

Social status determines sexual phenotype in the bi-directional sex changing bluebanded goby *Lythrypnus dalli*

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The behavioural mechanisms and patterns of protandrous sex change in bluebanded gobies *Lythrypnus dalli* were investigated and compared to the well-described behaviour patterns of protogynous sex change. To do this, unisex groups of males and females were established; behavioural and anatomical changes were recorded over a 42 day period as social status and sexual phenotype were determined. In all cases, social status, rather than the expression of a particular behaviour, accurately predicted final sexual phenotype. Rates of submissive behaviour, but not aggressive behaviour, were predictive of each discrete status class. Multiple individuals changed sex simultaneously if their sexual phenotype and social status were discordant, a novel finding suggesting that once a social hierarchy is established, individuals determined their sexual phenotype, regardless of initial sex, based on a simple operational principle: if subordinate express female, if dominant or not subordinate express male. This work demonstrates that similar mechanisms underlie sex change in both directions in *L. dalli* and potentially other sex changing species.

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INTRODUCTION

Dominance status has far reaching consequences for social animals with regard to the reproductive success they can achieve (Ellis, 1995). The relationship between dominance and reproductive success provides a unique set of challenges for species in which the social environment regulates functional sex (Kuwamura & Nakashima, 1998). In the context of a social group, individuals must make critical life-history decisions based on the accumulation and evaluation of local information. Questions regarding 'why' sex change would arise in certain species are addressed, in part, by the size advantage hypothesis (Warner *et al.*, 1975; Warner, 1988), which predicts the potential for, and direction of, adult sex reversal

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based on size-fecundity differences between the sexes. Because optimum 'size at sex change' refers to the relative size of individuals in any given social group, and not absolute size (Warner, 1988), an individual must make a decision on when to change sex based on local information (Muñoz & Warner, 2003). Although this information is inherently variable, because of potential immigration or emigration and lifelong growth, these decisions are of great importance, because fishes will subsequently invest heavily in a new suite of sex specific traits.

Examination of the proximate mechanisms directing sexual allocation decisions will aid in developing a more complete understanding of the sex change process (Munday *et al.*, 2006). One approach to better understand sex change is to identify the operating principles underlying allocation decisions, and in the case of bi-directional species, the degrees to which similar or different processes guide protogyny (female to male) and protandry (male to female). Agonistic interactions determine which individual will change sex in same-sex pairs of several bi-directional species (Sunobe & Nakazono, 1993; Nakashima *et al.*, 1995; Kuwamura *et al.*, 2002), and also direct protogynous sex change in *Lythrypnus dalli* (Gilbert), in both groups (Lorenzi *et al.*, 2006) and pairs (Rodgers *et al.*, 2005). Whether or not there is similar social regulation of protandrous sex change in *L. dalli* is not known. Tests of how dominance interactions regulate sexual transitions in both directions have been done only in pairs of fishes (Sunobe & Nakazono, 1993; Nakashima *et al.*, 1995; Kuwamura *et al.*, 2002), but many bi-directional species live in larger male dominated groups, which should pose a different set of challenges with regard to the reproductive payoffs associated with sex change. This study examines the behaviour and morphology of all members of unisex social groups, both male and female, to assess the effects of behaviour patterns, social status and initial sex on the expression of sexual phenotype.

During protogynous sex change in all-female groups, only the most dominant individual changes sex from female to male (Reavis & Grober, 1999). One possibility for protandrous change is that only the most subordinate male in unisex male groups becomes female, as has been seen in *Pseudolabrus sieboldi* Mabuchi & Nakabo (Ohta *et al.*, 2003) and is consistent with data from St Mary (1994) and Reavis & Grober (1999) in *L. dalli*. An alternative is that each individual responds to its own social status in a binary way, such that all individuals not achieving alpha status should change to female after social order is achieved. In this study, behavioural observations of unisex groups of males and unisex groups of females were used to evaluate whether the same cues regulate sex change in both directions.

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

In stable social groups, protogynous sex change in *L. dalli* can occur rapidly, with functional reversal (marked by presence of fertilized eggs in all-female groups) taking *c.* 2 weeks (Reavis & Grober, 1999; Rodgers *et al.*, 2005). The time to sex change varies in groups according to the size differential between the top ranking females; the closer in size the high ranking individuals are, the longer it takes for an individual to achieve

α status and to change functional sex (Reavis & Grober, 1999). Thus, hierarchy establishment influences the time required and propensity to change sex. In the bluebanded goby it has been shown that social dominance rather than size is an accurate predictor of which individual will change sex in the protogynous direction, when size asymmetries are minimized (Rodgers *et al.*, 2005). When size asymmetries are large, both size and social dominance are accurate predictors of protogynous change sex. To more closely examine the role of behaviour and social dominance in protandrous sex change in this species, one significantly larger individual was incorporated into each group to facilitate the emergence of a dominant individual in each test group.

SUBJECTS

Six unisex female groups composed of four individuals and six unisex male groups with four individuals were established. Prior to being placed into experimental groups all animals were housed in small social groups of between four and six individuals per group with only one male. Individuals used in the all-male groups were known to have spawned successfully with their group prior to inclusion in this experiment. Groups were housed in 38 l of salt water (c. 1.022 specific gravity) aquaria equipped with a gravel substratum, cylindrical nest tube (6 cm PVC pipe), biological filtration and fluorescent overhead lighting with a photoperiod of 12 L:12 D. Each group was completely (chemically and visually) isolated from the other groups. Fish were previously collected (California Fish & Game permit #803034-01) off the coast of Santa Catalina Island, California, U.S.A. (33°27'3" N; 118°29'15" W) using an anaesthetic solution of quinaldine sulphate (Sigma Chemical) and hand-nets. The fish were then transported back to the laboratory at Georgia State University, Atlanta. This research was carried out in accordance with the IACUC standards for use of animals in research at Georgia State University.

GROUP ESTABLISHMENT

All groups of *L. dalli* had one individual that was significantly larger (standard length, L_S) than the others (all-female: $F_{1,21}$, $P < 0.001$; all-male: $F_{1,21}$, $P < 0.001$) to facilitate one individual achieving dominance over the group. The L_S of the next two largest individuals in all-female groups were statistically indistinguishable (mean \pm s.e.: 30.2 ± 0.4 and 28.6 ± 0.1 mm; Tukey's HSD, $P > 0.05$) but were significantly greater than the smallest animal in the group (mean \pm s.e.: 26.8 ± 1.0 mm; Tukey's HSD, $P < 0.05$). In all-male groups, the L_S of the two smallest individuals were statistically indistinguishable (mean \pm s.e.: 32.8 ± 0.3 and 32.0 ± 0.3 mm; Tukey's HSD, $P > 0.05$) but were significantly smaller than the second largest animal of the group (mean \pm s.e.: 34.9 ± 0.7 mm). Variation in size among individuals was similar across groups (Levene HOV; all-female: $F_{5,17}$, $P > 0.05$; all-male: $F_{5,17}$, $P > 0.05$).

BEHAVIOURAL OBSERVATION AND STATUS DETERMINATION

Agonistic behaviour of each member of the all-female and all-male groups was observed during a 10 min focal period on 11 occasions throughout the experiment, and was recorded manually by one observer. 'Aggressive behaviour' represented a variety of behaviours: three forms of threat display (head-on and lateral display with fins and opercula flared, and 'headstand' display characterized by lifting of the tail off the substratum and wagging towards a nearby individual), and approach (orientation or slow movement towards another group member), as well as rapid approaches (attack) and bites towards another individual. Submissive behaviour included avoidance (move $< 2 L_S$) or retreat (move $> 2 L_S$) from an aggressive act by another group member. Courtship consisted of rapid, zigzag movements ('jerks') either towards another group member or

around the nest. For each status class in the all-female and all-male groups, the proportion of total behavioural acts dedicated by each individual to aggression, submission and courtship was calculated, and used for statistical analyses to control for differences in overall performance of behaviour across groups. Social rank was determined by directional aggression on each day of observation. In a given observation period, an animal was defined as dominant over another if it exhibited aggression towards, and elicited submissive behaviour from the other animal without receiving aggression in return. The sum of these individual interactions were used to determine the status of each animal in the group.

DETERMINATION OF SEXUAL PHENOTYPE

The genital papilla, an indicator of functional sex, was digitally imaged for each individual before the groups were established and every 2 weeks thereafter. The presence of fertilized eggs (visible appearance of eyes on the developing embryo) was used to verify that functional sex change had occurred within unisex groups (Reavis & Grober, 1999). For papilla measurements during the experiment, all individuals were removed simultaneously from the aquarium, their papillae photographed, and then returned simultaneously to the aquarium; these brief disturbances did not disrupt the social hierarchy. Because the papilla is not perfectly reflective of gonadal state (St Mary, 1994), assessment of sex by gonadal analysis was conducted at the conclusion of the experiment. Females possess a blunt papilla with a length to width ratio of *c.* 1.0, whereas the male papilla typically has a longer, more pointed morphology with a ratio >1.6 (St Mary, 1994; Rodgers *et al.*, 2005). All individuals of both sexes used in the experiment were within the normal papilla range for this species.

STATISTICAL METHODS

Mixed model repeated measures ANOVA were conducted to examine changes in papilla morphology (length: width ratio) and behaviour over time. One-way ANOVA was used to determine behavioural differences among the status classes. Because the various behavioural scores for each individual are not independent, multiple one-way analyses were conducted *in lieu* of a multivariate analysis; thus the *P*-values derived from the one-way analyses were subjected to sequential Dunn-Sidak adjustments to control for compounding type I error. All-female and all-male groups were treated separately because there was no significant status \times group-type interaction (all behaviour: $F_{3,38}$, $P > 0.10$). Tukey's HSD was used as a *post hoc* multiple comparisons test to determine status-dependent behavioural repertoires. All behavioural and morphological data were distributed normally without transformation (Shapiro-Wilk: $W > 0.92$, $P > 0.07$). Analyses were conducted using the JMP 5.0.1 statistical package and SAS version 8.2 (SAS Institute, Inc. 2002; Cary, NC, U.S.A.).

RESULTS

Male-typical courtship behaviour (jerks) was performed entirely by the α individual regardless of initial sex after hierarchy formation [Fig. 1(a), (b)]. On the first day of the experiment, an individual who would eventually become β , but whose status was unclear at the time, displayed jerking behaviour, which was not observed in any other observational period. The four status classes could not be distinguished on the basis of total aggression in all-female groups [ANOVA, $F_{3,19}$, $P < 0.001$ ($\alpha_{\text{adj}} = 0.017$), Tukey's HSD: $\beta = \alpha$; $\alpha = \gamma$, $\alpha > \delta$; $\beta > \gamma = \delta$; Fig. 1(a)], or all-male groups [ANOVA, $F_{3,19}$, $P < 0.001$ ($\alpha_{\text{adj}} = 0.01$), Tukey's HSD $\beta = \alpha > \gamma > \delta$; Fig. 1(b)]. Submission uniquely defined the four

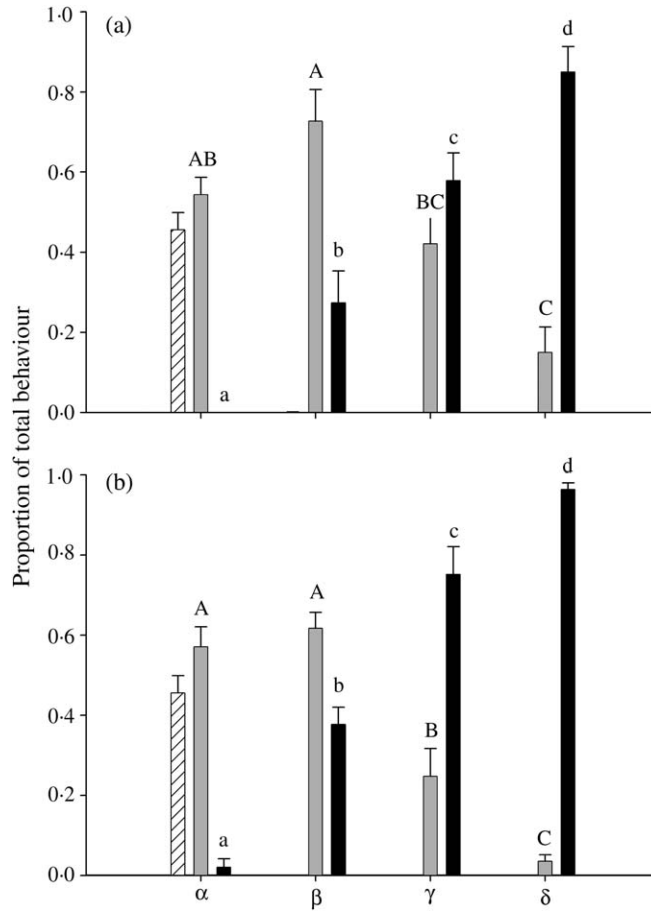


FIG. 1. Proportion (mean \pm s.e.) of total behaviour spent in each of three types of behaviour: aggression (■), submission (■) and courtship (▨). Groups that began as (a) all-female and (b) all-male. Aggressive behaviour alone does not uniquely define status class, whereas submission does. Values with different letters are significantly different at $P < 0.05$ using Tukey's HSD (aggression: upper case; submission: lower case).

status classes in both all-female [ANOVA, $F_{3,19}$, $P < 0.001$ ($\alpha_{\text{adj}} = 0.01$); Tukey's HSD: $\delta > \gamma > \beta > \alpha$; Fig. 1(a)] and all-male groups [ANOVA, $F_{3,19}$, $P < 0.001$ ($\alpha_{\text{adj}} = 0.01$); Tukey's HSD: $\delta > \gamma > \beta > \alpha$; Fig. 1(b)]. Status-dependent patterns of submission over time were used to ascertain hierarchy stability. There was no significant overall effect of time (repeated measures ANOVA, $F_{10,209}$, $P > 0.05$), and the relationship among the four status classes with respect to submission did not change with time (repeated measures ANOVA, time \times status: $F_{10,209}$, $P > 0.05$) or with group type (repeated measures ANOVA all-male v. all-female; sex \times time \times status: $F_{10,209}$, $P > 0.05$). Coupled with the fact that virtually no status reversals were observed, the analyses indicate that in the time frame of the experiment (42 days), *L. dalli* form stable linear hierarchies. None of the reversals that were observed involved individuals from the α status class.

Each group had only one dominant individual (α) emerge that remained or became male, indicated by no change in papilla ratio in male groups or a significant increase in papilla ratio in female groups, respectively [Fig. 2(a), (b)]. The papillae of all members of the female groups (ANOVA, $F_{3,22}$, $P > 0.05$) and male groups (ANOVA, $F_{3,20}$, $P > 0.05$) were not significantly different at the start of the experiment. For both male and female groups there was a significant status \times time interaction on the change in the papilla ratio (repeated measures ANOVA, females: $F_{3,18}$, $P < 0.001$; males: $F_{3,11}$, $P = 0.01$). There were no significant differences between status classes at day 0 (repeated measures ANOVA, females: $F_{3,22}$, $P > 0.05$; males: $F_{3,20}$, $P > 0.05$), but at 14 days (repeated measures ANOVA, females: $F_{3,22}$, $P < 0.001$; males: $F_{3,20}$, $P = 0.01$), 28 days (repeated measures ANOVA, females: $F_{3,22}$, $P < 0.001$; males: $F_{3,20}$, $P = 0.01$), 28 days (repeated measures ANOVA, females: $F_{3,22}$, $P < 0.001$; males: $F_{3,20}$, $P < 0.001$)

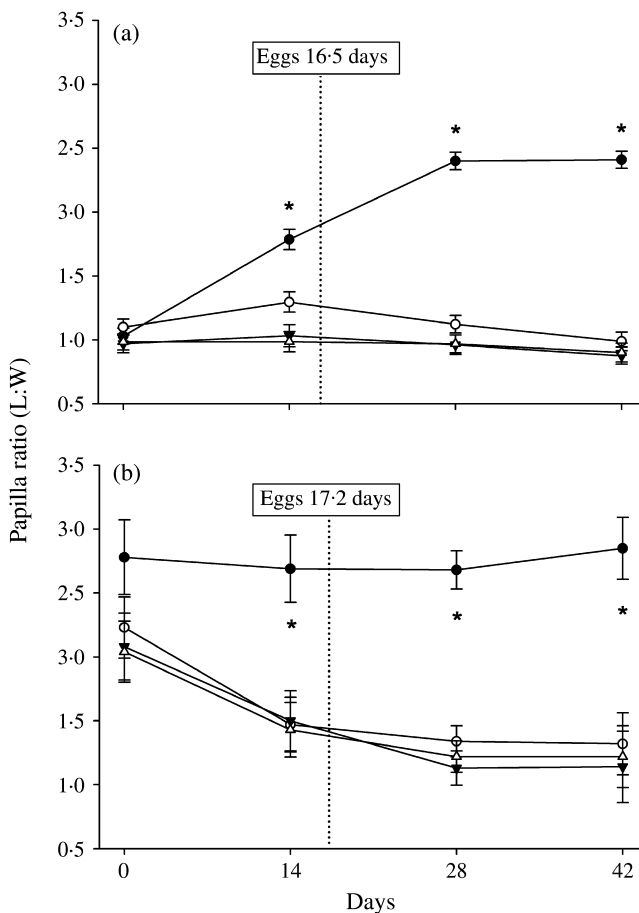


FIG. 2. Changes in papilla morphology (length: width ratio, L:W) over the course of the experiment in (a) all-female and (b) all-male groups. [α (●), β (○), γ (▲) and δ (△) class individuals]. Females possess a blunt papilla with a L:W of *c.* 1.0, whereas the male papilla typically has a longer, more pointed morphology with a ratio > 1.6 (St Mary, 1994; Rodgers *et al.*, 2005). The first appearance of fertilized eggs is marked with a dotted line. *, a significant difference (Tukey's HSD, $P < 0.05$) in L:W between α and all subordinates.

and 42 days (repeated measures ANOVA, females: $F_{3,21}$, $P < 0.001$; males: $F_{3,14}$, $P = 0.01$) there were significant differences between status classes. In both directions, beginning at day 14, α s had a greater papilla ratio than all other classes but there were no significant differences among the subordinate status classes.

There was a marked similarity between unisex groups in the mean time to first appearance of fertile eggs (functional sex reversal): 16.5 ± 1.0 (mean \pm S.E.) days for female groups and 17.2 ± 0.9 days for male groups. Five of six male groups produced fertile eggs. Alpha individuals from several of the male groups maintained eggs in their nests continuously for the remainder of the experiment, evidence of highly successful groups with multiple females contributing eggs. Subordinates (ranks β to δ) in all groups displayed female-typical papillae on the day eggs first appeared in the nest (Fig. 2). In the male groups, female-typical gonads were verified for 14 of 17 subordinates; three subordinates possessed ambiguous gonads (a mix of male and female tissue).

DISCUSSION

The design employed in this experiment reliably produced stable social hierarchies. Status within the social hierarchy dictated the expression of sexual phenotype: one individual per group, the α individual, remained or became male, while all subordinate individuals in the group (β to δ) remained or became female. Both sexes demonstrate equivalent potential to establish stable social groups and to alter their sexual phenotype accordingly under these conditions. The remarkable temporal symmetry for functional sex reversal (*c.* 17 days in both males and females) suggests that both sexes are equally sensitive and responsive to perturbations of their social environment.

In the unisex male and female groups a linear hierarchy was formed, wherein individuals tend to aggress towards those individuals to whom they were dominant and submit to those to whom they were subordinate. Proportions of submissive behaviour were highly correlated with the four discrete status classes (Fig. 1), and thus are likely to be an organizing principle of these small social groups. Notably, proportions of aggressive behaviour do not have the same predictive value. Courtship behaviour appears to be predictive of α status class, but is confounded by the fact that all α s are also males. As such, the expression of jerking (male courtship behaviour), while a hallmark of the α status class cannot be separated from status class itself in the context of this data set, and may not be required for the initial acquisition of α status. These distinct status classes gave rise to only two sexual phenotypes; thus status information, as it relates to sexual phenotype, is dichotomized. A simple organizing principle for the determination of sexual phenotype emerges: if subordinate express female, if dominant or not subordinate express male. While it has been shown in several species that sex change is under social regulation (Robertson, 1972; Fricke & Fricke, 1977), a simple yet powerful operating principle has been identified in *L. dalli* that determines the sexual phenotype of each group member and all group members respond in accordance with the principle regardless of initial sexual state or social status. *Lythrypnus dalli* uses a binary representation of social status to direct sex typical allocation of reproductive resources and behaviour, in both directions.

The results from this study contrast with earlier results in *L. dalli*, which indicated that sex change occurs more readily in the protogynous than in the protandrous direction (St Mary, 1994; Reavis & Grober, 1999). The discrepancy between previous research on protandrous sex change in *L. dalli* and the present study may stem from differences in group composition, with the present design facilitating the formation of a robust social hierarchy, which may be key to rapid changes in sexual phenotype. In social groups that are less tightly ordered, status ambiguity may result, which could explain instances of facultative sex change (Ross, 1990; Lutnesky, 1994).

The striking parallelism between sex change in the protogynous and protandrous directions with respect to both the time course and the use of social status suggest that a similar mechanism may underlie both processes. Throughout evolutionary history, control over sexual differentiation pathways has been entrained to a variety of different signals (Zarkower, 2001). Social status provides an ideal signal in that regard, because of a pre-existing relationship between dominance and reproductive success (Ellis, 1995), and because young animals are unlikely to be dominant or to reproduce effectively as the resource holding sex. Such a mechanism could allow individuals to take advantage of the differences in size-fecundity skew between the sexes originally described in the size advantage model (Ghiselin, 1969; Warner *et al.*, 1975; Warner, 1988). This suggests that sexual plasticity has been achieved over evolutionary history by shifting control of sexual differentiation from a static persistent genetic signal to a signal that may vary over the life of an individual: the variable signal model. Thus both unidirectional and bi-directional sex-changing fishes are likely to use the conserved process of sexual differentiation that is now linked to a variable signal (in this case social status) to direct adult re-allocation of the suite of traits that characterize the sexes. Entraining sexual differentiation pathways to signals that are predictive of changes in reproductive opportunity may explain the persistence of sex change across a range of species and mating systems that are not subject to dramatic size-fecundity skew (Kuwamura *et al.*, 1994) but where phenotypic flexibility would be reproductively advantageous, such as after mate loss or social disruption (Sunobe & Nakazono, 1993; Nakashima *et al.*, 1995; Munday *et al.*, 1998).

The operating principle described in *L. dalli* (if subordinate express female, if dominant or not subordinate express male) is unlikely to apply to all sex changing fishes (Fricke & Fricke, 1977; Shapiro, 1988), although it may apply to a large percentage of bi-directional species [similar social regulation has been documented in pairs of *Trimma okinawae* (Aoyagi) (Sunobe & Nakazono, 1993), *Labroides dimidiatus* (Valenciennes) (Kuwamura *et al.*, 2002) and possibly *Gobiodon histrio* (Valenciennes) (Munday, 2002)]. The principle, with its emphasis on subordination, will provide a model to examine the bi-directional potential of other sexually plastic species. The variable signal model, however, which allows for the control of sexual differentiation pathways by novel inputs, is likely to be broadly applicable to sexually plastic fishes.

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