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Stress inhibition of reproductive endocrine processes in a natural population of the spiny damselfish *Acanthochromis polyacanthus*

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**Abstract.** Stress-reproduction interactions were assessed by *in situ* sampling and laboratory manipulation of fish from a natural population of the tropical spiny damselfish *Acanthochromis polyacanthus*. Mean plasma cortisol concentrations for fish sampled immediately after capture underwater were < 11 ng mL\(^{-1}\) but individual values varied from <1 to 42 ng mL\(^{-1}\). This variation was not a function of disturbance prior to capture, with no differences in mean cortisol concentrations between 5 min time blocks for chase times of up to 30 min. Fish confined for 2 or 6 h after capture did show significant increases in plasma cortisol concentrations. Confinement resulted in depression of plasma T and E\(_2\) in females and T and 11KT in males in summer but not winter. There was, however, no correlation between plasma concentrations of cortisol and sex steroids at capture. Underwater treatment of fish with cortisol had no effect on brood-guarding or nesting behaviour. Injection of fish either at, or 24 h after capture with human chorionic gonadotropin or luteinizing hormone-releasing hormone analogue had no effect on plasma concentrations of gonadal steroids, which remained at low levels throughout. *In vitro* incubation of ovarian follicles from these fish showed that gonadal tissue was still steroidogenically active. This study confirms that stress inhibits reproduction in spiny damelfish, probably above the level of steroid biosynthesis.

*Extra keywords:* cortisol, oestradiol, testosterone, steroidogenesis, spiny damselfish.
Introduction

Stress inhibits reproductive function in all teleost species studied to date, with effects being detectable at all levels of the reproductive endocrine cascade (reviewed in Pankhurst and Van Der Kraak 1997; Pottinger 1999). The majority of studies have investigated this process by applying artificial stressors to captive or domesticated populations (eg Pickering et al. 1987; Campbell et al. 1994), or freshly captured wild fish (eg Carragher and Pankhurst 1991; Clearwater and Pankhurst 1997; Haddy and Pankhurst 1999), and measuring the associated changes in plasma concentrations of cortisol, gondal steroids and various indices of reproductive performance. Other studies have confirmed that the stress effect is likely to be mediated by cortisol (Carragher et al. 1989; Pottinger et al. 1991; Foo and Lam 1993; Pankhurst and Van Der Kraak 2000) although the mechanism by which cortisol acts is still unclear.

There is less information on the association between stress status or plasma cortisol concentrations and reproductive function in wild populations at rest. This partly results from the fact that capture, handling and sampling inevitably result in stress and consequent elevations in cortisol with possible alterations of reproductive endocrine status. The minimum latency for effects of stress on reproduction is unknown; however, effects on plasma concentrations of gonadal steroids occurred between 15 and 30 min after the imposition of stress in black bream *Acanthopagrus butcheri* (Haddy and Pankhurst 1999). This suggests that the assessment of correlations between plasma cortisol and gonadal steroid concentrations in normally active wild fish requires sampling strategies where there is very little delay between capture from the wild and sampling. There are several strategies for achieving this including line fishing (Pankhurst et al. 1992) and underwater capture and sampling (Pankhurst 1990; Pankhurst and Sharples 1992). Other techniques such as electrofishing can result in rapid capture but also appear to substantially alter commonly measured stress parameters (Barton and Grosh 1996; Barton and Dwyer 1997). Underwater sampling has been particularly useful for correlation of endocrine condition with immediate precapture behaviour (Pankhurst 1990; Pankhurst and Barnett 1993), and probably offers the best approach for assessing variation in cortisol and gonadal steroid concentrations in wild fish.
Underwater capture and sampling has been used previously to describe the association between reproductive condition, steroid hormone concentrations and behaviour in the coral reef-dwelling spiny damselfish *Acanthochromis polyacanthus* (Pankhurst *et al*. 1999). In that study, fish were assumed to have the endocrine condition of unstressed fish at capture. In support of this, mean plasma cortisol concentrations were always <14 ng mL$^{-1}$, and typically < 5-6 ng mL$^{-1}$, which is consistent with values described for unstressed fish from studies on a range of species (reviewed in Grutter and Pankhurst 2000). However, there were significant variations in plasma cortisol concentrations among females, with fish that were paired but without young having lower cortisol levels than unpaired fish, or fish protecting broods of young (Pankhurst *et al*. 1999). In addition, individual cortisol concentrations were quite variable, ranging from <0.6 ng mL$^{-1}$ to >30 ng mL$^{-1}$. These variations raised the question of whether the capture process was resulting in elevated cortisol concentrations despite the *in situ* sampling process, or whether the variability was an inherent characteristic of the population. In either case, it is possible that variable cortisol concentrations could generate effects such that plasma concentrations of gonadal steroids were not strictly correlates of gonadal condition or reproductive behaviour.

In the present study, the possibility that variability in time to underwater capture might generate differential cortisol concentrations in spiny damselfish was investigated by assessing plasma cortisol concentrations in relation to time since first disturbance by divers, in two consecutive years. The response to confinement was assessed by repeat sampling of fish captured underwater after a period of confinement in the laboratory. Confinement effects were assessed in terms of plasma cortisol and also concentrations of gonadal steroids (testosterone [T] and 17β-oestradiol [E$_2$] in females, and T and 11-ketotestosterone [11KT] in males) in winter when fish are generally sexually regressed, and also in early summer during the peak period of spawning activity (Pankhurst *et al*. 1999). Because there is a behavioural component to plasma steroid concentrations (Pankhurst *et al*. 1999), the effect of exogenous cortisol on behaviour was assessed by injecting fish underwater and releasing them back onto nest sites. Effects of injections on plasma cortisol concentrations were assessed using parallel experiments conducted in the laboratory. The effect of stress on the response to exogenous hormones was examined by
injecting fish either at capture or after acclimation to the laboratory with human chorionic
gonadotropin (hCG) or an analogue of mammalian luteinizing hormone releasing
hormone (LHRHA), and measuring subsequent changes in plasma concentrations of
gonadal steroids. Both hCG and LHRHA are routinely used to stimulate steroidogenesis
and gonadal maturation in other species (reviewed in Pankhurst 1998a). Finally, the
steroidogenic capacity of stressed fish was further assessed by in vitro incubation of
ovarian follicles with hCG or steroid hormone precursors using treatments previously
demonstrated to stimulate T and E2 production by spiny damselfish ovarian follicles
(Pankhurst et al. 2000).

**Materials and methods**

Spiny damselfish were captured by SCUBA divers using fence nets, from shallow
coral reefs (depth range 3-15 m) around Lizard Island (14° 40'S, 145° 27'E) in the
northern section of Australia’s Great Barrier Reef, during July and October-November in
correspond to austral winter and austral spring/summer, respectively. Immediately after
capture, a blood sample was taken underwater by caudal puncture as described in
Pankhurst (1990). Fish were then either killed by pithing the brain with a pair of scissors
and placed in labelled plastic bags for later examination, or held alive underwater in
plastic containers with a mesh covering on one end to allow water circulation. At the end
of the dive dead fish and blood samples were placed on ice, and live fish transferred to 40
L bins filled with sea water on the surface vessel for boat transport back to the Australian
Museum’s Lizard Island Research Station, for further experiment or processing as below.

Sampling was conducted over 36 dives in 1996, 31 dives in 1997 and 16 dives in 1998.
Water temperatures were 24-25°C, and 26-27°C in July and October-November 1996,

*Time taken to capture fish*

Measurements of time taken to capture fish were made in 1996 and July 1997 to
assess whether cortisol concentrations measured from fish underwater were partially a
product of response to disturbance by divers. In 1996 the time taken to capture fish was
measured as the time between successive samples. This slightly overestimated stalking
time as it included swimming time between netting sites (distances ranging from a few
metres to 50 m). In 1997, this protocol was refined by recording the time of first
approach when a diver first moved in to place the fence net near a targetted fish. Fish
were variably easy to catch depending largely on the complexity of the local reef
structures. This variation in capture time generated time-to-capture values ranging from
1-48 min.

*Effect of confinement on plasma steroid levels*

In July 1997, fish were blood-sampled underwater as described above then returned to
the laboratory and held in 400 L concrete tanks supplied with flow-through aerated
seawater, then sampled again after 2 or 6 h of confinement (time from underwater
capture), then killed for gonadal examination. The experiment was repeated in December
1997 when a greater proportion of fish was expected to be sexually mature, with fish
confined for 3 or 6 h respectively and sampled as before. All fish for this and other parts
of the study were assigned macroscopic gonad stages according to the maturity scale
described in Pankhurst *et al.* (1999).

*Effect of exogenous cortisol on behaviour*

In December 1997, single adults from nesting pairs were captured by fence netting as
before then injected intraperitoneally with 125 μL of either cortisol (Sigma, St Louis,
MO) in a 1:9 ethanol:suspension at 2 mg mL⁻¹, or the ethanol-saline mix as a control
(nine fish per group). Fish were assumed on the basis of previous measurements of wild
fish, to weigh 50 g to give an estimated dose of 5 mg kg⁻¹ body weight. After injection,
fish were fin-clipped for identification then released back onto the nest site, which was
marked with a small numbered float attached by cord to a lead weight. Where
logistically possible the behaviour of injected fish was assessed approximately 2 h after
injection, and in all cases 24 h after injection, by diver observation for 5 min. At the end
of the last observation period, an attempt was made to recapture all injected fish for sex
determination and blood sampling.
The effect of cortisol injection on plasma cortisol concentrations was assessed independently by capturing and transferring 14 fish to a 400 L tank on shore, and after leaving fish for 24 h to recover from capture stress, injecting them with the same volumes of solutions used for underwater injection. Fish were fin-clipped for identification then placed in the holding tank for subsequent blood-sampling at 1 and 3 h post-injection. After the second blood sample, fish were killed for gonadal examination.

Effect of capture stress on the response to exogenous hormones

In December 1998, fish were captured underwater, transferred to the field station and held overnight in a 400 L tank as before. The following day, fish were injected with either saline, hCG at 625 U kg\(^{-1}\) body weight, or Des-Gly\(^{10}\) (D-Ala\(^{6}\)) luteinizing hormone releasing hormone ethylamide (LHRHA) at 125 \(\mu\)g kg\(^{-1}\) (hormones purchased from Sigma, St Louis, MO). Fish were injected with 50 \(\mu\)L of 500 U mL\(^{-1}\) hCG or 50 \(\mu\)g mL\(^{-1}\) LHRHA to give the appropriate dose based on an estimated fish weight of 40 g. Doses were best-estimates based on the effective dose of hCG and LHRHA for stimulating gonadal steroid production in other species, (Pankhurst and Carragher 1992; Donaldson and Devlin 1996). Fish were blood-sampled at 3 and 6 h after injection then killed for gonadal examination. Distribution of males and females among treatments was 7, 5, 7 and 10, 9, 9 for saline, hCG and LHRHA respectively (fish were allocated to treatment groups on a rotational basis and their sex was not determined before injection).

The experiment was subsequently repeated using the same injection protocol but with injection of fish underwater at the time of capture and sampling at 6 h only (samples at 3 h were not logistically possible in fish injected before transport back to the field station). Distribution of males and females among treatments in this experiment was 5, 8, 5 and 11, 7, 8 for saline, hCG and LHRHA, respectively.

Ovarian follicle incubations

Ovarian follicles from the ovaries of two vitellogenic females from the exogenous hormone experiment (both injected with LHRHA 24 h after capture, fish chosen randomly on the basis of suitable sized follicles for incubation) were manually dispersed with 22 G syringe needles and placed at 10 per well in Leibowitz L15 medium (Sigma, St
Louis, MO) volume-adjusted for use with marine fish to 405 mOs kg$^{-1}$ (Pankhurst et al., 1995). Follicles from the first fish were incubated in either L15 alone, or L15 containing hCG at 100 U mL$^{-1}$ added as 100 μL of 1000 U mL$^{-1}$ in L15, 17α-hydroxyprogesterone (17P) or testosterone (T) at 100 ng mL$^{-1}$ added as 10 μL of a 10 μg mL$^{-1}$ solution in ethanol. Control and hCG wells received 10 μL of ethanol. In the second fish where fewer follicles were available for harvest from the ovary, follicles were incubated with either L15 or T as before. Follicles were incubated for 5 h at 27$^\circ$C at the end of which time media were aspirated into plastic test tubes and extracted as described below.

**Steroid hormone measurement**

Plasma steroids were extracted from 100 μL of plasma with 1 mL ethyl acetate, and in vitro media (1 mL) with 3 mL ethyl acetate, and the solvent fraction aspirated to fresh plastic vials or tubes and dried down for shipment to the University of Tasmania for analysis. In the laboratory, extracts were resuspended in assay buffer and assayed for E$_2$, T and cortisol in females, and T, 11KT and cortisol in males as described in Pankhurst et al. (1999). Detection limits were 0.3 ng mL$^{-1}$ plasma for gonadal steroids and 0.6 ng mL$^{-1}$ for cortisol. Interassay variability measured using pooled internal standards was (%CV[n]) 19[7], 16[13], 18[8] and 18[8] for E$_2$, T, 11KT and cortisol, respectively.

**Statistics**

Comparison of means was made by using one-way ANOVA following by Tukey’s b tests. Where necessary data were log ($x + 1$) transformed to satisfy homogeneity of variance requirements. Correlations between hormone concentrations were assessed using least squares regression. All tests were conducted using the SPSS for Windows computer package.

**Results**

Mean cortisol concentrations for all fish captured in 1996 were less then 10 ng mL$^{-1}$ (Fig.1). There were no differences between males and females (P>0.05) so the data were combined for analysis by capture time. There was no significant difference in cortisol
concentrations in relation to times taken to capture fish at times from <5 min up to in excess of 30 min. Data within same time periods were, however, quite variable with individual values ranging from as low as 0.6 ng mL\(^{-1}\) (assay detection limit) to a maximum of 42 ng mL\(^{-1}\).

In 1997, where stalking time was measured more precisely, the outcomes were very similar. Mean cortisol concentrations were all less than 11 ng mL\(^{-1}\) and there was no significant difference among stalking times from <5 to >25 min (Fig. 2). In contrast, a significant increase in plasma cortisol concentrations occurred after capture and 2 h of confinement, with a further increase in fish confined for 6 h (Fig. 2). Individual plasma cortisol values in freshly captured fish in 1997 ranged from 0.6 to 38 ng mL\(^{-1}\). The variation was not related to whether fish were territory holders or not (data not shown).

Confinement of fish captured in July 1997 for 2 or 6 h resulted in significant increases in plasma cortisol concentrations in both sexes (Fig. 3). There was no significant effect of confinement on sex steroids in either sex although all mean values were absolutely lower after confinement, and plasma E\(_2\) levels in females had become undetectable. In a repeat of the same experiment in October-November 1997 when fish were at a more advanced stage of sexual maturity, plasma cortisol concentrations rose significantly after confinement in females but not males (Fig. 4). In females, plasma E\(_2\) concentrations were suppressed after 6 h compared with control values, and plasma T levels after 3 and 6 h of confinement, and in males, both plasma T and 11KT levels were suppressed at 6 h.

There was little evidence of correlation between plasma concentrations of T or 11KT, and cortisol in male fish sampled at first capture from all three study years (Fig. 5). Regression coefficients (r) were 0.10 and 0.16 for T vs cortisol and 11KT vs cortisol respectively and the slopes of first or second order regressions were not significantly different from zero. Regression of plasma E\(_2\) and T against cortisol concentrations in females gave similarly weak correlations (r values of 0.25 and 0.36 for E\(_2\) and T, respectively) and regression slopes that were not significantly different from zero (Fig. 6). Only plasma values for vitellogenic females were assessed, as gonadal steroid concentrations can vary substantially with other reproductive stages (Pankhurst et al. 1999). Plasma E\(_2\) concentrations were at or below detection limits for all cortisol concentrations above 10 ng mL\(^{-1}\). This was not the case with plasma T concentrations in
females, which remained well above the detection limit across the whole range of plasma cortisol values.

All fish injected underwater with cortisol or saline were territorial, with two of nine saline and five of nine cortisol-injected fish not having visible broods. Nine fish were checked approximately 2 h after injection (five saline and four cortisol-treated) and of these, one cortisol-injected fish was absent and the remainder were involved in territorial behaviour. Twenty-four hours after injection all fish were present and guarding territories or broods, however, one saline-injected fish hid in a crevice on diver approach. All injected fish were extremely wary of diver presence and it was not possible to recapture any injected fish for sex determination or blood sampling for measurement of cortisol concentrations. Plasma cortisol concentrations in fish acclimated to the laboratory for 24 h before injection were significantly elevated by treatment with the same dose of cortisol used to treat fish underwater at 1 but not 3 h post injection (mean ± SE of 59 ± 6 vs 96 ± 14, and 40 ± 7 vs 35 ± 5 ng mL$^{-1}$ for saline and cortisol treated fish at 1 and 3 h post injection, respectively). Cortisol concentrations in saline-injected fish were similar to those seen in damselfish stressed by confinement.

Females injected with exogenous hormones or saline had undetectable plasma E$_2$ concentrations at all samples times, irrespective of whether they were injected at, or 24 h after capture (Table 1). Plasma T concentrations were generally above detection in females injected 24 h after capture but did not change in response to hormone treatment, whereas T concentrations in females injected at capture were at or below detection limits 6 h post injection. Female plasma cortisol concentrations were elevated at all sample times across all treatments and there were no differences between treatments at either injection time. Male T concentrations were detectable at all times but showed no change in response to hormone injection. In contrast, plasma concentrations of 11KT were never above the detection limit in any sample. As in females, plasma cortisol concentrations in males were high, and not different between treatments.

Ovarian follicles from two vitellogenic females from the hormone injection experiment were still steroidogenically active at the end of the experiment. In the first, there was spontaneous basal production of E$_2$ but not T (Fig. 7). HCG had no effect but T was converted to E$_2$, and 17P was converted to T. In the second fish where a smaller number
of suitable follicles precluded use of a complete protocol, there was no measurable spontaneous production of $E_2$ but exogenous T was converted by follicles to $E_2$. Plasma T and $E_2$ levels were below detection limits for both fish immediately prior to sacrifice, and plasma cortisol levels were 41.0 and 61.5 ng mL$^{-1}$ respectively.

**Discussion**

Mean plasma cortisol concentrations in freshly captured fish were typically below 10 ng mL$^{-1}$ and individual values were often undetectable (<0.6 ng mL$^{-1}$). These levels are within the range reported for other marine species captured and rapidly sampled from the wild (e.g., Pankhurst and Sharples 1992; Haddy and Pankhurst 1999; Grutter and Pankhurst 2000) and also for cortisol concentrations in unstressed, well maintained domestic fish stocks (Sumpter 1997). This further supports the view that unstressed tropical fish have basal corticosteroid concentrations that are very similar to fish from other habitats (Grutter and Pankhurst 2000). Stalking and chase times of up to 60 min of damselfish in the present study resulted in no significant increase in cortisol concentrations compared with fish captured quickly, indicating that the length of diver exposure to fish prior to capture had no effect on plasma cortisol concentrations. This was observed over two successive seasons using two slightly different measures of stalking or chase time. The first measure may have overestimated chase time where target fish were separated by any great distance on the reef, whereas the second recorded only the approach time to targetted fish and did not account for incidental effect on neighbouring fish that might have become subsequent targets. The fact that the outcomes were very similar in each sample year indicates that cortisol concentrations at capture were most likely reflections of corticosteroid status immediately before capture rather than artifacts of the capture process. This is an important distinction as it is clear that some high cortisol values reported by other workers for ‘wild’ fish are a consequence of capture stress and the delay between the onset of that stress and subsequent sampling, rather than a real measure of the normal range of plasma cortisol concentrations (Pankhurst and Sharples 1992).
Cortisol concentrations in damselfish that had been confined for up to 6 h post capture rose to concentrations well above those of freshly captured fish but peak values were towards the lower end of the envelope reported for stressed fish (Barton and Iwama 1991). This may reflect a specific characteristic of spiny damselfish, or that confinement was not perceived as a particularly severe stressor. The latter may be the case as considerably higher cortisol concentrations were recorded in the present study from damselfish that had been injected and serially bled. Despite mean cortisol concentrations from fish sampled at capture being generally low, some individual fish had concentrations as high as 30-40 ng mL$^{-1}$, similar to the concentrations recorded from fish stressed by capture, boat transport and confinement. This suggests that some fish in the natural state have plasma cortisol concentrations typical of fish under stress. The reason for this is not clear. It could mean that there are undetected physical or social stressors in the natural environment that act differentially on individuals, or that there is a small proportion of the population that has elevated cortisol concentrations for reasons not related to stress. An alternative explanation is that some but not all fish do respond to diver intrusion as a stressor. For this to be the case, the response latency would need to be very rapid as highest cortisol concentrations were detected in some of the fish exposed to the shortest stalking times (< 5 min). Examination of the response latency of plasma cortisol following exposure to stress in fish from a broad habitat and phylogenetic range including tropical reef fish, indicates that there is commonly a latency of 5-10 min (Grutter and Pankhurst 2000). Spiny damselfish would need to differ from this pattern for diver intrusion to account for the high cortisol concentrations in some individuals. Further assessment of this will require experiment under controlled laboratory conditions.

Confinement stress had no effect on plasma concentrations of sex steroids in spiny damselfish captured in austral winter (July), despite stress causing substantial increases in plasma cortisol. In contrast, confinement stress applied to reproductively active fish in early austral summer (October-November) when initial concentrations of gonadal steroids were higher than in July resulted in significant depression of gonadal steroids in both sexes. The generally lower concentrations of gonadal steroids in damselfish in winter than summer (Pankhurst et al. 1999) may explain the lack of a winter effect,
although there may still be effects indicated by the fact that \( E_2 \) concentrations from fish in winter fell below the assay detection limit following stress.

Stress-induced decreases in plasma concentrations of androgens and estrogens appear to be a universal phenomenon in teleost fishes (Pankhurst and Van Der Kraak 1997; Morehead 1998; Haddy and Pankhurst 1999; Pottinger 1999; Cleary et al. 2000) with decreases in gonadal steroids being recorded as little as 30 min after the imposition of stress in some species (Haddy and Pankhurst 1999). The effect was already present by 3 h after stress in female spiny damselfish in the present study but not until 6 h post stress in males which may indicate that male reproductive processes are less sensitive to disruption by stress. Alternatively, this may reflect the fact that male fish in this experiment had an ambiguous cortisol response to stress with apparently elevated but highly variable cortisol concentrations after confinement, and the highest baseline cortisol concentrations recorded in the study. In other species, stress inhibition of gonadal steroid concentrations has been found in both sexes without indication that differential sensitivity exists between males and females (Pankhurst and Van Der Kraak 1997; Haddy and Pankhurst 1999).

Regression of plasma sex steroid against cortisol concentrations in spiny damselfish at first capture showed little evidence of the negative correlation that might be expected if elevated cortisol in the wild was associated with suppressed gonadal steroidogenesis. However, no female fish with cortisol concentrations over 10 ng mL\(^{-1}\) had detectable plasma levels of \( E_2 \). With this proviso, it is clear that low cortisol concentrations were not requisite for measurable plasma levels of gonadal steroids to be present. Given the demonstrated effect of confinement stress on gonadal steroid concentrations, it appears that naturally elevated cortisol is not necessarily symptomatic of stress. Studies of other species show that differences in sex, gonadal condition, time of day and nutritional condition can all generate variation in plasma cortisol concentrations (reviewed in Sumpter 1997). Assessment of cortisol concentrations in spiny damsel in relation to sex, gonadal stage, and reproductive behaviour showed no differences among males whereas females had lower cortisol concentrations when defending territories with eggs than at all other times (Pankhurst et al. 1999). This difference does not explain cortisol variation in females in the present study, where vitellogenic females were predominantly egg
defenders. Instead, it appears, as discussed earlier, that undetected factors generate differences in corticosteroid status in wild fish, that may not be indicative of stress condition.

The usefulness of underwater experiments investigating the effect of artificial elevation of plasma cortisol concentrations on reproductive behaviour was limited by the difficulty in recapturing fish that had been previously handled. However, even without knowing the sex distribution of the fish injected, it is clear that acute exogenous elevation of plasma cortisol does not result in abandonment of either territory or partner. Territorial damselfish will abandon territories and broods temporarily in response to sustained challenge from divers or predators (Pankhurst et al. 1997). Acute elevations in plasma cortisol did not appear to generate the same effect. This suggests that territory abandonment is an avoidance response mediated by immediate sensory input rather than an endocrine modification of behaviour of the type seen in birds in response to unfavourable environmental conditions (Wingfield and Ramenofsky 1999). This does not discount the possibility that stress-mediated increases in cortisol concentrations may generate behavioural responses in spiny damselfish if the elevations are of longer duration.

Treatment of spiny damselfish with hCG and LHRHA had no effect on plasma steroid concentrations irrespective of the time of injection with respect to capture time. This suggests that the suppressive effect of stress includes inhibition of stimulation of native GtH release by LHRHA, and stimulation of steroidogenesis by exogenous GtH. A similar reduction in responsiveness to both LHRHA and hCG following stress has been described in the black bream Acanthopagrus butcheri (Haddy and Pankhurst 2000) and the snapper Pagrus auratus (Cleary et al. under review). However, in both these studies, fish were highly responsive to hCG and LHRHA in terms of increased plasma concentrations of T and E₂ and ovulation of mature females, when the treatment was administered at the time of capture. This suggests that either the effects of stress have a more rapid onset in damselfish so that even injection of hormones at capture is insufficient to stimulate steroidogenesis, or that the hormones used are generally ineffective in damselfish. HCG has been shown to be effective at stimulating in vitro production of T by ovarian follicles of spiny damselfish (Pankhurst et al. 2000) with
higher potency than either carp (*Cyprinus carpio*) or chinook salmon (*Oncorhynchus tshawytscha*) GtH indicating that damselfish ovarian tissue is responsive to hCG. It is still possible that the treatment dose chosen was insufficient, but on the basis of effective doses in a wide range of teleost species (Donaldson and Hunter 1983; Pankhurst and Caragher 1992), this seems unlikely. The failure of LHRHA to stimulate steroidogenesis may have resulted from tonic inhibition of pituitary release of GtH by dopamine as described for many cyprinid and salmonid species (reviewed by Peter and Yu 1997). However, studies on a variety of marine species show that dopamine inhibition is not universal, at least to the extent that there is an equivalent response to LHRHA alone or given in combination with dopamine antagonists (Zohar *et al.* 1987; Copeland and Thomas 1989; Poortenaar and Pankhurst 2000a). In this context it seems unlikely that damselfish would be unresponsive to LHRHA alone. Similarly, the dose chosen falls towards the upper range of effective doses in other species (Poortenaar and Pankhurst 2000b).

Ovarian follicles from stressed fish which had been treated with LHRHA but had undetectable plasma levels of T and E$_2$ in concert with high levels of cortisol, were unresponsive to hCG at a dose shown previously to be above the threshold for stimulating *in vitro* T production (Pankhurst *et al.* 2000). Follicles were capable of converting 17P to T, and T to E$_2$, indicating that the follicles were still steroidogenically competent and capable of converting steroid precursor substrates. If the observed lack of *in vivo* responsiveness described above results from stress-inhibition of the reproductive endocrine pathway then it appears that the effect is generated above the level of steroid synthesis in the ovary. Likely explanations would appear to be both reduced pituitary responsiveness to LHRHA, and ovarian responsiveness to GtH. A similar effect on ovarian responsiveness to GtH is found in rainbow trout (*Oncorhynchus mykiss*) where stress and cortisol suppress plasma concentrations of ovarian steroids but not GtH *in vivo* (Pankhurst and Van Der Kraak 2000), but ovarian steroidogenesis is robust in the face of elevated cortisol *in vitro* (Pankhurst 1998b). The most likely explanation for this is that stress interferes with either GtH-receptor binding, or the process of signal transduction. A similar affect seems to operate in damselfish.
**Acknowledgments**

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**References**


Table 1. Mean ± SE plasma steroid concentrations (ng mL\(^{-1}\)) at 3 or 6 h after injection in damselfish injected either at capture or 24 h post-capture with saline, hCG or LHRHA\(^1\).

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Females</th>
<th>Injection 24h post-capture</th>
<th>Treatment</th>
<th>E2</th>
<th>T</th>
<th>Cortisol</th>
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<td>hCG</td>
<td>LHRHA</td>
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<td></td>
<td></td>
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<td>0.32±0.01</td>
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<td></td>
<td>6 h</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injection at capture</td>
<td>6 h</td>
<td>0.3</td>
<td>0.3</td>
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</tbody>
</table>

<table>
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<th>Treatment</th>
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\(^1\)Doses: hCG = 625 U kg\(^{-1}\) body weight; LHRHA = 125 µg kg\(^{-1}\) body weight. \(^2\) 0.3 ng mL\(^{-1}\) = assay detection limit.
Captions for Figures

**Fig. 1.** Plasma cortisol levels (mean ± SE[n]) in fish captured and sampled underwater in 1996 in relation to the time between first disturbance and sampling. No value is different from any other (P > 0.05).

**Fig. 2.** Plasma cortisol levels (mean ± SE[n]) in fish captured and sampled underwater (clear bars) or following capture and transfer to the laboratory (black bars), in 1997 in relation to the time between first disturbance and sampling. Values with different superscripts are significantly different (P < 0.05). This figure was presented in similar form in Pankhurst et al. (1997).

**Fig. 3.** Plasma levels of cortisol, T and E$_2$ in females, and cortisol T and 11KT in males (mean ± SE) in fish at capture in winter (July) 1997, or after 2 or 6 h laboratory confinement. * = significantly different from 0 h sample (P < 0.05).

**Fig. 4.** Plasma levels of cortisol and gonadal steroids at capture and after 3 or 6 h laboratory confinement in early summer (October-November) 1997. Other details as for Fig. 3.

**Fig. 5.** Correlation of plasma cortisol and plasma T or 11KT levels in all freshly captured males sampled from 1996-1998.

**Fig. 6.** Correlation of plasma cortisol and plasma E$_2$ or T in all freshly captured vitellogenic females sampled from 1996 to 1998.

**Fig. 7.** *In vitro* production of T or E$_2$ (fish 1) or E$_2$ (inset - fish 2) by isolated ovarian follicles in response to incubation in L15 alone or containing hCG (100 U mL$^{-1}$), 17P or T (100 ng mL$^{-1}$). Values (mean ± SE[n=4]) that are significantly different (P <0.05) have different superscripts.