

Robert H. Reavis · Matthew S. Grober

## An integrative approach to sex change: social, behavioural and neurochemical changes in *Lythrypnus dalli* (Pisces)

Received: 17 March 1999 / Received in revised form: 15 May 1999 / Accepted: 28 May 1999

**Abstract.** This study examined three aspects of protogynous sex change in *Lythrypnus dalli* (Gobiidae): (1) social influences on the rate of sex change, (2) the sequence of behavioural changes, and (3) neuroendocrine changes. Social groups consisted of either four females, or four females with a male who was subsequently removed. Sex change occurred most rapidly in male-removed groups when the sex changer was larger than other females. Sex changers in female only groups and sex changers not larger than other females in male-removed groups changed sex at similar rates. These differences may be explained by two factors that affect dominance: prior knowledge of the social group and greater size. Sex changers were dominant to other females prior to male removal, and larger sex changers increased displacement rates three-fold immediately after male removal. Sex changers in the other groups did not show this increase in displacements. This early establishment of dominance accounts for the overall difference in the rate of sex change. Prior to spawning, however, all sex changers increased displacements and performed male-typical displays. Arginine vasotocin-immunoreactive forebrain cells of sex changers were similar in size to field-collected males, and larger than field-collected females. Previously nesting males also changed sex in male-only groups, but at slow rates. These data are combined with those of existing studies to generate an integrative model of sex change in this goby.

**Key words** Sex reversal · Reproduction · AVT · Gobiidae · Neuroendocrinology

### Introduction

Many teleost fishes change sex as adults (sequential hermaphrodites), and some species reproduce as simulta-

neous hermaphrodites (Shapiro 1989; Warner 1984). Much research has focused on the sex-allocation strategies of these fish (Fischer and Petersen 1987) and the proximate factors that initiate sex change (Lutnesky 1994; Ross 1990; Warner and Swearer 1991). Although common themes have emerged (e.g. the size-advantage hypothesis, Warner et al. 1975), the diversity of sex-change strategies continues to present challenges to researchers in this field (e.g. St. Mary 1997).

Of particular interest to ethologists are the behavioural interactions that initiate sex change. Two social factors commonly affect sex change: (1) inhibition by males, and (2) social stimulation by other females (reviewed in Shapiro 1979). Removal of the male from a social group 'disinhibits' the dominant female of the group, who then changes sex if other females are available as mates. Typically, this dominant fish is the largest in the group (e.g. Ross 1990); however, the relationship between size, dominance and the rate of sex change has not been examined in detail (Lutnesky 1996). In gonochoristic fishes, greater relative size and previous dominance status affect the outcome of contests and the rate at which contests are decided (e.g. Barlow et al. 1986; Keeley and Grant 1993). These factors may similarly affect the establishment of dominance associated with sex change.

In addition to behaviour, sex change involves modifications of gonads (Nakamura et al. 1984), hormone levels (Hourigan et al. 1991), brain chemistry (Grober and Bass 1991; Grober and Sunobe 1996) and several morphological characters (e.g. colour, Warner and Swearer 1991; genitalia, Cole 1983). Activation via the hypothalamic-pituitary-gonadal axis is a working hypothesis for the social regulation of sex change (Grober 1997). Although steroid treatment may induce partial or complete sex change in some species (reviewed by Reinboth 1985), whole-body titres of these hormones change days after behavioural changes in field populations (e.g. Godwin and Thomas 1993). Moreover, gonadectomized fish show the typical pattern of behavioural sex change (Godwin et al. 1996). These results suggest that the gonad does not initiate sex change. Pituitary regulation is an

R.H. Reavis (✉) · M.S. Grober  
Department of Life Sciences, Arizona State University West,  
P.O. Box 37100, Phoenix, AZ 85069-7100, USA  
e-mail: reavis@imap2.asu.edu

earlier step in the process and gonadotropins are known to stimulate sex change (Koulisch and Kramer 1989), but we have little information on early changes in gonadotropin levels. At the level of the hypothalamus, changes in the size, number, and/or activity of neuropeptide-producing cells have been associated with sex change (Grober and Bass 1991). Arginine vasotocin (AVT) is an hypothalamic peptide that regulates reproductive behaviour in all non-mammalian vertebrates (Moore 1992) and the size of AVT cells changes during sex reversal (Grober and Sunobe 1996).

Here, we focus on three aspects of sex change in the hermaphroditic fish, *Lythrypnus dalli* (Gobiidae): (1) social influences on the rate of sex change, (2) the behavioural sequence of sex change, and (3) changes in forebrain AVT-producing cells. We also tested the ability of functional males to change sex. Our results suggest that both size and prior knowledge of the social group affect the sex changer's ability to establish dominance, and thus affect the overall rate of sex change. Behavioural changes occurred in discrete stages that may reflect changes in the neuroendocrine system. Hypothalamic AVT cells increased to male-typical sizes by the end of sex change. These results provide the foundation for an integrative model of sex change in *L. dalli*, which is being tested in ongoing studies of this unusual hermaphroditic.

### Natural history of *Lythrypnus dalli*

St. Mary (1993, 1994) described *L. dalli* as the first known example of a fish that maintains both ovarian and testicular tissue, behaves as a sequential hermaphrodite, and provides male parental care. These small gobies (adult standard length 18–45 mm) inhabit rocky reefs from Morro Bay, California, to the Sea of Cortez (Miller and Lea 1972). They live at high densities (to 25 m<sup>-2</sup>) in mixed-sex groups; males guard nests where they care for eggs. After hatching, larval fish spend at least 2 months in the plankton before settling on reefs; little is known of this stage in *L. dalli* or other reef fishes (Behrents 1983).

Sex ratios in the field, size dimorphism, and experimental evidence suggest that most males derive from sex-changed females (protogyny; St. Mary 1994). However, these sex ratios were determined by their genital papillae (after Behrents 1983). St. Mary (1993) found that *L. dalli* with female-typical genital papillae allocated 95–100% of their gonads to ovarian tissue; however, those with male-typical genital papillae had a range of gonadal allocation from 95% ovarian tissue to 100% testicular tissue. Only those fish observed to spawn as males exhibited 100% testicular gonads. Furthermore, some 'males' later spawned as females (protandry, St. Mary 1994), although the behavioural sex of these fish was not confirmed prior to sex change. Thus, while the sex of females may be assigned based on their genital papillae, little can be said of the functional sex of individuals with male genital papillae in the absence of be-

havioural assays. St. Mary (1994) suggested that the extreme plasticity of *L. dalli* may be a response to rapidly changing social conditions. The unusual combination of sexual strategies exhibited by *L. dalli* provides a model system to test many of the ideas generated by hermaphroditic fishes.

### Methods

We collected *L. dalli* with either slurp guns or an anaesthetic solution of quinaldine sulfate (Sigma Chemical) from the area of Bird Rock, Santa Catalina Island, California (CFandG permit no. 802002-02) in May 1996 and March 1997. Both methods are commonly used in the capture of live fish (e.g. Wiley 1976). Captured fish were anaesthetized in MS 222 (tricaine methanesulfonate, Sigma Chemical), measured to the nearest millimetre standard length (SL), and initially sexed by genital papilla (after St. Mary 1993). Some fish collected in 1996 were immediately euthanized by an overdose of MS222 and immersion-fixed in 4% paraformaldehyde; brains from these fish provided a field sample for comparison with experimental animals. All others were initially housed in aquaria with flowing sea water at the Wrigley Institute for Environmental Studies (WIES), University of Southern California, Santa Catalina Island. Fish were later moved to aquaria at Arizona State University, West (ASU).

#### Sex change: hypotheses and methods

Protogynous sex change in *L. dalli* has been demonstrated in two disparate social contexts (St. Mary 1994). Within existing social groups, sex change follows the loss of the dominant male. Alternatively, upon the foundation of a new, all-female group, one to several females may initiate sex change. In either case, we hypothesize that an early step in sex change is the establishment of dominance over the entire social group and the rate of this process (establishment of dominance) should affect the overall rate of sex change.

We tested two hypotheses regarding the establishment of dominance in the context of sex change. First, we compared the rate of sex change between female-only groups versus groups that initially included females and a male who was later removed. Females in the male-removed groups had the opportunity to determine their dominance status prior to male removal. We hypothesized that this experience should speed the overall establishment of dominance by the sex changer in male-removed groups, relative to sex changers from the female-only groups. Second, we expected that sex changers with a size advantage would more rapidly establish dominance over the entire social group versus sex changers either similar in size or smaller than another female in the group. Moreover, if overall dominance is a prerequisite for sex change, the time to establish dominance should affect the time to change sex. This hypothesis also suggests that the earliest changes in behaviour will be related to dominance interactions, which may be measured by either the difference in or ratio of displacements given versus received (Shapiro 1981). Finally, by the completion of sex change, new males should exhibit male-typical patterns in AVT forebrain cells, and other neuroendocrine, gonadal, and genital characters.

Experimental groups consisted of either four females with no males (no male groups, NM), or one male with three five females (male-removed groups, MR; mean=4 females). Males remained in the MR groups at least 5 days prior to removal. Sex was assigned by genital papilla, a reliable indicator for females (see "Natural history"). Although male-typical genital papillae are not as reliable for assigning sex, these 'males' cared for nests and dominated their social groups, the two aspects of male behaviour critical for this experiment. For each type of group, we chose females that varied in SL and in banding pattern, to permit individual recognition. Banding patterns did not change with changes in either size

or sex, nor did they appear to affect social interactions in any way. Social groups were housed in compartments of covered sea-water tables that contained 23–38 l of flowing sea water (WIES), or in 37.5 l aquaria (ASUW). Fish at WIES received incidental day light. At ASUW, fish received artificial light 14 h day<sup>-1</sup>. All fish received commercially prepared food daily. Each group was provided with one or two pieces of PVC pipe as a nest site (after St. Mary 1994). Finally, some males removed from MR groups and some recently sex-changed males were housed in male-only (MO) groups to test whether individuals that actually spawned as a male could change sex and spawn as a female.

Both MR and NM groups were followed through sex change in spring 1996 at WIES, and summer 1996 and spring 1997 at ASUW. We measured the time to change sex from the day of male removal (MR groups; referred to as day 0) or from the first day groups were housed together (NM and MO groups; day 0). Note that MR groups had several days together prior to day 0, whereas the NM groups lacked this time together. This difference reflects the two social scenarios described above: (1) loss of a male from an existing group, and (2) formation of a new all-female group. Thus, day 0 for both groups represents the first day on which sex change might be initiated. Groups were observed daily to record behaviour and spawns. Female-to-male sex changers were first identified by their dominant behaviour and proximity to the nest site (below). We considered their sex change complete on the first day that eggs appeared in the nest. Although this time point also depends on the behaviour of a spawning female, it provides a conservative and definitive measure of male function (Sunobe and Nakazono 1993). Moreover, we directly observed the spawning act in 10 of 21 sex changes, and observed development to the eyed stage to verify fertilization in 12 cases (in six other cases observations were terminated by our move to ASUW, below). Finally, we examined the genital papilla of sex-changed fish after they received eggs. Observations of some initial groups at WIES were disrupted by the move to ASUW on or before day 14. Because this disturbance might influence the rate of sex change, their time to change sex is not included. Neither do we include time to change sex for other groups that did not change within 14 days. Male-to-female sex change was determined by the presence of eggs in MO groups, and female-typical genital papilla of the sex-changed fish.

We recorded behaviour by focal-animal samples of either 5, 10, or 15 min per individual. The duration of these intervals was predetermined before the start of each observation. Up to four observations were made per fish in any day. Here we report on observations of removed males and sex changers from 2 days prior to male removal until sex change was completed (i.e. eggs in the nest; up to day 14). We quantified dominance interactions into two classes, acts given (displacements) and received (avoids), defined by the approach of one fish to within 5 cm of another fish that resulted in the movement of the second fish away from the initiating fish. Approaches varied from rapid, forward swims to lateral approaches with the median fins erect. One type of approach combined a forward swim with jerky lateral displacements that is typical of male courtship ('jerks', Behrens 1983). This behaviour pattern was recorded separately in eight groups. We averaged behavioural observations on the same day to acts per minute for each focal fish. For some groups, we also estimated the distance of each fish to the nest (to the nearest centimetre) at either 1- or 5-min intervals by use of a repeating count-down timer. Alternatively, we identified which individual other than the male or sex-changing fish was closest to the nest when groups were first observed in the morning.

Observations focused on the female expected to change sex. Our predictions were not always correct, so data are missing for some sex changers early in the process of sex change. Thus, sample sizes vary between different statistical tests (e.g. rate of sex change versus behaviour; see Results). Similarly, we include data for females early in sex change that were not observed through spawning. We also observed eight males prior to removal and other females in the group. Observation data presented are from focal sex changers unless otherwise noted. Interobserver reliability was high for both measures of displacement and distance to the nest

( $r_s > 0.90$ , Spearman Rank correlation coefficient). Other behaviour was recorded opportunistically.

#### AVT forebrain nuclei

We used immunocytochemistry to examine changes in the size of AVT-immunoreactive (AVT-ir) cells in the forebrain magnocellular preoptic area (see Braford and Northcutt 1983 for neuroanatomical details). We compared the size of these cells in field collected males and females to those of fish that changed sex from female to male in experimental groups (defined above). We assigned sex to the field-collected fish by their genital papillae, although male-typical genital papillae are not unambiguous indicators of sex.

Following euthanization with excess MS 222, animals were immersion-fixed for 5–7 days in fresh 4% paraformaldehyde. Both eyes were removed to facilitate penetration of the fixative. Brains were then removed from the skull, postfixed for 1–2 h and stored at 4°C in 30% sucrose in 0.1 M phosphate buffer for cryoprotection. Brains were sectioned frozen at 15–25 µm on a cryostat, and mounted on chrom-alum coated slides, as two parallel series through the brain. Tissues were then either reacted immediately or stored at –20°C.

Following air-drying, the slides were treated using the methods outlined in Grober and Bass (1991), except that (1) a Streptavidin-Biotin Kit (KPL) was used and (2) a polyclonal antibody against AVT was used (courtesy of Lieve Moons, Zoological Institute, Belgium). Overnight preabsorption of the antiserum with 1 µg/ml synthetic AVT (Sigma) eliminated all immunostaining.

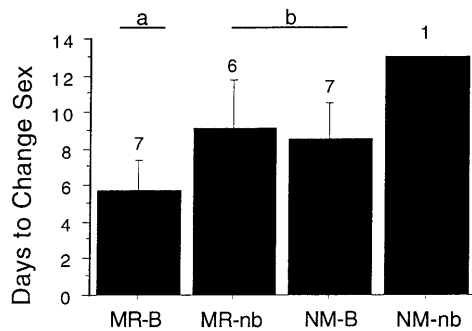
All slides were coded so that the individuals who quantified the size of cells did not know the identity of the fish to which each slide corresponded. Cell size was measured by capturing images and tracing the outside margin of the soma using NIH Image 1.55 (W. Rasband, NIH, Bethesda, Md.). After calibrating for magnification, the imaging program provided a measure of the area of each cell. For each fish in the study, we measured all magnocellular preoptic cells that exhibited a nucleus and at least one neurite. Magnocellular neurons could be separated from parvocellular neurons based upon their larger size and dorsolateral location. Differences among the treatment groups in the size of AVT-ir cells were determined using ANOVA. Relationships between body size and cell size were examined using simple linear regression and analysis of covariance.

## Results

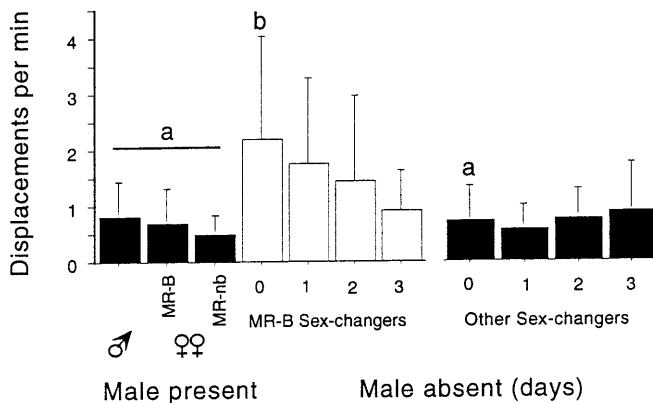
### Protogynous sex change: rates and behaviour

We observed 18 MR groups and 12 NM groups for at least 14 days or until one fish changed sex (i.e. received eggs in the nest). In 17 of the MR groups, 'males' were larger than all females within their group (males: 29–37 mm SL; females: 21–35 mm SL), and socially dominant. In the other MR group one female (33 mm SL) was larger than the male (28 mm SL); that group is not included here, but discussed below. Initial sizes of females in the NM groups (21–34 mm SL) were similar to MR females.

Sex change occurred in 13 of 17 MR groups (76.5%) and 8 of 12 NM groups (66.7%) within the 14 days of observation. In addition to the presence of eggs, we observed the spawning event in 10 cases and embryonic development to the eyed stage in 12 groups to verify that the sex-changed fish fertilized eggs. All sex-changed fish exhibited male-typical genital papillae. Only one fish per group changed sex; no others exhibited male-



**Fig. 1** Time to change sex (days) in *Lythrypnus dalli* for different social conditions and experimental outcomes. Group categories: MR-B (male removal group where the sex-changed female was bigger than all others), MR-nb (male removal with the sex changer not bigger than all others), NM-B (no male present, biggest female changes), and NM-nb (no male, and sex changer not bigger than all others). Columns and error bars represent the mean $\pm$ SD. The numbers above the columns represent sample sizes and letters denote statistical differences ( $P<0.05$ ).



**Fig. 2** Patterns of displacement behaviour in males and MR sex-changers before male removal, and all sex-changers in the first 4 days without a male present. Sex-changers are denoted as female (male present), MR-B or Other (MR-NB and NM-B). Abbreviations and notations as in Fig. 1

typical genital papillae even though some groups remained intact for up to 6 months.

Fish that changed sex were either the longest or the second longest individual in their group. Therefore, we split each treatment into two categories: those where the sex-changed female was bigger than all others (MR-B fish, NM-B fish) and those where the sex-changer was not bigger than all others (MR-nb fish, NM-nb fish).

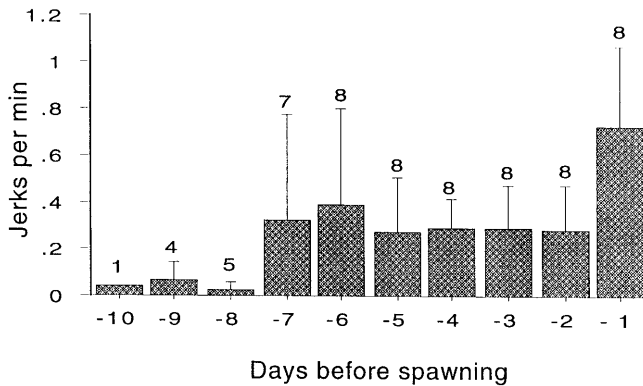
The rate of sex change differed significantly between types of sex-changers and by season (two-factor ANOVA: group,  $F_{2,14}=12.30$ ,  $P<0.01$ ; season,  $F_{1,14}=36.76$ ,  $P<0.01$ ; the single NM-nb fish excluded here because of sample size; Fig. 1). Sex change occurred most rapidly in MR-B fish (mean $\pm$ SD=5.71 $\pm$ 1.70 days,  $n=7$ ). Sex change was slower under other conditions: MR-nb fish=9.17 $\pm$ 2.64 days,  $n=6$ ; NM-B fish=8.57 $\pm$ 1.90 days,  $n=7$ , and one NM-nb fish=13 days. Post hoc comparisons found significant differences between MR-B and

MR-nb fish, and between MR-B and NM-B fish (both  $P$ -values  $<0.01$ , Bonferroni-Dunn tests adjusted for multiple comparisons). MR-nb and NM-B fish did not differ significantly ( $P=0.39$ ). Sex change was faster in mid-summer than at other times (5.5 $\pm$ 2.26 days,  $n=6$  vs 9.0 $\pm$ 2.20 days,  $n=15$ ). MR-B fish were proportionately represented in both seasons (2 of 6, summer; 5 of 15 at other times).

Based on their ratio of displacements to avoids, all MR fish that changed sex were dominant to other females in their group prior to male removal. Most dominant females were never observed avoiding other females. However, dominant females avoided males (0.31 $\pm$ 0.38 avoids min<sup>-1</sup>,  $n=15$ ). Males directed 30.7 ( $\pm 26.5$ ) % of all their displacements at dominant females (these data based on focal observations of the male;  $n=8$  males). These displacement rates towards dominant females were similar to the rate that males displaced other females in the group. Similarly, we ranked females by the amount of displacements received from the male; the female displaced the most by the male received a rank of '1'. Dominant females averaged a rank of 2.06 ( $\pm 0.95$ ) out of the four females in their group (range: 1–3;  $n=8$ ). Immediately after male removal, dominant females significantly reduced their rate of avoids (day 0; mean  $<0.01$  avoids min<sup>-1</sup>; one-way ANOVA:  $F_{1,28}=9.88$ ,  $P<0.01$ ,  $n=15$ ). All the above measures were similar for dominant females that changed sex, regardless of their relative size. Dominant females in NM groups exhibited similar low rates of avoidance from their first day together (day 0;  $<0.01$  avoids min<sup>-1</sup>,  $n=10$ ).

Displacement rates of MR-B fish increased within minutes after male removal (day 0; Fig. 2). Prior to male removal, MR-B fish displaced other females at a rate of 0.69 $\pm$ 0.64 displacements min<sup>-1</sup> ( $n=10$ ; Fig. 2); MR-nb fish displaced other females at a rate of 0.46 $\pm$ 0.51 displacements min<sup>-1</sup> ( $n=3$ ). Males displaced females at a similar rate (0.83 $\pm$ 0.62 displacements min<sup>-1</sup>,  $n=7$ ). On day 0, MR-B females increased their rate of displacements three-fold (2.18 $\pm$ 1.86 displacements min<sup>-1</sup>,  $n=13$ ). This rate was significantly greater than the displacement rates of males and these same females prior to male removal (Mann-Whitney  $U$ -test:  $U=58.5$ ,  $P<0.01$ ,  $n_1=13$ ,  $n_2=20$ ). Fish that changed sex in the other groups did not exhibit this high rate of displacements on day 0 (MR-nb fish=0.76 $\pm$ 0.89 displacements min<sup>-1</sup>,  $n=7$ ; NM-B fish=0.67 $\pm$ 0.28 displacements min<sup>-1</sup>,  $n=7$ ; Fig. 2). These rates were significantly lower than those of MR-B fish (Mann-Whitney  $U$ -test:  $U=37.5$ ,  $P<0.01$ ,  $n_1=13$ ,  $n_2=14$ ; MR-nb and NM-B fish pooled). By day 3, MR-B fish decreased their rate of displacements to 0.92 min<sup>-1</sup> ( $\pm 0.69$ ,  $n=9$ ), levels similar to males and their own displacement rates prior to male removal (Fig. 2). Displacements by other sex-changers increased slightly from days 0 to 3, but not significantly (Fig. 2). Across all groups, sex-changers increased displacements on the day before their first spawn as a male (1.37 $\pm$ 1.71 displacements min<sup>-1</sup>).

The approach component of displacements changed over the course of sex change. Initially, dominant fish



**Fig. 3** Jerk behaviour over the course of sex change. Time is denoted as the days before eggs were first seen in the nest. Columns and error bars represent the mean $\pm$ SD. Sample sizes vary due to differences in the rate of sex change. Sex-changing fish show a courtship peak in jerks that immediately precedes the first spawn as a male (day -1) and an earlier peak follows the establishment of dominance (around days -7 and -6, evident by both the higher mean number of jerks and standard deviation). This peak may indicate the initiation of sex change. The lower sample sizes for days -8 through -10 result from the fact that these fish took longer to change sex. Moreover, these data represent MR-nb and NM-b fish that jerked at low rates prior to their establishment of dominance and took longer to change sex

approached others with either direct swims or lateral displays. Later approaches were characterized by ‘jerk’ swims, a display typical of males but rarely seen in females in the presence of males. On day 0, sex-changing females exhibited few jerk approaches ( $0.05\pm 0.08$  min $^{-1}$ ,  $n=8$ ); these jerks tended to be poorly defined relative to typical male jerks. The frequency of jerks increased significantly over the course of sex change, and peaked on the day before spawning ( $0.73\pm 0.34$   $n=8$ ; Paired  $t$ -test:  $t_8=5.23$ ,  $P<0.01$ ). At this time the sex changer jerked intensely. It repeatedly jerked toward a female and back to the nest, apparently leading the female to the nest. Other jerks were made into and around the nest. An earlier peak in jerks occurred on day 2 for MR-B groups ( $n=3$ ) and days 1–5 for other groups ( $n=5$ ). Although the time to this initial peak ranged over 4 days, the subsequent time to change sex varied by only one day when corrected for season (see Fig. 3).

MR sex changers also showed a change in proximity to the nest site. Prior to removal, males were generally the closest fish to the nest, often perching on or within the nest. Dominant females tended to position themselves at intermediate distances from the nest (and male) relative to other females. For example, we recorded the female closest to the nest on the day prior to male removal ( $n=9$ ). In only one case was the dominant female the closest female to the nest (sign test,  $P<0.01$ ). From the day of removal, dominant females established positions near the nest except when chasing other fish. We observed the relative distance of fish to the nest on either day 0, day 1, or both. In all cases the sex changer was found closest to the nest (sign test,  $P<0.01$ ;  $n=11$ ). NM dominants also positioned themselves near the nest. Fol-

lowing the initial peak in jerk swims, sex changers spent much of their time in the nest, although the exact timing of this period varied. They rubbed themselves against the inside of the nest from this time through spawning. As spawning approached, they again left the nest to jerk and approach females.

We did not quantify egg care after sex changers spawned as a male. However, newly changed fish remained with the eggs and limited their other activities (e.g. jerks and displacements), as did other males with eggs in the nest.

#### Male-to-female sex change

One MR group contained a female (33 mm SL) longer than the male (28 mm SL). The large female changed sex and spawned as a male 11 days after the start of the group with the original male still in the group. The original male spawned as a female 3 days later (day 14). The longer fish was eventually removed from this group coincident with the move to ASUW. The original male finally spawned as a male in a new group at ASUW.

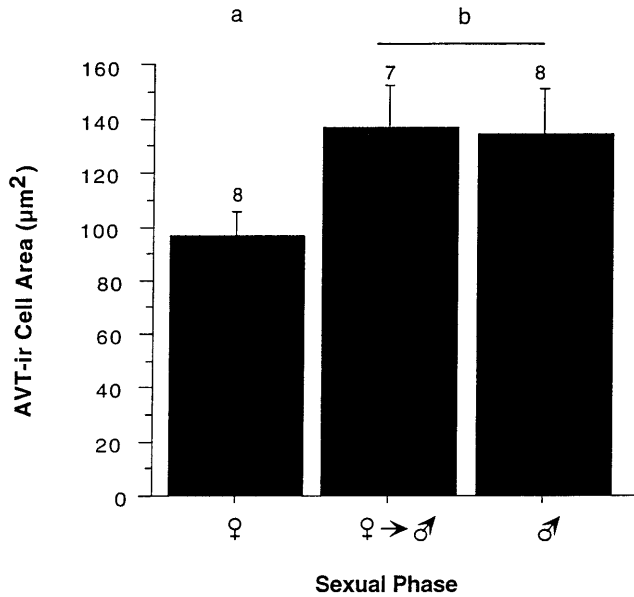
Four sex changers that received and cared for fertilized eggs (i.e. functional males) were later housed together as an MO group. Eggs first appeared 32 days after group formation, with additional spawns at approximately 2-week intervals. Only one of eight clutches developed to the eyed stage. We did not identify which fish spawned as the female, although one fish in this group had an unusual genital papilla.

A second MO group was constructed from four MR males known to receive eggs in the nest. They first spawned after 46 days together. These eggs developed. Two more clutches were laid at day 68 and day 93, but the eggs did not develop. One fish that died shortly after a spawned clutch had a female genital papilla. We did not examine the genital papilla of other fish in these groups.

A third MO group consisted of five males removed from MR groups. All of these ‘males’ had previously guarded nests, but not all received eggs. The first spawning occurred 53 days later. The eggs disappeared before development. One fish died in this group 4 days later; this fish had a male-typical genital papilla. No other spawnings occurred in this group (150 days together).

#### Changes in AVT

AVT-ir cells were located in the magnocellular (medial) and parvocellular (ventral) preoptic forebrain areas, as described in other gobies (Grober and Sunobe 1996). The mean cross-sectional area of AVT-ir magnocellular preoptic cells did not differ between males ( $134.6\pm 45.2$   $\mu\text{m}^2$ ,  $n=8$ , Fig. 4) and female-to-male sex changers ( $137.2\pm 39.4$   $\mu\text{m}^2$ ,  $n=8$ ). Females had significantly smaller mean cell areas ( $96.2\pm 27.9$ ,  $n=7$ ) versus the combined mean of both field caught and sex-changed males



**Fig. 4** A comparison of mean arginine vasotocin-immunoreactive (AVT-ir) cell size between female-to-male sex changers and field collected males and females. Columns and error bars represent the mean  $\pm$  SD. The numbers above the columns represent sample sizes and letters denote statistical differences (see text for a description of the analyses)

(ANOVA:  $F_{1,22}=5.9$ ,  $P<0.05$ , Fig. 4). Although the SL of these fish ranged from 21 to 37 mm, body size did not have a significant effect on AVT-ir cell size ( $R^2=0.05$ ;  $P=0.3$ ).

## Discussion

Both the rate of sex change and the behaviour of sex changers suggest that a fish must establish dominance over its social group as a prerequisite for sex change in *L. dalli*. The rapid rate of sex change in MR-B fish may be attributed to two factors known to affect contests in fishes: greater relative size and previous dominance status (e.g. Barlow et al. 1986; Keeley and Grant 1993). MR-B fish had both a size advantage and a minimum of 5 days to determine dominance relationships prior to male removal. They immediately established dominance over the entire social group after male removal. NM-B fish lacked the opportunity to determine dominance relationships in their groups prior to day 0, while MR-nb fish lacked a size advantage. Relative to MR-B fish, both NM-B and MR-nb fish took longer to establish dominance and change sex. The similar rates of the latter two groups suggest that prior experience with the social group and greater size have equivalent effects on the rate of sex change.

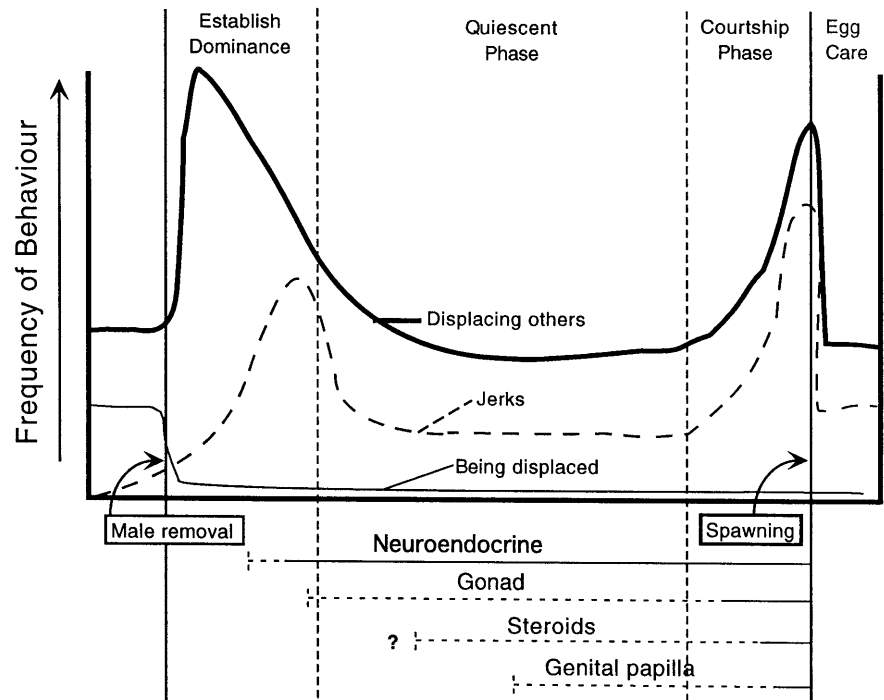
Shapiro (1981) suggested the critical cue that initiates sex change may be the profile of behaviour given (displacements) and received (avoids), rather than simply the rate of displacements given. This cue may be perceived

as either the ratio of displacements to avoids, or the difference between displacements and avoids. Our results are consistent with this hypothesis. Prior to male removal, MR sex changers avoided males at similar rates to other females, and were similar in their proximity to the male. However, from day 0 and beyond, sex changers of all types rarely avoided others, while other fishes in their groups continued to avoid. Thus, the low rate of avoids was the first behavioural change observed in all types of sex changers and indicates their new status as the dominant fish in their group.

We cannot compare the ratio of displacements to avoids for the different types of sex changers, because from the first opportunity to change sex (day 0) the rate of avoids approached zero (cannot divide by zero). However, the difference between their displacements and avoids is equivalent to their rate of displacements (because avoids  $\approx 0$ ), and early displacement rates by sex changers did correlate with the rate of sex change. MR-B fish initiated and won three-times as many contests on day 0 relative to other sex changers, and changed sex more rapidly. We interpret the abrupt rise and fall of displacement rates in MR-B fish as an indication that dominance was established (on day 0 or day 1). We did not define a time at which overall dominance was established for NM-B and MR-nb fish, and in several groups it was not clear for the first few days which fish would change sex. However, the peak of displacement rates for these other treatments occurred on day 3, which was 3 days later than MR-B fish (day 0), and these fish took an extra 3 days to change sex relative to MR-B fish. These correlations suggest the establishment of dominance is a prerequisite for sex change.

Jerk swims were the only male-typical behaviour pattern readily observed prior to nesting. Behrents (1983) associated jerks with courtship, and we observed a peak in jerks the day before spawning. A similar form of 'skipping courtship' occurs in a Japanese goby that exhibits serial sex change (Sunobe and Nakazono 1993). Jerks first appeared in sex changers after male removal and increased over the next few days. In MR-B fish, this initial rise in jerks peaked as their displacement rate began to decline (after the establishment of dominance, see Fig. 5). Moreover, this peak occurred earlier in MR-B females than in other sex changers, and correlates with the overall difference in the time to change sex both within and between treatments. Thus, while the initial decrease in avoids and increase in displacements (behaviour patterns common to both sexes) establish dominance and may be a prerequisite for sex change, the subsequent rise in jerks (a male-typical behaviour) may signal the onset of sex change. Robertson (1972) found a similar behavioural sequence in the sex-changing cleaner wrasse, *Labroides dimidiatus*: an initial peak in female-typical aggressive displays which waned after 30 min, followed by male-typical displays. For *L. dalli*, jerks may inhibit sex change in others; it may also provide a behavioural landmark indicative of changes in the neuroendocrine system.

**Fig. 5** An integrative model to describe the temporal characteristics of sex change in *L. dalli*. The behaviour profiles in this model represent a 'typical' group with a male initially present and the largest female changes sex (MR-B groups). The horizontal lines beneath the behaviour profiles indicate the timing of changes in several non-behavioural parameters. Dotted portions of this line represent our predictions for the earliest change in a specific characteristic. Solid portions of the line represent established changes in a characteristic based upon data from this study or previous studies on this or other sex-changing fishes. See text for further details



After the initial peak in jerks, sex changers exhibited low rates of both jerks and displacements, while spending greater time in the nest (the quiescent phase, Fig. 5). Similar decreases in aggression occur in many organisms after the establishment of dominance (Caldwell 1987; Keeley and Grant 1993). For *L. dalli*, this quiescent phase may also allow for the requisite physiological, anatomical, and morphological rearrangements to become a male. After the quiescent phase, sex changers courted females with increased jerks and combinations of jerks with displacements, which led to spawning. The rubbing behaviour in the nest has been associated with the deposition of sperm-laden mucous trails in other gobies (Marconato et al. 1995) and may be an additional indicator of sex change. Finally, the new males cared for the eggs, a male-typical behaviour.

By the time sex changers first spawned as a male, AVT cells in the magnocellular preoptic area of the hypothalamus were identical in size to field-collected males and larger than in field-collected female *L. dalli*. We did not control for the effect of captivity; however, the similarity between field-collected males (who probably underwent female-to-male sex change in the field; St. Mary 1994) and lab sex changers suggest that these patterns are not an artefact of our treatments. Moreover, sex-specific changes in the size of AVT cells has been established in a related goby (Grober and Sunobe 1996). Recent studies in a sex-reversing wrasse suggest that AVT expression changes early in the sex change process in parallel with behavioural changes (J. Godwin, R.R. Warner, D. Crews, R. Sawby and M.S. Grober, unpublished data). More generally, changes in AVT have been associated with the production of reproductive behaviour in a wide range of vertebrates, while the mammalian ho-

mologue to AVT (vasopressin) is associated with differences in sexual phenotype in a variety of mammals (Moore 1992). Comparative data on the regulation of this and other neuroendocrine factors will greatly facilitate our understanding of the general mechanisms driving the evolution of sexual plasticity in vertebrates.

#### Limitations and comparison to the field

Our experimental design confounds the presence of a male in the social group with the opportunity of females to interact (MR groups). The presence of a male may influence which fish changes sex, the rate of sex change, or both (Shapiro 1979). However, prior to the removal of the male, MR sex changers did not receive unusual behavioural profiles relative to other fish, and MR-nb fish changed sex at similar rates to NM-B fish. Differences in rate of sex change between these treatments and MR-B fish are more parsimoniously explained by the differences in relative size and prior knowledge of the social hierarchy.

MR-nb and the lone NM-nb fish also present a design problem. Initially, we did not expect these fish to be sex changers. In a sense, these groups were self-selected, rather than assigned to their treatment. However, this statement is also true for the MR-B and NM-B females, although we expected larger fish to become dominant and therefore change sex. Fish that changed sex without a length advantage may have been in better condition or had greater mass than other fish in their group. We did not quantify condition nor did we weigh these fish.

We did not control for the reproductive condition of either sex changers or other females in the social group,

and both of these factors may influence the time to change sex. In a related study, we observed sex changers that were gravid on day 0 that followed the normal sequence of sex change through the early stages: they established dominance, exhibited early jerks, then lowered their activity level. However, they remained inactive for many days before courtship. Their extended inactive period suggests that gravidity may require more time for gonadal rearrangement, relative to a female that just spawned (St. Mary 1996). Other fish may have changed sex earlier than recorded by our measure of new eggs in the nest, because they lacked a spawning partner to lay the eggs. Sex changers had access to only three females, and females spawn approximately every three weeks (Behrens 1983). As a result, this measure may overestimate the time to change sex. Several other factors may have influenced the sex change process in our study. Sex changers may have interacted with other females of their group in the holding tank prior to the start of treatments. All groups received visual stimuli from neighbouring aquaria, and the flowing sea-water system at WIES exposed groups to olfactory cues from males and other sex changers. Although any of these factors may affect the rate of sex change, all groups were subject to these potentially confounding factors.

Sex ratios and sizes in this study differed from those found in the field. Our groups consisted of only one male, either the initial large male (1 male: 4 females) or the large female that changed sex (1:3). Based on genital papillae, sex ratios in the field are less skewed (e.g. 1:1.61, Wiley 1976; 1:1.10, St. Mary 1994) and the size range of the sexes overlap, although the largest fish tend to be males. The confines of the lab environment might limit sex change either because the male effectively inhibited the females, or the absolute number of females available was not sufficient to stimulate sex change in others (for further discussion see Ross 1990; Lutnesky 1994). However, most individuals in the field with male-typical genital papillae maintain both testicular and ovarian tissues (i.e. tailed sperm and vitellogenic eggs; St. Mary 1993); they behave as either males or non-reproductive individuals (St. Mary 1994). Thus, sex ratios from the field as determined by genital papillae may differ significantly from the ratio of females to functional males. For instance, the ratio of successful males to all other fish in St. Mary's field groups was at least 1:8, by the end of her 2-week trials.

St. Mary (1994) previously demonstrated sex change from female to male and from male to female within 2 weeks. While we confirmed both types of sex change, our males changed sex at much slower rates than did females, or males that changed sex in her study. However, we studied established nesting males and males known to have spawned as males, rather than males defined by their genital papillae alone. Additionally, St. Mary (1994) found that males known to receive eggs in the nest were the only class of males that lacked ovarian tissue. Together, these studies suggest once a fish functions (spawns) as a male its ability to change sex is limited,

and individuals with both gonadal tissue types function only as females. Thus, females may prepare some testicular tissue between clutches in the event of an opportunity to spawn as a male, but retain the ability to return to female function (with loss of testicular tissue) if no opportunity arises. Consistent with this hypothesis is an ongoing study focused on *L. dalli* recruitment that finds a male bias (i.e. genital papillae) in fish that immigrate to vacant urchins (T. Hartney, personal communication). We predict most of these 'males' are transitional, and have yet to function as a male.

#### An integrative model for sex change

Our final goal is to integrate the present study with existing data to produce a general model that establishes the temporal relationships among the various characters that are known to change during sex reversal (Fig. 5). This model provides testable hypotheses for future study. First, we suggest a sequence of changes in morphological and neuroendocrine characteristics. Because the later occurring changes are best understood, we will present the process in reverse order. Second, we correlate these changes to the behavioural stages of sex change in *L. dalli*.

The well-established role of steroid hormones in the elaboration of secondary sexual characteristics suggests that changes in genital papilla morphology follow the onset of allocation to testes and concomitant increases in serum androgen levels. High androgen levels often occur late in the sex-change process of other fishes (Godwin and Thomas 1993; Hourigan et al. 1991), and Egami (1959) found male-like genital papillae in gobies following 30 days of steroid treatment. Implants of 11-ketotestosterone in female *L. dalli* caused transformation to male-type papillae within 3–5 days of treatment (Hernandez et al., unpublished data). Male-typical genital papillae in *L. dalli* correlate with the presence of testicular tissue (St. Mary 1994) and the genital papilla changes just prior to courtship (M.P. Black, R.H. Reavis and M.S. Grober, unpublished data; Fig. 5). Increased androgen production may also play a key role in the completion of male gamete production (Nagahama 1994).

Culture experiments suggest that gonadotropin regulation directly mediates the production of male versus female steroid profiles (Morrey 1997) and several studies (e.g. Chan and Yeung 1983; Koulis and Kramer 1989) demonstrated that gonadal transformation can be induced by exogenous gonadotropin administration. Finally, control of pituitary gonadotropin production rests with hypothalamic neuropeptides (Peter et al. 1990).

We suggest that changes in hypothalamic peptides occur during the early phase of sex change, coincident with early changes in behaviour (Fig. 5). Godwin et al. (unpublished data) found a concomitant rise in AVT with the initial behavioural change in sex in the bluehead wrasse. Moreover, it appears that androgens do not drive changes in forebrain AVT cells in this wrasse (McIntyre

1998). We hypothesize that *L. dalli* AVT cells will change early during sex reversal. Additionally, Grober (1997) suggested that brain aromatase activity could fall in response to early social cues (e.g. the rapid drop in avoids after removal of the dominant male) which would dramatically increase the ratio of testosterone to oestradiol within specific brain regions. This process could explain the changes in gonadotropin-releasing hormone producing cells in the bluehead wrasse (Grober et al. 1991) which initiate the cascade of changes in the hypothalamic-pituitary-gonadal axis.

The behaviour profile of sex changing *L. dalli* suggests three critical periods that may correspond to important neuroendocrine transformations (Fig. 5). The immediate increase in displacements and absence of avoids may be the initial trigger for the onset of sex change (as suggested by Shapiro 1981). Subsequent neural changes (e.g. AVT, aromatase) may initiate the early peak in jerks, which is the first male-specific behaviour produced during sex change. These jerks may signal to others that a new 'male' has established dominance over the group. Next, the sex changer enters a behavioural quiescent phase. During this phase the fish must transform its gonads, steroid hormone profiles and genital papilla. We suggest that the completion of these processes coincides with the initiation of courtship jerks, and is followed by the first spawn as a male.

**Acknowledgements** We thank J. Miranda, I. Reavis, M. Volkening, R. Host, A. Watkins, M. Black, P. Newberg, and S. Koeppl for many hours of observation. R. Sawby and S. Faha provided assistance with brain neurochemistry and P. Duncan assisted with data entry. We thank T. Sunobe, P. Gowaty, R. Warner and three anonymous reviewers and the Grober lab reading group from the fall of 1997 for helpful comments on previous versions of this manuscript. We thank Pete Pehl and the staff of the Wrigley Institute for Environmental Studies. This work was supported by NSF (IBN - 9309555 and 9723817 to M.G.) and Arizona State University (Seed Grants to both M.G. and R.R.).

## References

- Barlow GW, Rogers W, Fraley N (1986) Do midas cichlids win through prowess or daring? It depends. *Behav Ecol Sociobiol* 19: 1-18
- Behrens KC (1983) The comparative ecology and interactions between two sympatric gobies (*Lythypnus dalli* and *Lythypnus zebra*). Ph.D., University of Southern California, Los Angeles
- Braford MRJ, Northcutt RG (1983) Organization of the diencephalon and preteum of the ray-finned fishes. In: Davis RE, Northcutt RG (eds) *Fish neurobiology*. University of Michigan Press, Ann Arbor, pp 117-163
- Caldwell RL (1987) Assessment strategies in stomatopods. *Bull Marine Sci* 41: 135-150
- Chan STH, Yeung WSB (1983) Sex control and sex reversal in fish under natural conditions. In: Randall WSH, Randall DJ (eds) *Fish physiology*. Academic Press, San Diego, pp 171-222
- Cole KS (1983) Protogynous hermaphroditism in a temperate zone territorial marine goby, *Coryphopterus nichosi*. *Copeia* 3: 809-812
- Egami N (1959) Effect of testosterone on the sexual characteristics of the gobiid fish, *Pterogobius zonoleucus*. *Ann Zool Jpn* 32: 123-128
- Fischer EA, Petersen CW (1987) The evolution of sexual patterns in the seabass. *BioScience* 37: 482-489
- Godwin JR, Thomas P (1993) Sex change and steroid profiles in the protandrous anemonefish *Amphiprion melanopus* (Pomacentridae, Teleostei). *Gen Comp Endocrinol* 91: 144-157
- Godwin J, Warner RR, Crews D (1996) Behavioral sex change in the absence of gonads in a coral reef fish. *Proc R Soc Ser B* 263: 1683-1688
- Grober MS (1997) Neuroendocrine foundations of diverse sexual phenotypes in fish. In: Ellis L, Ebertz L (eds) *Sexual orientation: toward biological understanding*. Praeger, Westport, pp 3-20
- Grober MS, Bass AH (1991) Neuronal correlates of sex/role change in labrid fishes: LHRH-like immunoreactivity. *Brain Behav Evol* 38: 302-312
- Grober MS, Sunobe T (1996) Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry. *Neuroreport* 7: 2945-2949
- Grober MS, Jackson IMD, Bass AH (1991) Gonadal steroids affect LHRH preoptic cell number in a sex/role changing fish. *J Neurobiol* 22: 734-741
- Hourigan TF, Nakamura M, Nagahama Y, Yamauchi K, Grau EG (1991) Histology, ultrastructure, and in vitro steroidogenesis of the testes of two male phenotypes of the protogynous fish, *Thalassoma duperrey* (Labridae). *Gen Comp Endocrinol* 83: 193-217
- Keeley ER, Grant JWA (1993) Visual information, resource value, and sequential assessment in convict cichlid (*Cichlasoma nigrofasciatum*) contests. *Behav Ecol* 4: 345-349
- Koulish S, Kramer CR (1989) Human chorionic gonadotropin (hCG) induces gonad reversal in a protogynous fish, the bluehead wrasse, *Thalassoma bifasciatum* (Teleostei, Labridae). *J Exp Zool* 252, 156-168
- Lutnesky M (1994) Density-dependent protogynous sex change in territorial-harem fishes: models and evidence. *Behav Ecol* 5: 375-383
- Lutnesky M (1996) Size-dependent rate of protogynous sex change in the pomacanthid angelfish, *Centropyge potteri*. *Copeia* 1996: 209-212
- Marconato A, Rasotto MB, Mazzoldi C (1995) On the mechanism of sperm release in three gobiid fish (Teleostei: Gobiidae). *Environ Biol Fishes* 46: 1-7
- McIntyre KK (1998) Arginine vasotocin in the preoptic area of the bluehead wrasse and the effects of 11-ketotestosterone. M.S. thesis, Arizona State University, Tempe
- Miller DJ, Lea RN (1972) Guide to the coastal marine fishes of California. California Department of Fish and Game, Fish Bulletin 157
- Moore FL (1992) Evolutionary precedents for behavioral actions of oxytocin and vasopressin. *Ann N Y Acad Sci* 652: 156-165
- Morrey CE (1997) Ecological factors and potential endocrine mechanisms regulating sex change in *Thalassoma duperrey*. Ph.D. thesis, University of Hawaii, Manoa
- Nagahama Y (1994) Endocrine regulation of gametogenesis in fish. *Int J Dev Biol* 38: 217-229
- Nakamura M, Yamauchi K, Nagahama Y, Grau EG (1984) Endocrine-regulated morphological changes in the gonad during sex reversal in protogynous wrasse. *Zool Sci* 1: 967
- Peter RE, Yu KL, Marchant TA, Rosenblum PM (1990) Direct neural regulation of the teleost adenohypophysis. *J Exp Zool [Suppl]* 4: 84-89
- Reinboth R (1985) Ambosexuality in teleosts-a challenge to endocrinologists. In: Loft B, Holmes WN (eds) *Current trends in comparative endocrinology* University of Hong Kong Press, Hong Kong, pp 579-581
- Robertson DR (1972) Social control of sex reversal in a coral-reef fish. *Science* 177: 1007-1009
- Ross RM (1990) The evolution of sex-change mechanisms in fishes. *Environ Biol Fishes* 29: 81-93
- Shapiro DY (1979) Social behavior, group structure and the control of sex reversal in hermaphrodite fish. *Adv Study Behav* 10: 43-102
- Shapiro DY (1981) Size, maturation, and the social control of sex reversal in the coral reef fish *Anthias squamipinnis*. *J Zool* 193: 105-128

- Shapiro DY (1989) Sex change as an alternative life-history style. In: Bruton MN (ed) *Alternative life-history styles of animals*. Kluwer, Dordrecht, pp 177–195
- St. Mary CM (1993) Novel sexual patterns in two simultaneously hermaphroditic gobies, *Lythrypnus dalli* and *Lythrypnus zebra*. *Copeia* 4: 1062–1072
- St. Mary CM (1994) Sex allocation in a simultaneous hermaphrodite, the blue-banded goby (*Lythrypnus dalli*): the effects of body size and behavior gender and the consequences for reproduction. *Behav Ecol* 5: 304–313
- St. Mary CM (1996) Sex allocation in a simultaneous hermaphrodite, the zebra goby *Lythrypnus zebra*: insights gained through a comparison with its sympatric congener, *Lythrypnus dalli*. *Environ Biol Fishes* 45: 177–190
- St. Mary CM (1997) Sequential patterns of sex allocation in simultaneous hermaphrodites: do we need models that specifically incorporate this complexity? *Am Nat* 150: 73–97
- Sunobe T, Nakazono A (1993) Sex change in both directions by alteration of social dominance in *Trimma okinawae* (Pisces: Gobiidae). *Ethology* 94: 339–345
- Warner RR (1984) Mating behavior and hermaphroditism in coral reef fishes. *Am Sci* 72: 128–136
- Warner RR, Swearer SE (1991) Social control of sex change in the bluehead wrasse, *Thalassoma bifasciatum* (Pisces: Labridae). *Biol Bull* 181: 199–204
- Warner RR, Robertson DR, Leigh EG (1975) Sex change and sexual selection. *Science* 190: 633–638
- Wiley JW (1976) Life histories and systematics of the Western North American Gobies *Lythrypnus dalli* (Gilbert) and *Lythrypnus zebra* (Gilbert). *San Diego Soc Nat Hist Trans* 18: 169–184

Communicated by R.F. Oliveira