Water-borne and Tissue Endocrine Profiles of an Alternative Male Reproductive Phenotype in the Sex Changing Fish, *Lythrypnus dalli*

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In the bi-directionally hermaphroditic fish, *Lythrypnus dalli*, two distinct male phenotypes have been described. The more conspicuous parenting males are larger, establish breeding territories, and display courtship, mating, and parenting behaviors. The alternative males, called mini males, have been postulated to have a parasitic reproductive strategy, although the behavioral ecology of mini males is not well understood. The mini male morph has been characterized based on size and anatomical differences, including sperm-filled accessory gonadal structures (as opposed to mostly mucous in nesting males), consistent with parasitic male morphs in other gobiid species. Here, we determined the endocrine profiles of mini males to gain further insight into their phenotype. Systemic (water-borne) 17β-estradiol (E_2) concentrations were higher than testosterone (T), and 11-ketotestosterone (KT) concentrations were lowest. Mini males in *L. dalli* are similar to parasitic males of other species in having higher T:KT ratios than breeding males. In mini males, brain and reproductive tissue levels of T, E_2, and KT were higher than in the muscle. Among all the steroids, E_2 levels were high in all three tissues in mini males. Data from relative hormone levels in different tissues will lead to a better understanding of the endocrine regulation of behavioral, physiological, and morphological correlates of male sexual polymorphism.

TELEOST fishes exhibit a wide range of strategies to maximize reproductive success, and among these, male sexual polymorphism is very common. Males of many species exhibit alternate reproductive tactics (ARTs), which enable those of poorer competitive ability to procure fertilizations (Taborsky, 1994, 1998; Oliveira et al., 2001a). In general, two broad categories of male morphs have been described. 'Bourgeois' (parenting) males are the larger, competitively superior males that monopolize resources, aggressively defend territories, court females, and, in many fish species, invest in paternal care (Taborsky, 1998). 'Parasitic' males do not compete for mates; instead they employ different behavioral tactics, including satellites, group mobbers, streakers, sneakers, or female mimics, to steal fertilizations (Taborsky, 1998).

To date, several phenotype-typical morphological and physiological differences have been described in species with male ARTs (reviewed in Bass and Grober, 2009). For example, parasitic males have a high gonadosomatic index (gonad weight/body weight) compared to parenting males, a strong indication of sperm competition resulting from intrasexual selection (Taborsky, 1998). In addition to the testes, the reproductive organs of many male fishes also include a lobular organ called the accessory gonadal structure (AGS), seminal vesicle, or sperm duct gland. In the Gobiidae, parenting males have an AGS that is filled with pockets of sperm immersed in a matrix of mucin (sialoglycoproteins that increase ejaculate performance and longevity by extending the duration and reducing the rate of sperm release; Mazzoldi et al., 2005), while the AGS of parasitic males consists entirely of densely packed sperm (Scaggiante et al., 2006). Differences between ARTs are also reflected in brain structure, with parenting males having greater numbers of arginine vasotocin and gonadotropin-releasing hormone neurons in the brain relative to parasitic males (Bass and Grober, 2009). Additionally, in the mid-shipman fish, *Porichthys notatus,* there are brain region-specific differences in glucocorticoid, mineralocorticoid (Arterbery et al., 2010a), and estrogen receptors (Fergus and Bass, 2013), as well as androgenic enzyme (11β-hydroxysteroid dehydrogenase [11β-HSD] and 11β-hydroxylase [Arterbery et al., 2010b]) expression between the two male morphs. For example, the parasitic morph (Type II) has higher mRNA levels of the glucocorticoid receptor, 11β-HSD, and 11β-hydroxylase compared to the parenting morph (Type I) in the vocal-hindbrain and mid-CNS regions (Arterbery et al., 2010b). Finally, there are profound differences in circulating steroid hormones (addressed in detail below) and brain aromatase activity between male morphs in several species (Brantley et al., 1993; Schlinger et al., 1999; Knapp and Neff, 2007).

In the bi-directionally sex changing bluebanded goby, *Lythrypnus dalli*, there is evidence for the existence of two male morphs based on body size and AGS morphology (Drilling and Grober, 2005). Typically, the parenting male (usually >30 mm in standard length, SL) can be readily identified: he dominates his harem of females (usually smaller in size), defends his territory and nest, and displays courtship behavior and paternal care (Rodgers et al., 2006; Pradhan et al., 2014a). Alternative parasitic male morphs in *L. dalli* are smaller, which is why they have been called 'mini males.' Behavioral and ecological roles of this alternative morph have not yet been described. Mini males tend to overlap in size with small females, ranging in SL between 17–25 mm (Drilling and Grober, 2005), but have male-like external genitalia (genital papilla, GP). Male GPs are long and pointy (length to width [L:W] ratio > 1.5), while females have a wide and rounded GP (L:W ratio is close to 1; Carlisle et al., 2000). Histological analysis of the mini male gonad reveals the production of high quantities of mature sperm, indicating that these males are sexually mature (Drilling and Grober, 2005). The morphology of the mini male AGS also fits the description of the AGS in other parasitic morphs within the Gobiidae (Scaggiante et al., 2006).

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In this study, we measured water-borne and tissue steroid hormone levels in mini males. Steroid hormones have profound effects on behavior and morphology in vertebrates. In other species with ARTs, these males have phenotype-typical steroid levels (Brantley et al., 1993; Oliveira et al., 2001a, 2001b); therefore, it is possible that mini males of *L. dalli* also have phenotype-typical steroid levels. There are several methods of collecting steroids in organisms, which provide insight into distinct aspects of physiology. Measures of circulating steroid hormones, presumed to be of gonadal origin, are most often used to understand the endocrine regulation of behavior. Notwithstanding the potential role of circulating hormones, there is considerable evidence of differences in sex steroid levels in circulation relative to specific target tissues where hormones act (Schmidt et al., 2008). This is particularly relevant to brain levels of steroids, as behavioral change does not always correspond with changes in circulating steroid levels (Pradhan et al., 2010). Moreover, tissue-specific androgen and estrogen production are often regulated independently (Remage-Healey et al., 2008; Pradhan et al., 2010, 2014a). In *L. dalli*, when the dominant male is removed from the social group, all the females in the hierarchy show tissue-specific responses in hormone levels. In addition, the levels of steroid hormones are also different across tissues (Lorenzi et al., 2012). This independent regulation is important because the brain modulates behavior and must respond to rapid changes in social context, while other tissues (such as the gonad) have more delayed responses (Lorenzi et al., 2012). Brain aromatase, which is responsible for converting testosterone (T) to 17β-estradiol (E_2), has long been thought to be critical in locally regulating the expression of behavior (Adkins et al., 1980), and neural aromatase and 11β-HSD have both been implicated in several behavioral responses that are too rapid to be regulated by genomic mechanisms in the gonad (Black et al., 2005; Pradhan et al., 2014a). Given the substantial independent data indicating the utility of both systemic and tissue steroids, measuring tissue steroid levels in addition to circulating levels will provide a deeper understanding of bioavailability, production, and mechanisms by which specific behaviors are regulated (Pradhan et al., 2014b).

Mini males are found in low abundance and their number decreases in the general population over the course of the breeding season (Drilling and Grober, 2005). Hence mini males are not the focus of most studies in our laboratory. The present study was opportunistic, because we caught a number of mini males during the breeding season, and utilized these animals to address a very specific question about the endocrine correlates of ARTs in *L. dalli*. The goal of this study is to establish the endocrine profiles of androgens (T and 11-ketotestosterone [KT]) and E_2 in *L. dalli* mini males during the mid-breeding season. While endocrine profiles of females and parenting males are well established in *L. dalli* (Lorenzi et al., 2008; Lorenzi et al., 2012), little is known about the endocrinology of mini males. In addition to being a prohormone for E_2, T is also a precursor for KT, the more potent androgen of the two in many fishes (Borg, 1994). For example, systemic KT implants lead to the masculinization of female-typical GP (Carlisle et al., 2000; Pradhan et al., 2014b). Behavioral effects of KT are highly context dependent, such that it influences agonistic behavior only during social instability (Pradhan et al., 2014b), but promotes parenting behavior in males without affecting agonistic interactions (Pradhan et al., 2014a). Thus, the relationships among these steroids can provide insight into their physiological roles (Black et al., 2005). In the present study, water-borne steroids were quantified, which are a good representation of circulating or body-wide steroid levels (Rodgers et al., 2006; Lorenzi et al., 2008; Wong et al., 2008). This is a non-invasive method of collecting steroids that are exuded by fish into the surrounding water via gills and urination (Sebire et al., 2007). This method is particularly appropriate and useful for small fish due to the difficulty and/or impossibility of collecting sufficient plasma. We also quantified total extractable steroids from the brain, reproductive tissue (testes and AGS), and muscle to evaluate local tissue steroid levels (Schmidt et al., 2009; Lorenzi et al., 2012). Based on previous work on *L. dalli* (Lorenzi et al., 2012), we predict that levels of steroids will be different in all the tissues. In addition, we also predict that systemic hormone levels will be similar to what is seen in parasitic males of other species.

MATERIALS AND METHODS

Field protocol.—The habitat of *L. dalli* ranges from the benthic rocky reefs of the Gulf of California, Mexico, to Morro Bay, California (Miller and Lea, 1972). Free-living *L. dalli* live in mixed sex groups. Fish were captured in association with the crowned urchin, *Centrostephanus coronatus*, off the coast of Santa Catalina Island, California between July 9–17, 2009 using SCUBA diving and hand nets. Fish were then transferred to Nalgene bottles for transport to the boat, where they were then moved to a bucket filled with fresh seawater. Within 90 min of capture, fish were brought back to the laboratory at the USC Wrigley Institute for Environmental Studies.

Due to the lack of dramatic sexual dimorphism in this species, it is difficult to identify mini males in the field (see Drilling and Grober, 2005; for population estimates during the breeding season), so verification requires microscopic examination of the GP. To confirm phenotype, fish were immediately anesthetized (in water containing tricaine methanesulfonate MS-222; 0.5 mg/100 mL seawater) upon arriving back at the laboratory, and sex of the fish was determined based on the morphology of the GP (Drilling and Grober, 2005). The standard length (SL) was measured with a vernier caliper, and a digital photograph of the GP was obtained using an external camera that transferred images from the microscope to a laptop computer (Motic Images Software system running on a MacBook). A fish was considered a mini male (n = 9) if its SL was between 17–25 mm and if it had a male-like GP (L:W ratio >1.5 and a cone-like appearance; Drilling and Grober, 2005; Pradhan et al., 2014b). The subject was immediately resuscitated in a cup of fresh seawater and transferred to another beaker for hormone collection. This method of collection of steroids immediately after recovery from anesthesia does not affect systemic (water-borne) cortisol levels, indicating that this procedure does not represent a significant stressor for *L. dalli* (Solomon-Lane and Grober, 2012).

Steroid collection, extraction, and enzyme immunoassays.—Systemic sex steroid levels were determined by measuring water-borne steroid concentrations. Water-borne hormone samples were collected from each subject for a 2 h period between 1400–1700 h, according to the protocol described previously (Lorenzi et al., 2008). The only difference was

that we doubled the collection time from 1 h in the previous study to accommodate the possibly slower rate of steroids being exuded into water, considering that mini males are smaller (hence have a lower surface area) than nesting males and females. Briefly, each fish was placed in a 200 mL polypropylene beaker (cleansed with 100% ethanol, distilled water, and seawater prior to use) containing 100 mL fresh seawater, obtained from the tap supplying ocean water to holding tanks. To maintain a constant temperature during the sampling period, the beaker was placed in a water table with about 2 cm of flowing seawater. Individuals being sampled were in visual contact with each other. At the end of the sampling period, the water was poured through a net (rinsed with clean seawater) into a clean 200 mL polypropylene beaker.

Hormones were extracted from the water sample using C18 solid-phase extraction columns (Lichrolut RP-18, 500 mg, 3.0 ml; Merck) fitted to a 12-port vacuum manifold. This procedure has been validated in previous studies (Earley et al., 2006; Rodgers et al., 2006; Lorenzi et al., 2008). The extraction procedure was completed in two segments. First, water-borne steroid samples from the beakers were adsorbed to the C18 columns, both ends of each column were sealed with parafilm, and they were then stored at −20°C prior to shipment to Georgia State University (GSU; Atlanta, GA). Second, the extraction process was completed at GSU according to previous studies (Lorenzi et al., 2012; Pradhan et al., 2014b). The eluates were collected in 13 × 100 mm borosilicate test tubes and evaporated until dry under a gentle stream of nitrogen at 37°C.

Immediately following the 2 h sampling of systemic steroids, subjects were euthanized with an over-dose of MS-222 (1 mg/100 mL seawater) and tissues were immediately harvested. Brain, muscle tissue (located around the caudal penduncle), and reproductive tissues (testes and AGS) were collected and frozen rapidly on dry ice. Gross anatomical examination confirmed the presence of fully developed testes. Both testes and AGS were collected, as they comprise the total reproductive tissue in males and because both these tissues are known to produce sex hormones (Lahnsteiner et al., 1993; Lorenzi et al., 2012). Dissection times for each individual ranged between 6–8 min. Tissue samples were then stored at −80°C for six weeks, shipped to GSU on dry ice, and stored again at −80°C until further processing. Prior to extraction, each tissue sample was weighed and homogenized in 350 µL ice-cold buffer (brain and reproductive tissues in 0.1 M phosphate buffer; muscle, 0.1 M borate buffer) and 1500 µL HPLC grade methanol (denatures proteins) was added to stop steroidogenic enzyme activity. Steroids were then extracted from tissues according to previously described methods (Newman et al., 2008; Pradhan et al., 2014b). The final eluates were collected in 13 × 100 mm borosilicate tubes and dried in a gentle stream of nitrogen at 37°C.

Dried water-borne and tissue extracted steroids were then re-suspended to yield a final volume of 350 µL (5% ethanol and 95% EIA buffer supplied by Cayman Chemical kits: T: 582701, KT: 582751, E_2: 582251), which was enough for three hormone assays per tissue. Re-suspended samples were shaken on a multi-tube vortex for 1 h before beginning the EIA procedure. Each EIA for T, E_2, and KT was performed in duplicate, following the manufacturer's protocol. Validations for all these assays have been previously published (Lorenzi et al., 2008). The tracer for each steroid was a specific acetylcholine esterase conjugate (all the details are provided in the manufacturer's protocol). We included the following modifications from the assay manufacturer's protocol: first, following addition of the tracer and antiserum, incubations (T, 2 h; E_2, 1 h; KT, 18 h) were performed at 4°C on an orbital shaker; second, the plate was read at 45, 60, 75, and 90, min following the addition of Ellman's Reagent, and the readings that yielded the best standard curve ($r^2 > 0.9$) was used for further analysis of sample data. Intra-assay variations for T, E_2, and KT were 5.22%, 5.95%, and 2.68%, respectively. Systemic steroid concentrations were normalized to per hour of release. Lastly, all systemic steroid data are presented as pg/sample/hr (pg/mL multiplied by 0.35 mL, which was the amount of EIA buffer used to re-suspend the sample), while tissue steroids are further corrected by weight (pg sample/mg tissue).

Statistics.—Statistical analyses were conducted using Prism 4.0 for Mac OS X. Regression analysis was used to determine the relationship between GP ratio and SL. A within subjects design was used to examine the concentrations of steroids in each individual. For systemic steroids, the data did not fit the homogeneity of variance assumptions for parametric statistical tests, so we used Friedman's test. For tissue steroids, data were transformed [log (y)] to achieve homogeneity of variance. A two-way repeated measures ANOVA was then conducted with tissues (brain vs. reproductive tissue vs. muscle) and steroids (T, E_2, and KT) as within subject variables. Significant main effects were further analyzed by Wilcoxon-signed rank or Bonferroni tests. For each hormone, one-way repeated measures ANOVAs were used to analyze differences in tissues, and significant results were followed by Tukey tests. Regression analyses were performed to examine (1) the relationship between water-borne (systemic) and tissue (local) concentrations of steroids, (2) the relationship between systemic steroid concentration and GP ratio, and (3) the relationship between tissue steroid concentrations and GP ratio. To allow for objective between study and between species comparisons, we looked at ratios of hormones within studies (analyzed by one-way repeated measures ANOVA) and then compared these ratios between studies. For example, we computed the ratio of T:KT levels in the present study and estimated T:KT based on previously published hormone levels in nesting male and female *L. dalli* and in other species. We exercise caution when interpreting the ratio data estimates across studies because the relative averages from published work were used. All data (except for ratio analyses) are represented as mean±SEM, and α was set at 0.05.

RESULTS

Water-borne steroids.—There were significant differences among concentrations of systemic sex steroids (Friedman's $F = 18.00$, $P < 0.0001$, $n = 9$; Fig. 1A). *Post hoc* Wilcoxon signed rank tests revealed that E_2 concentrations were significantly higher than T and KT ($P = 0.004$), and T concentrations were significantly higher than KT ($P = 0.004$).

Tissue steroids.—Two-way repeated measures ANOVA revealed significant main effects of tissue ($F_{2,24} = 21.38$, $P < 0.0001$; Fig. 1B) and steroid hormone ($F_{2,24} = 37.71$, $P < 0.0001$), but there was no interaction ($F_{2,24} = 0.93$, $P = 0.46$). *Post-hoc* Bonferroni tests revealed no differences

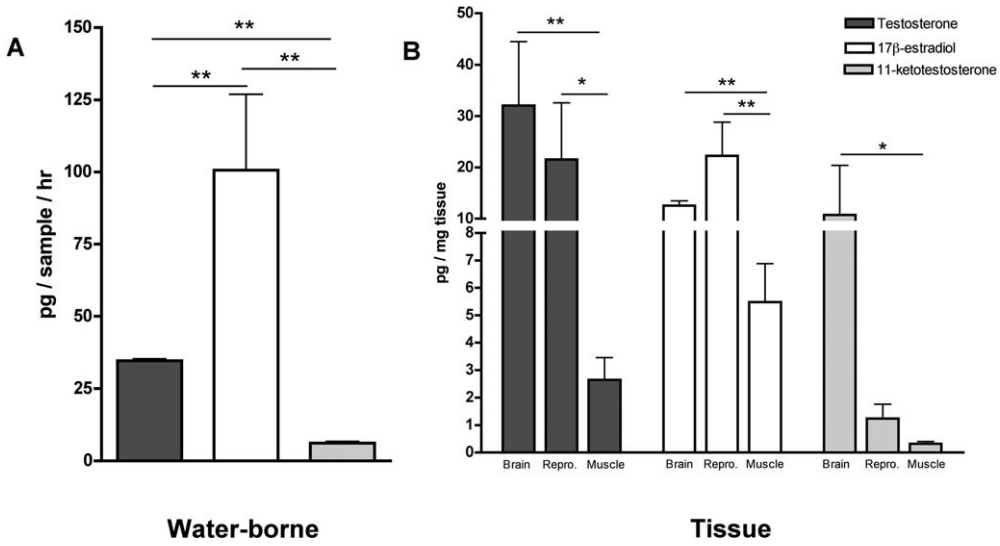

Fig. 1. Water-borne (A) and tissue (B) levels of sex steroids (mean±SEM) in mini males (n = 9). Repro., reproductive tissue (testes+AGS). * $P <$ 0.05, ** $P < 0.01$.

between T and E_2 levels in any tissue ($P > 0.05$). Levels of both T and E_2 were significantly higher than KT in all three tissues (T; brain: t = 4.24, $P < 0.001$; reproductive tissue: t = 4.26, $P < 0.001$; muscle: t = 3.54, $P < 0.01$; E_2; brain: t = 3.69, $P < 0.001$; reproductive tissue: t = 5.25, $P < 0.001$; muscle: t = 4.89, $P < 0.001$; Fig. 1B).

Levels of T in tissues were significantly different ($F_{2,8}$ = 8.41, $P = 0.0026$; Fig. 1B). There were no differences in T between brain and reproductive tissue ($P > 0.05$), but levels of T in brain and reproductive tissue were higher than in muscle (brain vs. muscle: q = 5.80, $P < 0.01$; reproductive tissue vs. muscle: q = 4.02, $P < 0.05$). Tissue E_2 levels also varied significantly ($F_{2,8}$ = 11.41, $P = 0.0009$; Fig. 1B). Again, there were no differences in E_2 between brain and reproductive tissue ($P > 0.05$), but both brain and reproductive tissue were higher than muscle (brain vs. muscle: q = 5.27, $P < 0.01$; reproductive tissue vs. muscle: q = 6.29, $P < 0.01$). Levels of KT in the tissues were also significantly different ($F_{2,8}$ = 5.58, $P = 0.0145$; Fig. 1B), but did not follow the same pattern as T and E_2. While KT was not significantly different between brain and reproductive tissue, and between reproductive tissue and muscle ($P >$ 0.05), brain KT was significantly higher than muscle (q = 4.68, $P < 0.05$).

Regression analyses.—There was a significant relationship between brain KT and water-borne KT ($r^2 = 0.56$, $P = 0.02$); however, there were no other significant relationships between any other water-borne steroids and tissue steroids (Table 1). There were also no significant relationships between steroids (water-borne and tissue) and SL of mini males (Table 2). Systemic levels of all three sex steroids were positively correlated with GP ratio (Fig. 2). For tissues, the only significant relationship was that brain KT levels positively correlated with GP ratio (Table 3).

Relationship between steroids.—In water-borne samples and in individual tissues, T:KT was the highest and significantly different from both T:E_2 and KT:E_2 ($P < 0.01$; Figs. 3, 4).

DISCUSSION

Mini males are difficult to identify in the field and this was an opportunistic study that allowed us to investigate water-borne and tissue levels of T, KT, and E_2 in mini males of *L. dalli*. This is the first study to address the alternative male morph in this species since its original description, which was based on external morphology/gonad histology (Drilling and Grober, 2005). The current study examined the endocrine traits (e.g., tissue and water-borne steroid hormones) in mini males to see if this species shows the suite of endocrine relationships that is well established in other fish species with ARTs (Bass and Grober, 2009).

Water-borne steroids.—Similar to female and parenting male *L. dalli* (Lorenzi et al., 2008), T was the predominant androgen in mini males, while KT was lower (Fig. 1A). As a result, water-borne T:KT ratio was highest in mini males (Fig. 3D). Of the two androgens, KT is considered more potent in teleosts, based on the greater phenotypic effects of KT and the greater binding affinity to the androgen receptor (Borg, 1994). In general, KT levels tend to be higher in

Table 1. Relationship between water-borne and tissue sex steroids in mini males of *L. dalli*. Results of simple regression analysis. n = 9 per group. *$P <$ 0.05.

	Water-borne vs. brain			Water-borne vs. reproductive tissue			Water-borne vs. muscle		
Steroid hormone	r^2	$F_{1,7}$	P	r^2	$F_{1,7}$	P	r^2	$F_{1,7}$	P
Testosterone	0.35	0.87	0.09	0.03	0.24	0.64	<0.01	0.01	0.92
17β-estradiol	0.04	0.30	0.60	<0.01	0.06	0.81	0.15	1.20	0.30
11-ketotestosterone	0.56	9.018	**0.02***	0.02	0.11	0.74	<0.01	0.01	0.92

Table 2. No significant relationship between sex steroids and standard length in mini males of *L. dalli*. Results of simple regression analysis. $n = 9$ per group.

	Water-borne			Brain			Reproductive tissue			Muscle		
Steroid hormone	r^2	$F_{1,7}$	*P*	r^2	$F_{1,7}$	*P*	r^2	$F_{1,7}$	*P*	r^2	$F_{1,7}$	*P*
Testosterone	0.12	0.92	0.37	0.06	0.49	0.51	<0.01	0.01	0.94	0.02	0.17	0.69
17β-estradiol	0.03	0.22	0.66	<0.01	<0.01	0.99	<0.01	<0.01	0.97	0.01	0.10	0.76
11-ketotestosterone	<0.01	<0.01	0.98	0.16	1.30	0.29	0.01	0.06	0.81	0.07	0.54	0.49

parenting males, perhaps for the regulation of male-specific courtship and parenting behavior (Rodgers et al., 2013; Pradhan et al., 2014a). This observation is consistent with studies from several phylogenetically distant species, such as plainfin midshipman (Brantley et al., 1993), bluegill sunfish (Knapp and Neff, 2007), and round gobies (Marentette et al., 2009). The ratio of T to KT is generally higher in parasitic than parenting males for all of the species that have been examined (Fig. 3). Of the three steroids measured, E_2 showed both the highest mean levels and the highest variation (Figs. 1, 2B). In other species, circulating E_2 levels are seldom measured and tend to be undetectable in nesting and parasitic males (e.g., plainfin midshipman; Brantley et al., 1993). However, timing is important to consider when sampling hormones from seasonal species, and E_2 was detectable in parenting male plainfin midshipman in a more recent study during one sampling period (Sisneros et al., 2004). In bluegill sunfish, *Lepomis macrochirus,* many parenting males have non-detectable E_2 and parasitic males also have generally low E_2 compared to females (Knapp and Neff, 2007). For these reasons, we were unable to include E_2 in the ratio calculations. Finally, an interesting insight from using ratios for these analyses is that we can compare across different sample collection and assay protocols (e.g., water-borne versus plasma). In mini males, T:KT was higher than both T:E_2 and KT:E_2, consistent with the high E_2 levels in this morph. Interestingly, KT:E_2 was lower compared to females, which is probably because while females have high KT, mini males have lower KT and higher E_2. Taken together, high systemic T:KT in mini males (mainly attributed to low water-borne KT), in comparison with parenting males, is consistent with the hypothesis that elevated KT is critical for regulating male parenting in *L. dalli* (Rodgers et al., 2006; Pradhan et al., 2014a), and that there are morph-specific systemic levels of sex steroids in *L. dalli*.

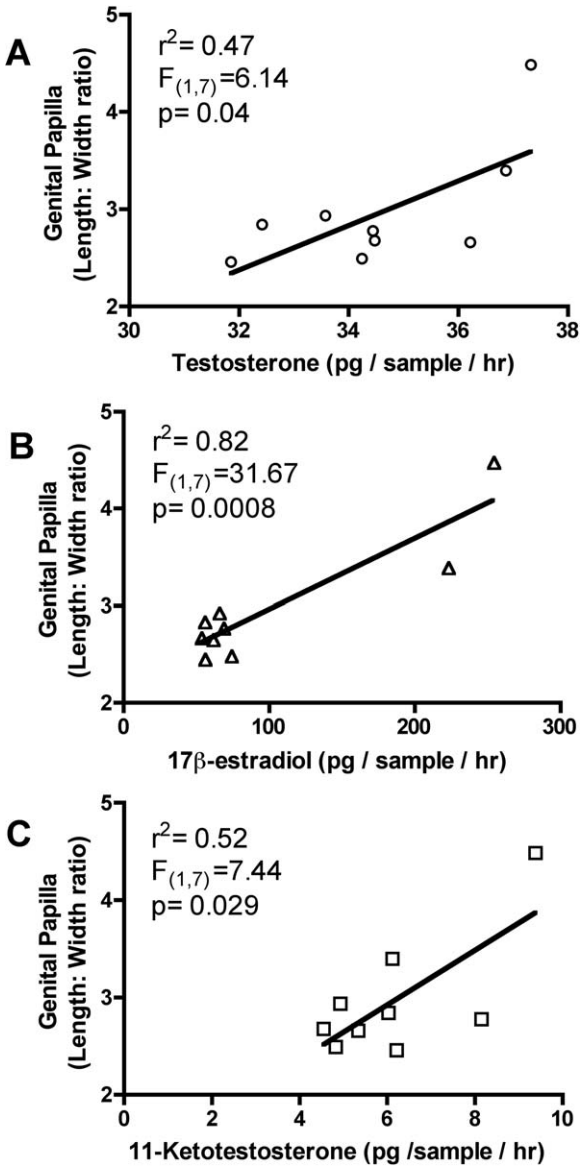

Fig. 2. Linear regressions between water-borne (A) testosterone, (B) 17β-estradiol, and (C) 11-ketotestosterone levels and genital papilla morphology (expressed as Length:Width ratio) in mini males of *L. dalli* (n = 9). Averages reported in Lorenzi et al. (2012) were used to estimate ratios for females and parenting males. T, testosterone; E_2, 17β-estradiol; KT, 11-ketotestosterone.

Tissue steroids.—A comparison of tissue-specific levels of T, E_2, and KT in mini males of *L. dalli* provides insights into the source of these hormones. If reproductive tissue is the primary producer of sex steroids, then levels should be highest in reproductive tissue. However, it is clear from the data that in mini males, both brain and reproductive tissues have similar levels of all three hormones (Fig. 1B), and these levels were, in general, higher than in muscle tissue. In contrast to the systemic data, T:KT was lower in brain, reproductive tissue, and muscle of mini males compared to nesting males and females (Fig. 4). This trend is also consistent with gonadal T:KT in peacock blennies, *Salaria pavo* (Oliveira et al., 2001a). In plainfin midshipman, the expression of mRNA coding for 11β-hydroxylase and 11β-HSD, the enzymes that convert T to KT, is higher in the central nervous system of parasitic males compared to parenting males, while these patterns are reversed in the gonad and sonic muscle tissue (Arterbery et al., 2010b). Measuring the rate of *in vitro* 11β-HSD activity in these tissues might further provide insights into the relationship

Table 3. Relationship between tissue steroid levels and genital papilla length to width ratio in mini males of *L. dalli*. Results of simple regression analysis. $n = 9$ per group. $^{**}P < 0.01$.

	Brain			Reproductive tissue			Muscle		
Steroid hormone	r^2	$F_{1,7}$	P	r^2	$F_{1,7}$	P	r^2	$F_{1,7}$	P
Testosterone	0.34	3.59	0.09	0.06	0.45	0.53	0.19	1.73	0.23
17β-estradiol	0.09	0.70	0.42	0.00	0.02	0.89	0.09	0.68	0.44
11-ketotestosterone	0.81	28.20	**0.001****	0.02	0.12	0.74	0.17	1.41	0.27

between T and KT, because tissues can also sequester hormones via binding to receptors (Shi et al., 2012).

The confirmation of tissue-specific steroid levels in this fish is important because it challenges the assumption that sex steroids are mainly produced by reproductive tissues and underscores the importance of properly interpreting hormone-behavior relationships based upon the specific proxy of steroid measurement being evaluated. In females of *L. dalli*, ovaries are likely to be the source of high systemic E_2 (Lorenzi et al., 2008, 2012) because aromatase activity in the gonad is higher than in the brain (Black et al., 2005). While the predominant source of E_2 is less clear in mini males, aromatase activity is high in diencephalon-midbrain and vocal hindbrain areas of parasitic (Type II) plainfin midshipman morph, suggesting a possible neural origin (Schlinger et al., 1999). In mini males, brain and reproductive tissue levels of T, E_2, and KT were similarly higher than in the muscle, but it is unlikely that the muscle produces steroids because it has relatively low levels of all steroids. Taken together, these results suggest that both the reproductive tissue and the brain could be significant sites of steroid production. Further studies of tissue expression and activity of steroidogenic enzymes in *L. dalli* would provide insight into the relationship between the sex hormone production and use in peripheral tissues (Pradhan et al., 2014a). Manipulation of steroids in mini males, as in previous

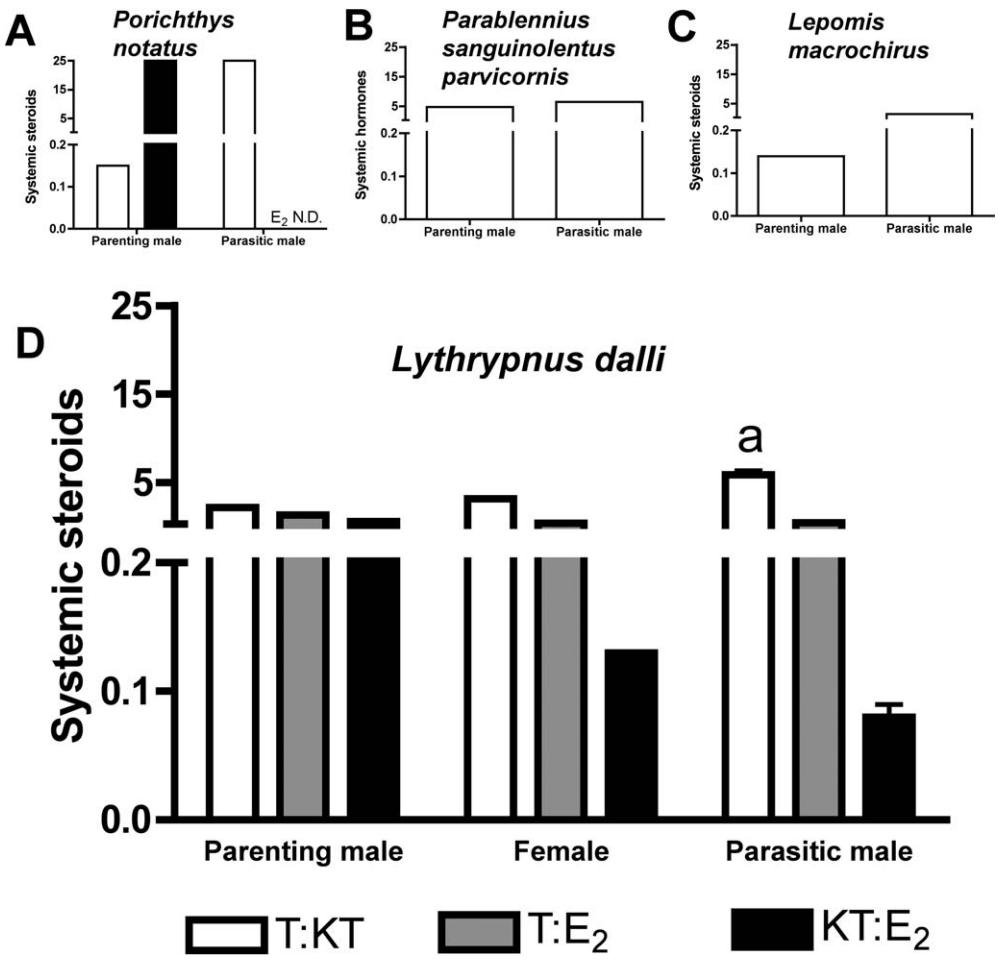

Fig. 3. Relative systemic sex steroid ratios from four species of teleost fishes. (A) *Porichthys notatus,* plainfin midshipman, (B) *Parablennius sanguniolentus parvicornis*, rock-pool blenny, (C) *Lepomis macrochirus*, bluegill sunfish, and (D) *Lythrypnus dalli*, bluebanded goby. Averages reported in Brantley et al. (1993), Oliveira et al. (2001b), Sisneros et al. (2004), Knapp and Neff (2007), and Lorenzi et al. (2008) were used to estimate ratios. Data for KT are not available for females of some species. T, testosterone; E_2, 17β-estradiol; KT, 11-ketotestosterone, N.D., non-detectable. For mini male data, the letter above the bar indicates that the mean is significantly different from the others.

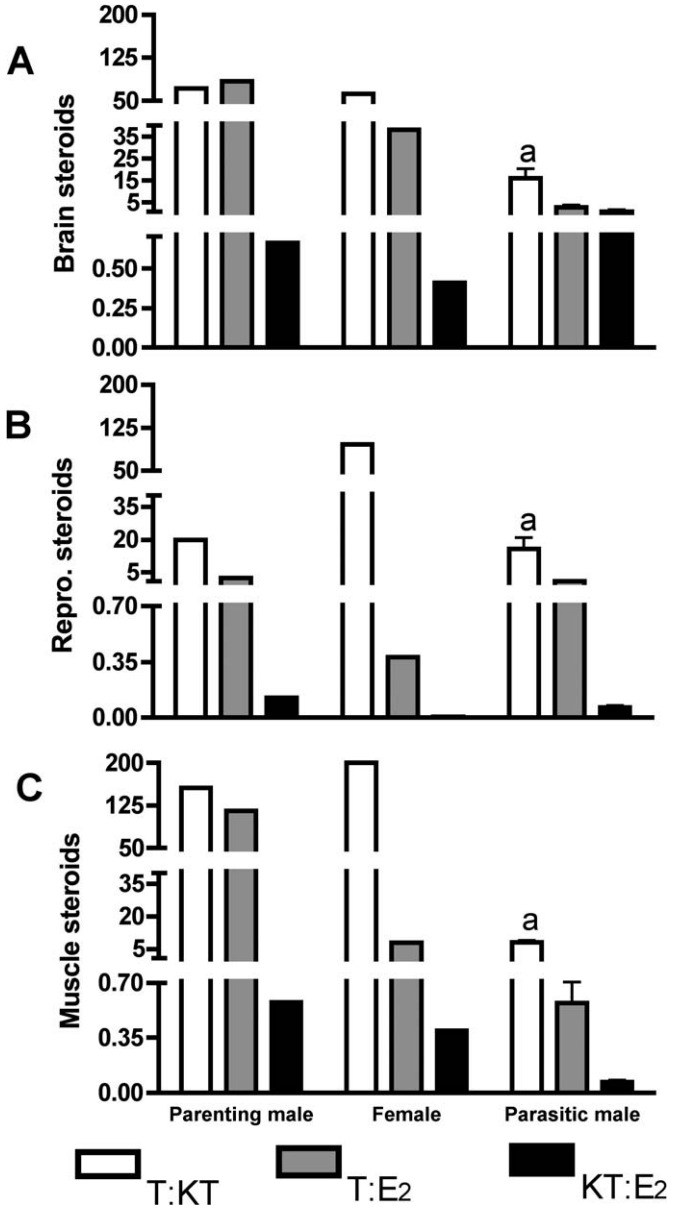

Fig. 4. Relative tissue sex steroid ratios from (A) brain, (B) reproductive tissue, and (C) muscle of parenting males, females, and mini males of *Lythrypnus dalli*. For mini males, mean±SEM is reported. For mini male data, the letter above the bar indicates that the mean is significantly different from the others.

studies involving alternative male morphs from other species (Goncalves et al., 2007; Oliveira et al., 2001b), will further elucidate the endocrine regulation of mini male phenotype.

Traditionally, gonads are assumed to be the source of sex steroids in circulation and target organs (Borg, 1994; Magee et al., 2006). Recent studies question whether tissues are passive recipients of sex steroids (via receptors), and/or also capable of synthesizing steroids *de novo* (Schmidt et al., 2008). Tissue-specific aromatization of circulating T has long been considered to be the mechanism for high local levels of E_2 in organs such as the brain (Adkins et al., 1980). In addition, recent studies provide evidence for androgen synthesis in tissues other than gonad, such as expression and activity of steroidogenic enzymes (Do Rego et al., 2009; London et al., 2009; Arterbery et al., 2010b; Pradhan et al., 2010). Thus, it is important to consider the endocrine profiles of other tissues in the regulation of sex-specific reproductive phenotype. Specifically, steroid loads in brain tissues may provide key insights into the regulation of morph-typical reproductive behavior.

Water-borne versus tissue steroids.—In this study, there was no relationship between water-borne and tissue sex steroids, except for brain KT and systemic KT (Tables 2, 3). If the traditional dogma that gonads are the primary site of steroid synthesis is true, then systemic levels should closely track gonadal steroid production or levels of hormone extracted from reproductive tissue, and this relationship should hold true for other target tissues as well. Thus, it is possible that the brain produces KT, which might account for high systemic KT. Based on previous studies, both males and females from social groups of *L. dalli* in the laboratory have high KT in the brain (Lorenzi et al., 2012) and brain KT can also be modulated to affect behavior (Pradhan et al., 2014a). Interestingly, the pattern of steroid levels in muscle matches the pattern of water-borne steroid levels (Figs. 1, 4), suggesting that muscle may represent a passive sink for steroids synthesized in other tissues and, therefore, might serve as a useful metric for systemic steroid levels.

Steroids versus morphology.—There was no relationship between SL and any water-borne or tissue sex steroid in mini males (Tables 2, 3), similar to a previous study for KT in nesting male and female *L. dalli* (Rodgers et al., 2006). In a cooperatively breeding cichlid, *Neolamprologus pulcher*, there is no relationship between SL and androgens (Bender et al., 2006). This finding is contrary to bluegills, where circulating E_2 and SL are negatively correlated (Knapp and Neff, 2007).

Systemic steroids were all positively correlated with GP ratio; however, E_2 had the strongest relationship ($r^2 = 0.82$; Fig. 2). Except for brain KT, there were no relationships between tissue steroids and GP ratio (Table 3). Visual inspection of the data reveals that two mini males have a high GP ratio, and these two males also have higher E_2 levels (Fig. 2). Due to the limited sample size in this study, we are unable to confirm the presence of a bimodal pattern, but we can speculate that there could be two different parasitic morphs.

Speculation on behavior.—Even though the endocrine profiles seem to be morph-specific (Figs. 3, 4), these data do not help elucidate the strategy that mini males take in order to obtain surreptitious fertilizations. The high variation in E_2 levels suggests a possibility of a bimodal distribution in E_2 levels and this can be confirmed in future studies with a greater sample size. In the laboratory, territorial males generally spawn with only one female at a time. In the field, we have not seen spawning; however, nesting sites of *L. dalli* are in small crevices or tubes, which reduce the likelihood of sneaking or streaking strategies. Like small females of *L. dalli* (18–24 mm SL), mini males might be less site-attached (Lorenzi et al., 2012) and, thus, could be adopting female mimicry. Under field conditions, it is challenging to reliably identify specific individuals as mini males because mini males are female-like in size and appearance. As a result, it is difficult to study the behavioral characteristics of these male morphs. However, mini male sized fish have never been observed defending a territory or dominating a harem of females. Preliminary studies in the

laboratory have revealed that when placed in a social group consisting of a nesting male and several females, mini males either remain mini males or transform to females (Lorenzi and Grober, unpubl. data).

Conclusions.—Here, we establish the endocrine profiles of mini males and these data are consistent with the previously published, morphologically based description of mini males of *L. dalli*. We show that brain KT is correlated with systemic KT and systemic KT is correlated with GP morphology; illustrating the importance of KT in these analyses. Out of the three steroids studied, only systemic and brain KT levels were associated. These data will help inform future studies on the role of hormones in mediating or responding to the social dynamics of mini males. Determining the local hormone profiles of tissues allows for more comprehensive understanding of endocrine regulation and corresponding phenotype. For example, data from tissue (e.g., local) can provide insight into where the site of action of steroids is and the mechanisms by which steroids have their phenotypic effects.

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