

Regulation of protogynous sex change by competition between corticosteroids and androgens: An experimental test using sandperch, *Parapercis cylindrica*

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Abstract

Cortisol, the dominant corticosteroid in fish, and 11-ketotestosterone (11KT), the most potent androgen in fish, are both synthesized and (or) deactivated by the same two enzymes, 11 β -hydroxylase and 11 β -hydroxysteroid dehydrogenase. Cortisol is synthesized in response to stress (such as that caused by interaction with a dominant conspecific), whereas 11KT is synthesized during protogynous sex change. It has been hypothesized that corticosteroids (such as cortisol) inhibit 11KT synthesis via substrate competition, thereby providing a mechanism for the regulation of socially mediated, protogynous sex change. We tested this hypothesis by administering cortisol (50 $\mu\text{g g}^{-1}$ body weight) to female sandperch (*Parapercis cylindrica*) under social conditions that were permissive to sex change (i.e. in the absence of suppressive male dominance). Twenty-one days later, mean physiological cortisol concentration in cortisol-treated fish was 4.2-fold greater than that in 'socially stressed' female fish maintained in a semi-natural system. Although the dosage of cortisol was therefore considered to be favorable for engendering competitive inhibition of 11KT synthesis, all cortisol-treated fish changed sex, as did all sham-treated and control fish ($n=7$ fish per treatment). In addition, there was no effect of cortisol treatment on the rate of sex change or on the pattern of steroidogenesis. Thus, our results refute the hypothesis that protogynous sex change is regulated by substrate competition between corticosteroids and androgens.

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Introduction

Unlike almost all other vertebrates, a significant number of fishes mature first as female and later change sex to male (protogyny), or mature as male and later change sex to female (protandry) (Francis, 1992; Devlin and Nagahama, 2002). Often, initiation of sex change is coincident with a shift in the local social environment. For example, in group-forming, protogynous species, the death or removal of a male fish typically stimulates a female fish to undergo sex change, thereby restoring the operational sex ratio of the social unit (Robertson, 1972; Mackie, 2003).

The proximate cause of sex change appears to be a shift in the pattern of gonadal steroidogenesis (Frisch, 2004). In particular, sex change involves reciprocal changes in the relative concentrations of estradiol-17 β (E_2), which tends to promote 'femaleness' (e.g. oogenesis), and 11-ketotestosterone (11KT), which tends to promote 'maleness' (e.g. aggressive behavior) (Nakamura et al., 1989; Cardwell and Liley, 1991; Cochran and Grier, 1991; Bhandari et al., 2003). Both E_2 and 11KT are synthesized from the same substrate, testosterone (T), which is typically found in all sexual stages of teleosts (Kime, 1993; Borg, 1994). The conversion of T to E_2 is catalyzed by the enzyme aromatase, while the conversion of T to 11KT proceeds via the intermediate 11 β -hydroxytestosterone (11 β OHT) and is catalyzed by the enzymes 11 β -hydroxylase (11 β H) and 11 β -hydroxysteroid dehydrogenase (11 β HSD) (Frisch, 2004). Not surprisingly, the relative activities of aromatase, 11 β H and

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11 β HSD in the gonad profoundly influence sexual status (Morrey and Nagahama, 2000; Bhandari et al., 2004; Sunobe et al., 2005).

Typically, sex change is limited to the highest ranking (or largest) individual within a social group (Munday et al., 2006). Thereafter, the dominant individual suppresses the transition of other (subordinate) fish in the social group, at least until that dominant individual dies or is removed (Ross, 1990). This suppressive effect is mediated via aggression and monopolization of resources, both of which cause stress to subordinates (Lutnesky, 1989; Fox et al., 1997; Perry and Grober, 2003). As in many other vertebrates, stress in fish manifests as, among other things, an increase in the synthesis of cortisol, the dominant corticosteroid in fish (Sumpter, 1997; Frisch and Anderson, 2000; Barton, 2002). This response presumably serves to increase the circulation of stored energy reserves in anticipation of sustained muscle activity (Barton, 2002).

Although the relationship between social interaction and sexuality has been well documented, little is known about the mechanisms by which social interactions influence gonadal steroidogenesis during sex change. This gap has been provisionally filled by a model that links stress-induced corticosteroid metabolism with inhibition of androgen synthesis – a prerequisite for protogynous sex change (Perry and Grober, 2003). Specifically, synthesis and deactivation of corticosteroids, such as cortisol, are catalyzed by 11 β H and 11 β HSD (respectively) – the same two enzymes responsible for the two-step synthesis of 11KT from T. The commonality of these enzymes to both steroid pathways creates the potential for competition between steroid substrates (Perry and Grober, 2003). It is hypothesized that female fish (under suppressive, male dominance) are unable to change sex because increased corticosteroid levels competitively inhibit the synthesis of 11KT. Conversely, the removal of suppressive dominance from a female fish is hypothesized to result in the clearance of corticosteroids and, ultimately, sex change via the synthesis of 11KT (and other androgens).

The objective of the present study was to test the above-mentioned model. To do this, we first assessed ‘natural’ levels of cortisol in females of protogynous sandperch (*Paraperca cylindrica*) that were assumed to be under chronic stress from suppressive (male) dominance (Experiment 1). We then artificially elevated cortisol levels in high-ranking female fish and, at the same time, relieved them of suppressive dominance, thus creating social conditions permissive to sex change (Experiment 2). Predictions based on the model suggest that cortisol-treated fish would not synthesize 11KT, but would instead continue to synthesize E₂, thereby failing to undergo sex change.

Materials and methods

Experimental animals

P. cylindrica is a group-forming, protogynous hermaphrodite that inhabits shallow coral reefs of the western Pacific Ocean (Randall et al., 1990). Social units typically contain one male and two or more females, and males suppress sex change in females via dominance interactions. Social rank is strictly size-based, and maturation occurs at ~70 mm total length (TL) (Walker and

McCormick, 2004). Importantly, *P. cylindrica* is well suited to manipulative experiments in captivity: individuals acclimate rapidly to tank conditions; males and females have sexually dimorphic coloration; and sex change occurs rapidly (~21 days) (Walker and McCormick, 2004).

Experiment 1: cortisol levels in a semi-natural system

To simulate the natural habitat of *P. cylindrica*, a generous quantity of coral rubble and algae-encrusted rock was added to seven 100-l plastic tanks, each supplied with high-quality circulating seawater (~2 l min⁻¹), supplemental aeration, and ambient light. Thirty-seven adult *P. cylindrica* (7 males, size range: 107–115 mm TL; 30 females, size range: 70–94 mm TL) were collected from the Great Barrier Reef, Australia using a dip-net and anesthetic (10% clove oil in ethanol). 1 male and ≥2 females were added to each of the seven tanks, after which all fish were left undisturbed, except for regular feeding with shrimp and fish flakes. Just as in the wild, a size-based social hierarchy was established within each group of fish (Walker and McCormick, 2004). This enabled social rank to be assigned based on TL (rank 1=largest fish [male], rank 2=second largest fish [dominant female], rank 3=third largest fish [subordinate female], etc.). After 30 days, all fish were captured with a dip-net and subsequently euthanized with an overdose of anesthetic (~1 min in 30 ppt clove oil). Each fish was captured and anesthetized as quickly as possible, thereby minimizing handling stress (Strange and Schreck, 1978; Morgan and Iwama, 1997; Wagner et al., 2003). To confirm each fish’s sex, the gonad was removed by dissection and subsequently stored in 10% buffered formaldehyde (pH 7.4) until histological sectioning (see below). The remaining body was frozen (–80 °C) until steroid extraction (see below).

Experiment 2: exogenous cortisol administration

Sixty-three adult *P. cylindrica* (21 males, size range: 106–123 mm TL; 42 females, size range: 73–106 mm TL) were captured (as described above) and placed into 21 tanks, with one male and two females per tank. Tank conditions were the same as in Experiment 1, except that algae-encrusted bricks and pots were used to simulate the natural habitat, and minced fish and squid were used as food. Daily observations confirmed that a size-based social hierarchy was established among each group of fish (Walker and McCormick, 2004). After 2 days, a dip-net was used to permanently remove all male fish. At the same time, dominant female (rank 2) fish were individually captured, briefly anesthetized (~1 min in 20 ppm clove oil), and restrained in a wet foam cradle. Dominant female fish were then randomly assigned to one of three treatment groups: ‘cortisol’, ‘sham’ or ‘control’ (7 fish per treatment). Fish in the cortisol group received an implant containing cortisol, while fish in the sham group received an implant without cortisol. Control fish did not receive an implant, but were restrained in the cradle for an equivalent amount of time as fish in the other two groups.

Previous studies have demonstrated that silicone implants are a reliable technique for chronic administration of exogenous steroids (Pankhurst et al., 1986; Gamperl et al., 1994; Shelton and Mims, 2003). In particular, cortisol implants are known to maintain elevated cortisol concentrations in the plasma of experimental fish for at least 20–30 days (Pickering and Duston, 1983; Pickering and Pottinger, 1985). In studies where the objective was to simulate chronic stress, the appropriate dose of cortisol was found to be ~18–60 $\mu\text{g g}^{-1}$ body weight (Pickering and Duston, 1983; Pickering and Pottinger, 1985; Carragher et al., 1989). In the present study, we chose to use cortisol at doses of 50 $\mu\text{g g}^{-1}$ body weight, which is in the upper part of this range.

To prepare the cortisol implants, 9.9 mg of cortisol (No. H4001, Sigma, Castle Hill, Australia) was added to 0.6 ml of medical grade silicone (No. Q7-4850, Dow Corning, Midland, USA) in a petri dish. After thorough mixing with a spatula, the cortisol–silicone composite was loaded into a 0.5-ml syringe and refrigerated (4 °C) overnight. A small, semi-solid implant (size range: 25–32 mm³) was then inserted into the ventral, posterior region of the peritoneal cavity of relevant fish using a short, blunt, 19-gauge hypodermic needle. The size of each implant was adjusted to ensure that each fish received the correct dose of cortisol. Sham implants were prepared in the same way but without the cortisol. The insertion of each implant was completed in ~1 min, after which each fish was returned to its tank. Within 24 h, dominant female fish (all three treatment groups) resumed normal behavior (e.g. feeding), and subsequent survival of these

fish was 100%. In contrast, three subordinate female fish died or went missing before the end of the experiment. To remedy this situation, 'new' female fish of similar size (and thus, equivalent social rank) were immediately added to the relevant tanks.

After 21 days (i.e. the approximate duration of natural sex change), dominant female fish were captured with a dip-net and subsequently euthanized with an overdose of anesthetic (~1 min in 30 ppt clove oil). Body size (mm TL) was measured with a standard rule and then converted to percent growth by dividing the change in body size by the initial body size, which was measured at the beginning of the experiment. Next, each fish was dissected to recover the implant and (or) to remove the gonad, which was placed in 10% buffered formaldehyde (pH 7.4) until histological sectioning. The remaining body was frozen (–80 °C) until steroid extraction.

All procedures were conducted in accordance with the 'Australian code of practice for the care and use of animals for scientific purposes'. In addition, permission to conduct the experiment was granted by the James Cook University Animal Experimentation Ethics Committee (Approval no. A902).

Gonad histology and steroid assays

The medial region of each gonad was embedded in paraffin wax, sectioned to a thickness of 5 µm, and mounted on a glass slide for staining with Mayer's hematoxylin and eosin (Blazer, 2002). Slides were examined in random order (to avoid possible biases in interpretation associated with prior knowledge of treatment group) using a high-power microscope, and gonads were categorized according to the most advanced male germ cell present (as per Frisch et al., 2007). The proportion–spermatogenic–tissue index (I_{ST}) was calculated using the formula $I_{ST} = S(O+S)^{-1}$ where S was the number of spermatogenic cysts and O was the number of oocytes in three randomly placed transects (Liu and Sadovy, 2004). Each transect was 1 mm in length and contained 20 equally spaced graduations.

Due to the difficulty in obtaining sufficient blood from *P. cylindrica*, steroid concentrations were measured in whole fish. To do this, fish bodies were cut into small pieces (~0.2 cm³) immediately prior to thawing, and subsequently homogenized in ethyl acetate (3 ml g⁻¹ body weight) using a mortar and pestle. The homogenate was transferred to a 100-ml beaker and mixed vigorously for 1 min. The mixture was allowed to settle for ~10 s, after which 2 ml of extract was transferred to an Eppendorf tube for centrifugation (3000×g for 5 min). Aliquots of supernatant (200 µl each) were transferred to duplicate polycarbonate vials for evaporation to dryness (~14 h). Steroid concentrations were then measured by specific radioimmunoassay, as per Frisch and Anderson (2005) (for cortisol) or Frisch et al. (2007) (for T, 11KT and E₂). Details of assay antibodies were as follows: anti-cortisol, No. 20-CG50, Fitzgerald Industries International, Concord, USA; anti-E₂, No. E2885, Sigma; anti-T, No. T4276, Sigma. Doctor David Kime (University of Sheffield, UK) kindly donated the anti-11KT, which has been previously described and validated (Kime and Manning, 1982). For each steroid, assay specificity was verified by confirming parallelism in the binding curves of serially diluted extracts and steroid standards. Extraction efficiency (mean recovery of [³H]-labeled steroid that was added to triplicate whole fish before homogenization) was 65%, and assay values were adjusted accordingly. A single assay was performed for each steroid, and assay detection limits were 0.60 ng g⁻¹ for cortisol and 0.08 ng g⁻¹ for E₂, T and 11KT. Intra-assay variability (coefficient of variance) was 4.1% for cortisol, 1.6% for E₂, 3.2% for T, and 4.7% for 11KT.

Statistical analyses

Homoscedastic data were analyzed by one-way analysis of variance (ANOVA) followed by *post hoc* comparisons of group means using Tukey's HSD test (Zar, 1999). Heteroscedastic data were transformed ($\log_{10}[x+1]$) and then analyzed as above (Zar, 1999). A χ^2 homogeneity test was used to compare the distribution of male germ cell types among treatment groups (Zar, 1999). To overcome bias associated with small cell expectations, the critical value of χ^2 was 'scaled' using the procedure of Lawal and Upton (1984). All statistical analyses were performed using SPSS computer software (Chicago, USA) and a significant difference was considered to exist if $p < 0.05$. All data given in the text and figures are the arithmetic mean ± standard error of untransformed data.

Results

Experiment 1: cortisol levels in a semi-natural system

The gender structure of each social group was confirmed by histological analyses; the gonads of male fish contained germ cells in all three stages of spermatogenesis (spermatocytes, spermatids, spermatozoa), while the gonads of female fish contained oocytes in a range of developmental stages (e.g. pre-vitellogenic, vitellogenic and hydrated oocytes). Male germ cells were not observed in the gonads of female fish, regardless of social rank.

Mean cortisol concentrations in dominant female fish (3.4 ± 0.6 ng g⁻¹) and subordinate female fish (3.7 ± 0.5 ng g⁻¹) were significantly greater than in male fish (1.1 ± 0.3 ng g⁻¹) (1-way ANOVA, $F_{2,33} = 6.3$, $p = 0.005$). Differences between dominant and subordinate female fish were not statistically significant (Tukey's HSD test, $q_{33,2} = 0.07$, $p = 0.99$). These results suggest that female fish experience higher levels of stress than male fish.

Experiment 2: exogenous cortisol administration

After 21 days, the external coloration of (formerly) dominant female fish (all three treatment groups) had changed to an intermediate or male-like state, thus suggesting that sex change was either underway or complete. This was confirmed by histological analysis, which revealed that gonads (all three treatment groups) contained clusters of spermatocytes, spermatids, or spermatozoa and degenerating oocytes. The distribution of male germ cell types among treatment groups was not significantly different (χ^2 homogeneity test, $\chi^2_4 = 4.0$, $p > 0.05$; Fig. 1). Furthermore, mean I_{ST} levels among treatment groups were not significantly different (1-way ANOVA, $F_{2,18} = 0.24$, $p = 0.79$; Fig. 2a). Together, these results indicate that cortisol administration did not influence the rate of sex change.

Administration of cortisol implants successfully elevated physiological cortisol levels; mean cortisol concentration in

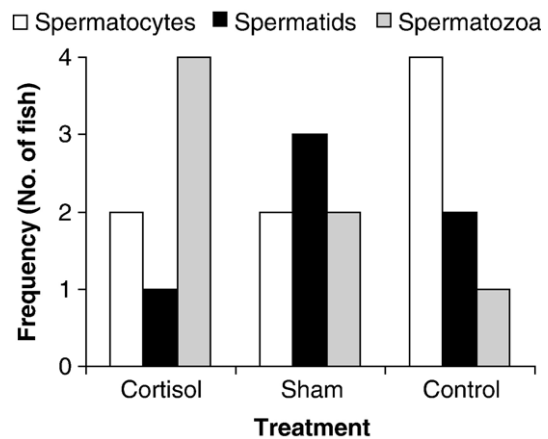


Fig. 1. The most advanced male germ cell type in the gonads of (initially female) *Paraperis cylindrica* that received a cortisol implant, a sham implant (no cortisol), or no implant (control) in Experiment 2 (seven fish per treatment). Sampling occurred 21 days after treatment. Differences between groups were not statistically significant.

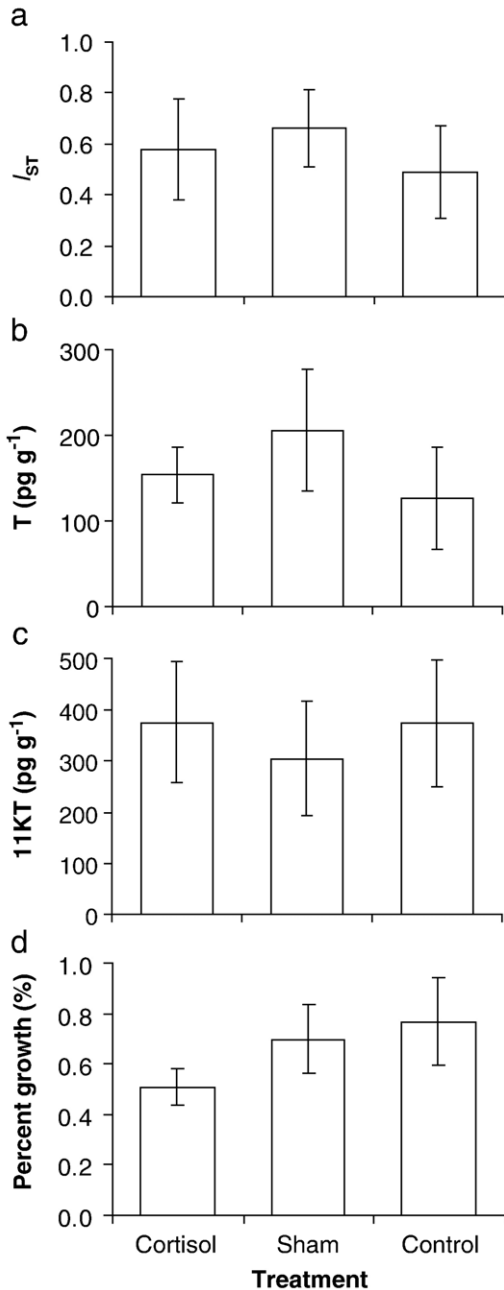


Fig. 2. (a) Mean proportion–spermatogenic–tissue index (I_{ST}), (b) mean testosterone (T) concentration, (c) mean 11-ketotestosterone (11KT) concentration, and (d) mean percent growth in (initially female) *Parapercis cylindrica* that received a cortisol implant, a sham implant (no cortisol), or no implant (control) in Experiment 2 (seven fish per treatment). All fish were sampled 21 days after treatment. Differences between groups were not statistically significant.

cortisol-treated fish ($14.4 \pm 4.1 \text{ ng g}^{-1}$) was significantly greater than that in both sham-treated fish ($3.5 \pm 0.8 \text{ ng g}^{-1}$) and control fish ($4.2 \pm 0.6 \text{ ng g}^{-1}$) (1-way ANOVA, $F_{2,18}=6.4$, $p=0.008$). Importantly, mean cortisol concentration in cortisol-treated fish was 4.2-fold greater than that in dominant female (rank 2) fish maintained in the semi-natural system (see above). Thus, cortisol levels in cortisol-treated fish were considered to be favorable for engendering competitive inhibition of 11KT synthesis, should substrate competition occur in this species.

For all treatment groups in Experiment 2, E_2 was either absent or undetectable (i.e. $<0.08 \text{ ng g}^{-1}$). In contrast, T was detected in cortisol-treated fish ($153.7 \pm 32.5 \text{ ng g}^{-1}$), sham-treated fish ($206.4 \pm 71.4 \text{ ng g}^{-1}$) and control fish ($126.1 \pm 59.9 \text{ ng g}^{-1}$), but differences between groups were not statistically significant (1-way ANOVA, $F_{2,18}=0.5$, $p=0.6$; Fig. 2b). Similarly, 11KT was detected in cortisol-treated fish ($304.7 \pm 111.8 \text{ ng g}^{-1}$), sham-treated fish ($375.9 \pm 117.2 \text{ ng g}^{-1}$) and control fish ($373.7 \pm 122.3 \text{ ng g}^{-1}$), but differences between groups were not statistically significant (1-way ANOVA, $F_{2,18}=0.1$, $p=0.9$; Fig. 2c).

Mean percent growth in cortisol-treated fish (0.51 ± 0.07) was generally less than that in both sham-treated fish (0.70 ± 0.13) and control fish (0.77 ± 0.18) (Fig. 2d). However, differences between groups were not statistically significant (1-way ANOVA, $F_{2,18}=1.0$, $p=0.39$).

Discussion

Our study is the first to test whether elevated cortisol levels inhibit protogynous sex change. Cortisol implants successfully increased physiological cortisol concentrations to levels well above those found in female fish exposed to suppressive (male) dominance. Although the dosage of cortisol was therefore considered to be favorable for engendering competitive inhibition of 11KT synthesis, all cortisol-treated fish exhibited signs of sex change, as did all sham-treated and control fish. In addition, there was no effect of cortisol treatment on the rate of sex change (Figs. 1 and 2a) or on the pattern of steroidogenesis (Figs. 2b, c). Thus, our results refute the hypothesis that increased circulating levels of cortisol inhibit protogynous sex change, at least in *P. cylindrica*.

Although Experiment 2 was relatively short in duration, we were able to observe evidence of sex change in at least three different ways. Firstly, experimental animals (all treatment groups) underwent changes in external coloration, a reliable indicator of sex in *P. cylindrica* (Authors' unpublished data). Secondly, gonads were found to contain proliferating testicular tissue in the presence of degenerating ovarian tissue (*sensu* Sadovy and Shapiro, 1987). Finally, the pattern of steroidogenesis was typical of that found in males of most teleosts (Kime, 1993; Borg, 1994; Frisch, 2004): 11KT was present (i.e. $>0.08 \text{ ng g}^{-1}$) whereas E_2 was low or absent (i.e. $<0.08 \text{ ng g}^{-1}$). Together, these results confirm that sex change was either underway or complete in all experimental animals.

The fact that cortisol treatment had no detectable effect on the pattern of steroidogenesis suggests that competition between cortisol and 11KT precursors was weak or absent. Interestingly, physiological cortisol concentrations in cortisol-treated fish were at least 20–30 times greater than those of T, a major precursor of 11KT (Frisch, 2004). How then is substrate competition limited or prevented during sex change? One possibility is that 11 β H and 11 β HSD have higher affinities for androgens than for corticosteroids, just as blood hemoglobin has a higher affinity for carbon monoxide than for oxygen (Randall et al., 2002). Alternatively, sites of androgen synthesis (i.e. endoplasmic reticulum of Leydig cells) may be spatially segregated from circulating corticosteroids.

The observation that female fish had higher cortisol concentrations than male fish conforms to conventional theory that males exert dominance over females in group-forming, protogynous species (Lutnesky, 1989; Ross, 1990; Perry and Grober, 2003). However, different cortisol titers among males and females do not necessarily indicate a causal role for this steroid in the regulation of sex change. Instead, such differences may reflect merely the behavioral or metabolic characteristics of the two groups. Determining whether or not cortisol has a role in the regulation of sex change therefore requires an evaluation of the effects of cortisol on the likelihood of sex change, and (or) its effects on the rate of sex change.

In *P. cylindrica*, both the occurrence and rate of sex change were apparently independent of the physiological concentration of cortisol. This suggests not only that competition between corticosteroids and androgens is unlikely to regulate sex change, but also that cortisol is unlikely to be involved in the regulation of sex change at all. Further support for the notion that sex change is independent of cortisol comes from studies of the protandrous anemonefish, *Amphiprion melanopus*. In particular, plasma cortisol concentrations in this species were found to be unchanged during the early stages of gonadal transition (i.e. until well after the onset of oogenesis) (Godwin and Thomas, 1993). Although plasma cortisol concentrations in the same fish were elevated after 20 days (i.e. when individuals became essentially female), this was probably due to heightened aggressive interactions among conspecifics (Godwin, 1994).

Whilst cortisol does not appear to be involved in the regulation of sex change in either *P. cylindrica* or *A. melanopus*, it is well established that, in other fishes, cortisol is capable of influencing both the levels of circulating reproductive hormones and the quality and quantity of gametes (Carragher et al., 1989; Pankhurst and Van Der Kraak, 1997; McCormick, 1998; Schreck et al., 2001). This influence is thought to be mediated via the hypothalamic–pituitary–gonadal (HPG) axis, since several of its components are known to contain glucocorticoid response elements (Mommensen et al., 1999; Teitsma et al., 1999; Gardner et al., 2005). Thus, it seems that cortisol is involved in the regulation of reproduction rather than the regulation of sex change *per se*.

Two alternative (non-cortisol) candidates for the regulation of sex change are arginine vasotocin (AVT) and gonadotropin releasing hormone (GnRH), both of which are synthesized in the brain – the apex of the HPG axis (Foran and Bass, 1999). Importantly, levels of both AVT and GnRH are known to be influenced by sociosexual stimuli (Bass and Grober, 2001), and both GnRH and AVT act as modulators of sociosexual behavior and (or) reproductive physiology (Foran and Bass, 1999; Semsar et al., 2001; Frisch, 2004). It is also noteworthy that a synthetic form of GnRH induces sex change in at least two protogynous species (Kramer et al., 1993; Tao et al., 1993). Future research on the role of these two hormones in the process of sex change is therefore greatly anticipated.

It is interesting that cortisol-treated fish tended to grow slower than sham-treated or control fish (Fig. 2d). Group differences, however, were small (and not statistically significant), presumably because of the experiment's short duration

(21 days). Reduced growth is a common response to cortisol treatment in a wide variety of fish species (Barton and Iwama, 1991; Pickering, 1993). However, since reduced growth is a tertiary response to stress (Barton, 2002), it is typical for cortisol-induced reductions in growth to take several months to manifest (Davis et al., 1985; Barton et al., 1987).

In summary, this study refutes the hypothesis that socially induced sex change is regulated via competition between corticosteroids and androgens, at least in *P. cylindrica*. Although males and females had different physiological cortisol concentrations, the process of sex change was apparently independent of circulating cortisol levels. Since this result contrasts with previous studies of the effects of cortisol on reproductive physiology (e.g. Carragher et al., 1989), it is hypothesised that the processes of reproduction and sex change are regulated by different hormonal pathways.

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