

Sex Reversal in Pairs of *Lythrypnus dalli*: Behavioral and Morphological Changes

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Abstract. In *Lythrypnus dalli*, the bluebanded goby, reproductive success is primarily determined by functional sex, and functional sex is determined largely by rank in the dominance hierarchy. In most natural social groups of *L. dalli*, one male is at the apex of the hierarchy, and 1 to 7 females are lower in rank. When a male exits the group, a female ascends to the top of the hierarchy and becomes a male. We have examined this process in a simplified environment—a pair of females—that allows us to identify behavior associated with the formation of a dominance relationship and any other phenotypic changes associated with dominance, sex change or both. We found that pairs of *L. dalli* females quickly and readily form stable dominance relationships, with the dominant fish changing sex into a male. This dominant animal also rapidly increases in body size and length of its dorsal fin. In summary, dominant *L. dalli* females change sex in this simplified environment, providing excellent opportunities to examine the early behavioral and morphological changes associated with dominance and sex change.

Introduction

Social context often determines how a given individual responds to behavioral stimuli, with individuals modifying their behavior on the basis of the behavior of the individuals around them. This is especially true in several species of protogynous (female to male) sex-changing fish, where sex reversal is socially controlled. In these species, a unique pattern of behavior stimulates one individual to change sex while inhibiting others (Robertson, 1972; Ross *et al.*, 1983). Two social factors are thought to be the primary regulators of sex change:

inhibition by males and stimulation from other females (Robertson, 1972; Shapiro, 1979). Without sufficient social stimulation (*i.e.*, in isolation), fish capable of sex reversal might not initiate sex change (Cole and Shapiro, 1995; Carlisle *et al.*, 2000). Size advantage (Warner *et al.*, 1975) contributes to the determination of which animal changes sex, but behavioral interactions are also critical determinants (Lutnesky, 1996; Munday, 2002). Larger size often equates with increased success in aggressive encounters and therefore social dominance, providing a proximate mechanism for the size advantage hypothesis. In protogynous sex changers, the most reproductively significant resource that dominance affords is “maleness”; thus the reproductive payoff for dominance is extremely large, and females would be highly motivated to increase their aggressive behavior in times of social instability (*i.e.*, in the absence of a dominant male).

The study of *Lythrypnus dalli* (Gilbert, 1890) had previously dealt exclusively with larger social groups (>3), and it was not known whether sex change could be induced in pairs of females. This study used pairs of fish to closely examine changes associated with the acquisition of dominance and, potentially, sex change. In a group environment, dominance relationships are more difficult to tease apart owing to multiple interactions between individuals in the group, whereas in pairs there are fewer social variables contributing to an animal’s behavior. We found that one of the pair quickly changes to male, as evidenced by male-typical behavior and male reproductive morphology.

Materials and Methods

We use four measures of “maleness” to determine if any of the fish changed sex: (1) display of male-typical courting behavior; (2) male-typical papilla ratio, defined as a length-

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to-width ratio greater than 1.6 (female ~ 1.0 l/w); (3) the presence of an accessory gonadal structure (AGS); and (4) the presence of fertilized eggs, an unequivocal indication of functional sex change.

Subjects

We selected 32 female specimens of *Lythrypnus dalli* with lengths between 23 and 30 mm for the study. Sex was determined by examination of the external genital papilla (Behrents, 1983). The papilla ratio in females of this species is about 1.0, compared with about 2.0 in the males (St. Mary, 1994); the largest ratio at the start of this experiment was 1.3. Fish with female-typical papilla have gonads consisting of more than 95% ovarian tissue (St. Mary, 1994). During the experiments, animals were housed in 33-l aquaria, each with an individual filter system (Marineland), at 20–21.1°C; fed twice daily using a commercially prepared diet (OSI Marine Labs); and kept on a 12-h light/dark cycle. One group of fish was collected (California Fish & Game permit # 803034-01) on Santa Catalina Island, California, in May 2002 (pre-breeding season), using an anesthetic solution of quinaldine sulfate (Sigma Chemical) and hand-nets. A second group of fish was collected, using the same methods, during the breeding season in late June 2002. Prior to the experiments, the animals were kept in 180-l holding tanks.

Experimental design

The study is divided into two experiments. In experiment 1, morphological traits and behavioral interactions were quantified between eight pairs of individuals identified as females. External morphological data were collected prior to pairing (standard length, mass, and papilla ratio, described below). The pairs were observed until eyed eggs (a marker of fertilization), indicating functional sex change, were seen, or until 14 days, an adequate amount of time for sex change in this species (Reavis and Grober, 1999), had elapsed. Experiment 2 was similar to experiment 1 and was designed to confirm a novel observation in experiment 1, that dominant fish exhibited disproportional growth of the longest rays of the dorsal fin. The behavior of the eight pair of fish in experiment 2 was not observed closely. In both experiments, the pairing and morphological measurements (with the exception of initial dorsal fin length) were identical (see below).

At the completion of each experiment, animals were given an overdose of tricaine methanesulfate (MS 222), and their morphological characters were measured again. This included measurement of the longest dorsal ray (see Results). The animals were placed in Bouin's fixative, the gonads were dissected out to examine internal morphology, and digital photographs of all the gonads were taken to aid in analysis.

During sex reversal, *L. dalli* undergoes gonadal reorganization (St. Mary, 1994), which in a number of sex-changing gobies involves degeneration of ovarian tissue (Sadovy and Shapiro, 1987) and generation of AGS and testicular tissue (Cole and Shapiro, 1990). In *L. dalli*, the presence or absence of the AGS is the primary indicator of functional sex; males have a highly developed AGS and females have none. Visual inspection was used to assess the composition of reproductive tissue, *i.e.*, presence of eggs and presence of AGS. Gonad sex was verified using standard paraffin histology followed by staining with hematoxylin and eosin.

Pairing

At the start of each experiment, animals were removed from the holding tank and anesthetized using MS 222. Mass and standard length were recorded. Papilla ratios were measured, and digital images were also taken. Animals were paired by standard length (within 2 mm of each other) and body mass (within 0.1 g). The fish were individually identified by their banding pattern, which did not appear to change nor have an effect on their behavior. Two animals were placed together into a 33-l tank with a single PVC tube to serve as the nest (St. Mary, 1994).

Observations and behavior

The behavior of each pair was observed twice daily, once in the morning and once in the afternoon, for 10 min each session. Data were recorded on paper and then transferred to a computer spreadsheet. The fish were given one day to acclimate before observations began. The observer recorded the number of approaches, displacements, jerks, bites, nips, solicitations, and tail-waggles. In addition, the observer noted which animal resided in the nest tube, if either animal was gravid, and whether eggs were present in the tube.

An approach is defined as a fish moving within 5 cm of the other fish. Movement away from the approaching fish is recorded as a displacement. Jerks are a male-typical behavior used during courtship (Behrents, 1983), and involve a saltatory swim motion with movement laterally as well. Because jerks can be directed either at the female or around the nest without being directed at a fish, a jerk towards a female is scored as both an approach and a jerk. Bites are aggressive interactions in which one fish bites the other after an approach. In contrast, nips follow a jerk to a female, with a male nipping the tail of the female. This behavior is also part of the courting process. Solicitations are produced by females when they move within 12 cm of the male in his line of view. Tail waggles are displayed by both sexes: the fish remains stationary while moving its tail back and forth.

Statistics

Morphological data were analyzed using parametric statistics. For traits that had "before" and "after" measures, we used paired student's *t*-tests (standard length and fin length in experiment 2). For fin length in experiment 1, we used an unpaired *t*-test to look at differences between males and females. We also used a *t*-test to examine whether papillar morphology was significantly different between new males and females. The distributions of the frequencies of the various behaviors for newly sex-changed males and females did not meet the criteria for parametric statistics (*e.g.*, normality, homogeneity of variance); thus we used nonparametric methods to examine group differences. All behavioral analyses were conducted with the Wilcoxon signed rank test. Values for mean and median were similar, indicating a symmetrical distribution. Simple linear regressions were employed to examine the relationship between known interdependent behaviors (*e.g.*, approaches regressed on displacements). Though the fish were size-matched, we used logistic analysis (SAS) to examine whether small differ-

ences in size predicted which individual would change sex. Significance level in all cases was set at $P < 0.05$. Mean values \pm standard error of the mean (SEM) are given. All analyses were carried out using the Statview 5.01 (SAS Institute Inc.) unless otherwise noted.

Results

In all cases, the pairs readily established a stable dominance relationship, defined as one individual (the dominant) instigating the majority of encounters (Fig. 1a) and exhibiting male courtship behavior (Fig. 1b), while the other individual (the subordinate) is displaced as a result of the encounter (Fig. 1c). In all cases the dominant individual initiated spawning behavior with the subordinate individual that resulted in a clutch of eggs. Of the 8 clutches, 4 were verified as fertile; in the other 4 cases, the male consumed the clutch before verification. At the completion of the experiment, all dominants had a male papilla ratio as well as an AGS (Figs. 2, 3). Using our criteria, we demonstrated

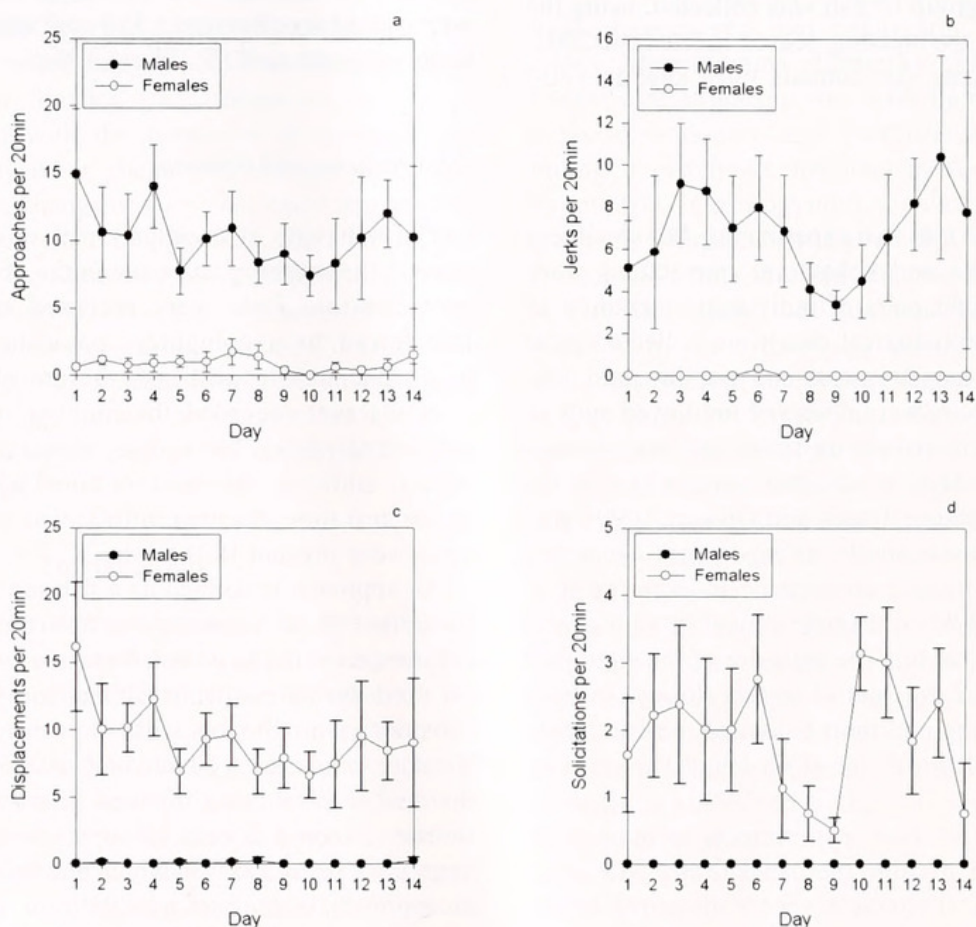


Figure 1. Changes in sex-typical behavior over the course of experiment. Animals that are labeled male are individuals that changed sex—*i.e.*, those that would become male. (a) Males approach and females rarely do, demonstrating dominance. (b) Jerking behavior, a major component of male courting behavior, is exclusively produced by males. (c) Females were displaced at high rates compared to males. (d) Solicitations, a type of female courting behavior, are exhibited exclusively by females. Note the similar pattern of behavior in b and d.

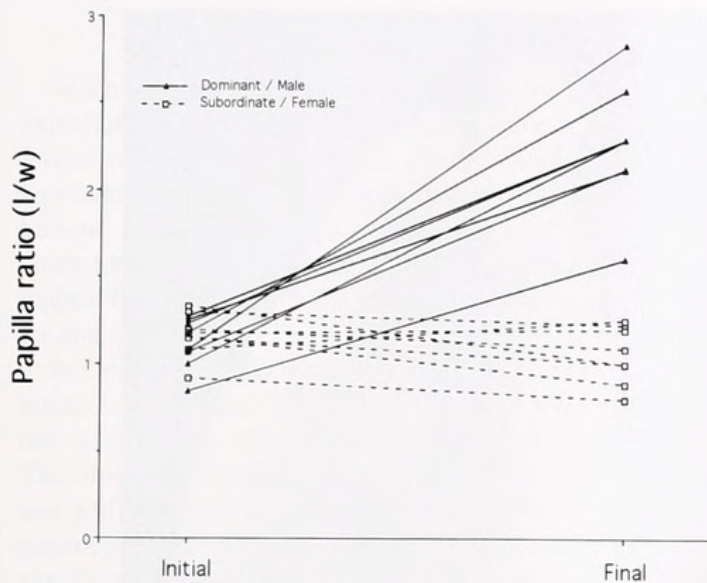


Figure 2. Papilla length/ width ratios at the start and conclusion for all animals in experiment 1. Dominants (males) had a significantly higher ratio than subordinates (females) at the end of the experiment ($t = 8.64$, $df = 14$, $P < 0.0001$).

that the dominant individual in a pair of females changed sex from female to male.

Morphological changes

All fish initially had a female-typical papilla ratio (mean \pm SEM: 1.15 ± 0.061). There was no difference in papilla ratio at the start of the experiment ($t = 0.732$, $df = 14$, $P > 0.05$) between fish that became male (mean \pm SEM: 1.15 ± 0.102) and those that remained female (mean \pm SEM: 1.17 ± 0.086). After sex change there was a significant change to a male-typical papilla in the fish that demonstrated male-typical behavior ($t = 8.64$, $df = 14$, $P < 0.0001$). Female papilla ratio remained the same, 1.09 ± 0.052 , whereas the ratio in sex changers more than doubled to 2.26 ± 0.127 (Fig. 2).

All dominant animals exhibited unambiguous male-typical gonad morphology that included the presence of sperm and an AGS. All subordinate individuals were gravid, and eggs were clearly visible within the ovary upon inspection. These results were verified histologically (Fig. 3).

The standard length of dominant individuals changed significantly over the course of the experiment ($P < 0.05$); that of subordinates did not ($P > 0.05$). When we examined the magnitude and direction of the change, we found that dominants grew significantly more relative to subordinates (paired t -test, $t = 4.528$, $df = 15$, $P = 0.0003$).

In experiment 1, we noted that the dorsal fin of the dominant fish was elongated. Although we had not measured initial fin length, at the conclusion of the experiment we measured the longest dorsal ray. Dominants had a mean fin length of 10.47 ± 0.539 mm, while subordinates had a

mean of 5.5 ± 0.269 mm ($t = 8.475$, $df = 14$, $P < 0.0001$). To control for dominant fish simply growing more, we used a ratio of fin length to body length. This ratio was significantly different: subordinates had a smaller fin with an average of 4.648 ± 0.137 fin lengths per body length, and dominants had a ratio of 2.64 ± 0.143 ($t = 10.129$, $df = 14$, $P < 0.0001$).

Experiment 2 verified the marked change in fin length observed in the first experiment. Future dominants and subordinates did not have different fin lengths at the beginning of the experiment ($t = 1.669$, $df = 8$; $P > 0.05$), but the dominant fish had a significantly longer fin at the end of the experiment ($t = 4.091$, $df = 8$; $P = 0.0035$). Dominant individuals exhibited a pronounced lengthening of their longest dorsal ray, while many of the subordinates showed a fin shortening.

We attempted to minimize size as a major contributing factor; however, small differences in size did exist between the paired animals. To assess whether these small differences in standard length were predictive of which animal changed sex, we ran a logistical regression. Our results suggest that the size differences present in this experiment were not a good predictor of which animal would become dominant (Wald $\chi^2 = 0.034$, $P = 0.8532$, $df = 1$, $\beta = -0.05006$).

Behavior

Verification of egg fertility in experiment 1 was difficult because of consumption of clutches by males (see below). Four of eight pairs completed sex change in less than 14 days, as determined by the presence of fertilized eggs. In the four remaining pairs, males exhibited all other sex-typical characteristics, but consumed at least one complete clutch of eggs. Because fish that parented and fish that consumed their eggs displayed similar rates of behavior in all measures, we grouped them in the behavioral analysis.

A clear dominance relationship was evident from the first observational session and persisted unchanged throughout the experiment (Fig. 1). The dominant fish produced male-typical behavior and began courting the subordinate. The dominant fish instigated the vast majority of behavioral interactions, evidenced by high rates of approach behavior. Dominants displayed significantly higher rates of aggressive and courtship behavior (approaches and jerks, $P < 0.05$, Fig. 1a, b). Subordinates displayed significantly more submissive and female-typical behavior (displacements and solicitations, $P < 0.05$, Fig. 1c, d). All dominants jerked and subordinates generally did not jerk, although there were rare individual displays over the course of the experiment. Subordinate fish were displaced at high rates (9.138 per 20 min, Fig. 1c) compared to dominants (0.062 per 20 min). There

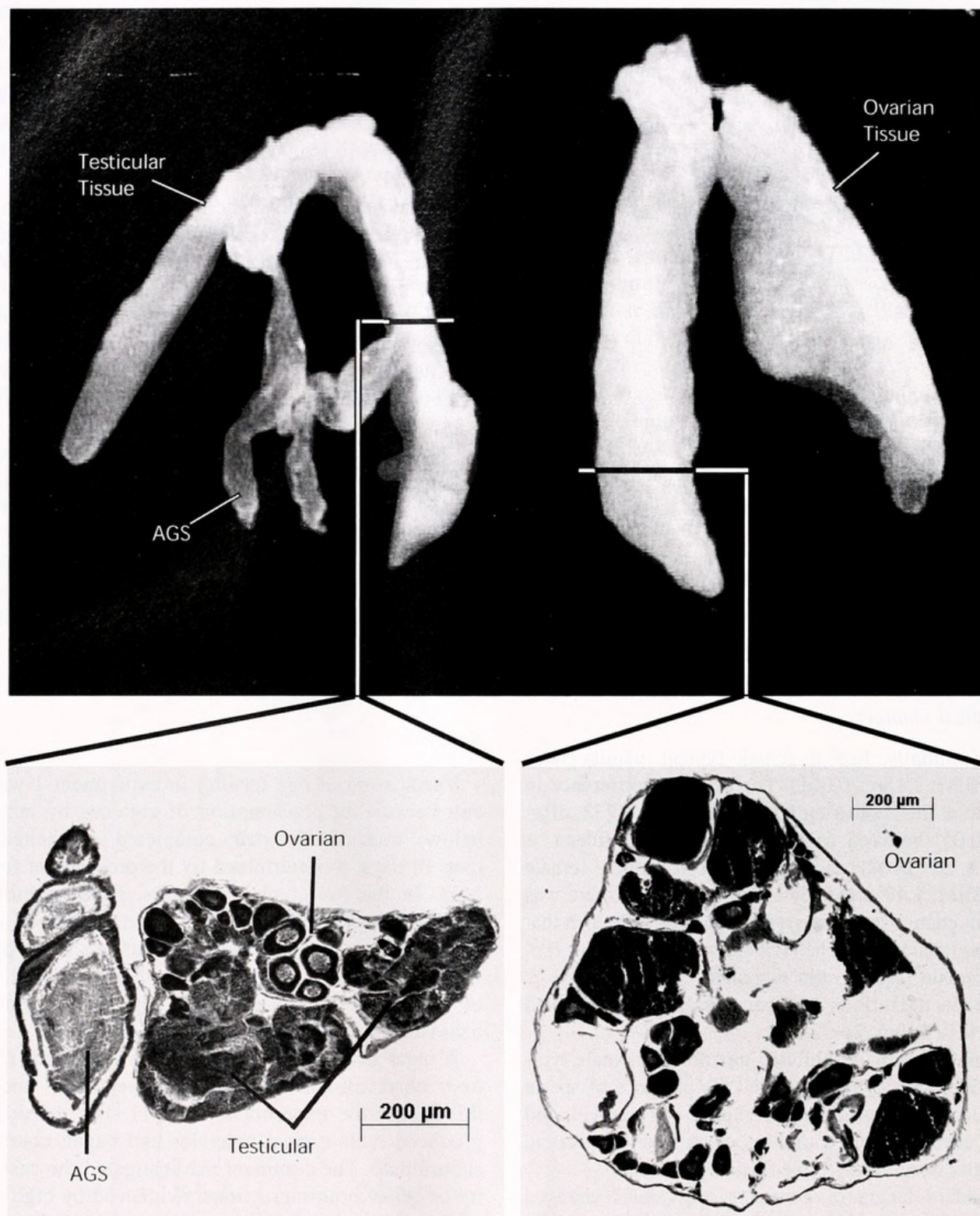


Figure 3. Gonad morphology of a typical male (left) and female (right). The cross-section of each type is shown underneath. Note that the male gonad contains mostly testicular tissue, some ovarian tissue, and an accessory gonadal structure (AGS). The female gonad contains only ovarian tissue and is visibly full of eggs.

was a significant association between approaches and displacements, such that when a dominant approached, the subordinate was usually displaced ($R^2 = 0.917$; $P < 0.001$). The dominant guarded or resided in the tube for most of the time.

Other behavior occurred at a far lower frequency, including bites, nips, and tail waggles. Dominant individuals produced bites and nips. Tail-waggle was the one behavior that did not show an overall sex/dominance bias, but was exhibited by both dominants and subordinates.

Discussion

When two female bluebanded gobies were paired, in our experimental conditions, in all cases, one fish established dominance over the other, with that fish instigating most of the encounters and winning nearly all of them. The dominant fish became male, exhibiting all behavioral and morphological characters associated with that sex. The subordinate fish remained female and exhibited all associated sex-typical behavior and morphology.

In *Lythrypnus dalli*, body size is sexually dimorphic, with males being larger (Wiley, 1976). In our work, dominant individuals grew more than subordinates and changed sex. The observed sexual dimorphism in growth rate is consistent with previous data from St. Mary (1994); however, we cannot address whether the increase in growth is a result of sex change or of dominance. Dominance rank has been shown to affect the growth rate of individuals within a hierarchy of sex-changing clown fish (Buston, 2003). We also found that the dorsal fin was elongated in the dominant, sex-changed fish. The dorsal fin is known to be sexually dimorphic (Wiley, 1976), but we did not expect the rapidity and magnitude of the changes we observed in the first experiment. Experiment 2 confirmed that the fin lengthened dramatically in the dominant individual over the course of the experiment. The dorsal fin shrank in many of the subordinate fish, but whether this is a natural process or the result of aggressive encounters with the dominant is unknown. As with the change in growth rate, we cannot say whether the fin elongation is caused by the sex-change process or is modulated by dominance status. If the latter is the case, then this trait can potentially be used as an index of rank within a hierarchy.

Simplification of the social group to a pair of animals, as in our study, revealed an interdependence of behavior between males and females that was not seen in previous studies using larger groups of fish. This is best illustrated in Figure 1b and d, where courtship behavior (solicitations and jerks) clearly shows the same pattern over time. Although the precise nature of the interaction is unclear, female behavior apparently had an impact on the rate of male behavior, an effect that would be difficult to examine in a group with multiple females.

Like Reavis and Grober (1999), we used fertilized eggs as a marker for complete sex change and allowed the experiment to run for a maximum of 14 days. All tanks had eggs in the nest within the 14 days, but only half of those nests had eggs that remained until they could be verified as fertile. In the four other groups, the males did not display paternal care. These males ate the eggs after day 10 of the experiment, making it impossible to use the presence of eyed eggs to verify fertility and thus terminate the experiment. The bulk of the data indicates that these eggs were viable. Clutch-consuming males exhibited all male-typical behavior

(approaches, jerks, bites, and nips). Morphological examination revealed no differences between males that demonstrated appropriate care and those that did not. The gonads of the two groups were indistinguishable from one another, and both groups developed an AGS, the hallmark of the male sex. There were no differences in the papilla between the two groups, and all males displayed sexually dimorphic elongation of the longest dorsal rays. In addition, parenting males had fertile clutches at the same time as egg consumption was occurring, suggesting that all of the males were capable of fertilizing eggs.

In experiments with groups of fish greater than two (Reavis and Grober, 1999), the amount of time required for full sex change depended on the size of the individuals in the group upon male removal. In groups where the size asymmetry between the top two females was greater than 10%, sex change occurred more rapidly than in groups where the size asymmetry was less than 10%. The observed delay was determined to be the result of an increase in time spent resolving the dominance relationship between the top two individuals. Individuals of similar size take longer to settle a conflict than individuals of different sizes (Enquist *et al.* 1990; Koops and Grant, 1993). In this experiment, we used pairs of size-matched individuals; thus we were surprised to find that the contestants quickly resolved the dominance relationship. One possible explanation is that in a group, individuals may receive contradictory signals. This could result from being dominated by one individual but also receiving positive stimulus by dominating other individuals in the group, thus prolonging conflict resolution. In dyadic contests, the signals are straightforward; one animal wins the encounters while the other loses them, thereby shortening the time and probably the number of interactions required to form a stable dominance relationship.

In summary, two females is a sufficient group size to induce sex change in *L. dalli*, as evidenced by one individual exhibiting male typical gonads, external genitalia, behavior patterns, and often fertilized eggs. When an *L. dalli* female becomes dominant, the first noticeable morphological change is the elongation of the dorsal fin. Dominant individuals also grow more rapidly.

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