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Influence of Cortisol on Developmental Rhythms During Embryogenesis in a Tropical Damselfish

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ABSTRACT Newly-spawned teleost eggs can vary widely in their maternal endowment of a variety of hormones, including cortisol. Field and laboratory experiments have shown that initial egg cortisol concentrations directly influence the size at hatching of the benthic spawning damselfish, *Pomacentrus amboinensis*. The present study examines the mechanism by which cortisol influences larval size at hatching by investigating the growth and developmental rhythms throughout embryogenesis. Newly spawned eggs of *P. amboinensis* were collected from natural benthic nests, and half of each clutch was incubated in a moderate level of cortisol (2.7×10^{-6} M, equivalent to a concentration of 0.79 pg/egg). Cortisol was found to have no affect on the rate of cell-pulsations up to epiboly (18 hr post-fertilization), with cells pulsing at a mean rate of 56–60 pulses/min. Cortisol had an affect on the relative growth rate from the start of gastrulation to knot formation. Growth in the cortisol-supplemented embryos was pulsed, with periods of fast growth punctuated by long periods of stasis. Overall growth rates during this period were lower in the cortisol-supplemented embryos despite their higher growth during active periods. Pulse rates of somite cells and contraction rhythms of myotomes and the heart were twice as high in cortisol-supplemented embryos than controls. Despite this, cortisol-supplemented eggs developed at the same rate as controls and hatched at the same time. This study suggests that the maternal endowment of cortisol to eggs plays a vital role in determining the embryonic rhythms by which embryos grow and may be directly influencing metabolism. *J. Exp. Zool. 293:456–466, 2002.*

Hormones play an important role as regulators and integrators of metabolism in developing fishes. During embryogenesis, the hormones that regulate and facilitate growth and development come to the embryo from the maternally derived yolk, where they are accumulated over the course of oogenesis (Tagawa et al., 2000). For most fish species, it is unknown when the embryo's hormonal systems become functional, although limited research suggests that, at least for the steroidogenic cells, it is generally after hatching and that the timing is likely to be species-specific (De Jesus et al., '91; De Jesus and Hirano, '92; Hwang et al., '92; Hwang and Wu, '93; Barry et al., '95; Sampath-Kumar et al., '97).

A series of studies has recently shown that levels of maternally derived hormones strongly influence embryogenesis in a tropical damselfish (McCormick, '98, '99). The level of cortisol in females is correlated with the densities of egg predators and conspecifics in the vicinity of the female's nest site. Field and laboratory experiments showed that supplementation of cortisol to females or directly to eggs reduced the size of larvae at hatching. Moreover, manipulations of cortisol during embryogenesis, within naturally occurring levels, resulted in larvae whose size at hatching spanned the complete size range found to naturally occur in the species at the study location. This is a surprising result, given the many factors that can influence offspring size (e.g., maternal size and nutritional condition, Kerrigan, '97). These findings suggest that behavioural interactions of females during their reproductive season influence the quality of their offspring and have the potential to determine an individual female's contribution to the next generation.

Currently, however, the mechanism by which cortisol influences larval size is unknown. Cortisol

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has diverse effects (Mommsen et al., '99) and interacts with developmental hormones, such as thyroid hormones (Kim and Brown, '97). It may directly influence metabolism, developmental rate, or growth. Cortisol is a glucocorticoid that plays an important role in energy metabolism and the response of fishes to stress (Gregory and Wood, '99). The goal of the present study is to examine the mechanism by which cortisol influences larval size during embryogenesis by examining developmental rhythms in a detail not previously attempted.

Previous studies have demonstrated that the movement of an embryo, and the rhythms these movements create, can be used to obtain detailed information on the timing of the development of systems during embryogenesis, and how this differs among and within species (Blaxter, '69; Kamler, '76; Nechaev et al., '92). Embryo motility is a general property of most vertebrate and invertebrate animals. Growth, respiration, and digestion of the yolk-products are all processes directly associated with a motor activity (Bautzmann, '56; Holliday et al., '64; Bell et al., '69; Saint-Amant and Drapeau, '98). However, few studies have examined the rhythmic processes accompanying fish embryogenesis despite movement playing important functional roles during embryo development (Reznichenko, '82; Richards and Pollack, '87; Warga and Kimmel, '90; Wood and Thorogood, '94; van Gestel et al., '98).

The present study documents the rhythmic processes associated with embryogenesis in a common tropical damselfish, *Pomacentrus amboinensis* (Pomacentridae). Using this foundation, we then explore in detail the influence of cortisol on embryogenesis.

MATERIALS AND METHODS

Study species

The damselfish, *P. amboinensis*, was chosen for the study since there already exists a good basis of ecological and physiological information. It is typical of most damselfishes, 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 3,000 eggs. Embryos hatch after 4.5 days (at 28°C), $\approx$15 min after sunset. McCormick ('98) previously found a five-fold difference in concentrations of cortisol in breeding females among groups of fish kilometres apart on the same coral reef. The strong positive relationship between ovarian and egg cortisol concentrations found in this species (McCormick, '98) suggests that these levels of variability in hormones are transferred to developing embryos.

Egg collection and embryogenesis

Experiments were conducted at Lizard Island on the northern Great Barrier Reef, Australia during December 2000. Large clutches of *P. amboinensis* eggs, which had been spawned naturally onto roughened acetate sheets clipped inside half-pipe nest-sites, were used in the experiments. Egg clutches were collected from the fringing reef and placed into an aerated tank of flowing seawater (28°C) within three hours of spawning. Eggs were photographed every 3–6 hours and samples were collected to describe embryogenesis.

Embryogenesis and developmental rhythms

Eggs for observation were removed from the main clutch using a fresh scalpel. Only the rhythms of intact eggs were recorded. These eggs were placed into an observation chamber and supplied with a constant flow of seawater. Movements of the embryo were recorded using a purpose-built microscope (details below). Observations were initiated from the blastomere's asynchronous division (3 hr post-fertilization). During the early developmental stages (before the appearance of the heart), eggs were replaced with fresh eggs from the main egg clutch every 2–3 hours. After the heart had developed, eggs were replaced every 30 min to 1 hr. Rhythmical processes in the eggs were recorded throughout embryogenesis (lasting 105–110 hours after fertilization at 28°C). In early developmental stages, the rhythmic pulsations of the blastodisc margin during epiboly were recorded. This was followed by the relative growth rate of the embryo (described below), the frequency of somites pulsations, the frequency of the myotomal contractions and, lastly, the frequency of lateral flexions by the embryo. Heart rate was also recorded as soon as the heart had developed.

Recording developmental rhythms

Movements of the embryo during development were recorded as a decrease of a light field (in mV)

in a window focused on the developing embyro. To accomplish this, a compound microscope was modified so it was mounted in a horizontal plane and alternations in light levels coming from a constant source (a cold light) behind the microscope stage were measured using a luminometer (Fig. 1). The signal from the luminometer was amplified and passed to the 24-bit analog-to-digital converter (AD7714, *Analog Device*) and stored digitally in the computer (40 times/s). In this way, both slow and fast rhythms associated with embryo development could be recorded. Each egg was placed into a purpose-built perplex chamber (ca. 0.7 ml volume) with flow-through seawater, which was fixed to the microscope stage (Fig. 1). An ocular diaphragm under $80\times$ magnification was used to focus the light on the portion of the embryo of interest. The luminometer converted changes in the light intensity caused by the embryo movement into electrical signals. All processes associated with the muscle movements, such as the separate somites pulsations (pulses/min), myotomes contractions (pulses/10 min), heart rate (beats/min), lateral flexions of the left side of the embryo (pulses/10 min), and general body movements (pulses/10 min) were recorded as the rhythmic changes of a light field. Relative embryo growth during the early observation periods was calculated as the difference in the intensity of illumination from the beginning to last record over the observation time interval.

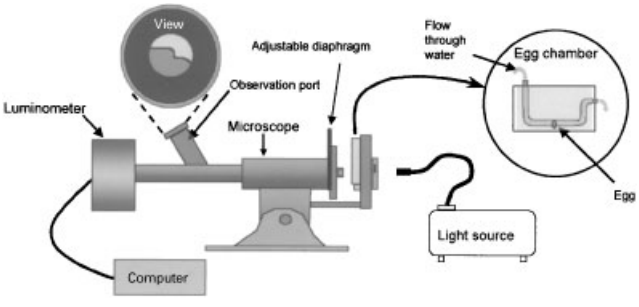

Fig. 1. Diagram of the apparatus used to record developmental rhythms of embryos. Embryos are placed in a purpose-built perspex chamber flushed by a small flow-through water system. The microscope focuses on the tissue or cells of interest and the diaphragm alters the light field so that only the growth plain of interest is in the field of view. The luminometer picks up small changes in the amount of light reaching the eye-piece, converts this into an electrical current and passes it to the computer, which interprets the signal.

To analyse the embryo's rhythmic processes, a power spectral analysis was performed by a Fast Fourier Transformation. This analysis simply determined the maximum frequency of the recorded rhythmical trace and the frequency of the dominant harmonic. All data were expressed as the mean pulse $\pm$ standard error. Data analysis was performed by a Student's *t*-test of means, at an α of 0.05.

Influence of cortisol on embryogenesis

Clutches of eggs were spawned naturally in the field on roughened acetate sheets. The newly spawned clutch was brought back to the laboratory, and the clutch was divided into two equal sized portions with a scalpel. These were then placed into one of two 3 l tanks of aerated seawater representing experimental treatments: a cortisol treatment and a disturbance control. The cortisol treatment involved adding cortisol (Sigma-Aldrich.com) at 2.7×0^{-6} M (10^3 ng/ml) to the seawater. Cortisol is lipophilic so it was dissolved in a small quantity of ethanol (0.3 ml) prior to being added to 200 ml of seawater. The ethanol was subsequently evaporated off and then added to additional seawater to make up the 3 l cortisol-treatment bath. Eggs were then placed within the aerated bath. The control consisted of eggs from the same clutch that were incubated in aerated seawater. Solutions were replaced twice per day.

To ascertain the extent to which cortisol within the eggs was elevated by the treatment bath, a small sample of eggs ($\approx$200) was removed from each control and cortisol-incubated clutch after three days of immersion. These eggs samples were immediately frozen and later processed using standard radioimmunoassay (RIA) techniques (detailed by McCormick, '99) to determine cortisol concentrations. Assays showed that at three days after the start of the treatments, the seawater control eggs had 0.35 pg/egg cortisol whilst the cortisol-incubated eggs had 0.8 pg/egg (t_{5df}=4.634, P=0.006).

To determine the residence time of cortisol in the aerated seawater, 1 ml water samples from the 3l treatment baths were collected, immediately frozen, and later assayed for cortisol using standard RIA techniques (detailed by McCormick, '99). There was no significant decrease in the concentrations of cortisol in the seawater among nine samples collected over a 24 hr period ($F_{8,32df}$=0.472, P=0.865).

RESULTS

Description of the embryogenesis of P. amboinensis

Twelve stages were distinguished in the *P. amboinensis* egg development (Fig. 2 illustrates six of these stages):

1. (3 hr post-fertilization). The cytoplasm has concentrated into a separate, nonyolky mass (blastula) that sits on the passive, noncleaving yolk mass (Fig. 2a). The blastodisc cleaves to form a small-cell morula, which expands to form a blastula. The individual blastomeres gradually join to form a dense epithelial layer, the blastoderm. At this stage, it is no longer possible to distinguish individual cells.

2. (12 hr post-fertilization). A knot-like formation develops at the thinnest part of the blastodisc. This is the embryonic shield, responsible for the formation of the neural plate and cord, the notochord, and somites. The blastoderm starts to invaginate along the surface of the yolk sphere toward the vegetal pole. The germ layers start to form: the ectoderm, the endoderm, and the mesoderm.

3. (18 hr post-fertilization). The embryonic disc continues to spread in a thin layer over the surface of the yolk. An unsegmented embryo body begins to form (Fig. 2b). Eventually, the edges of the blastodisc converge, surrounding the whole yolk, and the blastopore closes (epiboly). At this time, the rudiments of the brain, notochord, and somites have formed.

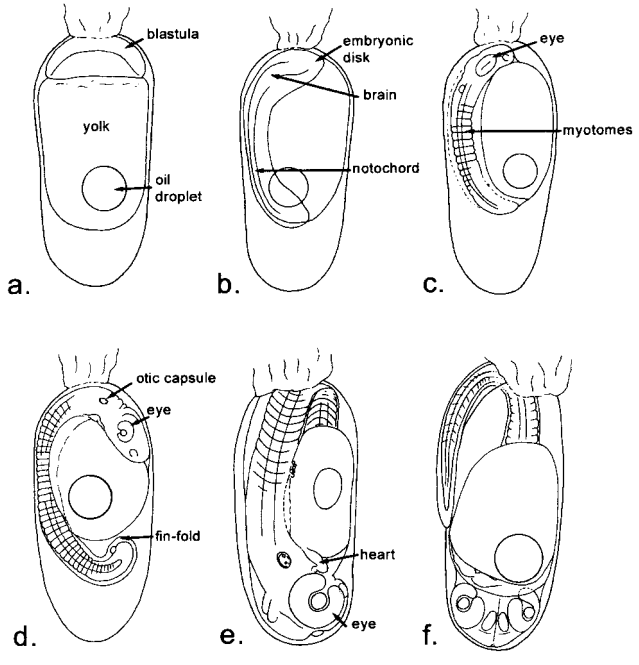

Fig. 2. Illustration of stages during the embryogenesis of *P. amboinensis*. **a**. blastula stage (stage 1); **b**. mid-gastrula (stage 3); **c**. formation of the first somites (stage 4); **d**. separation of the tail from the yolk sac; **e**. formation of main organs and systems; **f**. just prior to hatching.

4. (23 hr post-fertilization). Organogenesis occurs, with the formation of the organs of the head and trunk. Rudimentary eyes form as elongated vesicles (Fig. 2c), and within hours these become more rounded. The number of myotomes gradually increases, and the intestine and pericardial cavity become apparent.

5. (32 hr post-fertilization). Trunk segmentation is completed and the tail section displays rapid growth and begins to segment. The brain differentiates into the forebrain, midbrain, and hindbrain. The fin-fold forms around the tail and extends halfway down the elongated yolk sac (Fig. 2d).

6. (35 hr post-fertilization). The first movements of the heart are registered. Segmentation of the tail is complete. The anterior of the head separates from the yolk sac and embryonic respiratory organs develop. Subsequent to the development of a rudimentary heart, the state of heart development was used as a key criterion for the division of embryogenesis into further stages.

7. (40 hr post-fertilization). The heart is fully formed, consisting of the antechamber, the chamber, and the bulbus arteriosus. Also formed are the *aorta dorsalis*, the *arcus mandibularis*, the *arteria caudalis*, the *vena cardinalis anterior* and the *vena cardinalis posterior*, the *vena caudalis inferior*, the *vena caudalis posterior*. The head remains bent toward the yolk sac.

8. (52 hr post-fertilization). The head continues to separate from the yolk sac (Fig. 2e). The transition of the *arteria caudalis* into the *vena caudalis* has migrated to the last caudal myotome. The *vena caudalis* remains branched into the post-anal part of the fin-fold. Segmental veins and arteries continue to form and the vascular system of the head develops.

9. (67 hr post-fertilization). The head straightens and separates from the yolk sac. Gill arteries form and a network of branches of the *vena caudalis inferior* starts to form in the post-anal fin-fold. The *arteria caudalis* has developed, closely following below the notochord up to the last caudal myotome. Here, it changes into the *vena caudalis*, going in the opposite direction bordering the lower margin of the body up to where the caudal fin meets the trunk, and changes

into the *vena subintestinalis*. A clump of mesenchymal tissue is present where the anal and pectoral fins will later develop.

10. (80 hr post-fertilization). Muscular buds form inside the mesenchymal tissue in the vicinity of the dorsal and anal fins. The pericardial cavity continues to increase in size. The loop of the arteria caudalis in the caudal part of the body extends to the end of the notochord.

11. (95 hr post-fertilization). Pterigiophores form between muscular buds of anal and dorsal fins. First, lepidotrichias appear in the caudal fin. The yolk sac is rapidly being resorbed and the course of the *vena caudalis* changes.

12. (105 hr post-fertilization). The embryo is fully formed (Fig. 2f). A small portion of the larvae that hatch lie on their sides prior to initiating swimming, typical of the ''pre-larvae'' described by Kryzanovskij ('49) and Balon ('60, '85).

Influence of cortisol on embryogenesis

A comparison of the control and cortisol-treated eggs showed that during the early developmental stages (up to the flattening of blastodisc and beginning of the cell-material growth around the yolk surface), cortisol had no detectable effect on rates of cell pulsations (i.e., up to 18 hr post-fertilization). During the cleavage of blastula cells and before the beginning of asynchronous cell divisions, the blastodisc growth was characterized by pulsations of the individual cells. The frequency of these blastomere pulsations was 58 pulses/min ca 7 hr post-fertilization and 60 pulses/min ca 14 hr post-fertilization for eggs from both the control and cortisol treatments (Fig. 3).

The first difference in development of the control and cortisol treatment eggs was observed at the start of gastrulation ($\approx$9 hr post-fertilization). This difference was manifest in the relative growth rates of the embryos rather than their rates of pulsation. At this stage, the pulsations were characterized by periods of activity, interspersed by periods of inactivity (Fig. 4). During the active periods, the relative growth rates of embryos from cortisol-incubated eggs were twice as high as that of embryos from the control eggs. At 9 hr after fertilization, the cortisol embryos exhibited a mean relative growth rate (RGR) of 0.389 mV/min, whilst the control had a RGR of 0.156 mV/min. This difference was further emphasized at 18 hr post-fertilization, at which time the controls had a RGR of 0.048 mV/min compared to a RGR of 0.122 mV/min for the cortisol incubated embryos. However, not only did the cortisol-supplemented embryos exhibit higher growth during the active periods, but the periods of inactivity that followed each growth phase were far longer than shown by control embryos (e.g., at $\approx$9 hr after fertilization, cortisol-treated embryos were inactive for 96 min $\pm$ 30 min (n=22), whilst controls were inactive for 11 min $\pm$ 4 min (n=27) (Fig. 4). Averaged over the whole of gastrulation, therefore, control embryos had a slightly higher

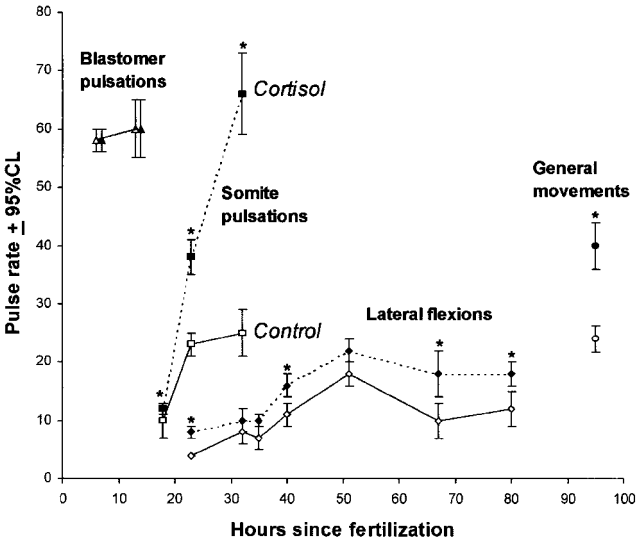

Fig. 3. Changes in the pulse rates of various tissue types over embryogenesis for control (solid lines, open symbols) and cortisol-supplemented (dashed lines, solid symbols) embryos. Tissues included are: somatic cells (pulses/min); lateral flexions of the whole embryo (that start as simple myotomal contractions, pulses/10 min); general body movements (pulses/10 min). Sample positions after 50 hr represent midpoints of 2–3 hr sampling periods. Asterisk displays significant differences between control and cortisol treatment means by Student's *t*-test.

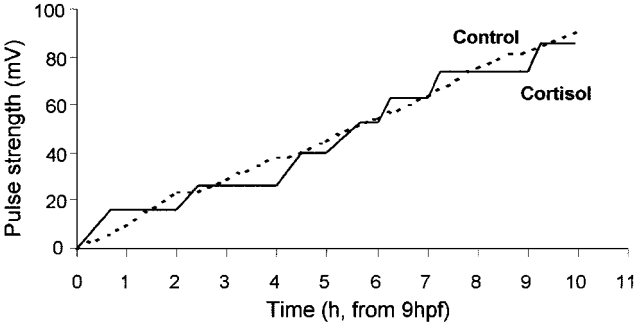

Fig. 4. The average relative growth (mV) of *P. amboinensis* from 9 hr post-fertilization for control and cortisol-supplemented embryos.

overall growth than the cortisol-supplemented embryos, despite the latter displaying much higher relative growth during the active periods. Interestingly, not only were the cells pulsing with a frequency of ≈ 60 pulses/min during early development, but the overall blastodisc exhibited slow rhythmic pulsations of 14–20 pulses/min.

Once the embryo axial had started to form, pulsations of the first pairs of somites were recorded (≈ 18 hr). In both the control and cortisol-incubated embryos, these pulsations took the form of rapid bursts of two to three pulses (lasting on average 4.1 sec $\pm$ 0.8 se), followed by an inactive period averaging 16 sec $\pm$ 4.4 se. There were 3–4 periods of activity per minute. At this time, there was no difference in somite pulse rate between control embryos and those from the cortisol incubation (Fig. 3).

At 23 hr after fertilization, the separate somites displayed regular pulses in embryos from both treatments (Fig. 5b,d). The frequency of the pulses in the control embyros (23 pulses/min) was significantly lower than that for the cortisol-incubated embryos (38 pulses/min) (Fig. 3).

At the same time, the myotomes formed and the first whole body movements were recorded (lateral flexions, Fig. 3). These movements consisted of lateral flexions of the body due to contractions in the first frontal myotomes. At this stage, the myotomes were not innervated, and the lateral flexions were irregular (Fig. 5a,c). Embryos from the control displayed a lower frequency of lateral flexions than the cortisol-incubated embryos (four flexions/10 min versus eight flexions/10 min, respectively, Fig. 3).

During the development of the myotome band (32–50 hr post-fertilization), the frequency of the somites pulsations increased significantly in the cortisol-treated embryos (Fig. 5h) compared to the controls (Fig. 5f), reaching up to 66 pulses/min (Fig. 3). At this stage, there was virtually no difference in frequency of embryo lateral flexions between control and cortisol-incubated embryos (Fig. 5e,g).

The frequency of flexions increased during development in both experimental and control embryos and reached a maximum mean value as the embryo head separated from the yolk sac at 50 hr after fertilization (Fig. 3) Two to 3 hr after the separation of the head, it was observed that the embryo's flexions were neither regular nor continuous in either group, with 2–3 movements in each active episode.

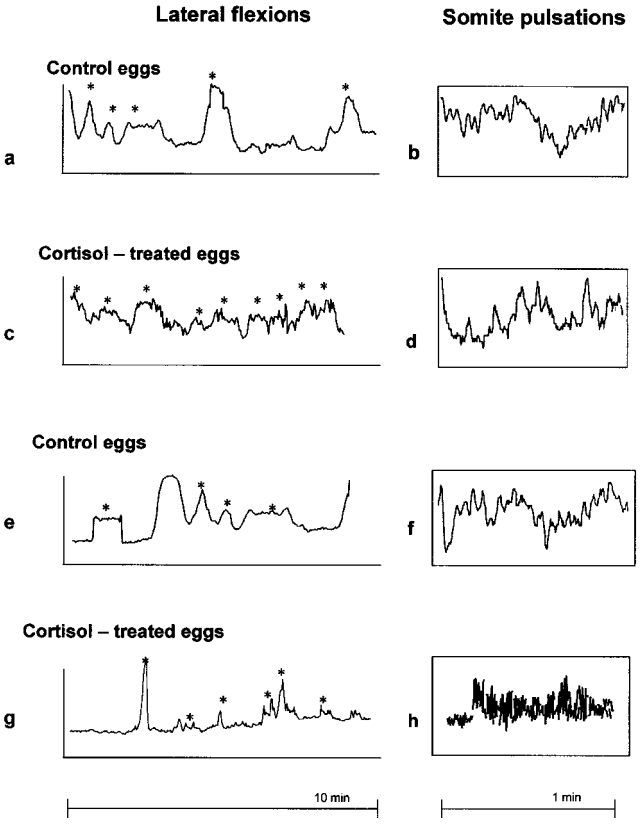

Fig. 5. Recordings of rhythms for *P. amboinensis* embryos at 23 hr (**a–d**) and 40 hr (**e–h**) after fertilization incubated in seawater (control) and incubated in a moderate concentration of cortisol. Typical examples of pulsation signatures of lateral body flexions (a,c,e,g) and individual somites (b,d,f,h) are displayed. Pulse strength (mV) is given on the y-axis. Stars indicate the timing of simple flexions.

Movements that had become characteristic during embryonic development changed six hours prior to hatching to overall embryonic movements. These movements increased in frequency up to hatching, with the frequency of embryo movements being lower in the control embryos (24 per 10 min) than those subjected to cortisol (40 per 10 min Fig. 3). Rhythmically, these movements were different from the lateral flexions that occurred at earlier developmental stages, being organized in bursts of 3–5 movements.

The heart appeared one to 1.5 hr early in the cortisol-incubated embryos than in the control embryos. Moreover, the initial heart rate in the cortisol-incubated embryos was higher than for the controls (Fig. 6). Interestingly, when the heart first developed, the heartbeat of the control embryos was considerably more arrhythmic than in the cortisol-incubated embryos (24% versus 8% instability, respectively).

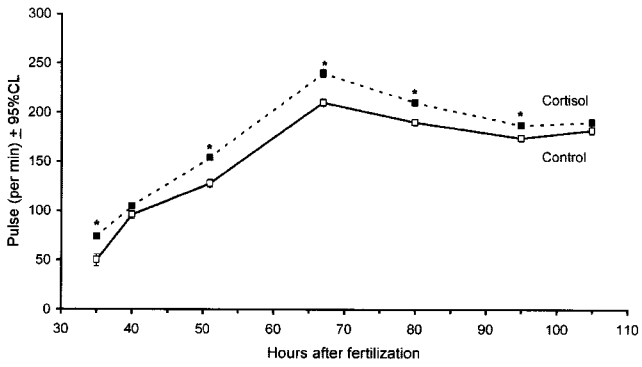

Fig. 6. Heart rates of *P. amboinensis* embryos incubated in seawater (controls) and a moderate concentration of cortisol. Asterisks displays significant differences between control and cortisol treatment means by Student's *t*-test. Note that some of the standard errors are obscured by the symbols.

Five hours after the heart appeared as a simple tube (≈40 hours after fertilization), the heart rate in both groups became stable. The standard deviation of heart rate, both for individual embryos and within treatments, was approximately eight beats per minute (bpm). At this stage, the differences in the heart rate between control and experimental embryos was minimal (Fig. 6).

The heart rate of both groups increased as the embryo circulatory system developed and the embryo body segmented (≈50 hr post-fertilization). The heart rate in all the embryos increased 2–3 hours after the circulatory system had started to form, reaching 154 bpm in the cortisol-incubated embyros and only 128 bpm for the controls (Fig. 6).

In all embryos, a sudden increasing of the heart rate at 66 hr after fertilization was associated with the completion of the embryonic circulatory system and a high embryo motor activity at this time. In the experimental embryos, the heart rate reached 240 bpm, in comparison to 210 bpm for the control embryos (Fig. 6). This high heart rate was maintained for 3–4 hours, after which it gradually decreased. Approximately ten hours before hatching, the heart rate was 174 bpm for the control embryos as compared to 187 bpm for the cortisol-incubated embryos (Fig. 6). There is a suggestion that the heart rate increased again just before the hatching, at the start of the egg membrane dissolution, reaching 182 bpm in the control and 190 bpm in the cortisol-incubated embryos, though these increases are not statistically significant. At this time, the difference in heart rate between cortisol-incubated and control embryos was also not significant (Fig. 6).

DISCUSSION

This study is the most complete description of the embryonic rhythms of any fish to date. Other studies have examined the rhythmical processes during small parts of embryology in isolation (e.g., Gideiri and Babiker, '66; Yusa, '67; Richards and Pollack, '87; Saint-Amant and Drapeau, '98). The present study has assessed the rhythms of embryos collected soon after fertilization in the field, where the parents have reproduced under natural conditions, rather than having been obtained from cultured stocks that have been subjected to unnatural levels of stress. Evidence from the present and previous studies (Campbell et al., '92, '94; McCormick, '98, '99) shows that any sort of maternal stress, be it naturally induced or from brood-stock maintenance, will influence cortisol levels within the eggs and hence developmental rhythms. The noninvasive technique used in the present study has the advantage of requiring minimal disruption of the embryos, in contrast to other techniques that require removal of the embryo from its egg case (e.g., Saint-Amant and Drapeau, '98).

Interestingly, individual embryos within a clutch of eggs, and among clutches laid under similar conditions, displayed similar developmental rhythms. Although the frequency of pulsations differed between the control and cortisol-supplemented treatments, the frequencies displayed the same trends in change through embryology. This suggests that the patterns in the pulse-frequencies displayed throughout embryology may be a characteristic of the species, rather than simply an individual. Earlier work on rhythms during embryogenesis supports the species-specific nature of these rhythms (e.g., Tracy, '25; Armstrong, '36; Tavolga, '49; Gideiri and Babiker, '66; Nechaev et al., '92).

A hierarchy of superimposed rhythms was evident in the tissues measured. Myotomal muscle blocks contracted irregularly at first, but then showed regular, spontaneous contractions of 4–10 contractions per 10 min. Meanwhile, within the muscle, the individual somatic cells pulsed at a rhythm of between 10–66 pulses/min. Recently, Saint-Amant and Drapeau ('98) also found that embryos of the zebra fish, *Danio rerio*, removed from their eggs and restrained by agar, exhibited spontaneous contractions of their tails at frequencies up to a maximum of 60 pulses/min. However, it was unclear from this study how the ontogenetic patterns in these contractions related to their

natural rhythms due to the extensive manipulation of the embryo prior to recording. Spontaneous movements have also been observed in chick embryos (Hamburger and Balaban, '63; Alconero, '65). For both the zebra fish and chick, manipulations showed that these rhythmical movements were mediated by spinal neurons (Ho and O'Donovan, '93; Saint-Amant and Drapeau, '98). In *P. amboinensis*, the initiation of spontaneous contraction is concurrent with the development of the notochord, and it is likely that the spinal cord is the site for precocious development of vertebrate locomotor functions (Saint-Amant and Drapeau, '98).

Supplementation of cortisol, within naturally occurring limits, directly increased the rhythms associated with the growth processes and motor activity of the embryos. Interestingly, cortisol had an impact on every rhythm measured including: the growth pulse of the first somite cells; growth and contraction of the myotomal muscle blocks; heart rates; and later, the flexion of the whole embryo body. By 23 hr after fertilization, the frequency of somite pulses in the cortisol-supplemented embryos deviated from the controls suggesting that just prior to this time, receptors for cortisol developed within the target cells.

It is noteworthy that the concentrations used in this study equate only to moderate levels of cortisol compared to the range of levels found in naturally spawned eggs. A comparison of cortisol concentrations induced by the incubation bath in the present study (0.8 pg/egg) to concentrations in wild eggs (McCormick, '99) indicates that the experimental concentrations are within the upper quartile of the distribution of cortisol concentrations for wild eggs. Higher concentrations (concentrations of 12.8 pg have been found in newly spawned *P. amboinensis* eggs, McCormick, '99) may be expected to produce a more marked response and emphasize the importance of maternally-derived cortisol in influencing embryonic developmental rhythms.

Growth in the early stages of development, from gastrulation to knot formation, occurred in stanzas, with alternating periods of growth and stasis. For *P. amboinensis,* this pattern of discontinuous growth was emphasized in the cortisol-supplemented embryos, which displayed short periods of active growth followed by long periods of stasis compared to the control embryos. Despite the cortisol-supplemented fish having higher relative growth rates during their active periods, the long rest periods resulted in lower overall growth up to knot formation. This complements the finding of our previous studies that *P. amboinensis* larvae hatch at smaller sizes when exposed to higher levels of maternal or experimentally manipulated cortisol (McCormick, '98, '99).

Research suggests that this discontinuous growth trajectory is due to the energetic requirements of active growth. The acceleration of embryo growth evoked by cortisol requires an increase in energy consumption, but the process of the energy supplementation may not be able to supply the demand. This supplementation rate may be limited by factors such as proteolytic activity of the ferments hydrolyzing yolk protein and the rate of O_2 consumption (Terner, '68, '79; Hamor and Garside, '77; Hughes, '80; Boulekbach, '81; Davenport, '83). Therefore, the pause in embryo growth (after the growth spurt) in the cortisol-supplemented embryos may be necessary to restore the equilibrium between anabolic and catabolic processes and this may be longer than in the controls.

The embryonic metabolism may be more sensitive to levels of cortisol than later in life. Cortisol within the egg, together with other lipophilic hormones (such as thyroxine), appears to be of maternal origin, being transferred into the oocyte during gametogenesis via vitellogenin, or by passive diffusion (Mommsen et al., '99; Tagawa et al., 2000). Studies of temperate flatfishes, tilapia, and salmonids suggest that corticosteroids, although metabolized, are not produced by young fish until after hatching (De Jesus et al., '91; Hwang and Wu, '93; Barry et al., '95; Tanaka et al., '95; Sampath-Kumar et al., '97). It is therefore unlikely that the hypothalamus-pituitary-interrenal axis, responsible for the production and regulation of cortisol, is fully functional during embryogenesis in many species. Since cortisol concentrations in the plasma are largely regulated through negative feedback of cortisol at the level of the hypothalamus and pituitary (Mommsen et al., '99), down-regulation of maternally- derived concentrations may not be possible.

Cortisol is a multifaceted hormone with an extraordinarily broad range of physiological and metabolic effects (Mommsen et al., '99; Schreck et al., 2001). Increased levels of cortisol can affect the utilization of yolk in many different ways. The yolk of teleosts is comprised mainly of protein ($\approx$65%), but it also contains moderate quantities of energy-rich lipid ($\approx$22%) and small quantities of carbohydrate ($<$3%) (Heming and Buddington, '88). Cortisol affects the metabolism of all of these

potential energy sources. It is generally agreed that cortisol exerts a proteolytic action as part of its widespread catabolic activity (Mommsen et al., '99). Plasma amino acids tend to increase in fish with cortisol implants (Vijayan et al., '97) and glucocorticoids are known to retard tissue growth (e.g., Lee et al., '86). Therefore, although cortisol potentially provides more amino-acid building blocks through its catabolic activity on the yolk protein, it lowers protein synthesis and so can be expected to reduce tissue growth. Cortisol also seems to possess a strong peripheral and hepatic lypolytic action, resulting in an increase in plasma fatty acids, though the fate of these fatty acids appears to vary among species (Wiegand, '96; Mommsen et al., '99). Lastly, cortisol modulates hepatic glucose metabolism (Vijayan et al., '94), generally stimulating gluconeogenesis and increasing liver glycogen (Mommsen et al., '99). Cortisol, therefore, plays a key role in mobilizing energy stores into forms readily available to the developing embryo.

Evidence presented in the current study suggests that much of the energy mobilized by the action of cortisol is allocated to motor activity, with the cortisol-supplemented embryos showing twice the levels of lateral contractions and whole body movement from 23 hr after fertilization than control embryos. Studies suggest that this embryo motor activity mixes the perivitelline fluid and increases circulation of water near the outer surface of the egg. This increased embryo activity may consequently enhance gas exchange near respiratory surface of the embryo body (Bautzmann, '56; Fry, '57). The finding of McCormick ('98, '99) that cortisol-treated *P. amboinesis* eggs yielded smaller larvae at hatching suggests that increased activity is at the expense of growth.

Surprisingly, cortisol did not affect the developmental rate of the embryo despite speeding up the metabolism. Eggs that had been incubated with additional cortisol reached each embryonic stage at the same time and finally hatched at the same time as the control embryos. Thus, although cortisol had a dramatic effect on the rhythms of growth and levels of movement of the embryos, it had little or no impact on the process of development. This finding supports the results of Stratholt et al. ('97) for coho salmon (*Oncorhynchus kisutch*), who found that elevated levels of cortisol in eggs did not alter time to hatching compared to controls. However, treatment of teleost eggs with cortisol after hatching produces slightly different and varying results. Kim and Brown ('97) found for the Pacific threadfin (*Polydactylus sexfilis*) that 1 hr post-hatch immersion in cortisol did not influence gut development compared to controls; however, it did reduce the frequency of spinal deformations, suggesting some influence on development. Mathiyalagan et al. ('96) immersed yolk-sac larvae of tilapia, *Oreochromis mossambicus*, for 2 wks in a range of concentrations of cortisol and found that cortisol not only influenced growth, but also accelerated yolk absorption and the onset of free-swimming activity. It is likely that the effect of maternal cortisol on larval development will depend strongly on the timing of the development of the hypothalamus-pituitary-interrenal axis (that regulates cortisol) and the extent to which excess maternal cortisol can be cleared from the embryo (Stratholt et al., '97).

In summary, cortisol-supplementation of fertilized damselfish eggs within naturally occurring limits dramatically affected embryo somatic rhythms including somite growth pulsations, myotomal contractions, lateral body flexions, and heart rates. Interestingly, although cortisol affected the periodicity of cell growth, it did not influence developmental rate. This study suggests that the maternal endowment of cortisol to eggs plays a key role in determining the rhythms by which the embryo grows. Since larval mortality agents are often selective for small size and slow growth (Meekan and Fortier, '96; Wilson and Meekan, 2002; Bergenius et al., 2002), the impact that maternal cortisol has on growth may have important consequences for larval survival.

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LITERATURE CITED

Alconero BB. 1965. The nature of the earliest spontaneous activity of the chick embryo. J Embryol Exp Morphol 13:255–266.

Armstrong PM. 1936. Mechanism of hatching on *Fundulus heteroclitus*. Biol Bull 71:407.

Balon EK. 1960. On the periodisation of the fish life and its terminology. Biologia 15:864–876.
Balon EK. 1985. Early life history of fishes: new developmental, ecological and evolutionary perspectives. Boston, W. Junk Publishers. p 280.
Barry TP, Malison JA, Held JA, Parrish JJ. 1995. Ontogeny of the cortisol stress response in larval rainbow trout. Gen Comp Endocrinol 97:57–65.
Bautzmann H. 1956. Fruckthullenmotorik und Embriokenese. Ihre Natur und ihre Bedeutung Fur eine physiologische Embrionalenwicklung bei Tier und Mensh. Arch Gynacol 187:519–545.
Bell GR, Hoskins GE, Bagahaw JW. 1969. On the structure and enzymatic degradation of the external membrane of the salmon egg. Can J Zool 47:146–148.
Bergenius MAJ, Meekan MG, Robertson R, McCormick MI. 2002. Larval growth predicts recruitment success of a coral reef fish. Oecologia 131:521–525.
Blaxter JHS. 1969. Development: eggs and larvae. In: Hoar WS, Randall DJ, editors. Fish physiology, vol 3. New York: Academic Press. p 178–252.
Boulekbach H. 1981. Energy metabolism in fish development. Amer Zool 21:377–389.
Campbell PM, Pottinger TG, Sumpter JP. 1992. Stress reduces the quality of gametes produced by rainbow trout. Biol Reprod 47:1140–1150.
Campbell PM, Pottinger TG, Sumpter JP. 1994. Preliminary evidence that chronic confinement stress reduces the quality of gametes produced by brown and rainbow trout. Aquaculture 120:151–169.
Davenport J. 1983. Oxygen and developing eggs and larvae of the lumpfish, *Cyclopterius lumpus*. J Mar Biol Assoc UK 63:633–640.
De Jesus EG, Hirano T. 1992. Changes in whole body concentrations of cortisol, thyroid hormones and sex steroids during early development of the chum salmon, *Oncorhynchus keta*. Gen Comp Endocrinol 85:55–61.
De Jesus EG, Hirano T, Inui Y. 1991. Changes in cortisol and thyroid hormone concentrations during early development and metamorphosis in the Japanese flounder, *Paralichthys olivaceus*. Gen Comp Endocrinol 82:369–376.
Fry FEJ. 1957. The aquatic respiration of fish. In: Brown ME, editor. The physiology of fishes-metabolism. Vol. 1 in a series.
Gideiri A, Babiker J. 1966. The behavior and neuroanatomy of some developing teleost fishes. J Zool 149:215–241.
Gregory TR, Wood CM. 1999. The effects of chronic plasma cortisol elevation on the feeding behaviour, growth, competitive ability, and swimming performance of juvenile rainbow trout. Physiol Biochem Zool 72:286–295.
Hamburger V, Balaban M. 1963. Observation and experiments on spontaneous rhythmical behavior in the chick embryo. Dev Biol 7:533–545.
Hamor T, Garside ET. 1977. Size relations and yolk utilization in embryonated ova and alevins of Atlantic salmon *Salmo salar L.* in various combinations of temperature and dissolved oxygen. Can J Zool 55:1892–1898.
Heming TA, Buddington RK. 1988. Yolk-absorption in embryonic and larval fishes. In: Hoar WS, Randall DJ, editors. Fish physiology, vol. 11. New York: Academic Press. p 407–446.
Ho S, O'Donovan MJ. 1993. Regionalization and intersegmental coordination of rhythm generating networks in the spinal cord of the chick embryo. J Neurosci 13:1345–1371.
Holliday FGT, Blaxter JHS, Lasker R. 1964. Oxygen uptake of developing eggs and larvae of the herring (*Clupea harengus*). J Mar Biol Assoc UK 44:711–723.
Hughes GM. 1980. Morphometry of fish gas exchange organs in relation to their respiratory function. Environ Physiol Fishes Lect NATO Adv Study Inst, Lennoxville, NY, 22–25 August 1979. New York. p 33–56.
Hwang PP, Wu SM. 1993. Role of cortisol in hypoosmoregulation in larvae of the tilapia (*Oreochromis mossambicus*). Gen Comp Endocrinol 92:318–324.
Hwang PP, Wu SM, Lin JH, Wu LS. 1992. Cortisol content of eggs and larvae of teleosts. Gen Comp Endocrinol 86: 189–196.
Kamler E. 1976. Variability of respiration and body composition during early developmental stages of carp. Polish Archs Hydrobiol 23:431–485.
Kerrigan BA. 1997. Variability in larval development of a tropical reef fish (Pomacentridae: *Pomacentrus amboinensis*): the parental legacy. Mar Biol 127:395–402.
Kim BG, Brown CI. 1997. Interaction of cortisol and thyroid hormone in the larval development of Pacific threadfin. Amer Zool 37:470–481.
Kryzanovskij SG. 1949. The rules of ecological and morphological development in Cyprinoidea and Siluroidea. J Inst Morphol AN SSSR 1:5–332 (in Czech).
Lee LEJ, Pochmursky V, Bols NC. 1986. Effect of corticosteroids on the morphology and proliferation of two salmonid cell lines. Gen Comp Endocrinol 64:373–380.
Mathiyalagan A, Reddy P, Lam T. 1996. Effects of cortisol on growth and development in tilapia larvae, *Oreochromis mossambicus*. Fish Physiol Biochem 15:453–458.
McCormick MI. 1998. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology 79:1873–1883.
McCormick MI. 1999. Experimental test of the effect of maternal hormones on larval quality of a coral reef fish. Oecologia 118:412–422.
Meekan M, Fortier L. 1996. Selection for fast growth during the larval life of Atlantic cod *Gadus morhua* on the Scotian Shelf. Mar Ecol Prog Ser 137:25–37.
Mommsen T, Vijayan M, Moon T. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Reviews in Fish Biology and Fisheries 9:211–268.
Nechaev IV, Labas Yu A, Denisov VA. 1992. Influence of catecholaminergic compounds of a bony fish *Aequidens pulcher* (*Cichlidae*). J Gen Biol 53:258–271 (in Russian).
Reznichenko PN. 1982. Development and changes of the functional mechanisms during ontogenesis of low vertebrate animals. Moscow: Nauka.
Richards C, Pollack ED. 1987. Development of spontaneous motility in the guppy embryo (*Lebistes reticulatus*) and the effect of spinal transection. J Exp Zool 244:223–229.
Saint-Amant L, Drapeau P. 1998. Time course of the development of motor behaviours in the zebra fish embryo. J Neurobiol 37:622–632.
Sampath-Kumar R, Lee ST, Tan CH, Munro AD, Lam TJ. 1997. Biosynthesis *in vivo* and excretion of cortisol by fish larvae. J Exp Zool 277:337–344.
Schreck CB, Contreras-Sanchez W, Fitzpatrick MS. 2001. Effects of stress on fish reproduction, gamete quality, and progeny. Aquaculture 197:3–24.

Stratholt M, Donaldson E, Robin LN. 1997. Stress induced elevation of plasma cortisol in adult female coho salmon (*Oncorhynchus kisutch*), is reflected in egg cortisol content, but does not appear to affect early development. Aquaculture 158:141–153.

Tagawa M, Suzuki K, Specker JL. 2000. Incorporation and metabolism of cortisol in oocytes of tilapia *(Oreochromis mossambicu*s). J Exp Zool 287:485–492.

Tanaka M, Tanangonan J, Tagawa M, de Jesus E, Nishida H, Isaka M, Kimura R, Hirano T. 1995. Development of the pituitary, thyroid and interrenal glands and applications of endocrinology to the improved rearing of marine fish larvae. Aquaculture 135:111–126.

Tavolga WN. 1949. Embryonic development of Platyfish (*Platypoecolis)*, the Swordtail *(Xiphophorus)* and their hybrids. Bull Amer Mus Nat Hist 94:1–229.

Terner C. 1968. Studies of metabolism in embryonic development. Glycogenesis and glyconeogenesis in trout embryos. Comp Biochem Physiol B 25:989–1003.

Terner C. 1979. Metabolism and energy conversion during early development. In: Hoar WS, Randall DJ, editors. Fish physiology, vol. 8. New York: Academic Press. p 261–278.

Tracy HC. 1925. The relation of carbon dioxide to spontaneous movements in the larva of *Opsanus tan*. Biol Bull 48: 408–431.

Van Gestel WJ, te Kronnie G, Stroband HW. 1998. Blastoderm structure, cell migration and formation of the embryonic shield during gastrulation in the carp (*Cyprinus Carpio*); a scanning electron microscopic study. Eur J Morphol 36: 65–75.

Vijayan MM, Pereira C, Moon TW. 1994. Hormonal stimulation of hepatocyte metabolism in rainbow trout following an acute handling stress. Comp Biochem Physiol 108C: 321–329.

Vijayan M, Pereira C, Grau E, Iwama G. 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp Biochem Physiol C Toxicol Pharmacol 116:89–95.

Warga RM, Kimmel CB. 1990. Cell movements during epiboly and gastrulation in zebrafish. Development 108:569–580.

Wiegand M. 1996. Composition, accumulation and utilization of yolk lipids in teleost fish. Reviews in Fish Biology and Fisheries 6:259–286.

Wilson DT, Meekan MG. 2002. Growth related advantages for survival to the point of replenishment in the coral reef fish, *Stegastes partitus*. Mar Ecol Prog Ser 231:247–260.

Wood A, Thorogood P. 1994. Patterns of cell behaviour underlying somitogenesis and notochord formation in intact vertebrate animals. Dev Dyn 201:151–167.

Yusa T. 1967. Embryonic development and larval of the atka mackerel, *Pleuzogramus azonus.* Jordan et Mets Sci Rep, Tohoku. Univ Ser 33:301–317.