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Experimental test of the effect of maternal hormones on larval quality of a coral reef fish

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Abstract Maternal hormones can play an important role in the development of fish larvae. Levels of the stress hormone, cortisol, in females are elevated by social interactions and transferred directly to the yolk of eggs, where they may influence developmental rates. In some vertebrates, prenatal exposure to high levels of testosterone determine early growth rates, social status and reproductive success. The present study examined whether post-fertilization exposure of eggs of the tropical damselfish, *Pomacentrus amboinensis* (Pomacentridae), to natural levels of cortisol or testosterone directly affects larval morphology at hatching. Maternal and egg levels of cortisol and testosterone varied widely among clutches of eggs from local populations around Lizard Island on the Great Barrier Reef. The morphology of larvae produced by these local fish populations also varied widely and differed significantly among sites (e.g., standard length: 2.6–3.4 mm; yolk sac area: 0.01–0.13 $\times 10^{-2}$ mm^2). Laboratory experiments showed that elevated cortisol levels in the egg reduced larval length at hatching, while slight elevations in testosterone increased yolk sac size. The influence of testosterone, and to a smaller extent cortisol, on larval morphology differed among egg clutches. These differences were partly explained by differences in initial egg hormone levels. Morphological changes induced by experimental hormonal regimes encompassed the entire range of variability in body attributes found in field populations. It is unclear whether cortisol influences growth alone or development rate or both. Testosterone appears to influence yolk utilization rates, and has no significant effect on growth, in contrast to its role in later developmental stages. Maternally derived cortisol and testosterone are important in regulating growth, development, and nutritive reserves of the embryo and larvae of this fish species. Factors that influence the maternal levels of cortisol and testosterone may have a major impact on larval mortality schedules and, therefore, on which breeding individuals contribute to the next generation.

Key words Maternal effects · Fish larvae · Larval quality · Cortisol · Testosterone

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Introduction

Maternal hormones can play an important role in the development of fish larvae (Brown et al. 1988; Tanaka et al. 1995), as they can in many other organisms (Adkins-Regan et al. 1995; Clark and Galef 1995; Schwabl 1996a,b). Recent studies have suggested that developmental hormones transferred to the egg during gametogenesis govern development rates prior to the production of these hormones by the embryo (e.g., Schreck et al. 1991; Hwang et al. 1992; Barry et al. 1995). Current evidence suggests a direct link between hormones in the maternal plasma and the eggs she produces, with developmental (e.g., thyroxine, triiodothyronine), reproductive (e.g., testosterone, estradiol) and metabolic (e.g., cortisol) hormones being transferred from female to egg (Brown and Bern 1989; Schreck et al. 1991; Hwang et al. 1992; Mylonas et al. 1994). Two steroid hormones that are of particular interest from an ecological perspective are the corticosteroid, cortisol, which has been used as an endocrinological indicator of stress (Pankhurst and Van der Kraak 1997), and the male reproductive steroid, testosterone (Staub and De Beer 1997).

Cortisol is not only important due to its coincidence with environmental and biotic stress, but also for its interaction with developmental hormones, such as thyroid hormones (De Jesus et al. 1990; Redding et al. 1991), and its suppression of reproductive activity when elevated (Pankhurst et al. 1995). Ecologically, there is the potential for stressful behavioral

interactions (e.g., territorial disputes, interference competition) to influence cortisol levels in breeding females and for these elevated maternal levels to affect the next generation by modifying the developmental rates of offspring (McCormick 1998).

A recent study has shown that cortisol can directly influence larval morphology in fish. McCormick (1998) sampled a natural population of tropical damselfishes and found a negative correlation between levels of cortisol in the ovaries of breeding females and the size of the larvae they produced. Elevation of female cortisol within the natural range by cortisol implants resulted in the production of smaller larvae. The mechanism by which this effect was elicited is unclear. It is unknown whether cortisol influences larval development indirectly by altering allocation of vitellogenin and levels of developmental hormones during gametogenesis, or whether cortisol has a direct effect on larval development. One of the objectives of the present study was to examine the direct effects of cortisol on larval morphology.

Cortisol is not the only maternal hormone found to have important ecological consequences for the next generation. Maternal levels of androgens, such as testosterone, have lasting effects on the biology of offspring. Testosterone is the androgen responsible for development of male state from the prenatal female condition in vertebrates, and high levels are often present in the blood of females of a broad range of taxa (Staub and De Beer 1997 for review). All sexually mature female vertebrates produce androgens in order to make estrogens. Despite this, it has only recently been suggested that female androgens may play an important biological role in development (Staub and De Beer 1997). Maternally derived testosterone influences the growth of hatchling canaries (*Serinus canaria*) and is positively correlated with aggressiveness of fully grown offspring (Schwabl 1993, 1996a,b). Similarly, the transfer of hormones in utero between litter mates in mammals affects development and in later life governs aggression, sexual attractiveness, and the length of the estrus cycle in females (Clark and Galef 1995). Given the similarity of the roles of many hormones across taxa, testosterone may have similar effects in marine fishes.

In the present study, I examined whether post-fertilization exposure of fish eggs to cortisol or testosterone at naturally occurring levels has direct effects on larval morphology at hatching in a common damselfish *Pomacentrus amboinensis* (Pomacentridae). Natural levels of variability in larval morphology produced from different local breeding populations around a single tropical reef were first described to allow interpretation of experimental results. Levels of cortisol and testosterone present in newly spawned eggs were also determined. These hormone levels in the eggs were then experimentally manipulated within naturally occurring limits and the effect on larval morphology was examined at hatching, 4.5 days later. The findings emphasize the potential for maternal physiology to influence the survival of offspring and maintain variability in morphology in an unpredictable larval environment. This mechanism has the end result of communicating environmental conditions from mother to offspring.

Materials and methods

The system

Damselfish are a superb model organism for examining the effect of cortisol and testosterone on offspring development. *P. amboinensis* is typical of most damselfishes in being a protogynous hermaphrodite with males guarding demersal nests during a summer breeding season. Associated with each nesting male are between one and six females in various states of reproductive condition. Eggs are laid in a single layer of approximately 40 cm^2, containing about 3000 eggs. Embryos hatch after 4.5 days (at 28°C), ≈15 min after sunset. During the breeding season, nest sites become centers of activity, with a myriad of egg predators (fish and invertebrates) waiting for a high-energy meal, and females competing for access to the nest site. McCormick (1998) previously found a five-fold difference in concentrations of cortisol and testosterone in breeding females among groups of fish kilometers apart on the same coral reef. The strong positive relationship between ovarian and egg cortisol concentrations found in this species (McCormick 1998) suggests that these levels of variability in hormones are being transferred to the developing embryo, where they may influence larval growth and development.

Monitoring and spawning substratum

To quantify the levels of natural variability in cortisol and testosterone in newly laid eggs, I monitored nests defended by male *P. ambionensis* daily during October–December 1994 at five locations around the Lizard Island fringing reef on the northern Great Barrier Reef, Australia (14°41′ S, 145°27′ E). Natural nests were replaced with artificial nesting surfaces consisting of half of an 18-cm diameter PVC water pipe (30 cm long), split lengthwise. These pipes presented a uniform concave nesting surface of similar dimension and defensibility as natural nests (upturned clam shells). From each egg clutch, samples of 500–1000 eggs were removed underwater with a scalpel within 4 h of spawning (at dawn) and preserved in liquid nitrogen for hormonal analysis.

Spatial patterns in larval morphology

To examine the extent to which larval morphology at hatching varied over the spatial scale of a whole reef, I collected larvae from 50 nests in the five monitored locations (separated by 1–7 km) around Lizard Island. Monitored nests containing eggs that were within a few hours of hatching were collected from the field and transferred to well-aerated aquaria in the laboratory. Larvae hatched ≈15 min after sunset. Individuals were preserved in 2.5% glutaraldehyde in seawater for 2 h at room temperature, rinsed in seawater, then transferred to fresh seawater and refrigerated for morphometric analyses. Standard length (SL), head depth (through the eye), eye diameter (maximum), and yolk sac area of 25–50 larvae from each nest were measured. Yolk sac area provided a measure of yolk reserves available for subsequent development. Measurements were made using a computer running image analysis software (NIH Image) linked to a binocular microscope.

Experimental protocol

Egg clutches used in these experiments were collected during November and December 1996 at one location on the Lizard Island

fringing reef. To facilitate easy removal and subsampling of egg clutches, I attached a roughened sheet of acetate to the inner surface of the upturned pipe nest sites with stainless steel clips. Nests were checked for new clutches ≈2 h after sunrise when the majority of spawning occurs (Meekan 1992). Large clutches of newly spawned eggs were brought back to the laboratory in plastic bags and placed in aquaria with a strong flow of seawater. Clutches were then divided into five (testosterone, T) or six (cortisol, F) equal portions with a scalpel and randomly allocated to four (T) or five (F) treatments, with one portion being frozen to assess the hormonal state of the embryos at the initiation of the experiment. Treatments consisted of 10-l aquaria containing aerated seawater with specific concentrations of cortisol or testosterone. Acetate sheets with the embryos were clipped to the sides of the tanks and a sheet of fine air bubbles was directed over the embryos to keep them well oxygenated and to avoid the formation of a hormone-depleted boundary layer. For the cortisol experiments, the five treatment concentrations were 0 (no cortisol added), 10, 100, 1000, and 10,000 ng F l^{-1}, while for the testosterone experiments, the concentrations were 0, 10, 100, and 1000 ng T l^{-1}. Tanks were flushed and cleaned daily for 30 min at 2100 hours and concentrations of cortisol immediately reestablished. The experiment was run independently on six clutches of eggs collected from nests guarded by six different males.

Newly hatched larvae were preserved in 2.5% glutaraldehyde buffered in seawater for 2 h at room temperature, rinsed with seawater, and stored in seawater at 4°C until measurement. As with larvae from monitored nests, I measured larvae from the experiments using an image analysis system linked to a binocular microscope. SL, head depth (through the eye), eye diameter (maximum), and yolk sac area of 20 larvae from each clutch were measured.

Experimental concentrations of F and T were chosen to span the range of variability present in newly spawned eggs in the field. Treatment levels were chosen using the known levels of variability in the hormones determined from field collection (see Results) and the estimation that ≈2000 eggs equated to 1 ml. To determine how immersion in the hormone baths actually influenced hormone levels within the eggs, an extra five clutches of eggs were subjected to the same experimental protocol as above, but sampled as eggs after 3 days of immersion. I determined F and T content of the eggs by radioimmunoassay (see below) and these concentrations were related back to the concentrations of T and F in the incubation baths.

Steroid content of eggs

Levels of variability in the concentrations of cortisol and testosterone in newly spawned eggs were determined by radioimmunoassay. Concentrations of these hormones were also determined for subsamples of eggs from clutches used in the hormone experiments, since initial hormone levels may influence whether or how the treatments affected larval morphology. I determined cortisol and testosterone concentrations using standard radioimmunoassay techniques (a modified version of the protocol of Pankhurst and Carragher 1992). A known number of eggs (500–650) were homogenized in 200 ml 0.05 M phosphate buffer containing 0.1% gelatine and 0.01% Thimerosal (Sigma). Ethyl acetate (1 ml) was added, vortexed, and centrifuged. Duplicate 150-µl aliquots were evaporated in assay tubes. Extraction efficiencies, determined by the recovery of ^{3}H-steroid added to triplicated egg homogenates, ranged between 84.1% to 93.1% (cortisol) and 84.4% to 96% (testosterone). Standards were run with each assay. Cortisol was measured in five assays using (1,2,6,7-^{3}H) cortisol (Amersham), with an antiserum produced by BioClinical Services, Cardiff. The antibody used was an antiserum raised in rabbits against a cortisol-3-(0-carboxymethyl) oxime-bovine serum albumin conjugate (BioClinical Services). This cortisol antiserum had the following cross reactivities: 11-deoxycortisol 25%; cortisone 8.5%; corticosterone 4.5%; 17α-hydroxyprogesterone, deoxycorticosterone 1.4%; progesterone 0.06%; Interassay variability for cortisol was 9.7%CV, $n = 5$. Testosterone was measured in five assays using (1,2,6,7-^{3}H) testosterone (Amersham). The testosterone antiserum was raised in rabbits against a testosterone-3(-0-carboxymethyl) oxime-bovine serum albumin conjugate (BioClinical Services). This testosterone antiserum had the following cross reactivities: 5α-dihydrostestosterone 16%; 5α-androstane-3α,17β-diol 5.8%; 5α-androstane-3β,17β-diol 3.7%; androstenedione 2.1%; cortisol $< 0.01\%$. Interassay variability for testosterone was 14%CV, $n = 5$. To avoid incorporating interassay variability into tests between experimental treatments, all egg samples from a single experiment were measured in the same hormone assay.

Analyses

I tested the effectiveness of the hormone baths in altering the hormone levels within the eggs using an analysis of variance to compare mean hormone concentrations among treatments. Clutches were identified in the analysis and used as a blocking factor to remove the variability due to differences in the initial hormone levels of the eggs among clutches.

Initially I tested for differences in aspects of mean body size (variables: SL, head diameter, yolk sac area, eye diameter) among treatments, clutches, and their interaction using analysis of variance (GLM; SAS 1987). Type IV sums of squares were used due to missing data in some treatment by clutch combinations. If the interaction was significant, separate analyses of variance were undertaken for each clutch using type III sums of squares, due to unequal sample sizes. Assumptions of homogeneity of variance and normality were examined for all tests by residual analysis and no data transformation was required. Tukey's (HSD) tests were used to compare means found different using analysis of variance.

Regression analysis was used to examine the relationship between SL and yolk sac area at hatching for each treatment. Homogeneity of the slopes of these relationships among treatments was examined. If the slopes did not differ significantly, analysis of covariance (ANCOVA) was used to test for differences in yolk sac area among treatments corrected for variable SL. Planned comparisons were used on least-square estimates of the mean yolk sac area (i.e., means adjusted for larval SL) to identify whether adjusted means differed among treatments (GLM; SAS 1987).

To summarize multidimensional trends, multivariate analysis of variance (MANOVA; Tabachnick and Fidell 1996) was used to test the hypothesis of no difference in larval morphology at hatching among the experimental treatment by clutch combinations. Canonical discriminant analysis (CDA) was used to identify and display the nature of the significant differences among clutches found by MANOVA. CDA identifies a number of trends in the dataset (canonical variates) that maximally discriminate among the identified groups (in this case, treatments) and sequentially explain less of the variance in the dataset. Trends in the original variables (SL, head depth, yolk sac area) are represented as vectors given by correlations of these variables with the canonical variates (also known as total structure coefficients). These vectors are plotted on the first two canonical axes, together with centroids of the treatment by clutch combinations. The strength or importance of each of the original variables in discriminating among groups is displayed graphically as the length of these vectors. The assumption of multivariate normality was validated prior to analysis.

Results

Natural levels of variation in larval morphology

The mean SL of larvae from clutches had a left-skewed length-frequency distribution (Fig. 1a) with a modal length of 3.1 mm and a range from 2.73 to 3.24 mm SL. When the variability attributable to individual larvae

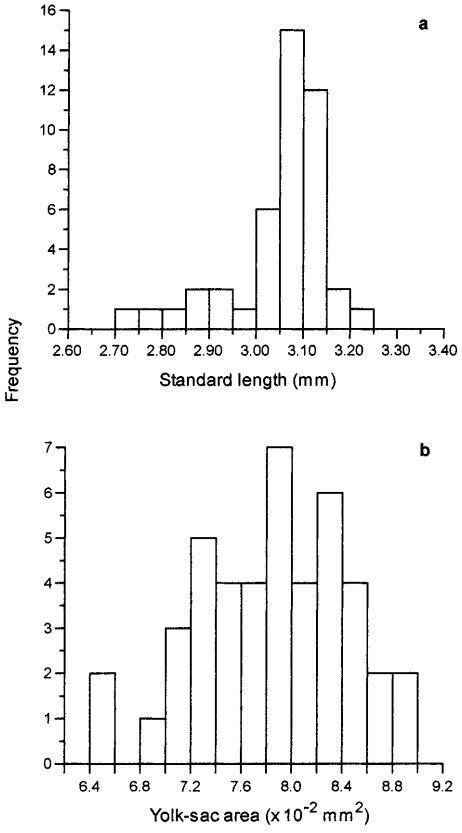

Fig. 1 Natural levels of variability in standard length (**a**) and yolk sac area (**b**) of *Pomacentrus amboinensis* larvae at hatching. Data represent mean morphology of 25–50 larvae measured from each clutch of eggs (n = 44 clutches) collected from nests around Lizard Island

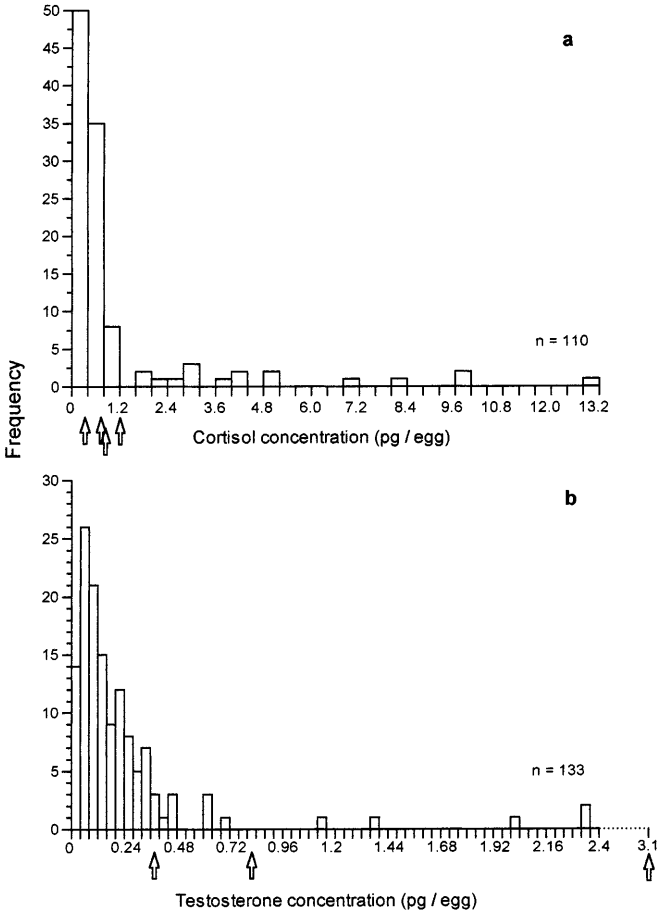

Fig. 2 Natural levels of variability in the concentrations of cortisol (**a**) and testosterone (**b**) in eggs of *P. amboinensis* collected within 4 h of spawning. *Arrows* indicate the mean effective concentrations of experimental treatments

within a clutch was included the range increased (2.56–3.39 mm SL), though this had a relatively low CV of 5.14%. More of the variability in larval length was attributable to differences among clutches within a location (47%) than was explained by variability within individual clutches (29%) or differences among localities (24%).

The mean yolk sac area from clutches exhibited a platykurtic size-frequency distribution with high variability and a range from 0.064 to 0.089 mm^2 (Fig. 1b). When variability within clutches was included, yolk sac area had a threefold range (0.04–0.13 mm^2), with a CV of 17.8%. Most of this variability was at the small scale, with sampling locality around the island only accounting for 2% of the total variation in yolk sac area. Most of the variability was attributable to differences among larvae within clutches (64%) and among clutches (35%).

Levels of cortisol and testosterone in newly spawned eggs

The concentrations of both hormones within the newly spawned *P. amboinensis* eggs displayed right-skewed frequency distributions (Fig. 2), being particularly accentuated for cortisol. Furthermore, it is likely that the lowest concentration class is underrepresented due to the lower limits of sensitivity of the radioimmunoassays. Modal concentrations within the eggs were 0.2–0.4 pg egg^{-1} for cortisol and 0.04–0.08 pg egg^{-1} for testosterone.

Effectiveness of immersion in F and T baths

Both cortisol and testosterone baths were successful in manipulating egg hormone levels within naturally occurring concentrations (Fig. 3). For the cortisol experiment, mean treatment concentrations spanned all but the upper 15% of the naturally occurring range (note placement of arrows on Fig. 2a). The highest concentrations of cortisol caused a fourfold elevation in hormone levels within the eggs compared to the controls. The testosterone treatments encompassed a slightly wider range of concentrations than occurred naturally, with the highest treatment (10^3 ng l^{-1}) inducing a hormone concentration within the eggs that was 28% higher than the highest value recorded in unmanipulated eggs (Fig. 3b). The 10 and 10^2 ng l^{-1} testosterone treatments,

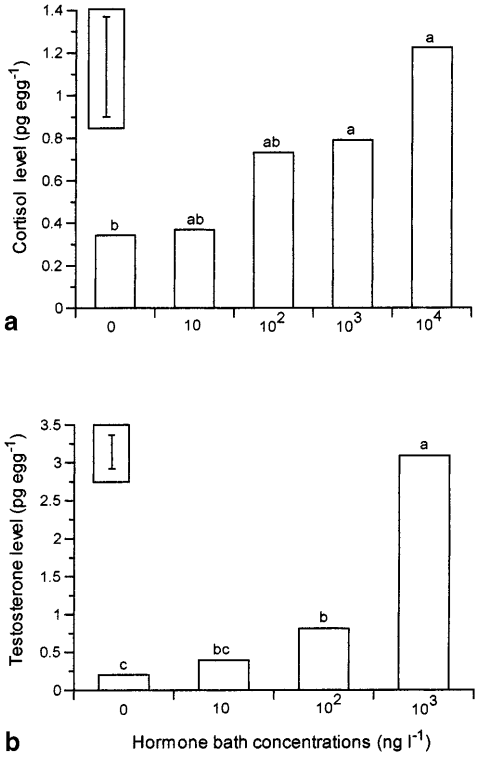

Fig. 3 Immersion of newly spawned eggs in hormone baths influences egg hormone levels 3 days later. Data represents back-transformed (antilogged) mean cortisol (**a**) and testosterone (**b**) levels for subsamples of egg clutches immersed for 3 days in experimental levels of hormones in aerated seawater ($n = 6$ clutches per hormone). Treatments differ significantly (cortisol: $F_{4,9} = 11.79$, $P = 0.001$; testosterone: $F_{3,14} = 13.98$, $P = 0.0002$; $\log_{10}$ transformed). *Letters* associated with bars represent Tukey's (HSD) groupings from means comparisons among treatments. The *error bar* is the back-calculated common standard error of the test

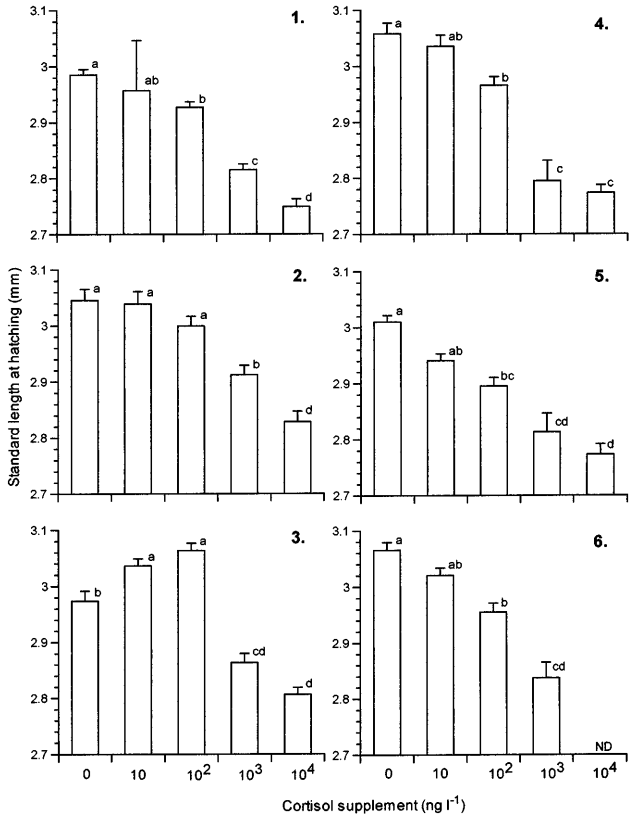

Fig. 4 Influence of cortisol at five experimental concentrations on the mean standard length at hatching of *P. amboinensis*. Results from six egg clutches are given, with ≈25 larvae measured per clutch by treatment combination. *Letters* associated with bars represent Tukey's (HSD) groupings from means comparisons within a clutch (*ND* no data due to embryo mortality)

however, encompassed 96% of the naturally occurring range (Fig. 2b, arrows).

Influence of cortisol on larval morphology

Elevation of cortisol in the developing embryo altered the SL of the larvae at hatching: in five of the six clutches SL decreased with exposure to increasing concentrations of cortisol (Fig. 4). One clutch showed a different trend, with larvae from the the 100 ng l^{-1} treatment being significantly larger than all treatments except for larvae from the 10 ng l^{-1} treatments (Fig. 4, clutch 3). Within the six clutches, mean length at hatching among the treatments ranged over 7.1 to 9.3% of the maximum mean fish size within the clutch.

Cortisol levels had a significant influence on the yolk sac size at hatching but this effect was not consistent among clutches (treatment × clutch, $F_{19,618} = 2.70$, $P = 0.0001$). There was a weak trend for fish in the 10^2 ng F l^{-1} treatment to have a slightly larger yolk sac than fish from eggs not exposed to cortisol supplementation (four out of six clutches), though this was only significant in one instance (clutch 5). Likewise, both head height and eye diameter of the fish at hatching showed significant differences among cortisol treatments within a clutch, but these were not consistent among clutches (treatment × clutch: eye diameter, $F_{19,618} = 2.502$, $P = 0.0004$; head diameter, $F_{19,618} = 8.804$, $P < 0.0001$).

Initial hormone levels in the eggs at the start of the experiment may have influenced the embyro response to the treatments. However, for all of the morphological variables measured, no relationship was found between the magnitude of the changes induced by the cortisol baths (quantified as the difference between measures from the 10^4 ng F l^{-1} treatment and the measures from the control fish) and the initial levels of T and F in the experimental egg clutches. It appears that the individual way that clutches responded to the cortisol treatments was not a simple function of the levels of maternally derived F and T in the yolk.

There was a significant and positive relationship between SL of the larvae and the size of their yolk sacs for all treatments (Fig. 5). The slopes of these relationships for the control and two lower cortisol supplementation treatments (10 and 10^2 ng F l^{-1}) did not differ from one another. However, these slopes were significantly lower than those displayed by larvae from the 10^3 and 10^4 ng F l^{-1} treatments (Fig. 5).

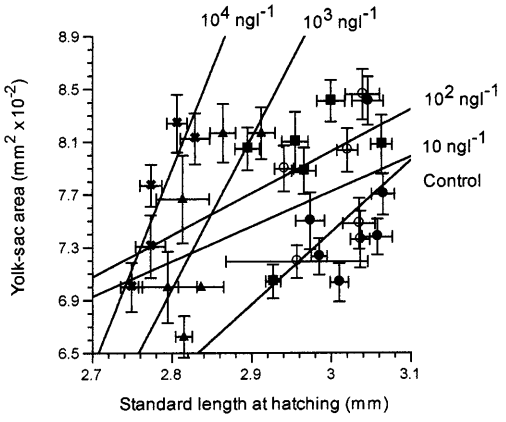

Fig. 5 Relationship between standard length and yolk sac area for larvae from five experimental cortisol treatments. Mean values for each egg clutch used in experiments have been plotted for clarity. A test of homogeneity of slopes was significant ($F_{4,637} = 3.322$, $P = 0.01$). *Error bars* are standard errors ($n \approx 25$)

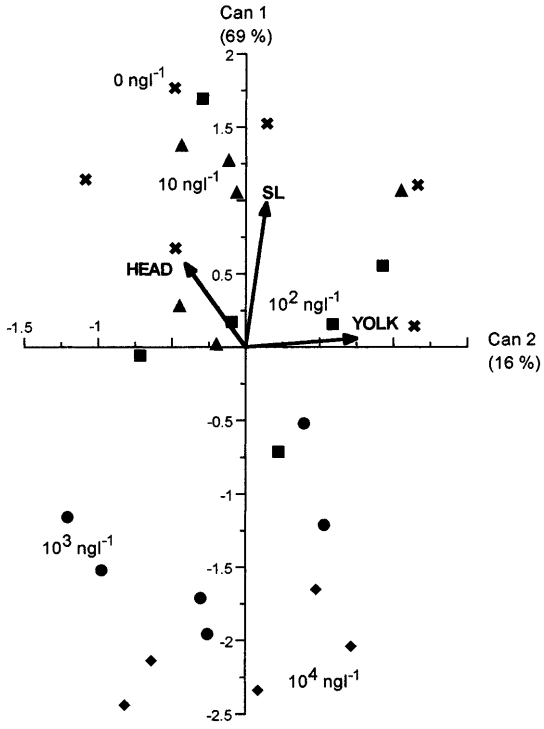

Fig. 6 Comparison of the mean morphology of larval *P. amboinensis* at hatching among cortisol treatment by clutch combinations. A canonical discriminant analysis (a type of multivariate means comparison) displays the difference in larval morphology among the treatments by clutch combinations (five cortisol treatments shown as different symbols). A MANOVA found treatment by clutch combinations to significantly differ from one another (Pillai's Trace, *df* 112,2472, $P < 0.0001$). The direction and importance (as indicated by vector length) of trends in measured larval attributes is also given (*SL* standard length, *YOLK* yolk sac area, *HEAD* maximum head depth). The mean 95% confidence circle for each treatment by clutch combination has a radius of 0.51 (Seber 1984). For clarity these have not been plotted

A comparison of the overall larval morphology from the four cortisol treatments and control shows considerable variation in the effect of cortisol amongst clutches, with the major effect of cortisol being its impact on larval length (Fig. 6). A MANOVA comparing the morphology of larvae among clutch by treatment combinations found significant differences (Pillai's trace, *df* 112,2472, $P < 0.0001$).

Influence of testosterone on larval morphology

Supplementation of testosterone to developing embryos appeared to reduce the SL of larvae at hatching. In all six clutches, the eggs not supplemented with testosterone (controls) produced the larger larvae on average than the testosterone-supplemented treatments, although this was only significant in four clutches. Within the testosterone-supplemented treatments, there were no consistent trends in the effects of differing levels of testosterone on larval length.

Testosterone supplementation markedly affected larval yolk sac area. Testosterone-supplemented eggs consistently produced larvae with larger yolk sacs than those of larvae from experimental controls (Fig. 7). However, there was no consistent effect of increased testosterone levels on yolk sac area among clutches.

In a similar way to SL, head height of the larvae at hatching showed significant differences among testosterone treatments within a clutch, but these were not consistent among clutches (treatment × clutch: $F_{11,417} = 2.35$, $P = 0.008$).

Eye diameter was the only aspect of larval morphology measured in which the trends in size among testosterone treatments were consistent among the six egg clutches (treatment × clutch: $F_{11,417} = 0.929$, $P = 0.512$). Testosterone supplemented at 10 ng l^{-1} reduced the eye diameter of the larvae at hatching, while there was a suggestion that the highest levels of testosterone (10^3 ng l^{-1}) increased eye diameter ($F_{3,417} = 2.928$, $P = 0.033$; Fig. 8). The identity of the clutch markedly affected eye diameter, regardless of treatment effects ($F_{5,417} = 9.471$, $P < 0.0001$).

Unlike the findings for the cortisol experiment, initial hormone levels in the eggs were found to have some influence on the way in which clutches responded to the testosterone treatments. Although data are limited, there was a negative relationship between the magnitude of the response of SL to the T treatments (as quantified by the difference between the 10^2 ng l^{-1} treatment and controls) and initial cortisol concentrations within the clutches ($r = -0.552$, $n = 6$ clutches). No other relationships were apparent for the morphological variables measured.

There was a positive linear relationship between larval length and yolk sac area within treatments (Fig. 9). A test of homogeneity of slopes found that this relationship did not differ among treatments ($F_{3,404} = 1.41$, $P = 0.241$) (note: the anomalous clutch 6 of the 10 ng l^{-1} treatment was excluded from this analysis). However,

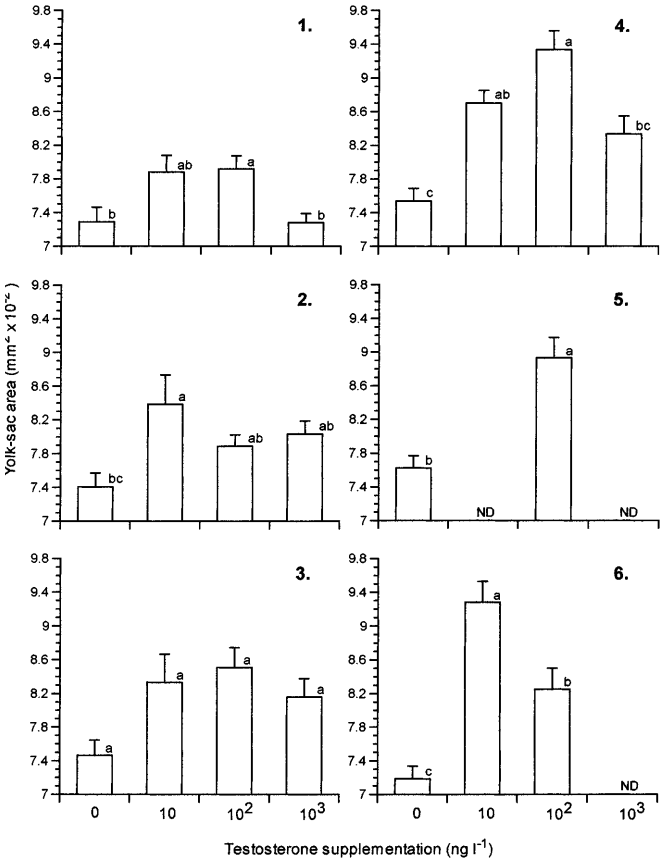

Fig. 7 Influence of testosterone at four experimental concentrations on the yolk sac area at hatching of *P. amboinensis*. Results from six egg clutches are given, with ≈25 larvae measured per clutch by treatment combination. *Letters* associated with bars represent Tukey's (HSD) groupings from means comparisons within a clutch (*ND* represents no data through embryo mortality)

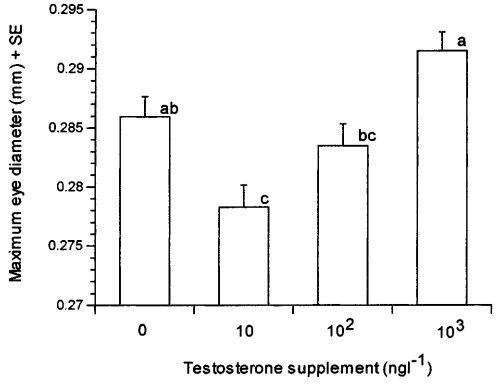

Fig. 8 Influence of testosterone on eye diameter at hatching. *Letters* associated with bars represent Tukey's (HSD) groupings from means comparisons among treatments

the intercepts of the relationships did differ among treatments (ANCOVA, $F_{1,407} = 7.66$, $P < 0.006$), with planned comparisons showing larvae from the controls having significantly smaller yolk sacs than larvae from the testosterone-supplemented treatments, once corrected for variable length (Fig. 9).

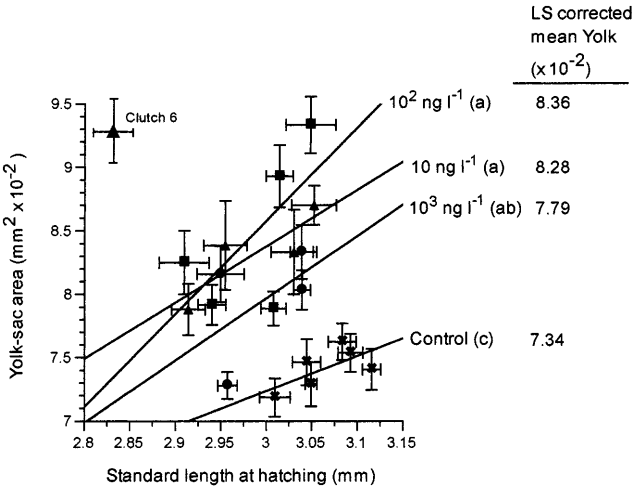

Fig. 9 Relationship between standard length and yolk sac area for larvae from four experimental testosterone treatments. Mean values for each egg clutch used in experiments have been plotted for clarity. *Error bars* are standard errors ($n \approx 25$). Treatment mean yolk sac sizes adjusted (least squares, *LS*) for standard length are also given

A comparison of the overall larval morphology from the three testosterone treatments and control clutches shows that testosterone levels had their greatest effect on larval yolk sac size (Fig. 10). A MANOVA comparing the morphology of larvae among clutch by treatment combinations found significant differences (Pillai's Trace, df 76, 1668, $P < 0.0001$).

Discussion

When levels of the ecologically important hormones, cortisol and testosterone, were elevated in newly spawned eggs of a tropical damselfish, the morphology of the larvae that hatched changed. Elevation of cortisol resulted in larvae that were markedly smaller at hatching than unmanipulated controls. In contrast, relatively small elevations of testosterone resulted in larvae of the same size as those released from control eggs, but that had larger nutritional reserves. Interestingly, the differences in morphology induced by hormonal manipulation spanned the range of variability in these body attributes that is found naturally. Since we know for this species that levels of both testosterone and cortisol are highly variable in breeding females, and that these are transferred to the eggs (McCormick 1998), the present evidence suggests that maternal hormonal levels may be responsible for much of the variability in larval morphology found at hatching.

The findings of this study have ramifications for determining the processes that ultimately control the numbers of larvae that survive to replenish the post-larval population. Current hypotheses suggest that as the growth rate of marine larvae increases, mortality due to starvation and predation decreases (Anderson 1988). A considerable body of evidence supports this

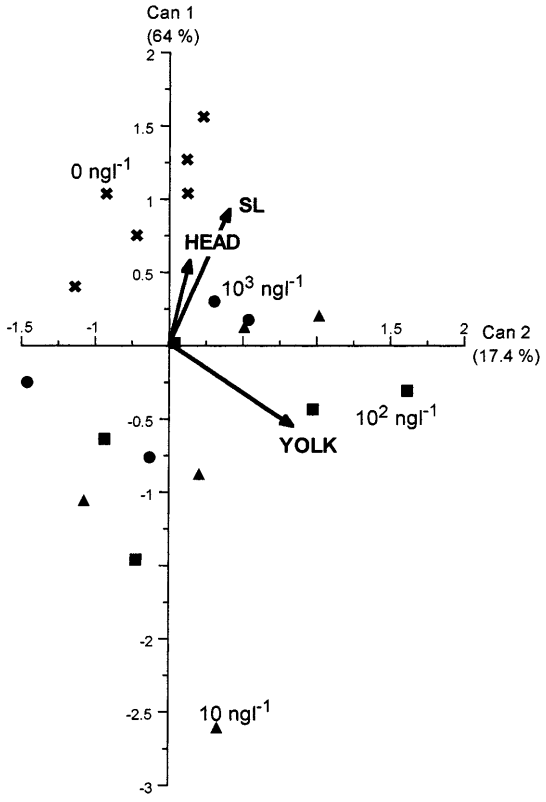

Fig. 10 Comparison of the mean morphology of larval *P. amboinensis* at hatching among testosterone treatment by clutch combinations. A canonical discriminant analysis displays the difference in larval morphology among the treatment by clutch combinations (four testosterone treatments shown as different symbols). A MANOVA found treatment by clutch combinations to differ significantly from one another (Pillai's trace, *df* 76,1668, $P < 0.0001$). The direction and importance (as indicated by vector length) of trends in measured larval attributes are also given: standard length (*SL*), yolk sac area (*YOLK*), and maximum head depth (*HEAD*). The mean 95% confidence circle for each treatment by clutch combination has a radius of 0.52 (Seber 1984). For clarity these have not been plotted

theory for marine fish larvae. Laboratory studies have found that larger larvae are better able to capture food, resist starvation, and avoid predators (Hunter 1981; Miller et al. 1988; Bailey and Houde 1989). Field studies have shown that surviving individuals from a larval year class were larger at younger ages compared to the population as a whole (e.g., Post and Prankevicius 1987; Tsukamoto et al. 1989; Meekan and Fortier 1996; Hare and Cowen 1997). Field studies also show that faster-growing cohorts of larvae exhibit higher rates of survival (e.g., Graham and Townsend 1985; Castrow and Cowen 1991). If fast-growing, well-provisioned larvae maintain their growth advantage through the larval stage, larvae spawned from females with low levels of cortisol, but high levels of testosterone, are likely to have higher probabilities of survival.

Limited evidence suggests that being large at hatching may translate to enhanced survival. Campbell et al. (1992) found that repeated episodes of chronic stress (3 min of emersion) randomly applied to rainbow trout (*Oncorhynchus mykiss*) over a 9-month period caused elevations in plasma cortisol. Stressed fish produced smaller eggs than controls in keeping with the present finding that smaller larvae are produced from eggs with higher concentrations of cortisol. Moreover, Campbell et al. (1992) found that larvae from stressed parents displayed higher mortality during the early post-hatching stage than those from control parents. Similar experiments on brown trout (*Salmo trutta*) supported these results (Campbell et al. 1994). In these laboratory examples at least, higher maternal stress led to increased cortisol levels, and smaller offspring with higher levels of mortality. With the damselfish studied here, it is not known whether smaller larvae produced from eggs with high cortisol have higher mortality, and this warrants further investigation.

The present experiments show that cortisol directly influenced larval size at hatching but did not influence the size of the yolk sac. Cortisol may directly influence developmental rates of the larvae or may influence larval metabolism to affect changes in growth rate. Increased cortisol reduces growth rates in post-larval fish (Pankhurst and Van der Kraak 1997). However, information on the role of cortisol in embryonic development in fish is scarce (De Jesus et al. 1991; Hwang et al. 1992).

The action of cortisol on development in fish may vary with ontogeny. In general, cortisol increases metabolic rate in fishes (Chan and Woo 1978; Vijayan et al. 1991; Vijayan and Moon 1994), being important in gluconeogenesis (Vijayan et al. 1996). In fish and amphibians, corticosteroids (cortisol and corticosterone) have also been shown to enhance the action of thyroid hormone (T_3) during late larval development to speed up metamorphosis (Kaltenbach 1985; De Jesus et al. 1991; Denver 1997). In contrast, cortisol suppresses thyroid hormones (T_3 and T_4) in adults eels (Redding et al. 1986) and T_3 in a number of salmonids (Vijayan and Leatherland 1989; Brown et al. 1991).

Cortisol may also influence growth by interacting with growth hormone. Stress changes the levels of growth hormone in adult fish, although whether levels are depressed or elevated seems to depend on the level of stress imposed (Pankhurst and Van der Kraak 1997). Evidence suggests that cortisol interacts with growth hormone to influence the production of insulin-like growth factors that directly affect growth (Pankhurst and Van der Kraak 1997).

In contrast to the present experiments, Mathiyalagan et al. (1996) found that treatment of 1-day-old yolk sac larvae of tilapia (*Oreochromis mossambicus*) by immersion in cortisol solution for 2 weeks significantly enhanced growth. SL, tail length, head width, and wet weight showed dose-dependent increases at concentrations of cortisol (hydrocortisone) from 0.05 ppm to 0.5 ppm, with no further increase at 1.0 ppm. However, cortisol at 5.0 ppm retarded the growth of the larvae without causing any morphological abnormality. The

onset of free-swimming activity in the larvae was also accelerated by cortisol treatment, suggesting that cortisol was accelerating development as well as growth in this species. It is unclear whether the marked difference between the tilapia and the present study are due to ontogenetic differences in the action of cortisol or represent species-specific differences in developmental physiology. The closeness of the two groups of fish phylogenetically (cichlids and pomacentrids) suggests it may be an ontogenetic difference in the action of cortisol.

In contrast to the action of cortisol on embryo growth and development, testosterone had no consistent effect on size at hatching. Instead, eggs reared in high levels of testosterone produced larvae with larger yolk sacs. Because treatments were imposed randomly on newly laid eggs this result suggests that testosterone must be increasing the efficiency with which the yolk is being utilized by the embryos. Few comparative studies exist, and evidence from fish and other taxa on the general effects of testosterone on growth and development are contrary to the present findings. Schwabl (1996b) manipulated testosterone levels in the yolks of canaries and found that testosterone directly enhanced nestling growth. Furthermore, because chicks with high growth had a higher metabolism, they hatched with smaller yolk reserves compared to chicks that hatched synchronously from eggs with lower testosterone concentrations. This contrasts with the findings of the present study, where yolk size increased with relatively small increases in testosterone concentrations and there was no change in fish size. Androgens are known to promote tissue differentiation and development including that of muscle (Sassoon et al. 1987; Joubert and Tobin 1995), cartilage (Schwartz et al. 1994), bone (Weisman et al. 1993; Lieberherr and Geosse 1994) and the nervous system (Breedlove 1992; Forger et al. 1992). It is presently unclear what role maternally derived androgens have during embryogenesis, but this study emphasizes that it is of physiological importance and may differ from the role played by androgens in later developmental stages.

The determinants of testosterone levels in females fishes are poorly understood. Aggression may lead to increased levels of testosterone in males (Wingfield et al. 1987; Pankhurst 1995), although not all aggression is testosterone activated (Staub and De Beer 1997), and a similar mechanism may occur in females. In hermaphrodites, the change of sex from female to male is also known to be linked to an increase in the level of plasma androgens (Mims et al. 1995). Since the damselfish in the present study, *P. amboinensis*, is a protogynous hermaphrodite, testosterone levels may vary with size and be closely related to the position of a fish in a dominance hierarchy. Social rank has been correlated with testosterone levels in parrotfish, male rainbow trout, and cichlids (Cardwell and Liley 1991; Cardwell et al. 1996; Oliveira et al. 1996). In the present case, dominant females may not only have greater access to the nest site, but may produce eggs with larger yolks by virtue of the higher maternal testosterone endowment. This effect may be further accentuated by high social dominance leading to access to greater, or better-quality, food resources. The link between female androgen levels and the social systems of fish has yet to be examined for any fish species.

In contrast to the uncertainty about the factors influencing maternal testosterone levels, the factors that influence cortisol levels have been well studied. Stress is well known to elevate cortisol levels in the blood of fishes and other organisms (Pickering 1993; Saltzman et al. 1994; Astheimer et al. 1995; Coddington and Cree 1995; Pankhurst and Van Der Kraak 1997). In fish, elevations in plasma cortisol may be caused by mechanical disturbance (Young and Cech 1993; Lowe and Wells 1996), emersion (Campbell et al. 1992), environmental pollution (Stephens et al. 1997), or osmotic challenge (Langhorne and Simpson 1986). Social interactions may also elevate cortisol levels (Iwata 1995) and this effect is accentuated at high population densities (Pottinger and Moran 1993). In a field study of the damselfish *P. amboinensis*, McCormick (1998) found that densities of key egg predators explained 38% of the variation in ovarian cortisol levels of monitored females. It was proposed that dominant females played a role in defense of the nest and that the relationship between predators and female cortisol levels was stress related.

This study has demonstrated that maternal influences through the hormonal system may cause much of the variability in larval morphology found at hatching. Variability induced through maternal effects may maintain levels of variability in larval characteristics despite growth and size-selective processes that seem prevalent in the planktonic environment (Hare and Cowen 1997). In the system examined here, it is presently unclear whether the hormonally driven changes in body morphology have consequences for survival or performance in later larval stages. In higher vertebrates, the embryonic hormonal regime has the potential to influence the behavioural regime in later life stages and this may also be found for fish (vom Saal 1984; Clark and Galef 1995; Casolini et al. 1997). Unfortunately, we currently know little about the factors that influence maternal hormone levels. Once these factors have been identified, we may begin to explore the evolutionary implications of how maternal interactions influence larval attributes over and above their importance in maintaining phenotypic variability in a changeable environment.

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