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## Diurnal patterns and sex differences in cortisol, 11-ketotestosterone, testosterone, and 17 $\beta$ -estradiol in the bluebanded goby (*Lythrypnus dalli*)

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### Abstract

The primary goals of this study were to evaluate diurnal patterns of and sex differences in the levels of cortisol, 11-ketotestosterone, testosterone, and 17 $\beta$ -estradiol in the sex-changing bluebanded goby *Lythrypnus dalli*. Steroid hormones were collected from water samples and analyzed by enzyme immunoassay. During the breeding season, hormones were sampled from both males and females at seven time points between 0600 and 2000 h. When comparing each time point separately, there were significant overall time effects for cortisol and 17 $\beta$ -estradiol. Cortisol concentrations were lowest at the 0800–1000 h sampling point and showed a qualitative peak in late morning (1000–1200 h). Concentrations of 17 $\beta$ -estradiol were elevated at the last sampling point (1800–2000 h). Broader temporal trends were revealed for testosterone and 11-ketotestosterone concentrations, both of which were elevated in the morning. There were no sex differences in overall hormone concentrations or temporal profiles for cortisol, 11-ketotestosterone, or testosterone. Males and females showed similar diurnal patterns of 17 $\beta$ -estradiol but females had significantly higher water-borne 17 $\beta$ -estradiol levels than males. The results show the presence of diurnal changes in steroid hormone levels in male and female bluebanded gobies. The lack of sex differences in androgens suggests that males of this species, and perhaps other bi-directional sex-changing species in which males do not exhibit prominent secondary sexual characteristics, do not require persistent elevations in 11-ketotestosterone or testosterone to maintain the male phenotype. Although the role of 17 $\beta$ -estradiol in maintaining sex differences in sexually plastic species is unclear, our results suggest that, of the hormones measured, 17 $\beta$ -estradiol has the greatest potential for future studies interested in this question.

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**Keywords:** Diurnal pattern; Cortisol; 11-Ketotestosterone; Testosterone; 17 $\beta$ -Estradiol; *Lythrypnus dalli*

### 1. Introduction

A central goal of research in behavioural endocrinology is to understand the relationships between circulating hormone levels and behavioural traits. Fluctuations in hormone levels in the same individual over time can be associated with a variety of non-behavioural factors including sex, season, and reproductive status (e.g., Goymann et al., 2004). Independent of these factors, hormones also

follow endogenous diurnal rhythms in many vertebrates (Meier, 1975). Therefore, determining the relationship between environmental or behavioural variation and individual differences in endocrine profiles requires an understanding of diurnal patterns in hormone secretion.

Diurnal rhythms of cortisol and corticosterone have received significant attention, in part because it is important to account for daily variation in these hormones when evaluating the effects of stressors such as handling and sampling. In fishes, there is evidence that cortisol presents a diurnal rhythm characterized by higher values at night (Rance et al., 1982; Pickering and Pottinger, 1983; Nichols and Weisbart, 1984; Laidley and Leatherland, 1988), but

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the results during the day have shown tremendous variability. For example, no significant diurnal variation in cortisol exists in pre-spawning chum salmon (*Oncorhynchus keta*; Saito et al., 2004), while studies that have manipulated photoperiod length indicate that cortisol increases at the onset of the light phase in rainbow trout (*Oncorhynchus mykiss*; Reddy and Leatherland, 2003) and European sea bass (*Dicentrarchus labrax*; Cerda-Reverter et al., 1998). In male swordtails (*Xiphophorus helleri*), corticoids are significantly higher at 0800 h than in the afternoon (Hannes and Franck, 1983). There also is clear evidence that daily cortisol peaks occur after feeding in rainbow trout (Bry, 1982) and brown trout (*Salmo trutta*; Pickering and Pottinger, 1983).

Variation in sex steroid levels, such as 17 $\beta$ -estradiol (E2), testosterone (T), and 11-ketotestosterone (KT) have been thoroughly investigated in fishes with regards to how circulating concentrations fluctuate with spawning cycle (Scott et al., 1984; Prat et al., 1990; Barnett and Pankhurst, 1994) but less in terms of diurnal rhythms. Most work on daily rhythms of sex steroids has been conducted in salmonids and other aquaculture species, while studies of this sort have been conducted less often in other fish models. As with cortisol, studies vary greatly in the demonstration of diurnal sex steroid rhythms. For instance, Bayarri and colleagues (2004) found a significant daily rhythm for T but not KT in male European sea bass; T levels peaked at 0700 h while levels of KT did not vary significantly during the day. In male cichlids (*Oreochromis mossambicus*) both androgens were higher at 0900 h than the rest of the day (Oliveira et al., 2001a,b). Conversely, water-borne KT and T in common dentex (*Dentex dentex*) were higher in the afternoon and early evening (Pavlidis et al., 2004), concordant with plasma T results in male swordtails (Hannes and Franck, 1983). In the Japanese charr (*Salvelinus leucomaenis*) T showed a peak at 1800 h in both sexes and KT at 1500 h in males while E2 fluctuated without a significant pattern in females (Yamada et al., 2002). In the belted sandfish (*Serranus subligarius*), a simultaneous hermaphrodite, no daily rhythms were found for T or E2 (Cheek et al., 2000).

We explore daily variation in concentrations of the stress and sex hormones—cortisol, E2, T, and KT—in the sex-changing bluebanded goby, *Lythrypnus dalli*. The bluebanded goby is predominantly a protogynous fish (St. Mary, 1994; Reavis and Grober, 1999) but is capable of bi-directional sex change (St. Mary, 1994; Reavis and Grober, 1999; Rodgers et al., 2007). The well-established role of hormones in the process of sex change in this and other fishes (Carlisle et al., 2000; Frisch, 2004; Black et al., 2005a) suggests that understanding sexual and diurnal variation in hormone production is essential for generating a comprehensive picture of the behavioural endocrinology of sexually plastic fishes. The bluebanded goby is a social species living on rocky reefs from southern California to the west coast of Mexico (Wiley, 1976). The breeding season of the bluebanded goby ranges from

May to September (Wiley, 1976). Hormones from both males and females were collected at intervals throughout the day during the breeding season using a non-invasive sampling technique. Our aims were to test the hypotheses that there are diurnal changes in endocrine profiles and sex differences in steroid hormone production.

## 2. Methods

### 2.1. Study organism: collection and maintenance

Fish were caught off the coast of Santa Catalina Island, CA at 4.5–11.3 m below the surface on the southeast and southwest points of Bird Rock using SCUBA (California Department of Fish & Game permit #: 803024-05 to VL). Briefly, groups of sea urchins (*Centrostephanus coronatus*) occupied by bluebanded gobies were located and the fish inhabiting the urchins were anesthetized with 10% quinaldine (30 ml quinaldine; 270 ml acetone), captured by hand netting, and transported in large plastic bottles back to the laboratory at the USC Wrigley Institute for Environmental Science. The fish were held in a large (60  $\times$  94 cm<sup>2</sup>) holding tank for 3 weeks until further processing; the holding tank was supplied continuously with seawater pumped directly from the cove adjacent to Bird Rock, and stocked with abundant shelter (8 cm PVC tubes and kelp). The fish were fed either rehydrated brine shrimp or marine pellets twice daily between 0800–0900 h and 1700–1800 h. The research conducted herein was approved by the Georgia State University IACUC Protocol No. A06004 (0708) and University of Southern California IACUC Protocol No. 10262.

### 2.2. Sampling methods

All hormone samples were obtained on 7 July 2005, 1 day after the new moon; air temperature on this day ranged from 16.1 to 22.8  $^{\circ}$ C (www.weather.com) and water temperatures (San Pedro buoy #46222; [http://www.ndbc.noaa.gov/station\\_page.php?station=46222](http://www.ndbc.noaa.gov/station_page.php?station=46222)) ranged from 18.2 to 19.2  $^{\circ}$ C over the course of the day. No artificial lights were operating in the laboratory and the fish were exposed only to the light penetrating large panel windows adjacent to the holding tank.

Eleven fish were removed from the holding tank with a net at each of seven time points ( $N = 77$  animals) from sunrise (0550 h) to sunset (2006 h): 0600, 0800, 1000, 1200, 1400, 1600, and 1800 h (Pacific Standard Time). All fish that were used for a given time point were removed from the tank within 2 min; capture of males was especially rapid because they occupied the PVC shelters, and could be drawn into the net by tilting the shelter. Furthermore, it is important to note that all fish resumed normal perching (on the shelters or kelp) behaviour and social interactions minutes after collection, which indicates that the procedure did not visibly disturb the fish for any length of time. We attempted to gather approximately equal numbers of males and females for each time point based on visual inspection of size; we chose not to closely inspect the genital papilla of each fish (using stereomicroscopy) prior to hormone sampling to minimize handling stress.

Individual fish were placed in 200 ml polypropylene beakers filled with 100 ml seawater (the same seawater as was pumped into the holding tank) at each time point for a 2 h hormone collection period; all beakers had been rinsed with ethanol and distilled water beforehand. The hormone collection beakers were placed in 2 cm of seawater to maintain temperatures approximating those in the holding tank and the beakers were spaced to eliminate visual contact between fish. Once the 2 h collection period was finished, the hormone collection water was poured through a net (rinsed with clean seawater) into a new, clean 200 ml polypropylene beaker, and fish were measured for standard length and their genital papillae were inspected to verify sex. The genital papilla is thin and pointy in males, and wide and rounded in females (Wiley, 1976). All animals used in this study had unambiguous genitalia; of the 77 individuals,  $N = 32$  males and  $N = 45$  females (see Fig. 1 legend for sex distributions across time

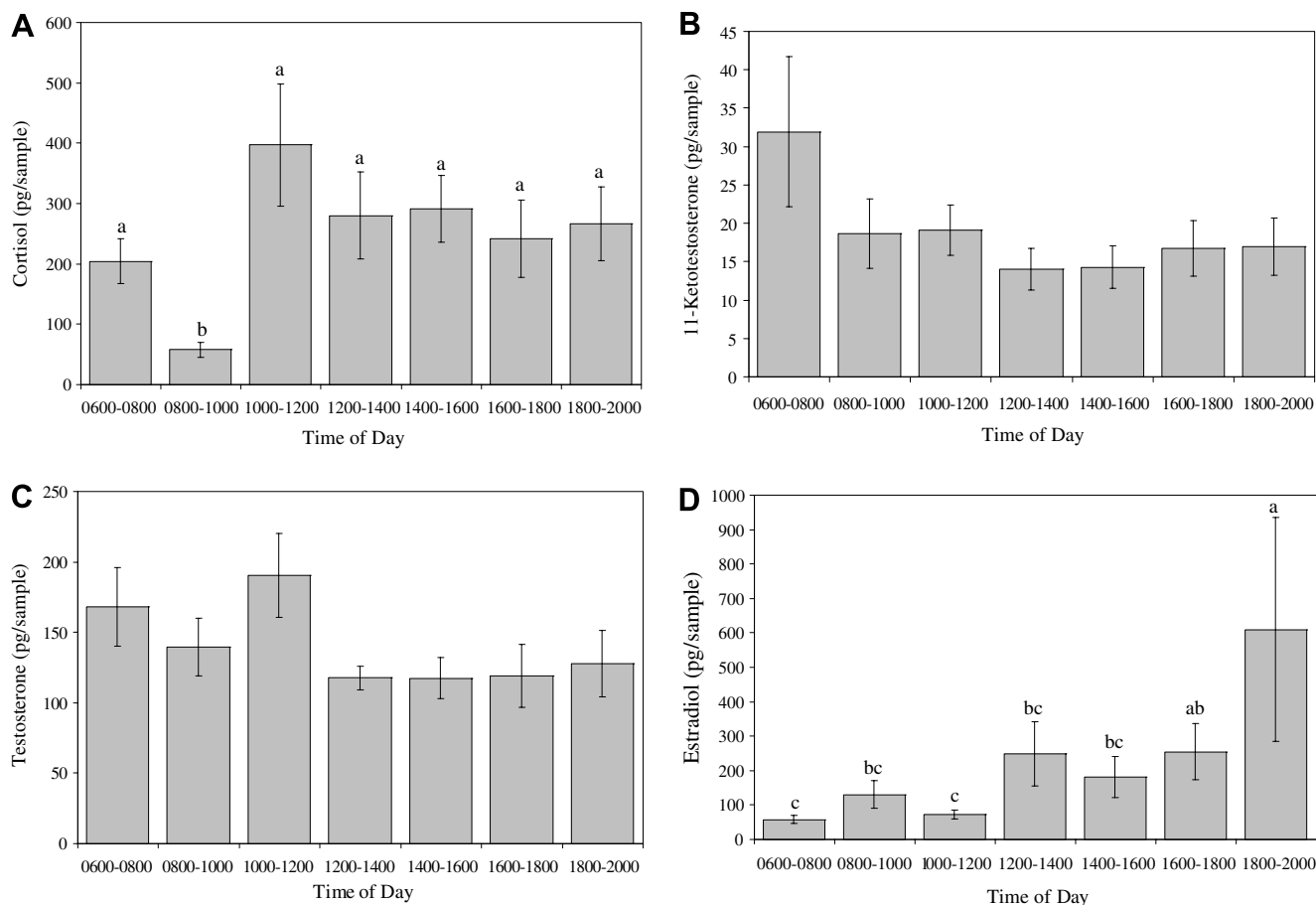


Fig. 1. Time course of water-borne hormone concentrations (pg/sample) for (A) cortisol (B) 11-ketotestosterone (C) testosterone, and (D) 17 $\beta$ -estradiol. Water-borne hormone concentrations were pooled for males and females because there was no sex difference in temporal profile (Table 1; time  $\times$  sex). Despite all hormone values being transformed for analysis, the histogram bars depict untransformed means  $\pm$  1 SEM.  $N = 11$  for each bar in each graph. The sex distribution is as follows, with F = female, M = male: 0600–0800 (3F, 8M), 0800–1000 (7F, 4M), 1000–1200 (9F, 2M), 1200–1400 (9F, 2M), 1400–1600 (8F, 3M), 1600–1800 (5F, 6M), 1800–2000 (4F, 7M). For cortisol and E2, histogram bars with different letters are significantly different (Student's  $t$ -test;  $P < 0.05$ ); for KT and T, see Section 3 for comparisons across broader time scales.

167 points). Mean standard length (mm)  $\pm$  1 SEM of females (F) and males  
 168 (M) at each time point were: 0600–0800 (F: 24.03  $\pm$  0.33; M:  
 169 30.09  $\pm$  1.04), 0800–1000 (F: 26.24  $\pm$  1.24; M: 33.18  $\pm$  1.37), 1000–1200  
 170 (F: 25.29  $\pm$  0.73; M: 31.2  $\pm$  2.10), 1200–1400 (F: 25.44  $\pm$  0.50; M:  
 171 32.35  $\pm$  2.05), 1400–1600 (F: 26.73  $\pm$  0.48; M: 34.87  $\pm$  1.03), 1600–1800  
 172 (F: 25.68  $\pm$  0.56; M: 33.85  $\pm$  1.95), 1800–2000 (F: 26.65  $\pm$  0.30; M:  
 173 31.79  $\pm$  1.06). Following measurement, fish were placed in a new holding  
 174 tank with the same dimensions as described above to prevent using the  
 175 same fish multiple times.

176 Hormone was extracted from the water samples using C18 solid phase  
 177 extraction columns (Lichrolut RP-18, 500 mg, 3.0 ml; Merck) fitted to a  
 178 12-port manifold (Earley et al., 2006; Rodgers et al., 2006). Columns first  
 179 were primed using two consecutive washes with 2 ml HPLC grade methanol  
 180 (MeOH) followed by two consecutive washes with 2 ml distilled water.  
 181 Tubing was then fastened to the top of each column and was placed into  
 182 the polypropylene beaker containing a water sample collected from the  
 183 fish. The vacuum was engaged and the water sample was drawn up the  
 184 tubing, passed through the column and the wastewater was collected in  
 185 a bin inside the manifold. Salts were removed from the columns with  
 186 two consecutive 2 ml washes of distilled water. The columns were then fro-  
 187 zen and shipped to Georgia State University (GSU) for further processing.  
 188 Freeze storage of water samples and columns has been determined not to  
 189 impact steroid concentrations (Ellis et al., 2004). At GSU, the columns  
 190 were thawed and purged with two consecutive 2 ml washes of distilled  
 191 water. Hormone was eluted from the columns into 12  $\times$  75 mm (6 ml)

borosilicate vials by two consecutive 2 ml washes with HPLC grade  
 MeOH. The 4 ml of eluted solvent was evaporated at 40  $^{\circ}$ C (water bath)  
 with a gentle stream of nitrogen ( $\sim$ 0.7 bar), which was passed over the  
 samples through an evaporating manifold. The resulting hormone pellet  
 was resuspended in 840  $\mu$ l of enzyme-immunoassay (EIA) buffer supplied  
 with the kits (see below), and the samples were stored at  $-20$   $^{\circ}$ C until  
 assayed.

Cayman Chemicals Inc. enzyme-immunoassay kits were used for all  
 hormones (cortisol, KT, T, and E2). All samples were assayed in duplicate  
 on two plates. All four hormones were assayed for each individual. Briefly,  
 50  $\mu$ l of each sample was pipetted into the wells followed by 50  $\mu$ l of acetyl-  
 cholinesterase tracer and 50  $\mu$ l of antiserum. Cortisol and KT plates  
 were incubated overnight (18 h) on an orbital shaker at 4  $^{\circ}$ C; T plates were  
 incubated for 2 h on an orbital shaker at room temperature; E2 plates  
 were incubated for 1 h on an orbital shaker at room temperature. The  
 plates were then washed five times with wash buffer (provided with the  
 kits), blotted dry, and 200  $\mu$ l of Ellman's reagent was added to each well.  
 The plate was wrapped in aluminum foil and placed on an orbital shaker  
 for 60–120 min depending on the assay. Plates were read at 405 nm on a  
 BioMek microplate reader. Two bluebanded goby pooled water extracts,  
 which were derived from non-experimental fish (see next paragraph) and  
 assayed in duplicate, were used as controls on each plate. Intra-assay coef-  
 ficients of variation were (assay 1, assay 2): cortisol (6.58%, 1.24%), KT  
 (2.67%, 10.3%), T (7.91%, 20.4%), and E2 (7.36%, 4.22%). Inter-assay  
 coefficients of variation were: cortisol (6.78%), KT (5.38%), T (12.28%),

and E2 (6.36%). All antisera are reported by Cayman Chemical Inc. to have 100% specificity for the focal steroid hormone. The cortisol antiserum cross reacts with prednisolone (22%) and cortexolone (6.1%), cortisone (2%), and corticosterone (1.3%); all other cross-reactivity values were <0.2%. The KT antiserum shows no cross-reactivity with other androgens (e.g., testosterone <0.01%); see also Cuisset et al. (1994) for a report of low cross-reactivity (<0.01%) of this antiserum with cortisol and cortisone. The T antiserum cross reacts with 5 $\alpha$ -dihydrotestosterone (27.4%), 5 $\beta$ -dihydrotestosterone (18.9%), androstenedione (3.7%), and 11-ketotestosterone (2.2%); all other cross-reactivity values were <0.51%. The E2 antiserum cross reacts with estradiol-3-glucuronide (17%), and estrone (4%); all other cross-reactivity values were <0.57%.

The kits were validated for bluebanded goby water-extracted hormones by assessing parallelism of a serial dilution curve with the standard curve and quantitative recovery. Hormones were obtained from 48 non-experimental fish (males and females) using collection (8 h) and extraction methods similar to those described above. Evaporated samples were then resuspended in 60  $\mu$ l of 0.1 M phosphate buffer and combined into a pool of 2.9 ml. The pool was kept either at 1:1 (for serial dilutions) or diluted 1:16 in EIA buffer (for quantitative recovery), aliquoted and frozen.

Serial dilutions used 250  $\mu$ l of the pooled, 'neat' (1:1) control. Briefly, 125  $\mu$ l of this sample was transferred to a 1.5 ml microcentrifuge tube and mixed (by vortexing) with 125  $\mu$ l of EIA buffer to create a 1:2 dilution; 125  $\mu$ l of 1:2 dilution was mixed with an equal volume of EIA buffer to create a 1:4 dilution, and so on until 1:64 (cortisol, KT) or 1:128 (T, E2). The serial dilutions were assayed in duplicate using the EIA protocol described above. The log-logit transformed dilution curve was constructed using average % maximum binding and pg/ml concentrations for the eight dilution samples. The dilution curve was parallel to the standard curve for all hormones (comparison of slopes, Zar, 1996, p. 355; cortisol:  $t_{11} = 0.002$ ,  $P = 0.99$ ; KT:  $t_{11} = 0.001$ ,  $P = 0.99$ ; T:  $t_{12} = 0.089$ ,  $P = 0.93$ ; E2:  $t_{12} = 0.210$ ,  $P = 0.84$ ).

A large (560  $\mu$ l) sample of the bluebanded goby pooled control was used for quantitative recovery. One hundred microliters of this large sample was pipetted into a tube to constitute the 'neat' control, and 70  $\mu$ l of the large sample was then pipetted into eight additional tubes and mixed with an equal volume of the standards provided with each enzyme-immunoassay kit. Expected recovery concentrations were based on the known amount of cortisol, KT, T, or E2 in the bluebanded goby control sample. Minimum observed recovery for cortisol, KT, T, and E2 was 93.3%, 92.6%, 62.3%, and 76.5%, respectively. The slopes of the observed versus expected curves were 0.995 (cortisol), 1.029 (KT), 0.815 (T), and 0.762 (E2), suggesting linear relationships between expected and observed values for all hormones. The sensitivities of the assays (plate 1, plate 2) were as

follows: cortisol (15.20, 19.18 pg/ml); KT (1.37, 1.06 pg/ml); T (8.53, 8.17 pg/ml); E2 (11.30, 18.69 pg/ml). All data are presented as pg/sample (pg/ml multiplied by 0.84 ml, which was the amount of EIA buffer used to reconstitute the sample).

### 2.3. Statistical analyses

Two-factor analysis of covariance (ANCOVA) with standard length as a covariate was used to assess differences in hormone concentrations (pg/sample) among the seven time points and between the sexes; the use of different animals at each sampling period precluded within-subjects analyses. Interactions between the main effects (time and sex) and standard length were not significant for any of the hormones and thus were eliminated to produce a reduced model (time  $\times$  standard length; cortisol:  $F_{6,54} = 0.97$ ,  $P = 0.45$ , KT:  $F_{6,54} = 0.63$ ,  $P = 0.71$ , T:  $F_{6,55} = 0.38$ ,  $P = 0.89$ , E2:  $F_{6,55} = 0.38$ ,  $P = 0.89$ ; sex  $\times$  standard length; cortisol:  $F_{1,54} = 0.31$ ,  $P = 0.58$ , KT:  $F_{1,54} = 1.39$ ,  $P = 0.25$ , T:  $F_{1,55} = 0.09$ ,  $P = 0.76$ , E2:  $F_{1,55} = 0.07$ ,  $P = 0.78$ ). Variance in standard length was homogeneous among time points (Brown–Forsythe test:  $F_{6,70} = 1.43$ ,  $P = 0.21$ ). T was natural-log transformed, E2 was  $y^{0.25}$  transformed, cortisol was square root transformed, and KT was inverse transformed to achieve normality (Shapiro–Wilk goodness of fit,  $P > 0.13$ ). Linear contrasts were used to investigate broader differences in the time course of hormone release (e.g., morning versus afternoon).

### 3. Results

Comparing all seven sampling points, there were significant overall time effects only for cortisol and E2 (Table 1). Cortisol concentrations showed a significant nadir at the 0800–1000 h sampling point (Student's  $t$ -test:  $P < 0.05$ ) and a qualitative peak in late morning (1000–1200 h). E2 concentrations were significantly elevated at the last sampling point (1800–2000 h) relative to all others (Student's  $t$ -test:  $P < 0.05$ ), except the one that immediately preceded it (1600–1800 h); E2 concentrations at the 1600–1800 h sampling point were significantly greater than at 0600–0800 h and 1000–1200 h (Student's  $t$ -test:  $P < 0.05$ ).

Broader analyses of differences in the time course of hormone release indicated that cortisol concentrations were

Table 1  
The effects of time, sex, and standard length on hormone concentrations derived from a reduced ANCOVA model

Hormone	Effect	F-value	P-value	Means
Cortisol	Time	$F_{6,61} = 2.76$	0.019	Female: 251.01 $\pm$ 32.67, male: 246.13 $\pm$ 41.58
	Sex	$F_{1,61} = 0.13$	0.720	
	Time $\times$ sex	$F_{6,61} = 0.46$	0.833	
	Standard length	$F_{1,61} = 0.73$	0.397	
11-Ketotestosterone	Time	$F_{6,62} = 0.39$	0.881	Female: 19.07 $\pm$ 2.96, male: 18.44 $\pm$ 1.92
	Sex	$F_{1,62} = 0.55$	0.459	
	Time $\times$ sex	$F_{6,62} = 0.44$	0.846	
	Standard length	$F_{1,62} = 1.71$	0.196	
Testosterone	Time	$F_{6,62} = 1.21$	0.311	Female: 156.71 $\pm$ 12.62, male: 116.44 $\pm$ 9.27
	Sex	$F_{1,62} = 1.70$	0.197	
	Time $\times$ sex	$F_{6,62} = 1.02$	0.421	
	Standard length	$F_{1,62} = 0.04$	0.842	
17 $\beta$ -Estradiol	Time	$F_{6,62} = 4.23$	0.001	Female: 332.7 $\pm$ 86.69, male: 66.53 $\pm$ 8.13
	Sex	$F_{1,62} = 9.88$	0.003	
	Time $\times$ sex	$F_{6,62} = 1.78$	0.119	
	Standard length	$F_{1,62} = 0.01$	0.925	

Mean (pg/sample)  $\pm$  SEM hormone concentrations are displayed for the sex effect ( $N = 32$  males;  $N = 45$  females in all cases).

significantly lower in early morning (0600–1000 h) than in late morning, afternoon, and evening combined (1000–2000 h) (linear contrast:  $F_{1,59} = 6.62$ ,  $P = 0.013$ ). KT concentrations were significantly higher at the 0600–0800 h time point than all other time points combined (linear contrast:  $F_{1,59} = 5.83$ ,  $P = 0.02$ ). T concentrations were significantly higher in the morning (0600–1200 h) than in the afternoon and evening combined (1200–2000 h) (linear contrast:  $F_{1,60} = 4.81$ ,  $P = 0.03$ ). Lastly, E2 concentrations were significantly higher in the late afternoon (1600–2000 h) than in all other time points combined (linear contrast:  $F_{1,62} = 21.41$ ,  $P < 0.0001$ ).

There were no sex differences in overall hormone concentrations or temporal profiles (time  $\times$  sex) for cortisol, KT, or T (Table 1). There was, however, a pronounced sex effect for E2 (Table 1) with females having significantly higher overall water-borne E2 levels than males; males and females showed similar diurnal patterns of E2 (time  $\times$  sex interaction not significant). Hormone concentrations did not covary significantly with standard length (Table 1).

#### 4. Discussion

Our results demonstrate prominent diurnal patterns of water-borne E2 and cortisol secretion in the sexually plastic bluebanded goby (*Lythrypnus dalli*), and broader daily variations in T and KT. Although our data suggest that these steroid hormones follow a discernable temporal pattern, we sampled hormones over only a 14 h period between 0600 and 2000 h. To reveal a true diurnal cycle would require at least 2 days of continuous hormone sampling and the demonstration that similar peaks and troughs in steroid hormone concentrations occur across days.

Cortisol concentrations were lowest at the 0800–1000 h sampling point, exhibited a qualitative peak in late morning (1000–1200 h) and intermediate values the rest of the day. We fed our fish between 0800 and 0900 h suggesting that the observed increase in cortisol between the 0800–1000 h and 1000–1200 h time periods could represent a post-feeding cortisol response, which is typical in other fish species (Bry, 1982; Pickering and Pottinger, 1983). We also observed low cortisol concentrations before the feeding peak, which corroborates results of a study in immature rainbow trout (Laidley and Leatherland, 1988); the exact time of the cortisol trough in immature rainbow trout ranged between 0800 and 1200 h depending on feeding time and season. It is interesting to note, however, that we did not see a peak in cortisol concentrations following the afternoon feeding period (1700–1800 h), suggesting at least three alternative explanations: (1) cortisol increases are not tightly coupled with feeding *per se*, (2) there may be differential cortisol responses to feeding in the morning and afternoon due to the time interval between feeding bouts (e.g., 8–10 h between morning and afternoon; 14–16 h between afternoon and next morning), or (3) afternoon feeding-induced peaks of cortisol are masked by diurnal

peaks during this time period (i.e., there is a confound between feeding time and endogenous diurnal patterns).

One study in the European sea bass showed that cortisol levels peak with the onset of the light phase, decline in early afternoon, and increase again in the evening (Cerdeira-Reverter et al., 1998). In juvenile rainbow trout cortisol levels were very low in early morning just before the light phase, peaked in late morning, and dropped in the afternoon (Reddy and Leatherland, 2003). These results are somewhat similar to the pattern we observed in the bluebanded gobies with respect to an early morning trough and an increase in late morning. The decline in cortisol that we observed, however, occurred after the onset of light (0550 h). In European sea bass and juvenile rainbow trout cortisol decreased or peaked again in the afternoon, respectively, while in bluebanded gobies cortisol concentrations remained quite constant and moderate in the afternoon.

There also is evidence for seasonal effects on diurnal patterns of cortisol secretion. During the summer, cortisol levels of immature rainbow trout peaked at midnight and between 0700 and 0900 h. The same species tested in the winter did not present any diurnal pattern in cortisol levels (Rance et al., 1982). In the future, it will be interesting to ascertain whether diurnal patterns of cortisol in bluebanded gobies are contingent upon season and associated features such as reproductive versus non-reproductive periods or differences in water temperature.

Regarding diurnal patterns of sex steroid secretion in bluebanded gobies, E2 levels were elevated in the late evening while androgens (T and KT) tended to be higher in the morning. These findings parallel those in male swordtails, cichlids, and European sea bass where androgens are high in the morning and decrease in the afternoon (respectively: Hannes and Franck, 1983; Oliveira et al., 2001a,b; Bayarri et al., 2004). Our results for E2 differ from those found in the belted sandfish, a simultaneous hermaphrodite, for which no daily E2 rhythms were uncovered (Cheek et al., 2000). Our results are, however, very similar to those found in *Pseudolabrus sieboldi* (Sundararaj et al., 2003), which like the bluebanded goby, is predominantly a protogynous sex-changing fish. Sundararaj and colleagues (2003) demonstrated in male *P. sieboldi* that T and KT levels were higher in the morning until 0900 h, and declined at the next sampling point of 1200 h. They also found that E2 levels were very low except for a peak at 1500 h. Despite that study examining only males, it is consistent with our results that show higher androgens levels in the morning and a peak in E2 in the afternoon in both sexes. A previous study on bluebanded gobies found higher KT values in the morning but these were not significantly different from afternoon samples in newly sex-changed individuals (Black et al., 2005b). In light of our data on daily hormone patterns, the data from Black et al. (2005b) is not surprising because their morning sampling occurred after 0900 h when KT concentrations should already have dropped markedly. In the same study Black et al. (2005b) found that morning, but not total, KT levels correlated with aggressive behav-

our. Oliveira et al.'s (2001) work on the relationship between social interactions and androgen levels also testifies to the importance of controlling for time of day when studying hormone–behaviour relationships. In that study, androgen levels in control animals declined as the day progressed, making it essential to account for time of day in fish that were exposed to a social challenge and sampled up to 6 h following the interaction.

The significant peak of E2 in bluebanded gobies after 1800 h might indicate ovulation and precede spawning. Bluebanded gobies spawn in captivity at different times of the day (personal observation, VL) but likely in late evening or early morning; most spawnings occur outside the daily behavioural observations (0800–1800 h), as evidenced by the appearance of eggs the next morning. Indeed, female *P. sieboldi* show E2 peaks at 0300 h and this corresponds with vitellogenic follicle activity (Ohta et al., 2001). Both E2 and T peak during vitellogenesis in the proterandrous Asian seabass (*Lates calcarifer*; Guiguen et al., 1993), and in the spiny damselfish (*Acanthochromis polyacanthus*), females have the highest E2 values when oocytes are at the final stage of maturation or ovulated (Pankhurst et al., 1999). Also, E2 levels correlate with an increase in vitellogenic oocytes in the bi-directional sex-changing broad-barred goby (*Gobiodon histrio*; Kroon et al., 2003). Although the spawning status of females in our study was unknown, it is possible that the late afternoon peak in E2 represents spawning anticipation. We noted substantial variation in untransformed E2 levels at this time point (Fig. 1). Perhaps this is the result of sampling females with oocytes at different stages of development, a hypothesis that will require confirmation in future experiments.

In our study we found a large peak in E2 in the afternoon and we would expect also a corresponding peak in T because E2 released by fish ovaries typically is synthesized via aromatization of T (e.g., Lee et al., 2006). We did not observe an afternoon increase in T so this might indicate that E2 is following a different synthetic pathway. In fact, in the protogynous *P. sieboldi*, E2 is produced from estrone instead of T (Ohta et al., 2001). This alternative pathway also could operate in bluebanded gobies, perhaps in tandem with aromatization of T; female bluebanded gobies do show higher aromatase activity than males (Black et al., 2005a). The possibility that bluebanded gobies uses divergent pathways for E2 synthesis deserves further investigation because it could represent a shared mechanism for E2 production in hermaphroditic species, and because it has implications for understanding seemingly discordant diurnal patterns of biosynthetically related steroid hormones.

When examining sex differences in hormone levels, we revealed that there was a sex difference only in E2. We did not find any differences in cortisol levels between males and females, which corroborate the lack of sex differences in cortisol levels in the proterandrous anemonefish (*Amphiprion melanopus*; Godwin and Thomas, 1993). In bluebanded gobies, as in many other sex-changing species

(Nakamura et al., 1989; Guiguen et al., 1993; Kroon and Liley, 2000; Lone et al., 2001; Bhandari et al., 2003; Kroon et al., 2003), females have higher E2 levels than males. Studies using exogenous administration of E2 and/or aromatase inhibitors have indeed shown that E2 plays an important role in the regulation of sex change (for a review see Frisch, 2004).

KT has been considered the primary male androgen in fishes and typically is higher in males than females (reviewed by Borg, 1994). This also is true for some protogynous sex-changing species such as the blackeye goby (*Coryphopterus nicholsii*; Kroon and Liley, 2000) and the honeycomb grouper (*Epinephelus merra*; Bhandari et al., 2003), and some proterandrous species such as the sobaity (*Sparidentex hasta*; Lone et al., 2001), the seabass (Guiguen et al., 1993), and the anemonefish (Godwin and Thomas, 1993). Interestingly, bluebanded goby females and males have comparable KT regardless of the time point at which sampling occurred. This result parallels those found in the bi-directional sex-changing broad-barred goby (Kroon et al., 2003), where KT levels were very low or undetectable in all sexual stages, and in the protogynous saddleback wrasse (*Thalassoma duperrey*) where circulating levels of KT were not significantly different between females and males (Nakamura et al., 1989). On the other hand, Nakamura and colleagues (1989) found that even if there were no sex differences in circulating hormones, the testis show a greater capacity for in vitro KT production than the ovary so perhaps tissue specific differences play a major role. Our results confirm those of Rodgers et al. (2006) who found no sex difference in bluebanded goby KT levels unless the males were sampled while they were parenting a clutch of eggs. KT in bluebanded gobies is known to affect genital papilla masculinization (Carlisle et al., 2000) and correlate with percent testicular tissue and the size of the accessory gonadal structure (Black et al., 2005b) indicating a putative role for KT in supporting the initial development of the male phenotype during protogynous sex change. The importance of KT in maintaining the male phenotype, however, might be negligible given the lack of sex differences observed in these studies. Persistent elevations in KT, an androgen known to potentiate the development of exaggerated male phenotypic traits (Oliveira et al., 2001a,b; Mayer et al., 2004) may be unnecessary in bluebanded gobies and other bi-directional sex changers because of the absence of dramatic male secondary sexual characteristics. It also is possible that KT serves more general purposes such as tissue growth and development (Thorarensen et al., 1996) that are sex-independent, thereby precluding dramatic sex differences in hormone concentrations (Schultz et al., 2005). It also is important to note that bluebanded goby females can often have small pockets of testicular tissue present in the gonad (St. Mary, 1993) even while they are reproducing as female and have vitellogenic oocytes. Because KT is associated with spermatogenesis, this might explain why female KT levels are comparable to those found in males.

The lack of sex difference in T levels is not surprising because in fishes T is present in both males and females, and females often have higher levels (Borg, 1994) especially during the spawning season because T is used as a precursor for E2 during aromatization (see above for alternative mechanisms). For example, no sex differences in T were found in the protransdrone sobaity (Lone et al., 2001), in the protogynous saddleback wrasse (Nakamura et al., 1989) and honeycomb grouper (*E. merra*; Bhandari et al., 2003), or in the bi-directional broad-barred goby (Kroon et al., 2003). It should be noted, however, that there are also cases in fish where males have higher T than females (blackeye goby, Kroon and Liley, 2000; common dentex, Pavlidis et al., 2000).

We did not find any evidence of a sex difference in diurnal hormone patterns (time  $\times$  sex interaction). In gulf killifish (*Fundulus grandis*) there is some evidence of a sex difference in diurnal pattern with males having two peaks in cortisol (at 0600 and 1400 h) and females having only a peak at 0600 h (Garcia and Meier, 1973). Nevertheless, most other studies on diurnal patterns examine either males or females.

In the present study, we used a technique that entailed extracting steroid hormones from the water. This technique has the advantages of reducing handling stress and allowing for non-sacrificial hormone sampling in small fishes. Because steroid hormones released through the gills, feces, and urine of fishes can be sampled reliably from the water, and because water-borne steroid concentrations appear to be indicative of circulating levels, this non-invasive sampling technique has become increasingly popular for measuring stress and sex hormones, including all of those measured in this study (Scott and Sorensen, 1994; Greenwood et al., 2001; Scott et al., 2001; Hirschenhauser et al., 2004; Ellis et al., 2004; Pavlidis et al., 2004; Black et al., 2005b; Seibre et al., 2007). As we discussed above, our results are consistent with previous studies in the bluebanded goby and with studies in other species where hormones were measured from blood giving further support to this valuable technique.

In conclusion, we have demonstrated robust diurnal patterns of sex and stress hormones in a sexually plastic goby using a non-invasive sampling technique. Cortisol and E2 showed marked changes in the morning and evening, respectively, while both androgens showed gradual changes detectable only with broader scale analyses. These results suggest that steroid hormones, even those that are intricately linked (e.g., T and E2) follow independent diurnal trajectories, thereby necessitating standalone diurnal assays for each steroid. Our data correspond well with those of other fish species but we still are unable to make comparative generalizations about diurnal hormone patterns owing to tremendous variability across studies in the techniques, contexts, and times of day at which hormones are sampled. It is nonetheless essential that we understand diurnal changes in hormone levels, and properly account for time of day in studies of hormone–behaviour relationships. We confirmed previous reports (Rodgers et al., 2006) that KT concentrations

do not differ between male and female bluebanded gobies. This result contrasts a body of literature implicating KT as the primary male hormone in fishes (but see Schultz et al., 2005 and references therein). Some unique features of the bluebanded goby—notably sexual plasticity and lack of dramatic secondary sexual characteristics in males—may preclude sex differences in KT. Lastly, E2 concentrations were strongly sexually dimorphic, with females consistently (throughout the day) having higher levels than males. This suggests that, of the hormones measured in this study, E2 has the greatest potential for being involved in the maintenance of divergent phenotypes in a bi-directional sex-changing species.

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