for a complete description, see Semsar and Godwin 2004). These behavioral changes occur rapidly and are controlled by social cues. The absence of larger, dominant males in the bluehead wrasse (Warner and Swearer 1991) and other protogynous species, for example, lyretail anthias (*Anthias squamipinnis*; Fishelson 1970; Shapiro 1980) and bluestreak cleaner wrasse (*Labroides dimidiatus*; Robertson 1972), can be considered a ''stimulus'' or ''trigger'' because it creates a permissive social environment for sex change in the largest, highest-ranking females of a social group (Robertson 1972; Ross et al. 1983; Warner and Swearer 1991). Sex change may also depend upon the presence of at least one smaller conspecific in at least some species, for example, saddleback wrasses (*Thalassoma duperrey*; Ross et al. 1983) and Potter's angelfish (*Centropyge potteri;* Lutnesky 1994). Size or social rank therefore serves as a ''primer'' or ''prerequisite'' for behavioral sex change.

Visual and chemical cues are important sensory systems regulating reproductive and agonistic behaviors in fishes (Stacey and Sorensen 2011; O'Connell et al. 2013), including sex-changing species. Ross et al. (1983) demonstrated that visual signals are crucial for sex change in saddleback wrasses, but tactile cues are not. A study in bridled gobies (*Coryphopterus glaucofraenum*) showed that chemical signals from females are an important stimulus of sex change. Isolated female gobies that received water from a tank holding a group of conspecific females were more likely to change sex than were isolated females who received water from tanks holding no fish, conspecific males, or females from a different species (Cole and Shapiro 1995). It is likely that chemical cues, in addition to visual cues, are important in a variety of sex-changing fishes.

Characteristics of gonadal sex change in protogynous fishes

Gonadal sex change in protogynous fishes involves radical morphological changes and the restructuring of tissues from a functional ovary to a functional testis. As a fascinating example of sexual lability and phenotypic plasticity, gonadal sex change has been intensely investigated in several protogynous species (reviewed by Godwin 2010; see also Muncaster et al. 2013; Nozu et al. 2013). Following removal of TP male bluehead wrasses, sex-changing females establish dominance, ovarian tissues regress, and testicular tissues can develop within 4–5 days (Figs. 1 and 2) (Warner and Swearer 1991). Sex changers may possess fully functional testes and be capable of fertilizing eggs by 8–10 days (Warner and Swearer 1991).

Six structural stages describe protogynous gonadal sex change in wrasses, based on histological characteristics of the gonad (see legend for Fig. 1) (Nakamura et al. 1989; Nozu et al. 2013). The process of gonadal sex change appears comparable across species based on histological characteristics. For example, in the honeycomb grouper (*Epinephelus merra*), early sex change is comparable histologically to Stage 3–4 in *Thalassoma* wrasses, while late sex change is similar to Stage 5–6 (Bhandari et al. 2003, 2005).

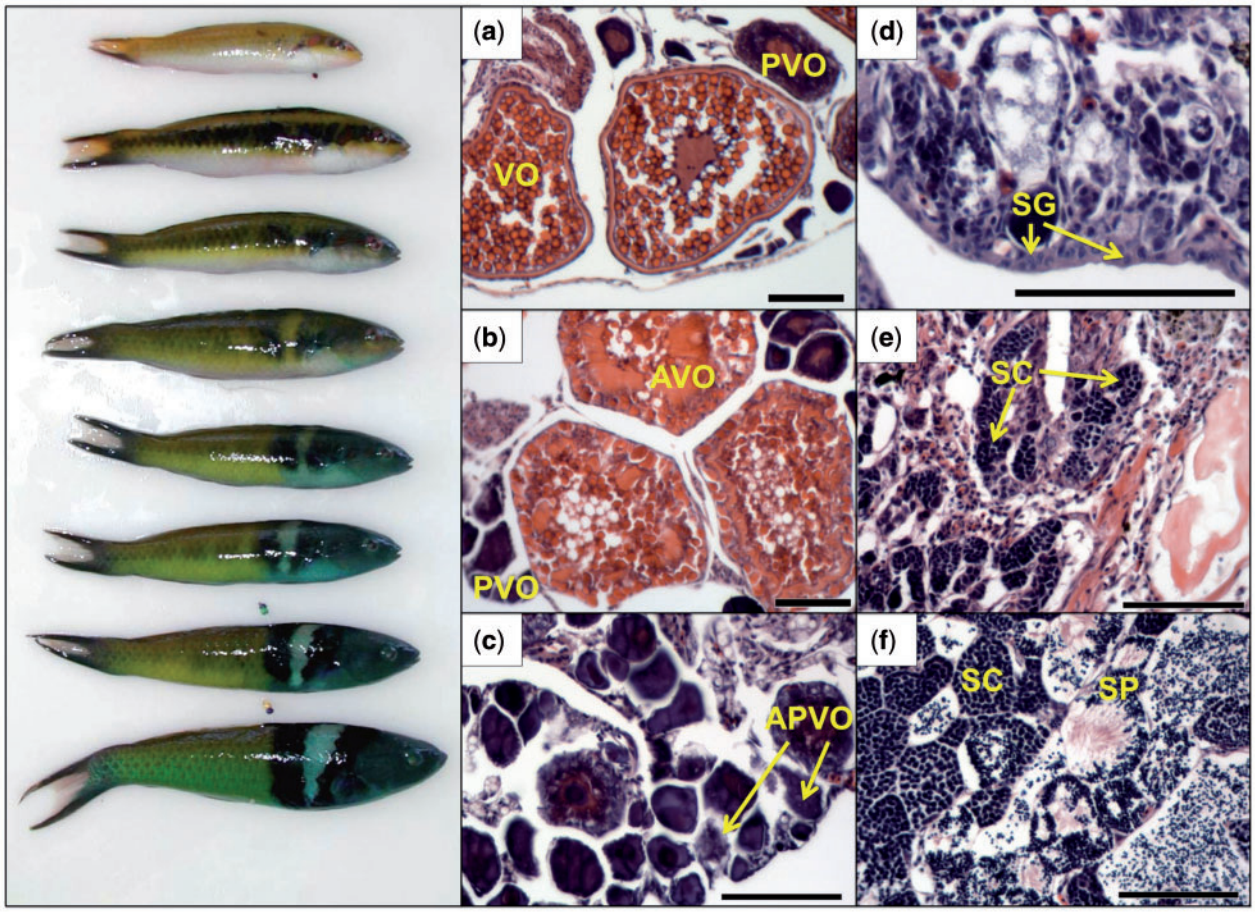

Fig. 1 Morphological and gonadal sex change in the bluehead wrasse. Left: morphological changes from female (top) to TP male (bottom) (images by J. Godwin). Right: six gonadal sex-change stages from ovary to functional testis (images by M. Lamm and J. Godwin). (**a**) Stage 1: mature ovary with healthy vitellogenic oocytes (VO) and pre-vitellogenic oocytes (PVO); (**b**) Stage 2: atretic vitellogenic oocytes (AVO) with degraded *zona pellucida*; (**c**) Stage 3: atretic previtellogenic oocytes (APVO); (**d**) Stage 4: proliferation of presumed spermatogonia (SG) and Leydig cells; (**e**) Stage 5: onset of spermatogenesis, indicated by spermatocytes (SC) in spermatocysts; (**f**) Stage 6: presence of mature, tailed sperm (SP). Based on classification by Nakamura et al. (1989). Scale bar: 50 μm. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

Hormonal alterations during sex change

Estrogens and androgens are known to regulate ovarian and testicular differentiation across vertebrates, and gonadal steroids also play central roles in gonadal sex change (reviewed by Devlin and Nagahama 2002; Guiguen et al. 2010; Piferrer 2011). In teleost fishes, 17β-Estradiol (E_2) and the non-aromatizable androgen 11-ketotestosterone (11KT) function as the major estrogen and androgen, respectively (Devlin and Nagahama 2002). Although present in both sexes, E_2 levels are generally higher in females than in males, while 11KT shows the opposite pattern. Testosterone (T) is also often found at high levels, even in females (Borg 1994; Piferrer 2011). However, this may be due to the role of T as a "prohormone" in the production of either E_2 through gonadal or brain aromatase (Cyp19a1a and Cyp19a1b, respectively) or 11KT by 11β-hydroxylase (Cyp11b) and 11β-hydroxysteroid dehydrogenase 2 (HSD11B2) (reviewed by Baroiller et al. 1999; Devlin and Nagahama 2002; Frisch 2005).

Changes in plasma concentrations of gonadal steroids accompany gonadal sex change. E_2 decreases and 11KT typically increases during protogynous sex change, while protandrous species show the opposite pattern (reviewed by Godwin 2010; Guiguen et al. 2010). Interestingly, gobies that change sex bidirectionally appear to show changes in E_2 and not in 11KT across sex change, for example, broadbarred gobies (*Gobiodon histrio*; Kroon et al. 2003) and bluebanded gobies (*L. dalli*; Lorenzi et al. 2008). This suggests a central role for E_2 in sex change, but the role of 11KT remains unclear in these gobies. These researchers suggest that low and

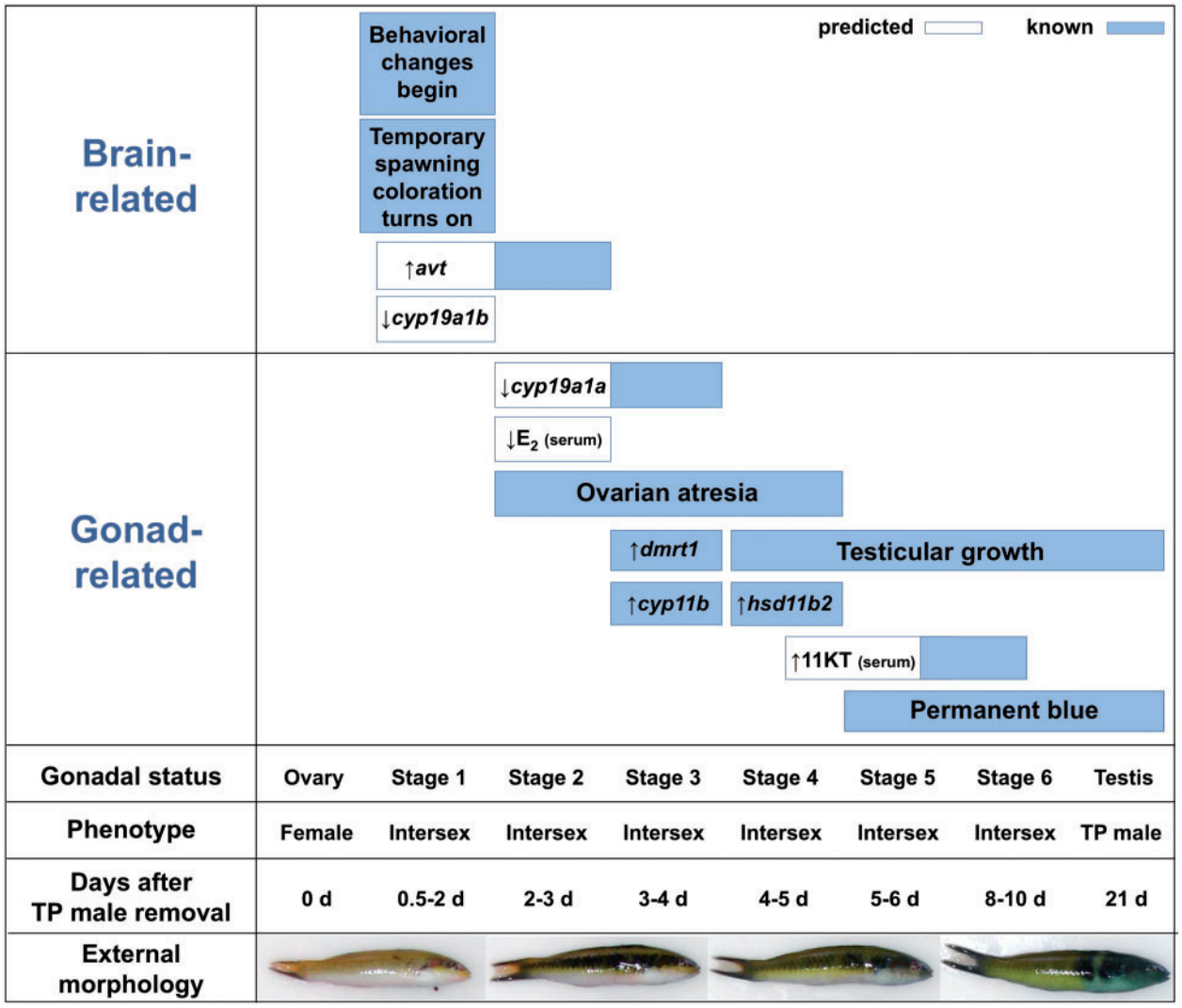

Fig. 2 Timeframe of physiological and behavioral changes during female-to-TP-male sex change in the bluehead wrasse. Shown are the earliest appearances of selected known (solid bars) and predicted (open bars) changes during sex change. Behavioral changes, consisting of aggression and sexual behaviors toward conspecifics during the spawning period, are observed as early as minutes to hours after removal of TP males (Warner and Swearer 1991). Temporary spawning coloration, composed of darkening of the pectoral fins and translucency of the head, occurs concurrently with sexual behaviors typical of TP males during the spawning period (Godwin et al. 1996). avt mRNA abundance in the magnocellular preoptic area (mPOA) of females increases within 2–3 days of removal of TP males (earliest time measured; Godwin et al. 2000) but is predicted to increase within hours into behavioral sex change because AVT signaling is necessary for behavioral changes (Semsar et al. 2001). Abundance of cyp19a1b mRNA in the POA is significantly lower in TP males than in females (Marsh-Hunkin et al. 2013) and likely decreases very early into sex change, as suggested by decreases in brain aromatase activity in female sex-changing bluebanded gobies (*Lythrypnus dalli*) within hours of males being removed (Black et al. 2005a). cyp19a1a gonadal mRNA decreases by Stage 3 (earliest stage sampled; Liu et al. unpublished) and is predicted to decrease by Stage 2 of sex change, concurrently with falls of E_2 in the serum. Serum E_2 has not been measured in bluehead wrasses but, based on patterns in the congener *Thalassoma duperrey* (Nakamura et al. 1989), is predicted to decrease by Stage 2 of sex change and remain low. Ovarian atresia is evident by Stage 2 (degeneration of vitellogenic oocytes) and continues through Stage 3 (degeneration of pre-vitellogenic oocytes) and often Stage 4 (degeneration of remaining cysts of oocytes; classification by Nakamura et al. 1989). dmrt1 gonadal mRNA increases by Stage 3 of sex change (earliest stage sampled; Liu et al. unpublished), just prior to the appearance of spermatogonia and Leydig cells in Stage 4. Testicular growth begins at Stage 4 of sex change and continues through Stage 5 (spermatogenesis inside spermatocysts) and Stage 6 (presence of tailed sperm). *cyp11b* gonadal mRNA begins to rise at Stage 3 and remains elevated compared to values for females (Liu et al. unpublished). *hsd11b2* gonadal mRNA rises at Stage 4 and remains elevated (Liu et al. unpublished). Serum 11KT is higher in TP males than in females (Grammer 1998) and rises within 6–7 days into sex change (earliest time measured; Godwin et al. unpublished). 11KT is predicted to increase around Stage 4 of sex change due to the presence of Leydig cells and elevated levels of *cyp11b* and *hsd11b2* mRNA. Permanent blue coloration is dependent on 11KT signaling (Godwin et al. 1996; Semsar and Godwin 2003) and first appears on the ventral surface of the body 5 days into sex change (Warner and Swearer 1991), corresponding to Stage 4–5. (This figure is available in black and white in print and in color at *Integrative and Comparative Biology* online.)

indistinguishable levels of 11KT in males and females may be due to a lack of dramatic differences in the secondary sexual characteristics of males (Lorenzi et al. 2008), and may also facilitate bidirectional sex change (Kroon et al. 2003). In addition to absolute levels of gonadal steroids, the ratio of 11KT to E_2, expression levels of androgen and estrogen receptors, and other factors are important considerations for the regulation of sex change.

Manipulative studies also support a role for E_2 and 11KT in sex change. Working with three-spot wrasses (*Halichoeres trimaculatus*), Higa et al. (2003) showed that treatment with either the aromatase inhibitor (AI) fadrozole or the 11KT induced gonadal sex change, but co-administration with E_2 blocked these effects. Results of *in vivo* or *in vitro* manipulations in other protogynous fishes have produced similar overall results (reviewed by Devlin and Nagahama 2002; Guiguen et al. 2010; see also Marsh-Hunkin et al. 2013). Taken together, patterns across a variety of sex-changing species are consistent with a critical role for estrogens in this process.

Gonadal E_2 levels are well-correlated with the expression of gonadal aromatase (*cyp19a1a*) mRNA in fishes (reviewed by Guiguen et al. 2010). What factors down-regulate *cyp19a1a* and therefore E_2 levels during sex change? In females, the pituitary gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) bind to their receptors in the gonad to regulate transcription of *cyp19a1a* (reviewed by Devlin and Nagahama 2002), but it is unknown whether/how LH/FSH signaling induces changes in the expression of *cyp19a1a* during sex change. In the gonochoristic Nile tilapia (*Oreochromis niloticus*) and Japanese flounder (*Paralichthys olivaceus*), *cyp19a1a* was shown to be regulated by the transcription factors forkhead box protein L2 (FoxL2) and doublesex and mab-3 related transcription factor 1 (DMRT1), which act antagonistically to up-regulate and down-regulate the expression of *cyp19a1a*, respectively, and regulate ovarian (FoxL2) or testicular (DMRT1) differentiation (Wang et al. 2007; Yamaguchi 2007; Wang et al. 2010). However, their roles in sex-changing fishes are poorly understood. Cortisol and certain DNA methyltransferases (DNMTs) have also been shown to down-regulate the expression of *cyp19a1a* (Yamaguchi et al. 2010; Navarro-Martín et al. 2011; Zhang et al. 2013). Both these factors may be important regulators of *cyp19a1a* during protogynous sex change.

In honeycomb grouper, the expression of *dmrt1* mRNA was higher and *foxl2* lower in late-stage sex changers and males, but not in early-stage sex changers (Alam et al. 2008), suggesting that these factors may not critically regulate the expression of *cyp19a1a* during sex change because E_2 levels (and therefore the expression of *cyp19a1a*) decrease in early sex change, for example, saddleback wrasses (Nakamura et al. 1989) and Mediterranean red porgies (*Pagrus pagrus*; Kokokiris et al. 2006). Our findings in the bluehead wrasse are largely consistent with these patterns over the course of sex change (Fig. 2) (Liu et al. unpublished). Surprisingly, Kobayashi et al. (2010b) found no difference in gonadal *foxl2* expression among three-spot wrasse females, IP males, and TP males. Treatment with an AI to induce sex change in females increased the levels of *foxl2* mRNA during sex change (Kobayashi et al. 2010b), possibly due to a feedback response to declining E_2 levels. Together, these findings suggest that *cyp19a1a* expression may be decoupled from FoxL2 regulation during protogynous sex change. While DMRT1 may not significantly down-regulate *cyp19a1a* in early protogynous sex change, DMRT1 is likely important for testicular development in mid-late sex change (reviewed by Herpin and Schartl 2011).

Rapid epigenetic methylation of *cyp19a1a* could also be responsible for early decreases in the expression of *cyp19a1a*. Temperature-induced DNA methylation of the *cyp19a1a* promoter in European sea bass (*Dicentrarchus labrax*) induced the formation of testes in fish reared at different temperatures and caused *cyp19a1a* to be less responsive to FoxL2 protein *in vitro* (Navarro-Martín et al. 2011). Similarly, working with the protogynous ricefield eel (*Monopterus albus*), Zhang et al. (2013) found greater methylation in the promoter region of *cyp19a1a* in testes and in the ovotestes of sex changers than in ovaries. Treatment with gonadotropins *in vitro* was only able to increase the expression of *cyp19a1a* in ovaries. In our studies employing RNA-sequencing, we found increased expression of some genes encoding DNMTs, including *dnmt3ab* and *dnmt4*, in the gonads of bluehead wrasses with advanced ovarian regression and the beginnings of testicular proliferation (Liu et al. unpublished). It is unknown whether these particular DNMTs interact with *cyp19a1a*.

Finally, cortisol can also inhibit transcription of *cyp19a1a* and cause masculinization, for example, in flounder species (Yamaguchi et al. 2010; Mankiewicz et al. 2013). Because E_2 positively up-regulates the expression of *cyp19a1a* (Guiguen et al. 2010), lower E_2 levels due to down-regulation of *cyp19a1a* in early sex change may help to maintain

low expression of *cyp19a1a* throughout the completion of sex change.

Rapid neuroendocrine mechanisms mediating protogynous sex change

The neuroendocrine mechanisms by which social cues are ''transduced'' into rapid behavioral and gonadal changes are intriguing, but poorly understood. However, significant progress has been made in recent years, and that progress is the focus of this section.

Behavioral sex change may promote gonadal transition because changes in behavior usually precede gonadal changes. However, because behavior and gonadal change can be decoupled in some cases (Godwin et al. 1996), and because gonadal sex change can also occur without changes in the behavioral phenotype, for example, changes from female to IP male in some wrasses and parrotfishes, it is therefore also possible that these processes are regulated through different pathways. Male-typical behaviors are influenced by fast-acting neurochemicals, such as vasotocin (Semsar et al. 2001; Semsar and Godwin 2003, 2004), while reproduction and likely gonadal sex change are regulated by gonadotropins and gonadal steroid hormones via the hypothalamo–pituitary–gonadal (HPG) axis. There is also potential crossover between the systems controlling behavioral and gonadal sex change. We discuss these possibilities in detail.

Fast-acting neuropeptides, monoamines, and neurosteroids

The neuropeptide arginine vasotocin (AVT; non-mammalian homolog of vasopressin) is a key mediator of male-typical behaviors in vertebrates generally (recently reviewed by Godwin and Thompson 2012; Kelly and Goodson 2013). For sex-changing species, actions and expression of AVT have been best studied in the bluehead wrasse, in which the expression of *avt* appears independent of gonadal influences and 11KT does not affect its expression (McIntyre 1998; Semsar and Godwin 2003). Abundance both of *avt* mRNA in the magnocellular preoptic area (mPOA) and the *v1a2* AVT receptor subtype in the hypothalamus was higher in TP males than in females and increased during socially-induced sex change (Godwin et al. 2000; Lema et al. 2012). In the presence of T-TP males (inhibitory environment), injection of AVT increased aggression and courtship in NT-TP males (Semsar et al. 2001), but not in females or IP males (Semsar and Godwin 2004). However, the AVT receptor antagonist Manning compound blocked behaviors typical of T-TP males in all phenotypes (Semsar et al. 2001; Semsar and Godwin 2004). These findings indicate that AVT signaling is necessary for behavioral sex change in females. However, alterations in AVT signaling alone are not sufficient, suggesting that other neurochemical changes are also critical for behavioral sex change.

The neuropeptides isotocin (IST; non-mammalian homolog to oxytocin) and neuropeptide Y may also mediate sex change, but information is limited. Injection of NPY in female bluehead wrasses induced clear gonadal sex change in some individuals in tanks (Kramer et al. 1997), presumably through stimulation of the HPG axis (reviewed by Van Der Kraak 2009). However, expression and activity of NPY during protogynous sex change remain uncharacterized. IST is also poorly studied with respect to sex change, although Black et al. (2004) found that in bluebanded gobies the number of IST-ir neurons was lower in the preoptic area (POA) of males and late-stage sex changers than of females. Given the importance of oxytocin in social interactions in mammals (reviewed by Donaldson and Young 2008), IST signaling might be a particularly interesting area of inquiry.

Monoamine neurotransmitters can change rapidly in different areas of the brain at the beginning of protogynous sex change. In the saddleback wrasse, activity of norepinephrine (NE) peaked in the POA 3 days into sex change and returned to baseline levels by Day 5, while activities of dopamine (DA) and serotonin (5-HT) showed the opposite pattern, decreasing by Day 3 and returning to baseline levels by Day 7 (Larson et al. 2003a). Consistent with a functionally important role in sex change, elevating the activity of NE with implants of the NE agonist ephedrine, or the NE reuptake inhibitor maprotiline, induced sex change in female saddleback wrasses under inhibitory conditions, that is, in isolation, after 4 and 8 weeks. In contrast, elevating the activities of DA and 5-HT with implants of the DA agonist apomorphine, or the selective serotonin reuptake inhibitor (SSRI) sertraline, reduced rates of protogynous sex change under permissive conditions, that is, a smaller female present (Larson et al. 2003a). In a complementary finding in bluehead wrasses, the SSRI fluoxetine decreased both the aggressive behavior and the abundance of *avt* mRNA in TP males (Perreault et al. 2003; Semsar et al. 2004). Blockade of behavioral or gonadal sex change in female bluehead wrasses was not tested. In contrast, protogynous sex change in bluebanded gobies was not affected either by augmenting or by antagonizing serotonergic signaling (Lorenzi et al. 2009).

Local production of steroid hormones in the brain is of increasing interest with respect to control of reproductive behavior in a variety of species. E_2 and 11KT can produce rapid changes in behavior in teleosts (on the order of minutes) (Remage-Healey and Bass 2007), although this is not well-explored in sex-changing fishes. Both *hsd11b2* and *cyp19a1b*, the aromatase gene that is predominantly expressed in the brain of teleosts, are expressed widely and abundantly in the brain of male and female adult teleosts (Marsh et al. 2006; Alderman and Vijayan 2012). In the bluehead wrasse, aromatase-ir glial cells were found in close association with AVT-ir and tyrosine hydroxylase-ir neurons in the POA (Marsh et al. 2006), suggesting potential interactions among E_2, AVT, and DA in controlling behavior. Abundance of *cyp19a1b* mRNA in the POA was lower in bluehead wrasse TP males than in females, and implants of E_2 in females both elevated the abundance of *cyp19a1b* mRNA in the preoptic area and blocked behavioral sex change under permissive social conditions (Marsh-Hunkin et al. 2013). Similarly, female bluebanded gobies showed decreases in whole-brain activity of aromatase within hours of the removal of males and the initiation of sex change (Black et al. 2005a). However, it was later shown that behavioral changes preceded changes in whole-brain activity of aromatase during sex change in this species (Black et al. 2011).

11KT is also produced in the brain by the activity of local HSD11B2. In zebrafish (*Danio rerio*), *hsd11b2* was abundantly expressed in every major region of the brain (Alderman and Vijayan 2012), often overlapping areas of known *cyp19a1b* expression. In protogynous fishes, it is unknown if, or how rapidly, the activity of HSD11B2 might change during sex change. It is known that changes in the activity of HSD11B2 can produce rapid behavioral changes in bluebanded gobies, in which injection of an HSD11B2 inhibitor into the brain eliminated parental behavior within 20 min, while co-injection with 11KT rescued these behaviors (Pradhan et al. 2014). It is possible that rapid increases in the activity and transcription of HSD11B2 occur very early during protogynous sex change, increasing local levels of 11KT in the brain and potentially promoting behaviors typical of dominant males. In female bluehead wrasses, exogenous 11KT could induce opportunistic displays of courtship behavior typical of T-TP males, but these implants were intraperitoneal (Semsar and Godwin 2004). Because elevations of 11KT levels (as evidenced by typical coloration for TP males) are not seen until mid-to-late sex change and behavioral sex change can occur in the absence of gonads, neural synthesis of 11KT that exposes the brain to elevated 11KT earlier in the sex-change process could still be important.

The HPG axis, kisspeptin, and GnIH

Social cues are transduced through a series of neurotransmitter and neuroendocrine systems, including the systems described in section 3.1, which relay these signals to the HPG axis to regulate gonadal steroid-hormone synthesis and sex change. The evolutionarily-conserved HPG axis controls reproduction in males and in females, but in different ways. In females, the hypothalamic-releasing form of gonadotropin-releasing hormone (GnRH), typically GnRH1, from the POA regulates release of FSH and LH from the anterior pituitary for follicular development and production of estrogens and other gonadal steroids, while GnRH also stimulates surges in LH that lead to ovulation. In males, GnRH activates both FSH and LH to regulate spermatogenesis and production of androgens and other steroids (reviewed by Devlin and Nagahama 2002; Zohar et al. 2010; Maruska and Fernald 2011).

Consistent sexual dimorphism of GnRH neurons in the POA has been found in both protogynous and protandrous species: males typically have larger and/or a greater number of GnRH neurons than do females (reviewed by Godwin 2010). In bluehead wrasses, 11KT implants increased the number of GnRH neurons in the POA of females and IP males while inducing changes in sex or role to the TP-male phenotype (Grober et al. 1991). It is unclear if the actions of 11KT on the number of GnRH neurons are caused by direct effects of 11KT, suppression of E_2 production, or both. While there are currently no data for any species on the changes in GnRH during sex change, the very rapid activation of GnRH1 neurons during males' role change in the gonochoristic cichlid fish *A. burtoni* shows that this system can respond rapidly to social cues (Burmeister et al. 2005).

Our knowledge of changes in gonadotropin hormones (GtHs; LH/FSH) in sex-changing species is mostly based on the detection of pituitary mRNA expression of GtH subunits: *lhb*, *fshb*, and glycoprotein hormone α subunit (*cga*), the latter of which is part both of the mature LH and FSH peptides. However, there is presently no consistent pattern in the expression of GtH-subunits during sex change across species (e.g., Ohta et al. 2008; An et al. 2009, 2010; Kobayashi et al. 2010a; Hu et al. 2011).

Genes encoding GtH receptors, *fshr* and *lhr*, showed parallel gonadal expression in the

bidirectional goby *Trimma okinawae* (Kobayashi et al. 2009). This species possesses an ovotestis, in which maturation of the ovarian or testicular portion of the gonad depends on social status. Expressions of *fshr* and *lhr* were high in the active part of the gonad and very low in the quiescent portion. As individuals underwent sex change (activation of either the ovarian or the testicular tissue), *fshr* and *lhr* expression increased in the newly active gonadal tissue within 12–24 h, and these newly-activated tissues became responsive to GtHs within 24 h. The mechanisms underlying the shift in GtHR expression remain unknown, and there are no comparable data for sex-changing species that do not possess ovotestes.

Manipulations of GnRH or GtH signaling can induce partial-to-complete sex change in a number of species. Bovine FSH (but not LH) triggered sex change in female honeycomb groupers (Kobayashi et al. 2010a). LH or human chorionic gonadotropin (analog of LH) exerted the same effect in female rainbow wrasses (*Coris julis*; Reinboth and Bruslé-Sicard 1997), bluehead wrasses (Koulish and Kramer 1989), and ricefield eels (Tang et al. 1974; Yeung et al. 1993). The observed effects of GnRH in induction of sex change have been less striking than those of GtHs, perhaps because GnRH is upstream to GtHs and a variety of neuropeptides and neurotransmitters can modulate the stimulatory effects of GnRH on GtHs. For example, GnRH analogs typically need to be applied with a dopamine receptor antagonist to induce sex change (Kramer et al. 1993; Ravaglia et al. 1997), with an exception being ricefield eels (Tao et al. 1993). While there is not a clear understanding of their role, there is nevertheless considerable evidence indicating that GtHs are important in regulating sex change.

Kisspeptins, a conserved group of RF-amide peptides encoded by the *kiss1* and/or *kiss2* genes in nearly all vertebrates, typically stimulate expression and release of GnRH in the POA of fishes (reviewed by Elizur 2009; Oakley et al. 2009; Oka 2009), though effects can depend on the gonadal state of individuals. For example, intramuscular injections of kisspeptins in gonochoristic female and male hybrid bass (*Morone saxatilis* × *M. chrysops*) induced release of LH at both pre-puberty and recrudescence, but reduced the expression of *gnrh1* mRNA during recrudescence while increasing the expression during pre-puberty (Zmora et al. 2012). In the protogynous orange-spotted grouper (*Epinephelus coioides*), mature females had higher expression of hypothalamic *kiss2* than did males (Shi et al. 2010). During 4 weeks of methyltestosterone treatment to induce sex change in females, *kiss2* and *gnrh1* mRNA levels decreased during the first 3 weeks, but increased two- and eight-fold, respectively, during week 4. Shi et al. (2010) suggest that lower *kiss2* and *gnrh1* expression during early sex change may be important for ovarian atresia, and levels may increase during testicular development. We found that implantation of kiss2 for 9–10 days in females with either active or regressed ovaries at sampling was not sufficient to override social inhibition and induce sex change in the presence of TP males (Lamm et al. unpublished). Effects of blocking the action of kisspeptin have not been tested in any sex-changing species. Interactions of AVT with the kisspeptin and GnRH1 systems could also be important, potentially as a connection between AVT-controlled behavioral changes and HPG-controlled gonadal changes. Studies in fishes have found connections between the AVT and GnRH1 systems, for example, the rockhind grouper (*Epinephelus adscensionis*; Kline 2010), and studies in mammals found connections between AVP and kisspeptin in mice (Vida et al. 2010) and Syrian hamsters (Williams et al. 2011). It is possible that interactions between AVT and kisspeptin also occur in fishes, including sex-changing species.

Another recently discovered RF-amide peptide, gonadotropin-inhibitory hormone (GnIH), also known as neuropeptide VF (NPVF) or LPXRF in fishes, also interacts with GnRH neurons and GtH gonadotropes to regulate the release of GtHs in vertebrates (reviewed by Ogawa and Parhar 2014). GnIH and its receptor have been found in a variety of fishes, but *in vivo* and *in vitro* studies in gonochoristic fishes have shown complex and even conflicting results of GnIH on the synthesis and release of GtHs (Zhang et al. 2010; Moussavi et al. 2012; Qi et al. 2013; Biran et al. 2014; reviewed by Ogawa and Parhar 2014). Ogawa and Parhar (2014) suggested that, like kisspeptins, the effects of GnIH signaling depend on the reproductive state of the fish and on the concentrations of circulating gonadal steroids at the time of treatment.

To our knowledge, the role of GnIH in sex-changing fishes has not yet been investigated. In birds, intracerebroventricular injection of GnIH inhibited male-typical sexual and aggressive behaviors in male quail (*Coturnix japonica*) and increased estrogen synthesis in the POA via aromatase activation within 30 min of injection, whereas GnIH RNA interference increased these behaviors 1 day after injection (Ubuka et al. 2014). GnIH RNA interference also induced sexual and aggressive behaviors 2 days after injection in male white crowned sparrows

(*Zonotrichia leucophrys gambelii*) (Ubuka et al. 2012). These studies suggest a potential for rapid modulation of behavior and gonadal function by GnIH in fishes.

Additional signaling systems and future directions

The focus of this symposium was on rapid neuroendocrine mechanisms mediating behavior and physiology. Therefore, we present information on two additional fast-acting neuroendocrine influences, the hypothalamo-pituitary-interrenal/adrenal axis and hypothalamo–pituitary–thyroid axis, which could play important roles in behavioral and gonadal sex change. We also propose promising future directions for elucidating the sex-change process.

The HPG axis has been the primary focus in studies of sex change. However, other systems, including the hypothalamo–pituitary–interrenal (HPI) axis, have also been proposed to play key roles in the regulation of sex change (Perry and Grober 2003; Gardner et al. 2005, Solomon-Lane et al. 2013). The HPI axis in fishes is homologous to the hypothalamo–pituitary–adrenal (HPA) axis of tetrapods, being composed of corticotropin-releasing hormone (CRH) neurons in the POA, adrenocorticotropic hormone (ACTH) cells in the anterior pituitary, and glucocorticoid-producing cells in the interrenal gland. As with tetrapods, levels of cortisol in the plasma of fishes rise rapidly on exposure of the fish to environmental stressors (reviewed by Pankhurst 2011), making cortisol a potentially reliable physiological signal for adaptive reproductive responses.

Until recently, it was thought that glucocorticoids might inhibit protogynous sex change (Perry and Grober 2003; Frisch et al. 2007b). However, a recent study of the bluebanded goby showed that cortisol is elevated during the first 3 days of protogynous sex change (Solomon-Lane et al. 2013). This suggests that an early rise in glucocorticoids could be important for initiating sex change in females. AVT has been shown to have a synergistic effect on CRH-stimulated release of ACTH, and hence on the release of cortisol, in some fishes, for example, rainbow trout (*Oncorhynchus mykiss*; Baker et al. 1996). It is possible that AVT signaling helps to maintain elevated cortisol levels during the first few days of sex change.

Frisch et al. (2007b) tested whether treatment with cortisol could inhibit protogynous sex change under permissive social conditions in the sandperch (*Parapercis cylindrical*) but found that sex change proceeded normally. To date, no study has tested whether cortisol can initiate protogynous sex change under inhibitory social conditions, or whether its presence is necessary. However, studies in gonochoristic fishes have shown that during development cortisol can induce the development of testes and secondary sexual characteristics in genetic females, for example, pejerrey (*Odontesthes bonariensis*; Hattori et al. 2009; Fernandino et al. 2012), medaka (*Oryzias latipes*; Hayashi et al. 2010), mosquitofish (*Gambusia affinis*; Knapp et al. 2011), Japanese flounder (*Paralichthys olivaceus*; Yamaguchi et al. 2010), and Southern flounder (*P. lethostigma*; Mankiewicz et al. 2013). The masculinizing actions of cortisol are likely not confined to gonochoristic species. If cortisol signaling does help to initiate sex change, it may do so by targeting different levels of the HPG axis.

Cortisol may contribute to gonadal sex change by decreasing ovarian E_2 production, thus reducing vitellogenesis and contributing to the ovarian collapse that characterizes the beginning of sex change (Carragher and Sumpter 1990; Berg et al. 2004; reviewed by Milla et al. 2009). Cortisol can decrease E_2 production by down-regulating transcription of *cyp19a1a*. In Japanese flounder, cortisol inhibited transcription of *cyp19a1a* and masculinized the gonad (Yamaguchi et al. 2010). Interestingly, the *cyp19a1a* gene in the bidirectionally sex-changing broad-barred goby possesses two putative glucocorticoid response elements (GREs) in the promoter region (Gardner et al. 2005), suggesting that *cyp19a1a* is subject to direct modulation by cortisol. Furthermore, based on findings in gonochoristic fishes, it is possible that, during protogynous sex change, cortisol helps to increase circulating levels of 11KT via up-regulation of expression of *hsd11b2* in the gonads (Fernandino et al. 2012) or via catabolism of cortisol in the liver into androgens within the 11KT synthesis pathway (Kime 1978; Schulz 1986). Finally, as demonstrated in gonochoristic species, cortisol could contribute to promotion of testicular tissue via induction of apoptosis of primordial germ cells and/or somatic cells (Strüssmann et al. 2008; Hattori et al. 2009; Fernandino et al. 2011; Yamamoto et al. 2013), as well as through suppression of the proliferation of primordial germ cells (Hayashi et al. 2010). In the protogynous three-spot wrasse, oocytes underwent apoptosis during aromatase-inhibitor-induced sex change, but somatic cells survived and proliferated during the transition from ovary to testis (Nozu et al. 2013). It is unknown whether cortisol played a role in these processes. At this point, the

involvement of cortisol in protogynous sex change is speculative and based primarily on studies in gonochoristic fishes, but is an area worthy of further investigation.

The hypothalamo–pituitary–thyroid (HPT) axis offers another promising avenue of research. As in other vertebrates, the enzyme deiodinase 2 (Dio2) converts the less active thyroxine (T4) to the active triiodothyronine (T3), while the Dio3 enzyme converts T3 to less active metabolites. There is evidence of sexual dimorphisms in this system in fishes, including sex-changing species. In the diandric protogynous striped parrotfish (*Scarus iseri*), females and IP males had similar circulating levels of T3 and T4, but IP males had higher gonadal expression of *dio2*, *dio3*, and thyroid hormone receptors (Johnson and Lema 2011), while females had greater expression of *dio2* in the brain. Females and TP males were not compared in this study. Higher thyroid activity in testes than in ovaries is consistent with a role of the thyroid axis in testicular development and cross-talk with androgens and the HPI/A axis (reviewed by Flood et al. 2013; Castañeda Cortés et al. 2014). However, higher levels of *dio2* mRNA in the brains of females is inconsistent with our RNA-sequencing findings in zebrafish and bluehead wrasses (Wong et al. 2014; Lee 2015; Liu et al. unpublished).

Thyroid hormones also target the HPG axis at the level of the brain. Intraperitoneal injection of T3 directly increased *gnrh1* and indirectly increased *kiss2* mRNAs in the brains of male Nile tilapia, while decreasing thyroid-hormone activity by treatment with methimazole lowered levels of *gnrh1* and *kiss2* (Ogawa et al. 2013). Whether thyroid hormones are necessary and/or sufficient for protogynous sex change has not been tested, but evidence suggests a potentially crucial role.

Although this has not been examined to our knowledge in fishes, *dio2* and *dio3* gene expression is sensitive to social stimulation and changes rapidly in songbirds. In European starlings (*Sturnus vulgaris*), naïve females exposed to males for 7 days during the breeding season had higher expression of *dio2* in the hypothalamus, lower *dio3*, and increased gonadal activity relative to females without exposure to males (Perfito et al. 2015), suggesting up-regulation of the thyroid and gonadal axes. This finding suggests that social modulation of thyroid signaling could be a productive line of investigation in sex-changing fishes.

Finally, examining sex change at global genetic levels within tissues could provide insight into the involvement of a variety of genes in the sex-change process. High-throughput technologies have been largely unexplored in sex-changing fishes, though exceptions include protandrous sharpsnout seabream (*Diplodus puntazzo*; Manousaki et al. 2014) and protandrous Barramundi (*Lates calcarifer*; Ravi et al. 2014). Genomics in non-model species is becoming increasingly more affordable and common. Whole-genome sequencing, epigenetic sequencing (including bisulfite sequencing), RNA sequencing, and chromatin/RNA immunoprecipitation sequencing (ChIP-seq/RIP-seq) are several technologies that can detect epigenetic, transcriptional, and/or translational changes over the course of sex change. Experiments incorporating these techniques should yield promising data regarding which genes may be involved in sex change, how rapidly these genes are up-regulated or down-regulated, and how gene networks co-vary. Because epigenetic (Bilang-Bleuel et al. 2005), transcriptional (Burmeister et al. 2005), and translational (Knight et al. 2012) events can occur rapidly, on the order of minutes to hours, following environmental stimuli, these high-throughput technologies would allow for detection of global changes that occur upon initiation of socially-induced behavioral and/or gonadal sex change.

High-throughput experiments also allow for genome/transcriptome-wide comparisons of genetic events during sex change across species, allowing assessment of those genetic patterns that have been evolutionarily conserved and therefore more likely to be critical mediators of sex change. We are currently using comparative genomic approaches on several distantly-related sequential hermaphrodites to identify the key genes involved in protogynous sex change in fishes. From this set of genes, we hope to be able to investigate the role of candidate genes in sex change with hypothesis-driven manipulative studies incorporating technologies such as vivo morpholinos (Moulton and Jiang 2009) and the CRISPR-Cas system (Hwang et al. 2013). This emerging combination of high-throughput genomic investigation and direct genetic manipulation for non-model species promises to yield powerful, novel results into a process that is as fascinating as it is mysterious. Such work should greatly enhance our knowledge both of the genes and of the coordinated pattern of gene expression involved in socially-controlled sex change.

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