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Juvenile social status predicts primary sex allocation in a sex changing fish

Tessa K. Solomon-Lane,[a,*] Polina Shvidkaya,[b] Alma Thomas,[c] Megan M. Williams,[c] Andrew Rhyne,[d] Lock Rogers,[c] and Matthew S. Grober[b,e]

[a] Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712, USA
[b] Department of Biology, Georgia State University, Atlanta, GA 30303, USA
[c] Department of Biology, Agnes Scott College, Decatur, GA 30030, USA
[d] Department of Biology and Marine Biology, Roger Williams University, Bristol, RI 02809, USA
[e] Neuroscience Institute, Georgia State University, Atlanta, GA 30303, USA

*Author for correspondence (e-mail: tksolomonlane@utexas.edu)
Address at the time the work was conducted (if different from current): Solomon-Lane: Neuroscience Institute, Georgia State University, Atlanta, GA 30303.

SUMMARY Both individual sex and population sex ratio can affect lifetime reproductive success. As a result, multiple mechanisms have evolved to regulate sexual phenotype, including adult sex change in fishes. While adult sex change is typically socially regulated, few studies focus on the non-chromosomal mechanisms regulating primary sex allocation. We investigated primary sex determination in the bluebanded goby (*Lythrypnus dalli*), a bidirectionally sex-changing fish. Of the studies investigating primary sex determination in species with adult sex change, this is the first to incorporate the roles of social status and size, key factors for determining adult sex allocation. For *L. dalli*, adult sex is regulated by social status: dominants are male; subordinates are female. In social groups of laboratory-reared juveniles, we demonstrate that status also predicts primary sex. Dominant juveniles developed male-typical genitalia, and their gonads contained significantly less ovarian tissue than subordinates, which developed female-typical genitalia. To better understand natural development, we quantified the distribution of juveniles and adults on the reef and analyzed genital papilla and gonad morphology in a sample of wild-caught juveniles. Juveniles were observed in various social environments, and most grouped with other juveniles and/or adults. The majority of field-caught juveniles had female-typical genitalia and bisexual, female-biased gonads. These data are consistent with a single mechanism that regulates sexual phenotype throughout life. Social status could first cause and then maintain through adulthood a female-biased population, allowing individuals to regulate sex based on local conditions, which is important for optimizing lifetime reproductive success.

INTRODUCTION

Fisher's principle states that population sex ratio should, over time, reach a stable equilibrium at ~1:1 (Fisher 1930), but there are notable examples of selection for skewed ratios (Trivers and Willard 1973). Sex ratios are influenced by a variety of factors (Charnov 1982; Charnov and Bull 1989; Hardy 1997), including the mechanism of sex determination (e.g., genetic or environmental) (Kobayashi et al. 2013) and differential survival of the sexes (Donald 2007). An individual's sex relative to the population sex ratio has a profound effect on lifetime fitness, especially in skewed populations. Members of the rarer sex may be more likely to find a mate, choosier in mate selection, and require less investment post-fertilization (Fisher 1930; Hardy 1997). Multiple mechanisms have evolved to influence sex allocation, which describes the allocation of resources to female versus male reproduction. In many species, parents influence the sex of their offspring so as to gain a fitness advantage (Hardy 1997). In eusocial insects, for example, queens influence the hive sex ratio, in part by regulating which eggs get fertilized and develop as females (Passera et al. 2001).

While optimizing offspring sex ratio promotes parental fitness, changing sex in response to population structure allows individuals to optimize their lifetime fitness. Adult sex change is favored in species in which the expected fitness for each sex differs across life history stages, usually increasing more rapidly with age or size for one sex (Ghiselin 1969; Warner 1975). Teleost fishes exhibit extraordinary sexual plasticity, including protogynous (female to male), protandrous (male to female), and bidirectional sex change (Kobayashi et al., 2013). In a haremic species with territorial males, for example, young, small males may be less competitive. To optimize reproductive potential, an individual should reproduce as female when young and small and as male when older and larger (Ghiselin 1969; Warner 1975). This sexual reorganization is usually socially regulated: after removing the dominant fish from a social group,

the individual that establishes dominance changes sex through a cascade of behavioral, physiological, and morphological changes (Reavis and Grober 1999; Godwin 2009).

Primary sex allocation decisions in sex changing species have received little attention relative to the mechanisms underlying adult re-allocation. The sex of an individual at first reproduction, however, is critical to evaluating reproductive potential over a lifetime (McGraw and Caswell 1996; Charnov 1997). Evidence from several species of sex changing fishes suggests that primary sex, like adult sex, is influenced by the social environment (e.g., Bruslé-Sicard et al. 1994; Hobbs et al. 2004; Iwata et al. 2008). For example, the proportion of juvenile bluehead wrasse (*Thalassoma bifasciatum*) (Munday et al. 2006a) and chocolate hind (*Cephalopholis boenak*) (Liu and Sadovy 2004b) that developed as male versus female differed between isolated compared to group-reared juveniles. Identifying the specific social cue that regulates initial sex allocation will be critical to determining whether the mechanisms underlying sexual plasticity are conserved across life history stages and identifying the mechanisms that allow social information to direct developmental pathways.

We test the hypothesis that social status, the cue that regulates adult sex, also determines primary sex allocation in the bluebanded goby (*Lythrypnus dalli*), a fish capable of bidirectional sex change. In nature, *L. dalli* functions primarily as a sequential, protogynous hermaphrodite (St. Mary 1993), and the population is female-biased with sex ratios ranging from 1.1:1 to 4:1, female to male (Wiley 1976; Behrents 1983; Drilling and Grober 2005). Adults form linear social hierarchies composed of a large, dominant male and multiple subordinate females, and following male removal, the dominant female changes sex (Reavis and Grober 1999). In experimental all-male groups, the dominant male remains male, while all subordinates become female (Rodgers et al. 2007). Males reproduce with the females in their harem and provide sole parental care for the eggs (Behrents 1983). Once hatched, larvae undergo a planktonic stage, and following metamorphosis, settle on rocky reefs. During the reproductive season, juveniles recruit in large numbers and are often interspersed with adults. Juveniles are always subordinate to adults and, over time, have opportunities to ascend in status. Our understanding of the ecological, behavioral, morphological, and neuroendocrine basis of adult sex change in this species (Behrents 1983; St. Mary 1993; Reavis and Grober 1999; Black et al. 2005; Rodgers et al. 2007; Lorenzi et al. 2012) provides a strong foundation for comparison to primary sexual development.

Working with *L. dalli* allows us to expand on previous studies of initial sex allocation in several important ways. Because juvenile *L. dalli* can be reared in the laboratory (Archambeault et al. 2015), we can control all social experience. This is critical because the social environment influences juvenile sex allocation in other species; however, neither early social experience nor age can be controlled fully with field-collected animals (Bruslé-Sicard et al. 1994; Hobbs et al. 2004; Liu and Sadovy 2004b; Munday et al. 2006a). Natural populations of *L. dalli* juveniles are also easily accessible for observation and collection, providing ethological context for laboratory studies. Finally, studies of this kind rarely present data on juvenile social behavior (but see Iwata et al. 2008) and may conflate the importance of status with size. Direct behavioral observations allow us to disentangle the importance of social behavior and status from the social environment, in general, or body size.

We completed three studies investigating primary sexual differentiation in *L. dalli* juveniles. First, using laboratory-reared juveniles, we tested the hypothesis that social status determines primary sex allocation. We predict that in all-juvenile social groups, the dominant fish will develop as male and subordinates will develop as female, resulting in a skewed sex ratio. Second, we used transects to map the distribution of wild juveniles and adults to better understand the social environments in which juveniles develop. Third, genital papilla (GP) morphology and gonadal sex allocation were determined for wild-caught juveniles across a range of sizes and developmental stages. We hypothesized that both tissues, which are sexually dimorphic in adulthood (St. Mary 1993), are female-biased in differentiating juveniles because most juveniles are subordinate, either to nearby adults or other juveniles. Therefore, the majority should develop in a feminizing social environment.

MATERIALS AND METHODS

(a) Study organism

L. dalli is a small (<45 mm standard length, SL), marine fish that inhabits rocky reefs in the Pacific Ocean, from Morro Bay, California as far south as the Galapagos Islands, Ecuador (Miller and Lea 1972; Béarez et al. 2007). The reproductive season lasts approximately from April to September, and females lay multiple clutches throughout the season. Larvae (9–11 mm SL) settle on the reef between June and January, with the peak occurring July–August (Behrents 1983). Natural social groups range from small (3–10 fish), isolated groups to large aggregations (up to 120 fish/m^2). Within aggregations, high-ranking females associate closely with the territory of one male, while lower-ranking females move among territories (Lorenzi 2009).

(b) Study 1: development of laboratory-reared *L. dalli* in all-juvenile social groups

Juvenile L. dalli

Fish were reared at Roger Williams University (Bristol, RI) as previously described (Archambeault et al. 2015). The larval

phase is planktonic and non-social. When larvae were clear and competent to settle, larvae of the same developmental stage and size were transferred into individual tanks in groups of four. Newly settled fish started to gain color within approximately 2 days. This manipulation limited all juvenile social experience to the experimental social group. The fish were then shipped in their groups (1 L Nalgene bottles: 400 ml salt water, 600 ml O_2) to Georgia State University (GSU; Atlanta, GA) for the remainder of the experiment. There was less than 3 weeks variation in the time/age when groups were shipped. Fish were housed in 38 L aquaria with gravel substrate and a PVC tube (7.62 cm long × 3 cm diameter) that adult males use as a nest. The fish facility was maintained on a 12:12 light/dark cycle at 18–20°C. Fish were fed daily with New Life SPECTRUM Small Fish Formula (Homestead, FL) early on, substituting brine shrimp as they grew.

Social groups and behavioral observations

This study was completed in two blocks. In 2013, social groups ($n = 11$) arrived at GSU between March 6 and March 23. Weekly behavioral observations began on April 2 and continued through May 21 (7 weeks). In 2014, social groups ($n = 10$) arrived at GSU on March 20. Because all groups formed clear hierarchies in 2013, only four observations were conducted in 2014: April 8, April 15, April 22, and June 9. In the event of a death, social groups were maintained as groups of three or as pairs (groups of four: $n = 11$, groups of three: $n = 7$, pairs: $n = 2$). All behavioral observations lasted for 10 min per group and were conducted in the afternoon. We recorded all agonistic interactions. An approach was recorded when a fish swam, within two body lengths, directly at another fish. If the approached fish swam away, it was scored as a displacement. Displacements are a common measure of aggression, and being displaced is a signal of submission (Rodgers et al. 2007). Hierarchies were simple to construct using patterns of submission, and juveniles were assigned a rank from 1 (dominant) to 4 (most subordinate). Status was assigned prior to analyzing GP or gonadal morphology. Behavior data were averaged (±standard error of the mean; SEM) by year across all weeks of observations. Status differences in rates of submission were analyzed using a repeated-measures ANOVA, to account for multiple measures of behavior sampled from the same group (also for size/growth, GP ratio, and gonad morphology below). Tukey HSD tests were used for post hoc analysis. See Supporting Information for data on the behavior of dominant and subordinate juveniles in adult groups ($n = 4$, 2013).

Growth; GP and gonad morphology

We processed juveniles twice (April 9 and May 9, 2013; April 10 and May 9, 2014) to measure SL (to the nearest 0.1 mm) and mass (to the nearest 0.1 g) and digitally image the GP. Juveniles were briefly anesthetized in tricaine methanesulfonate (MS-222; 500 mg/L salt water) for processing, and the few fish that did not recover were preserved for analysis. Genital papilla length-to-width ratio was analyzed using ImageJ software (Rasband 1997–2014). Male papillae are long and pointed (ratio >1.4), while female papillae are short and rounded (ratio <1.4) (St. Mary 1993). Growth between processing dates (1 month) was calculated by subtracting the first mass measurement from the second. At the end of the experiment (May 31, 2013; June 13, 2014), fish were sacrificed in an overdose of MS-222, preserved in 4% paraformaldehyde for 48–96 h, and then stored in 0.1 M phosphate buffer. The gonads were removed by dissection.

We analyzed whole gonads stained with hematoxylin and eosin using squash preparations (Fig. 1). We stained the gonad by submerging it sequentially in: ultrapure H_2O (1 min), hematoxylin (50 sec), ultrapure H_2O (1 min), eosin (1 min), ultrapure H_2O (3 × 5–10 sec washes). We wet mounted the gonad and used a light microscope for analysis. We examined all regions of the gonad and made detailed drawings, delineating regions of sperm and eggs. From the drawings, we estimated the proportion of ovarian versus testicular tissue in the gonad. These data are presented as the proportion of ovarian tissue, and the remaining proportion can be assumed to be testicular. While this method was being established, two researchers analyzed each gonad independently. Once the researchers consistently estimated the proportion of ovarian versus testicular tissue within 5% of each other, the remaining gonads were analyzed by one researcher.

We compared GP ratio across statuses using nonparametric Skillings–Mack tests (10,000 replications), an alternative to the Friedman Test that is appropriate when there are missing data resulting from a death in the social group (Chatfield and Mander 2009). Bonferonni–Dunn tests were used for post hoc analysis. A Kruskal–Wallis test, with a Tukey HSD test for post hoc analysis, was used to analyze status differences in the proportion of ovarian tissue. A portion of juvenile gonads (2013) was not included because some fish were given additional social experience (see Supporting Information). A limited number of gonads were also lost during dissection. χ^2 tests were used to compare the proportion of males across status classes ($\alpha = 0.0071$ following Bonferonni correction for multiple tests). We used repeated-measures ANOVAs to compare mass and growth across social statuses, with Tukey HSD tests for post hoc analysis of significant results. Correlation analysis was used to identify associations between GP ratio and the proportion of ovarian tissue.

(c) Study 2: local social environment of wild juveniles

We completed twenty 60-meter transects, ten on Isthmus Reef (33.44711 latitude, −118.49302 longitude) and ten at Bird Rock

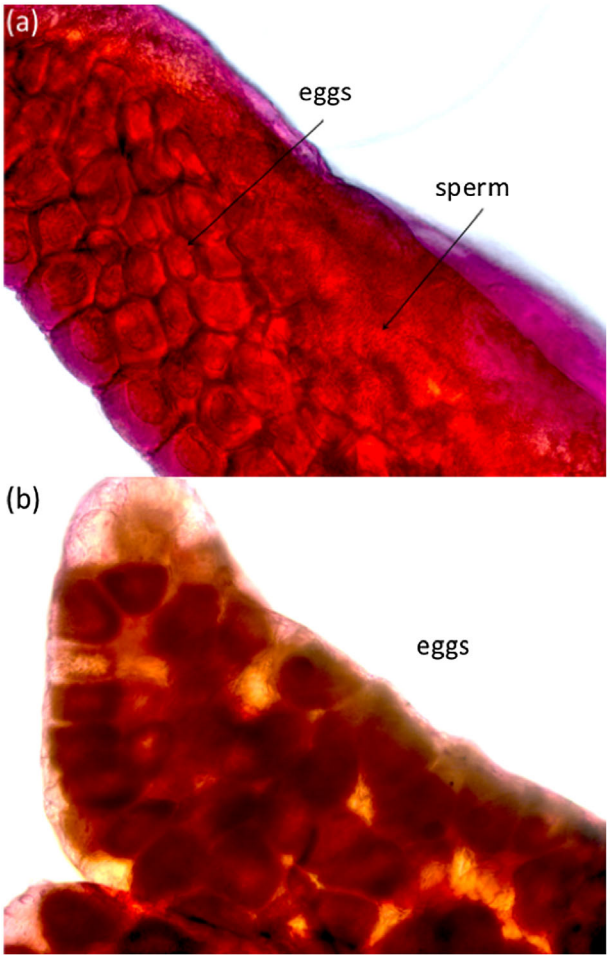

Fig. 1. Whole gonads stained with hematoxylin and eosin. (a) Field-caught juvenile gonad with nucleated eggs and sperm present. (b) Laboratory-reared juvenile gonad with developed eggs and little to no visible sperm.

(33.45047 latitude, −118.48657 longitude) (Catalina Island, CA). We haphazardly chose transect locations and used tape measures to mark the transect length. While SCUBA diving, we recorded the location and sex/developmental stage (adult male, adult female, or juvenile) of every *L. dalli* observed within 0.3 m on either side of the transect line. These categorizations were made visually based on morphological and behavioral characteristics. Adult males are typically the largest in their social group and remain more hidden/closer to their nests than the adult females and juveniles, which perch on rocks in the open for extended periods of time. Morphologically, adult males have thin bodies relative to the width of their head, musculature on the top of their head that is distinct from adult females, and long dorsal fins. Adult females have rounder bodies that are as wide as their heads and shorter dorsal fins. Eggs inside of the ovary also cause visible distention in the body wall, even for females that are only slightly gravid. Juveniles were easily distinguished from adults by size and had no visible ovarian distension. Rocky reefs contain numerous crevices into which can fish retreat; therefore, a limited number of fish were overlooked using this method.

We calculated fish density by dividing the number of fish observed per transect within the sampling area (36.6 m^2). We also categorized juveniles based on the number observed together (1–2, 3–10, 10–31 juveniles) and the number of adults nearby. Each occurrence is represented on a frequency distribution. These groupings of juveniles were assigned by the divers while completing the transects. Groupings were distinguished from each other by areas with no visible juveniles. All adults within the bounds of the transect containing the juvenile group were considered to be nearby. These metrics do not account for the distance over which juveniles and/or adults may interact socially.

(d) Study 3: sex allocation in juvenile population samples

We sampled juveniles during the reproductive season (July 2013) from Isthmus Reef and Bird Rock (no overlap with Study 2 transects). Juveniles ($n = 238$) were collected haphazardly using hand nets while SCUBA diving (California Fish and Game permit no. SC-11879). Juveniles were euthanized immediately with MS-222 and preserved in 4% paraformaldehyde. At the Wrigley Institute for Environmental Studies (Catalina Island, CA), we measured SL and mass and digitally imaged the GP. All juveniles were then dissected and, if present, the gonads were removed ($n = 76$). Although all juvenile *L. dalli* have primordial gonadal tissue (Lorenzi and Grober, unpublished data), we were interested in the development of the bi-lobed gonadal structure characteristic of adults. Gonads were stained and analyzed as in Study 1. Linear regression analysis was used to identify an association between GP ratio and the proportion of ovarian tissue in the gonad. We present the frequency of GP ratios in wild-caught and laboratory-reared (Study 1) juveniles on a frequency distribution.

RESULTS

(a) Study 1: juvenile status was associated with initial sex allocation

All juvenile social groups formed hierarchies, indicated by significantly different rates of submission across statuses (repeated measures ANOVA, square root transformation: 2013: $F_{3,23} = 36.56$, $P < 0.0001$; 2014: $F_{3,26} = 24.98$, $P < 0.0001$). In 2013 groups, post hoc analysis showed that ranks 3 and 4 submitted significantly more than both rank 1 (rank 3: $P < 0.0001$; rank 4: $P < 0.0001$) and rank 2 (rank 3: $P = 0.027$; rank 4: $P = 0.0007$). Rank 2 also submitted significantly more than rank 1 ($P < 0.0001$). In 2014 groups,

post hoc analysis showed that rank 2 ($P = 0.0001$), rank 3 ($P < 0.0001$), and rank 4 ($P < 0.0001$) all submitted significantly more than rank 1. There were no significant differences in submission among the subordinate ranks (rank 2 vs. 3: $P = 0.66$; rank 2 vs. 4: $P = 0.21$; rank 3 vs. 4: $P = 0.81$) (Fig. 2, a).

Genital papilla ratio already differed significantly across statuses by the first processing date (April 9, 2013; April 10, 2014) (Skilling–Mack: SM = 34.98, d.f. = 3, $P < 0.0001$). Post hoc tests showed that rank 1 GP ratios were significantly higher (more male-typical) than the subordinate ranks ($P < 0.0001$). There were no significant differences among subordinates ($P > 0.10$) (Fig. 2, b). These differences were maintained through the second processing date (May 9, 2013; May 9, 2014) (Skilling–Mack: SM = 33.85, d.f. = 3, $P < 0.0001$), with significantly higher GP ratios in rank 1 ($P < 0.0001$) and no differences among subordinates ($P > 0.37$) (Fig. 2, b). The same status/sex differences were reflected in the gonad. The proportion of ovarian tissue differed significantly across social statuses (Kruskal–Wallis: $H = 21.52$, d.f. = 3, $P < 0.0001$). Rank 1 gonads had significantly lower ovarian content than subordinates (rank 2: $P < 0.001$; rank 3: $P < 0.001$; rank 4: $P < 0.01$), and there were no differences among subordinates ($P > 0.94$) (Fig. 2, c).

Using GP ratio to define maleness (>1.4), there were significant differences in the proportion of males in each status (second processing date: Chi-squared: $\chi^2 = 45.63$, d.f. = 3, $P < 0.0001$). Rank 1 had significantly more males than the subordinate ranks (vs. rank 2: $\chi^2 = 30.71$, d.f. = 1, $P < 0.0001$; rank 3: $\chi^2 = 29.68$, d.f. = 1, $P < 0.0001$; rank 4: $\chi^2 = 25.44$, d.f. = 1, $P < 0.0001$), and there were no differences among subordinates (rank 2 vs. 3: $\chi^2 = 0.004$, d.f. = 1, $P = 0.95$; rank 2 vs. 4: $\chi^2 = 0.39$, d.f. = 1, $P = 0.53$; rank 3 vs. 4: $\chi^2 = 0.31$, d.f. = 1, $P = 0.58$). Thus, the sex ratio in all-juvenile groups was female-biased, or 37.5 ± 3.5% male (expected proportion if only rank 1 was male: 30.0 ± 3.75%).

At both processing dates, mass differed significantly across statuses (repeated-measures ANOVA, square root transformation: first: $F_{3,55} = 61.54$, $P < 0.0001$; second: $F_{3,51} = 46.26$, $P < 0.0001$). For the first processing, post hoc analysis showed that all of the ranks differed significantly from each other (ranks 1 vs. 2: $P < 0.0001$; 1 vs. 3: $P < 0.0001$; 1 vs. 4: $P < 0.0001$; 2 vs. 3: $P = 0.025$; 2 vs. 4: $P < 0.0001$; 3 vs. 4: $P = 0.0013$) (Fig. 3, a). For the second processing, post hoc analysis again showed that rank 1 was significantly heavier than the subordinates ($P < 0.0001$). Rank 2 was still significantly heavier than rank 4 ($P = 0.0005$) but did not differ from rank 3 ($P = 0.85$). Rank 3 was significantly heavier than rank 4 ($P = 0.0092$) (Fig. 3, a). Growth also differed significantly across statuses (repeated-measures ANOVA, natural log transformation: $F_{3,44} = 10.62$, $P < 0.0001$). Rank 1 gained significantly more mass than all subordinate ranks (vs. rank 2: $P = 0.0004$; rank 3: $P = 0.029$; rank 4: $P < 0.0001$), but subordinate growth did not differ across ranks ($P > 0.14$) (Fig. 3, b). Status differences in SL were similar to mass (data not shown).

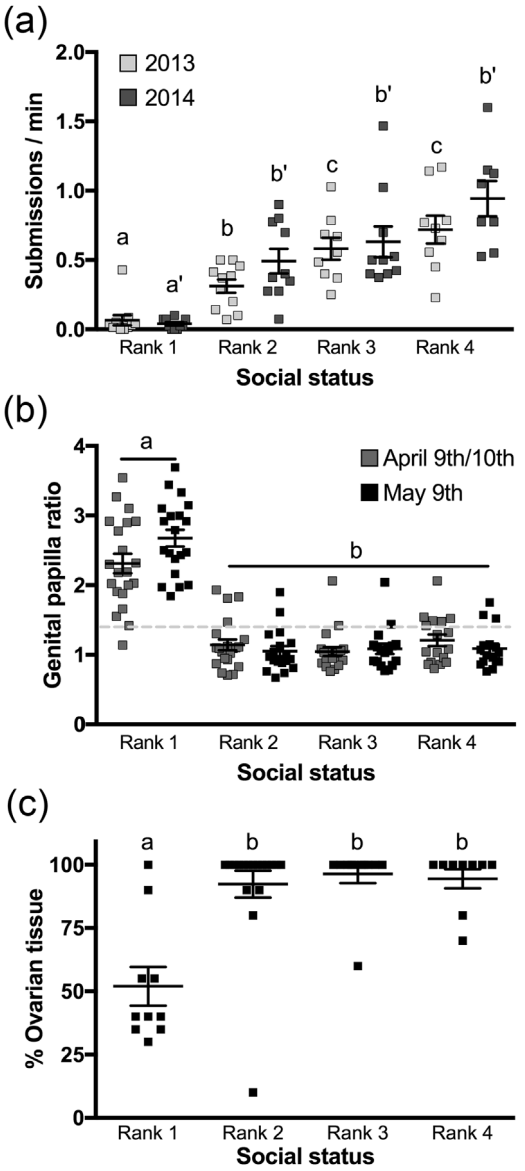

Fig. 2. (a) Average (±SEM) rates of laboratory-reared juvenile submissions. 2013: rank 1 ($n = 11$), rank 2 ($n = 11$), rank 3 ($n = 9$), rank 4 ($n = 9$). 2014: rank 1 ($n = 10$), rank 2 ($n = 10$), rank 3 ($n = 10$), rank 4 ($n = 9$). (b) Average (±SEM) genital papilla length-to-width ratio of laboratory-reared juveniles across social statuses. First processing: April 9, 2013, April 10, 2014 (rank 1: $n = 21$; rank 2: $n = 21$; rank 3: $n = 20$; rank 4: $n = 17$). Second processing: May 10, 2013, May 10, (rank 1: $n = 20$; rank 2: $n = 18$; rank 3: $n = 17$; rank 4: $n = 16$). Dashed line indicates the division (1.4) between male and female-typical GP. (c) Average (±SEM) proportion of the gonad comprised of ovarian tissue across social statuses in laboratory-reared juveniles (rank 1: $n = 10$; rank 2: $n = 17$; rank 3: $n = 11$; rank 4: $n = 9$). Different letters indicate significant differences ($P < 0.05$).

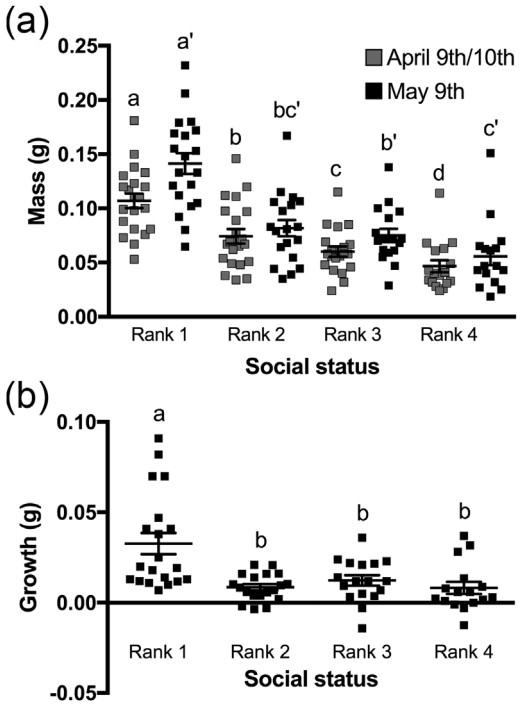

Fig. 3. (a) Average (±SEM) laboratory-reared juvenile mass at two different processing dates across social statuses. (b) Average (±SEM) laboratory-reared juvenile growth over the 1 month period between processing dates across social statuses. Rank 1 ($n = 20$), rank 2 ($n = 18$), rank 3 ($n = 17$), rank 4 ($n = 16$). Different letters indicate significant differences ($P < 0.05$).

(b) Study 2: juvenile social environment varied substantially in the wild

We recorded a total of 1,318 *L. dalli*, including 85 adult males, 358 adult females, and 875 juveniles, in our transects. The adult sex ratio was 4.21:1, female to male. Adult density was 0.032 fish/m^2 (females: 0.26 fish/m^2; males: 0.0062 fish/m^2), and juvenile density was 0.061 fish/m^2. Juveniles were observed in a range of social environments, from solitary individuals to one group of 31 juveniles. On average, we observed 5.18 ± 0.42 juveniles together, with 2.48 ± 0.22 adults nearby (Fig. 4, a).

(c) Study 3: wild-caught juveniles were bisexual and female-biased

We collected 238 juveniles ranging in size from 7.1 to 21.8 mm SL (14.44 ± 0.21 mm) and 0.0071 to 0.16 g (0.056 ± 0.0022 g). Similar to laboratory juveniles (Study 1), the distribution of GP ratios in wild-caught juveniles were female-biased with a peak around 1 (Fig. 4, b). Seventy-one of the wild-caught juveniles had a gonad sufficiently developed that it could be removed by dissection. The smallest fish with a gonad was 13.4 mm SL, and the largest fish without a gonad was 18.1 mm SL. The majority of gonads (91.5%) were bisexual, containing both eggs and sperm (e.g., Fig. 1, a). Just one gonad contained only sperm, and five gonads contained only eggs. Gonadal allocation was female-biased; however, the distribution was less dramatically skewed than for laboratory-reared juveniles (Fig. 4, c). Genital papilla ratio was significantly and negatively associated with the proportion of ovarian tissue for both laboratory-reared ($r = 0.64$, $P < 0.0001$) and wild-caught juveniles ($r = 0.55$, $P < 0.0001$).

DISCUSSION

Primary sex allocation in juvenile *L. dalli* was predicted by social status. In the laboratory, each all-juvenile social group formed a hierarchy, and by the second processing date, 100% of the dominant fish had male-typical genitalia, compared to 11% of rank 2, 12% of rank 3, and 0% of rank 4 subordinates (Fig. 2, b). Dominants also had significantly higher proportion of testicular tissue (Fig. 2, c). These data indicate that the same social cue, status, can both create and maintain through adulthood a skewed population sex ratio by regulating sex allocation at multiple life history stages (Rodgers et al. 2007). Social status has also been implicated in the regulation of primary sex in other sex changing species. In all-juvenile groups of false clown anemonefish (*Amphiprion ocellaris*), the proportion of testicular tissue in the developing gonad differs across statuses (Iwata et al. 2008). Juvenile coral-dwelling gobies (Hobbs et al. 2004) and anemonefish (Bruslé-Sicard et al. 1994) also develop into the opposite sex of their adult pair, which can be assumed to be dominant. In other species, social factors and size, but not status, specifically, were implicated in a regulatory role (Francis and Barlow 1993; Liu and Sadovy 2004a; Munday et al., 2006a). Diverse species have evolved mechanisms that allow for environmental cues to regulate sex allocation throughout life (Policansky 1982; Munday et al. 2006b), including teleosts (e.g., Godwin 2009), mollusks (e.g., Coe 1943), crustaceans (e.g., Bauer 2000), echnioderms, and annelids (e.g., Policansky 1982). The ability to adjust sex allocation in response to population structure at multiple life history stages allows individuals to increase their current reproductive success without compromising future/lifetime fitness.

The social regulation of primary sex allocation can provide a framework for understanding skewed population sex ratios observed in nature. Juvenile *L. dalli* develop in highly variable social environments (Fig. 4, a), but most should lead to a female-biased population. In the presence of adults, which are always dominant, all juveniles should develop as female. In the absence of adults, the dominant juvenile should develop as male, and subordinates should be female, as in the laboratory groups (Fig. 2). Our empirical data support these predictions. The majority of wild-caught juveniles had GP ratios within the female-typical range (Fig. 4, b), and gonadal sex allocation was skewed toward ovarian tissue (Fig. 4, c). Based on GP and

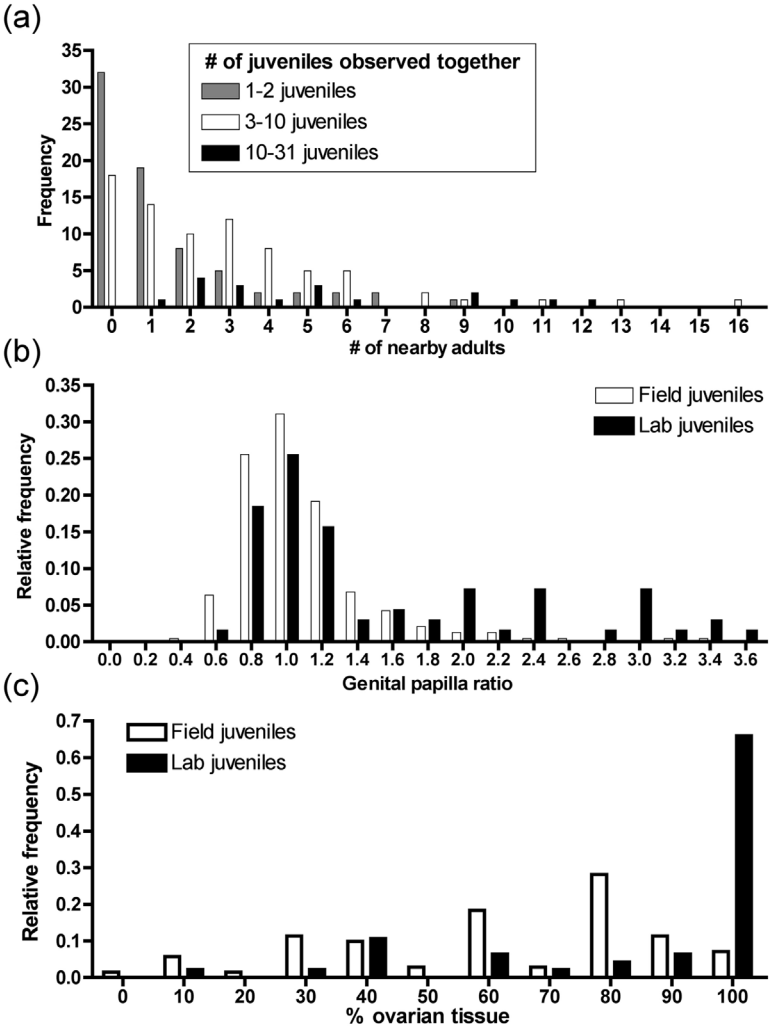

Fig. 4. (a) Frequency distribution of the number of juveniles observed together on the reef (1–2 juveniles, gray bars; 3–10 juveniles, open bars; 10–31 juveniles, black bars) by the number of adults observed nearby. (b) Relative frequency distributions of laboratory-reared ($n = 71$) and wild-caught juvenile ($n = 238$) genital papilla ratio. (c) Relative frequency distributions of laboratory-reared ($n = 47$) and wild-caught juvenile ($n = 71$) proportion of ovarian tissue in the gonad.

gonad morphology, some juveniles were developing initially as male (Fig. 4, b and c), and we predict that these individuals were locally dominant. Young males may also become mini males, an alternative reproductive morph that is consistent behaviorally and morphologically with the few subordinates developing as male in the laboratory groups (Drilling and Grober 2005). Laboratory-reared juveniles were also more likely than wild-caught juveniles to be strongly biased toward one sex (Figs. 1, b and 4, c). This provides further support for social regulation over a mediator such as size, because when size differences are small, sexual phenotype should be less dimorphic; however, the juveniles that were initially size-matched (laboratory) were more differentiated. The social stability of laboratory groups may also accelerate sexual differentiation.

For sex changing fishes, the interactions among sex, status, and size are complex. Large body size increases the probability that an individual will have the opportunity to change sex if/when a permissive environment arises (Ghiselin 1969), while social status can serve as a direct regulator of sex change (Rodgers et al. 2007; Godwin 2009). Size can also bias status outcome, and dominance can lead to differential growth (Buston 2003; Rodgers et al. 2005). By grouping undifferentiated laboratory-reared juveniles of the same developmental stage and size, with no previous social experience, we have the opportunity to tease apart the origins of sex, status, and size differences in a way that has not yet been possible. For example, previous studies that have used wild-caught (e.g., Munday et al. 2006a) and laboratory-reared (e.g., Iwata et al. 2008) juveniles did not control for influential social experiences in the first weeks of life. The most parsimonious interpretation of our data is that social status was established first, leading to differences in sexual differentiation and size/growth. Dominance relationships form between unfamiliar fish faster than size differences among same-sized fish or sex in immature juveniles, including in groups of juvenile *A. ocellaris* (Iwata et al. 2008). Furthermore,

size-matched *L. dalli* adults can establish social status within minutes to hours, while size differences become evident only after days to weeks (Rodgers et al. 2005).

The time at which sex is determined is not straightforward because sexual differentiation is a process, and for *L. dalli*, sex is never fixed; however, social status establishment and/or size differences should precede sexual maturation. At metamorphosis, juveniles do not have a gonad. The size range of wild-caught juveniles without gonads, and the fact that nearly all wild juveniles were observed in a social context (Fig. 4, a), demonstrates that there is a developmental window during which juveniles are social and grow, but do not yet have a gonadal sex. As a hierarchical species, all social interactions occur within a dominance structure, including between juveniles and adults (Supporting Information Fig. S1). Once the gonad forms, it develops initially in a bi-potential state. Nearly all wild-caught juvenile gonads contained eggs and sperm (e.g., Fig. 1, a), and both gametes were present in most laboratory-reared juveniles, especially rank 1 males (Fig. 4, c). Other species of sex changers also have bisexual gonads initially, including multiple species of fish (Bruslé-Sicard et al. 1994; Miura et al. 2003; Liu and Sadovy 2004b; Iwata et al. 2008). Allocating to both male and female tissue may allow juveniles to reproduce more quickly once mating opportunities arise, decreasing their age at first reproduction and increasing lifetime reproductive potential (McGraw and Caswell 1996; Charnov 1997; Wiegmann et al. 1997). The earliest *L. dalli* juveniles have been observed laying eggs in the laboratory is 90 days post-hatching (A. Rhyne, personal observation). More than half of adult *L. dalli* gonads also contain both ovarian and testicular tissue, with sex allocation strongly biased towards the functional sex (St. Mary 1993). Similar to initial sexual development, simultaneous allocation in adulthood may facilitate rapid reproduction following sex change (St Mary 1994).

It was not feasible for us to determine empirically whether social status was established before the laboratory-reared juveniles differentiated in size. While the groups were initially matched for size, dominant juveniles were already significantly bigger than subordinates by the first time they were measured (Fig. 3, a). We did not measure size earlier to avoid handling injury or stress. Dominants also grew at a significantly faster rate (Fig. 3, b), similar to other developing sex changers, including *C. boenak* males (Liu and Sadovy 2004a) and dominant *A. ocellaris* (Iwata et al. 2008). Juvenile *T. bifasciatum* that develop as male also tend to be the largest in their group (Munday et al. 2006a). However, the question of status vs. size has been answered unambiguously for *L. dalli* adults: social status regulates sexual phenotype (Reavis and Grober 1999; Rodgers et al. 2007), and it is more parsimonious that the direction of causation in this complex, biological pathway is conserved across life history stages rather than reversed following initial differentiation.

In nature, a combination of behavioral, morphological, and physiological traits may bias an individual toward early dominance, which can impact lifetime reproductive potential. Although fish in Study 1 were matched for size and developmental stage, the age at which larvae in the laboratory metamorphose and settle varies considerably, 40–100 days post-hatching (Archambeault et al. 2015). Size at settlement and early growth could impact the probability of being locally dominant (Ochi 1986). Planktonic larval reef fish utilize a variety of cues in choosing a settlement location (Lecchini 2004), including social cues from conspecifics (Lecchini 2004; Ben-Tzvi et al. 2009). The social environment into which juveniles settle, including whether it is early or late in the reproductive season (Ochi 1986) and the local density of juveniles and adults, should affect initial status and, thus, primary sex allocation.

Here, we show for the first time for *L. dalli* that primary and adult sex allocation follow a similar rule: if dominant, be male (Rodgers et al. 2007). In a population with a skewed sex ratio, an individual's sex has a profound impact on reproductive potential and, thus, fitness. The ability of *L. dalli* juveniles to modify individual sex allocation, and thus population sex ratio, during early development without limiting future reproductive plasticity should allow individuals to optimize fitness, a hypothesis that will be important to test directly. This life history strategy is in stark contrast to species that become fixed as one sex, or even species that can sex change only once as adults. Our work highlights the potential for future research to elucidate the evolution of social and sexual plasticity, and its underlying mechanisms, using species such as *L. dalli*. By manipulating the status, age, size, or neuroendocrine state of juveniles in a social group, at or before the time of metamorphosis, for example, we can uncover the biological origins of social status, which has remained elusive despite the fundamental importance of status to fitness for diverse social species (Ellis 1995). The initial sexual differentiation of the brain that must precede gonadal sex (Francis 1992) can also elucidate the neurochemical pathways responsible for translating early-life social information into biological decisions that set individuals on dramatically different life history trajectories.

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