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Sex Differences in Steroid Hormones and Parental Effort across the Breeding Cycle in *Amphiprion ocellaris*

Ross S. DeAngelis[1] and Justin S. Rhodes[1]

While it has traditionally been viewed that high androgens are a hindrance to male parental care, recent studies in several vertebrate taxa have shown the opposite pattern, where high androgens either co-occur with, or are necessary for high parental investment. These inconsistencies suggest that in order to develop a complete understanding of the role sex steroids play in parental care, it is important to study multiple species with varying life-history characteristics. Anemonefishes of the genus *Amphiprion* provide a useful complement to more classically studied systems within vertebrate, and more specifically teleost models of parental care. Therefore, parental behaviors and blood plasma levels of 11-ketotestosterone (11-KT), estradiol (E2), and cortisol were measured in five breeding pairs at three points in the spawning cycle. Males displayed 5.6-fold more parental behaviors and spent 67% of their time in the nest as compared to 12% in females, along with significantly higher levels of 11-KT (males = 0.75±0.076; females = 0.02±0.005 ng/ml). Alternatively, females displayed higher levels of E2 (males = 0.09±0.009; females = 3.65±0.655 ng/ml), and E2 fluctuated across the breeding cycle with low levels on the day eggs were laid and higher levels as eggs developed. Cortisol tended to be higher in males, and higher in breeders than non-breeders, though these differences were not significant (males = 35.8±11.01; females = 16.9±3.58 ng/ml). Results suggest *A. ocellaris* may be a useful model for studying paternal behavior in the presence of high androgens.

In many species, across a wide range of taxa, parental care is essential for offspring survival. Parental effort is often divided between the sexes in a sex-specific manner, and steroid hormones play a critical role in the regulation of these behaviors (Kelley, 1982; Ball et al., 2002; Champagne et al., 2003; Munakata and Kobayashi, 2010). In most mammals, females are the primary caregivers, and high estrogens and low androgens are associated with high parental investment (Trivers, 1972). Moreover, numerous studies among birds, mammals, and fishes support an inverse relationship between androgens and parental care in males (Wingfield et al., 1990; Pall et al., 2002; Hirschenhauser and Oliveira, 2006). On the other hand, many teleosts show the opposite pattern with males performing the majority of parental care, and in these species, high parental investment is associated with high androgens and low estrogens.

Within males, variation in circulating steroid hormones have been associated with parental effort, but in different ways depending on the species. For example, in male Garibaldi (*Hypsypops rubicundus*) and also the Three-spined Stickleback (*Gasterosteus aculeatus*), 11-ketotestosterone (11-KT; the teleost male bioactive androgen) levels were higher during courtship and lower during parental care. Similarly, in male damselfish (*Chromis dispilus*), a subset of gonadotropin-releasing hormone (GnRH) mediated androgens was higher during courtship and lower during the egg brooding period (Pankhurst and Peter, 2002). Other species have shown the opposite pattern. In the Blue-banded Goby (*Lythrypnus dalli*), experienced parental males have higher 11-KT levels than non-brooding males (Rodgers et al., 2006), and administration of carbenoxolone (CBX), an antagonist that blocks the conversion of 11-keto-androsterone to 11-KT, significantly reduced paternal effort (Pradhan et al., 2014).

In the Bluegill Sunfish (*Lepomis macrochirus*) and Plainfin Midshipman (*Porichthys notatus*), relationships between 11-KT, egg care, and embryo care change across the breeding cycle (Knapp et al., 1999). Levels of 11-KT in the Bluegill Sunfish were highest prior to spawning, then lowered during brood care, before increasing again prior to egg hatching (Magee et al., 2006). Taken together, results suggest that the pattern of sex-specific roles in parental care and underlying steroid hormone levels in teleost fishes is highly species specific and in some species changes across the breeding cycle. Therefore, more species with varied life histories and social dynamics are needed to gain a comprehensive understanding of the relationship between sex steroid hormones and parental care across vertebrate taxa (Amundsen, 2003).

Anemonefishes from the genera *Amphiprion* and *Premnas* represent an unusual social system, complementary to other more studied species such as the Three-spined Stickleback and Blue-banded Goby. Anemonefish display monogamous pair bonds between a dominant alpha female and beta male, which form long before mating occurs and can last for decades (Ross, 1978a; Fricke, 1979). Furthermore, anemonefish are protandrous hermaphrodites. These fish hatch with both ovarian and testicular tissue within the gonads, but during ontological development female gonads change to predominantly ovarian while males retain both testicular and ovarian tissue (Fricke, 1983; Godwin, 1994a). Thus, the protandrous, female-dominant anemonefish provides an interesting contrast to the other protogynous or bi-directional male-dominant species (Semsar et al., 2001; Rodgers et al., 2006), and research in this group may elucidate the dynamic relationship between steroid hormones and parental care.

Among the anemonefish species, *Amphiprion ocellaris* is particularly felicitous for laboratory research. *Amphiprion ocellaris* is one of the smallest anemonefishes, and is highly dependent on its protective host sea anemones (Santini and Polacco, 2006). Small size and high host dependence has led to spatial restriction, and consequently small home ranges. While small in comparison to other species of *Amphiprion*, they are still large enough to enable blood draws to be easily performed without sacrificing the animal. Together, these conditions enable semi-natural behavior to be observed in the laboratory while at the same time facilitating accurate determination of circulating steroid hormones.

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Only a handful of studies have measured sex steroid levels and behavior in mature anemonefish. In field studies of the Cinnamon anemonefish (*A. melanopus*), males were the predominant caretakers and increased the number of fanning acts, as well as time in the nest over the eight-day incubation period of the eggs (Ross, 1978b). In Clark's anemonefish (*A. clarkii*), males spent over 65% of their time in the nest by day 7 of the incubation period (Yanagisawa and Ochi, 1986). Finally, both male and female *A. ocellaris* display substantial parental care of the eggs, and male care increases as eggs approach hatching (Madhu et al., 2006); however, this effort was not quantified. In association with this greater caregiving, male Cinnamon anemonefish displayed 3-fold higher levels of 11-ketotestosterone (11-KT), the putative bioactive androgen in species of *Amphiprion*, as compared to females. In contrast, estradiol (E2) was 7-fold higher in females than in males (Godwin and Thomas, 1993). In teleost species where spawning is seasonal, such as the Plainfin Midshipman and catfish, E2 covaries with seasonal changes and/or lunar cycles, and direct, mechanistic studies have established that E2 concentrations directly influence vitellogenisis (Lamba et al., 1983; Sisneros et al., 2004). However, in non-seasonal, continuously spawning species, such as anemonefish, patterns of E2 variation across the spawning cycle are less well understood.

In addition to the sex steroids, circulating glucocorticoid levels are associated with reproductive behaviors across taxa, but few studies in teleosts have characterized the relationship between cortisol and parental care between the sexes (Magee et al., 2006; Dey et al., 2010). In Cinnamon anemonefish, levels of cortisol were reported to be similar in males and females (Godwin and Thomas, 1993). However, animals may not have been collected at a time when the pair was currently caring for a brood. In paternal Bluegill Sunfish, cortisol was higher in males caring for larger broods than in males with fewer eggs (Magee et al., 2006). Parental care requires increased metabolism, effort, and attention, and thus may be perceived as a stressful experience. Hence, if anemonefish were sampled during egg tending, cortisol might be expected to display a similar pattern as sunfish, with higher cortisol levels in individuals displaying relatively more parental effort.

The first aim of this study was to characterize sex differences in parental care during egg development in laboratory reared, reproductively active pairs of *A. ocellaris*. We hypothesized that males would display more care than females and that care would increase as eggs develop. The second aim was to characterize sex differences in circulating levels of 11-KT, E2, and cortisol across the breeding cycle. We hypothesized that females would display higher E2 and lower 11-KT. We further hypothesized that actively parenting males would display higher cortisol levels than females, associated with greater parental effort.

MATERIALS AND METHODS

Animals and husbandry

Amphiprion ocellaris were obtained from ORA (Oceans Reefs and Aquariums, Fort Peirce, FL) as juveniles and raised in 25 gallon tanks (18″x18″x18″) in pairs until reaching sexual maturity. Tank conditions were set to mimic natural environmental conditions with a temperature of 79°F, photoperiod of 12:12 (lights on at 0700 hr and off at 1900 hr), pH of 8.2, and specific gravity of 1.025. Length and weight measurements were taken prior to the onset of experimental procedures, and again at the conclusion of this study. Fish were fed twice daily with a variety of fresh, frozen, and dried foods. One clay pot was placed in each tank as surrogate for a host sea anemone. Behavioral observations between pairs in anemones versus those pairs used in this experiment showed no overt behavioral differences (unpubl. obs.). The fish also deposit eggs in the clay pots, and hence the clay pots serve as the 'nest' in behavioral analyses described below. All procedures were approved by the University of Illinois Institutional Animal Care and Use Committee and adhered to NIH guidelines. All measures were taken to minimize the number of fish used as well as the pain and suffering of the animals. The University of Illinois at Urbana-Champaign is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Experimental design

Five spawning pairs of *A. ocellaris* were used in this experiment. Pairs were selected based on two factors. First, the size of the male needed to be large enough for repeated blood draws and a blood collection volume of over 25 μl. In preliminary trials this weight was usually just over two grams. Second, pairs needed to be regularly spawning (spawn period of less than 25 days). Spawning pairs are difficult to produce due to the length of time it takes for sexual maturation (Iwata et al., 2008), and out of more than 20 spawning pairs at the time of this experiment, only five pairs had consistent spawn intervals of under 25 days. Although other pairs in the laboratory were spawning, they were relatively novice in their reproductive behavior. After several spawns, intervals decrease and parenting effort increases as individuals become more experienced (unpubl. obs.). Eggs are laid in the evening hours in the clay pot and consequently the nest was visually inspected for the presence of eggs every morning prior to any experimental procedures. If eggs were identified, the experiment began that day at 'day 0.' Eggs took from 8–10 days after deposition to hatch into the planktonic larval phase at which experimental procedures for that spawn period ended. From the five selected pairs, video recordings were analyzed for behavioral displays at three separate time points in separate spawn periods during egg rearing. Following video recordings, blood draws were performed.

Behavior

The behavior of the pairs was video recorded on the day the eggs were laid (day 0), as well as in two additional spawning periods, three, and six days following the spawning event for a five-minute period. Number of parental behaviors as defined by the sum of fanning (fanning the eggs with pectoral fins or caudal fin), nipping events (cleaning the eggs with the mouth) and duration spent in the nest were recorded for both the male and the female using JWatcher behavioral event recording software (Blumstein et al., 2006). In addition to parental behaviors, agonistic and affiliative behaviors were recorded, including number of bites on the partner, number of chases (charge toward partner), and submissive displays (quiver display or fleeing from partner).

Blood sample collection

The first blood sample collection occurred when animals were not tending eggs, exactly three days after the eggs hatched from the previous spawn, and 11 days after the eggs were laid from the previous spawn. The second sample was taken on the day the next batch of eggs was laid (day 0). The third and fourth samples were taken three and six days following egg deposition. Consecutive spawning events were used unless the previous blood draw had occurred within less than 12 days (a period previously established to be critical for recovery; unpubl. data), in which case the following spawn period was used.

All blood sampling was performed between 0200 and 1500 hr in order to control for any diurnal changes in steroid hormone levels. Fish were placed between two heavy paper towels wetted with seawater with only the caudal region exposed. Blood samples were taken from the lateral caudal vein using a 27-gauge heparinized butterfly needle (Terumo Medical Products) mounted on a 1 ml syringe (BD Syringe). As much blood as would freely flow into a 1 ml syringe was drawn (50 µl to 300 µl). The time from which the capture net entered the tank (start time), the time the fish was netted (catch time), and the time final collection was finished (blood time) were recorded for all individuals. Steroid hormones can undergo rapid changes due to stress (Kammerer et al., 2010); hence, it is critical to isolate samples quickly, and only blood samples collected within three minutes of catching the fish from the home tank were used.

Immediately following collection, blood was dispelled into a 0.6 ml centrifuge tube and placed on ice until the daily collection was finished (no longer than 10 min). Samples were then immediately centrifuged (Eppendorf Centrifuge 5417R) at 4000 rpm for 15 minutes following established protocols (Kidd et al., 2010). The plasma supernatant was extracted with a 100 µl pipet (avoiding the red hematocrit layer), isolated, and stored at –80°C until circulating levels of 11-KT, E2, and cortisol were measured via Enzyme Immunoassay (EIA). All EIA measurements were performed in duplicate, and the average of both technical replicates was used to calculate plasma hormone concentrations.

Validation of EIA kits

EIA kits are only capable of measuring concentrations of the hormones within a specific range. Plasma concentrations of the hormones of interest for *A. ocellaris* were not previously known. Hence, appropriate dilutions of the plasma samples that produce hormone concentrations within range of the kits was established. A pooled plasma sample across individuals was serially diluted, measured for hormone concentration, and then compared to serially diluted standards (Mills et al., 2010; Fischer et al., 2014). The pooled sample (total 600 µl) consisted of blood plasma from at least five individuals from the following groups: non-reproductive juveniles (200 µl), reproductive males and females tending eggs (200 µl), and reproductive males and females not tending eggs (200 µl). This pooled sample was combined this way in order to reflect hormone values unbiased by sex, breeding status, or position in the social hierarchy.

11-KT.—For the 11-KT assay (EIA kit from Cayman Chemical, Item No. 582751), a sample containing a known concentration of 1000 pg/ml standard was serially diluted eight times giving a range of 1000 pg/ml to 7.8 pg/ml. The pooled plasma sample was diluted five times (from 1:1 to 1:256). All samples were measured for 11-KT concentration as indicated by percent bound following instructions in the kits.

E2.—For the E2 assay kit (Calbiotech Mouse/Rat Estradiol Kit, Lot No. ESG4324), six standards were provided containing known concentrations of E2 ranging from 0 pg/ml to 300 pg/ml. The pooled plasma sample was diluted five times (from 1:1 to 1:256). All samples were measured for E2 concentration as indicated by percent bound following instructions in the kits.

Cortisol.—For the cortisol assay (Cortisol ELISA kit, Cat. No. ADI-900-071), a sample containing a known concentration of 10,000 pg/ml was serially diluted seven times giving a range of 10,000 pg/ml to 156 pg/ml. The pooled plasma sample was diluted five times (from 1:1 to 1:256). All samples were measured for cortisol concentration as indicated by percent bound following instructions in the kits.

Statistical analysis

Data were analyzed using R (3.1.1). $P < 0.05$ was considered statistically significant. Sex differences in body mass and length were analyzed using paired t-tests. Behavioral measures (e.g., total duration in the nest, total number of parental acts, fanning or nipping eggs) were not normally distributed and hence were analyzed for sex differences using the non-parametric Wilcoxon Rank Sum Test. Differences in behaviors across the three days of the breeding cycle were analyzed using the Kruskal-Wallis test.

Validation of the EIA kits was accomplished using the following procedure. First, percent bound (as measured using the kits) was plotted against the natural logarithm of the dilution ratios of both standards and the pooled plasma samples. Data points were then analyzed by analysis of covariance to determine the extent to which the two lines (sample and standard) were parallel. The natural logarithm of the dilution ratio was entered as the continuous predictor and the standard versus pooled sample as the categorical variable. A non-significant interaction was taken to indicate that the slopes were not significantly different from each other and that the slope of the given standards can accurately predict samples.

Sex differences in steroid hormone concentrations were analyzed using unpaired t-tests. For these analyses, the multiple hormone concentration measures per individual across the breeding cycle were averaged together to generate one number per individual. Samples were also analyzed by repeated measures ANOVA with sample day relative to the spawning event (0, 3, or 6) as a within-subjects factor and sex as the between-subjects factor. These analyses also include start time, catch time, and blood time entered as covariates (one at a time).

To determine how the hormones may have fluctuated across the breeding cycle, plasma hormone concentrations were analyzed using polynomial regression with sample day (0, 3, 6, and 11) and the square of these values entered as continuous predictors. Note that sample day 11 occurred three days after eggs from the previous batch hatched.

RESULTS

Sex differences in body mass, body length, and frequency of spawning events

Females were larger than males in both standard length and weight. Mean standard length of females was 72.2±0.42 mm (SEM; range 68–78 mm), whereas in males it was

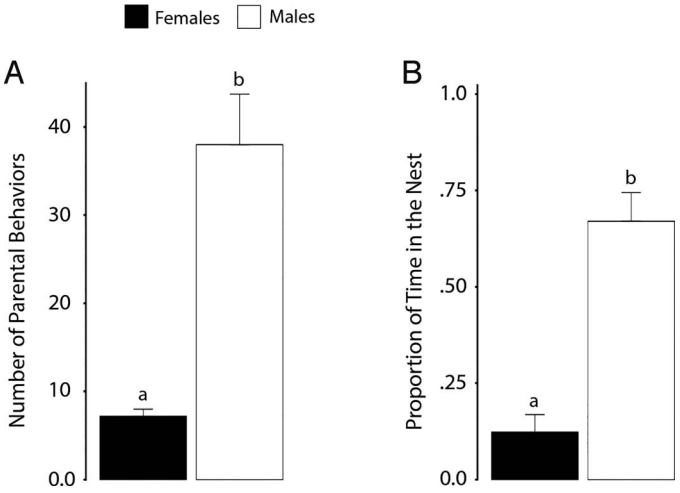

Fig. 1. Males display greater parental effort than females. (A) Average total number of combined nips and fans displayed by females (black bars) and males (white bars). (B) Average duration spent in the nest expressed as a percentage of total sample time. Standard error bars shown. Letters 'a' and 'b' denote significant differences.

55.6±0.56 mm (SEM; 50–62 mm; t = 5.25, df = 4, $P < 0.007$). Mean weight of females was 7.09±0.405 g (6.58–7.65 g), whereas in males it was 3.33±0.78 g (2.75–4.23 g; t = 5.29, df = 4, $P < 0.007$). The average number of days between adjacent spawning events for the pairs used in the study was 21.7 (±3.17 SEM), 18.7 (±5.50), 15.7 (±3.21), 13.7 (±3.06), and 14.0 (±2.00).

Sex differences in parenting behavior

None of the behaviors changed significantly across the breeding cycle (day 0, 3, and 6; all *P*-values greater than 0.19). However, there was a tendency for males to spend more time in the nest as eggs developed ($P = 0.19$). Collapsed across the breeding cycle, males displayed 5.6-fold more total parental behaviors than did females (W = 156.5, $P < 0.0003$, $n = 5$; Fig. 1A). Males also spent 9.3-fold more time in the nest than did females (W = 19, $P < 0.0004$, $n = 5$; Fig. 1B). No aggressive or submissive behaviors were observed in any of the videos.

Validation of EIA kits

For each kit, the relationship between the dilutions (both sample and standard) and percent bound followed a sigmoidal curve suggesting that the upper and lower dilutions were at or exceeded the kits' maximum and minimum measurement capabilities. Those points that did not fit the linear portion of the curve were removed.

11-KT.—Sample dilutions of 1:16 to 1:128 were found to be linear ($F = 388.3$, df = 1,4, $P < 0.0001$) and parallel to the standard ($F = 0.187$, df = 1,4, $P > 0.65$; Fig. 2A). Hence, for measuring 11-KT in individual *A. ocellaris*, plasma samples were diluted 1:30 (by mixing 7 µl of plasma with 210 µl of assay buffer) to ensure the concentrations would fall well within the measurable range and in the linear portion of the standard curve. The average intra-assay coefficient of variation from the two technical replicates per sample was 4.4 (±0.68 SEM).

E2.—Plasma dilutions from 1:8 to 1:128 were found to be linear ($F = 877.7$, df = 1,6, $P < 0.0001$) and parallel to the

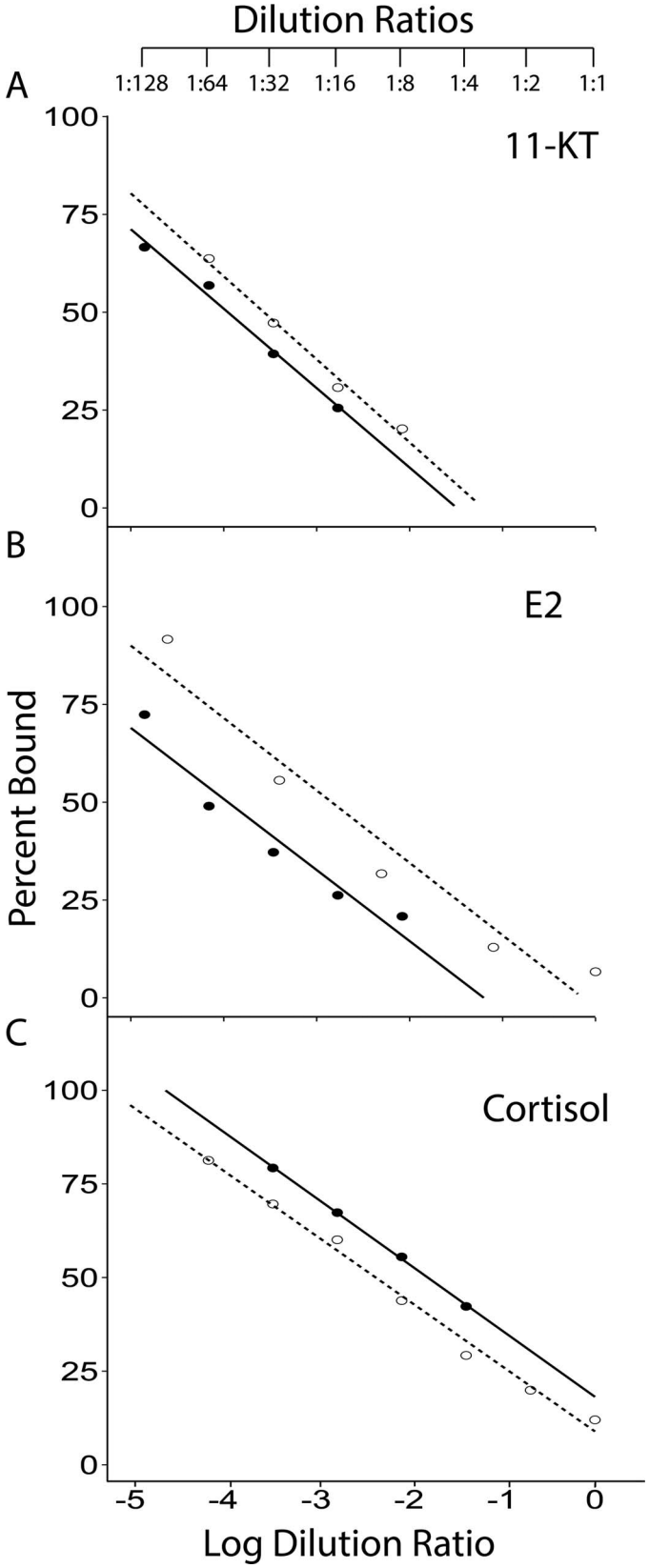

Fig. 2. Validation of EIA kits. Percent bound as measured by the kits is plotted against the natural logarithm of the dilution ratio (expressed as a percentage, i.e., 1:4 = 0.25). The standard dilutions are shown as open circles, whereas the pooled sample dilutions are shown as filled circles. Simple linear regression lines are shown. (A) 11-KT kit. (B) E2 kit. (C) Cortisol kit.

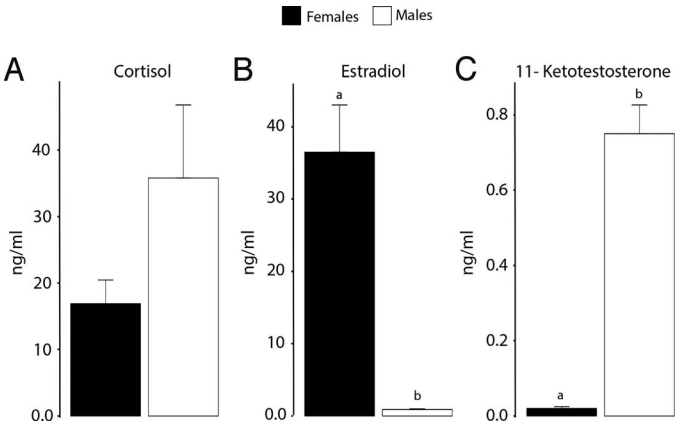

Fig. 3. Sex differences in circulating steroid hormone levels. (A) Average concentration of cortisol in the plasma of females ($n = 5$; black bars) and males ($n = 5$; white bars). (B) Same as A for E2. (C) Same as A for 11-KT. Standard error bars for males and females reflect individual variation. Standard error bars for the pooled sample reflect technical replication. Letters 'a' and 'b' denote significant differences.

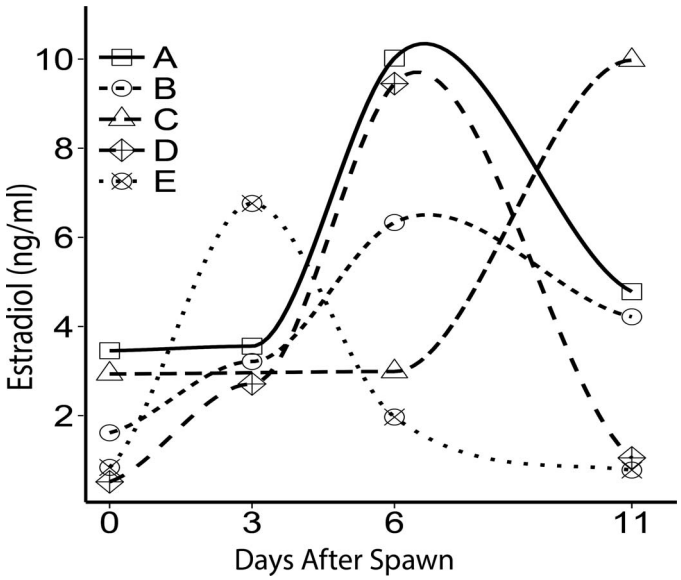

Fig. 4. Rising and falling E2 levels across the breeding cycle. Concentration of E2 plotted against days after spawning for each of five separate females. Each female is represented by a different symbol. A loess smoothing function was applied to connect the data points separately for each female to facilitate differentiating the individuals.

standard curve ($F = 0.013$, df = 1,6, $P > 0.9$; Fig. 2B). Plasma samples of individual *A. ocellaris* were diluted 1:35 (7 µl of plasma mixed with 245 µl of assay buffer) for analysis of sex differences. The average intra-assay coefficient of variation was 7.5 (±1.08 SEM).

Cortisol.—Plasma dilutions from 1:4 to 1:32 were found to be linear ($F = 648.9$, df = 1,4, $P < 0.0001$) and parallel to the standard curve ($F = 0.221$, df = 1,4, $P > 0.22$; Fig. 1C). Plasma samples of individual *A. ocellaris* were diluted 1:30 (7 µl of plasma mixed with 210 µl of assay buffer) for analysis of sex differences. The average intra-assay coefficient of variation was 6.7 (±0.90).

Sex differences in plasma hormone concentrations

No relationship between start time, catch time, or blood time and the levels of any the circulating hormones were observed in analyses of covariance (all *P*-values greater than 0.24); hence, only results of unpaired t-tests are shown.

11-KT was significantly higher in males than females ($t = 10.4$, df = 9, $P < 0.0001$; Fig. 3A). In contrast, E2 was significantly higher in females than males ($t = 4.9$, df = 9, $P = 0.0008$; Fig. 3B). Cortisol was slightly higher in males than females, though this effect was not statistically significant ($t = 1.88$, df = 9, $P = 0.09$; Fig. 3C).

Correlations between hormones and behavior across the breeding cycle

Plasma levels of 11-KT and cortisol were not correlated with any of the behaviors and showed no obvious changes across the breeding cycle (all *P*-values greater than 0.46). Plasma levels of E2 were not correlated with any of the behaviors (all *P*-values greater than 0.68). However, E2 levels significantly rose following a spawning event (during egg production) and subsequently fell prior to egg deposition (Fig. 4). This was indicated by significant linear ($F = 5.5$, df = 1,17, $P = 0.032$) and polynomial ($F = 4.6$, df = 1,17, $P = 0.047$) coefficients in the polynomial regression of E2 concentration by day in the spawning cycle.

DISCUSSION

Information on sex differences in parental behavior and bioactive steroid hormones during egg care are missing from the literature for *A. ocellaris*. In this study, documentation of sex differences in parental effort (Fig. 1) and circulating levels of E2, 11-KT, and cortisol in reproductively active male and female *A. ocellaris* were quantified for the first time (Figs. 2–4). In populations of actively breeding pairs, males spend the majority of their lives with eggs present in the nest and devote an impressive amount of parental effort, continuously fanning and nipping eggs, cleaning them from debris, and keeping them free of fungus. During the three, five-minute behavioral observation periods, males display an average of almost 40 parental behaviors (e.g., nipping or fanning their eggs) and spend nearly 70% of their time in the nest tending the eggs. This level of effort is high in comparison to some teleost species and more comparable to others. In the Azorean Rock Pool Blenny, males display roughly two acts of nest cleaning per 20 minutes (Ros et al., 2004), and in the Convict Cichlid, paternal acts occur at 12 per ten minutes (O'Connell et al., 2012). The effort displayed by Blue-banded Goby and Three-spined Stickleback males is comparable to the data presented in this study (Pall et al., 2002; Pradhan et al., 2014); time in the nest and the number of behavioral acts are also high in these species. Females, although not absent in parental care, display few parental behaviors and spend less than 15% of time in the nest (Fig. 1).

While there were a high number of parental care behaviors, other social interactions were infrequent. Males and females form pair bonds several months before spawning occurs; therefore, there were no agonistic behaviors and only one event of affiliative behavior. During initial pair formation, there are many such interactions, but that period was not recorded for this experiment, and previous work has demonstrated that during initial pair formation, high levels of aggression are apparent (Yaeger et al., 2014). This lack of aggression may provide a useful context for future studies examining the role of androgens in paternal care, without

the confounding effects of aggression on circulating androgens.

We hypothesized that the high level of paternal care displayed by males would result in higher levels of cortisol, a hormone critical for stress response and energy mobilization. While no significant difference in cortisol between males and females was observed in our study, males tended to have higher cortisol values. The lack of significance may be due to the high variability within females and small sample size of this study. Cortisol levels in field measurements in *A. melanopus* were at around 15 ng/ml in males and females, but around 60 ng/ml in sexually transitional individuals. The lack of sex differences in mentioned field studies might be attributed to the fact that the breeding status of individuals measured was unknown, or non-breeding following female removal. If cortisol levels reflect parental effort, then it should be higher during the egg brooding period. Unlike E2 and 11-KT, where the pooled species sample approximated an average between males and females, cortisol values were higher in reproductively active males and females in comparison to the pooled species sample (data not shown). This result is consistent with the notion that parenting is metabolically expensive (Nunes et al., 2001). Breeding individuals are expending more energy during parental care than are those without a brood, and the trend toward higher cortisol values in males may reflect increased parental effort during the egg brooding period.

Males displayed higher levels of parental effort than females (Fig. 1). Males also showed higher circulating levels of 11-KT and lower E2 relative to females (Fig. 3A, B). The simultaneously occurring high paternal effort and high levels of 11-KT are inconsistent with the hypothesized tradeoff between high androgens and parental care observed in avian species (Wingfield et al., 1990). However, this result is consistent with recent studies showing high androgens do not disrupt parental care in gobies and mice. In the Blue-banded Goby, males that were experienced fathers showed higher 11-KT during care than inexperienced males, and when no eggs were present that difference disappeared (Rodgers et al., 2006). Moreover, in the monogamous California mouse, high androgens were necessary for paternal care (Trainor and Marler, 2001). In these species, males are also defending territories and may show aggression towards conspecifics at the same time that they are caring for their offspring, potentially confounding the relationship between androgens and paternal behavior. In Pradhan et al. (2014), blockade of 11-KT synthesis specifically reduced parental care without affecting other social behaviors such as aggression. These results suggest a direct causal connection between 11-KT and paternal care that is not a consequence of other simultaneously occurring behaviors. Our results in males of *A. ocellaris* are consistent with these findings, in that they provide complementary evidence that high androgens can be associated with high paternal behavior and not aggression or other social interactions.

11-KT has never been measured in adult *A. ocellaris* prior to this present study. In previous research where steroid hormones were measured in *A. ocellaris*, the samples were collected from non-reproductive juveniles (Iwata et al., 2008) or in natural field populations of closely related *A. melanopus* (Godwin and Thomas, 1993; Godwin, 1994b). In laboratory studies exploring the relationship between circulating steroid hormones and dominance status in juveniles, 11-KT was highest in α, and lowest in β individuals (Iwata et al., 2008). This is an interesting result as the α eventually becomes the dominant female with lower 11-KT levels than β-males, or subordinates.

Results from this study demonstrate that laboratory reared, captive bred *A. ocellaris* display sex differences in hormones in a manner expected from measurements performed in other closely related species from the field. In field studies of another anemonefish, *A. melanopus*, E2 was approximately 7-fold higher in females than in males, a pattern also supported here (Fig. 3B). Opposite to E2, 11-KT was much higher in male *A. ocellaris* than in females (Fig. 3A). The sex difference was larger than that reported for *A. melanopus* (Godwin, 1994b). While 11-KT occurs at comparable levels in males of *A. ocellaris* and *A. melanopus*, it is much lower in females of *A. ocellaris* than females of *A. melanopus*. However, measurements are still reasonably within range in comparison to all species of *Amphiprion* reviewed (Godwin, 1994a, 1994b; Iwata et al., 2008, 2012; Mills et al., 2010).

In addition to establishing sex differences in circulating E2 and 11-KT, we also wished to determine whether the levels of these hormones fluctuated across the breeding cycle in a manner consistent with other species. E2 measurements from four time points during the spawning cycle suggest that E2 rises following the spawning event during egg production and then falls prior to the next spawn (Fig. 4). This pattern is consistent with other teleosts. In the Amago salmon, E2 levels rise during vitellogenesis and fall in ovulated fish (Kagawa et al., 1981). The data here also show that spawning periods are variable, and between individuals the trajectory of E2 vacillation does not follow the same chronology. In some females, E2 rises more quickly following a spawning event, making it difficult to discern patterns across individuals. This is further complicated by the fact that our samples were taken across four separate spawn periods, and E2 variation likely occurs within individuals during each cycle. In the future, time course studies will be performed using waterborne hormone analysis (Kidd et al., 2010). This method will allow repeated hormonal measurements over a single spawning cycle, which could provide more accurate insight into the pattern of E2 variation.

Results suggest 11-KT does not change across the breeding cycle in males, which reflects the lack of behavioral changes in this study. However, as stated above, this result may be confounded by measurements across spawning periods, and future studies using waterborne methods could yield different results. Alternatively, in species such as *A. ocellaris* where eggs are almost always present and spawning non-seasonal, 11-KT may not fluctuate as is required for timed spawning events to regulate sperm production (Malison et al., 1994; Pankhurst and Peter, 2002; Fine et al., 2004), secondary male reproductive behaviors (Knapp and Neff, 2007), and territory defense (Cardwell and Liley, 1991).

In our laboratory, social groups of *A. ocellaris* consist of a dominant α female, subordinate β male, and zero or one non-reproductive individuals. The lack of predation risk, anemone defense, territory defense against conspecifics, and decreased overall group size are all limitations of this current study. However, in natural populations of *A. ocellaris,* their host anemones are spatially isolated, and it is very common for there to be only two individuals in a group (Mitchell and Dill, 2005). Anemonefishes also have unusually long lifespans exceeding 30 years, 2–3 times higher than similarly related damselfishes, which has been interpreted to be a result of the efficient protection of predation by their anemone hosts (Buston and Garcia, 2007). Thus, the lack of predation risk in this study may reflect natural conditions.

Our results establish robust sex differences in steroid hormones and parental care. Given the high suitability of the species as a laboratory model, we believe our findings consistent with sex differences in hormones and behavior seen in the natural environment.

In summary, results demonstrate sex differences in parental behavior and circulating sex steroids in *A. ocellaris*. Males displayed greater parental behavior than females, and this sex difference in parental investment occurred concurrently with higher circulating 11-KT and lower E2 in males as compared to females. Results establish *A. ocellaris* as a relevant model in social neuroscience for studying paternal behavior in the presence of high 11-KT and without the confounding influence of aggression and behavioral dominance.

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