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Behavioural sex change in the absence of gonads in a coral reef fish

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SUMMARY

It is an axiom of vertebrate behavioural endocrinology that full expression of a male behavioural phenotype depends on testicular influences during development, in adulthood, or both. Sex change in fishes challenges this necessity: behavioural changes are often rapid and greatly precede gonadal changes. However, steroid hormones can have fast actions on the nervous system, so gonadal influences on behavioural sex change cannot be excluded based solely on the speed of these changes. We report that surgical gonad removal does not prevent or discernibly alter female-to-male behavioural sex change in a protogynous coral reef fish. Male behaviour assumption is instead purely dependent on attaining social dominance. This is the first example of a vertebrate fully expressing a male behavioural phenotype without current or previous exposure to a functioning testis or testicular products.

1. INTRODUCTION

While a diversity of mechanisms control mating behaviour in vertebrates (Crews & Moore 1986; Crews 1993), there are no descriptions of a vertebrate fully expressing a male behavioural phenotype without exposure to a testis or its products at some point in life. The only exception to this generality may be socially-controlled sex change in coral reef fishes. Behavioural changes during sex change are rapid and can occur within minutes (Robertson 1972; Warner & Swearer 1991, Godwin 1994), calling into question any causal role of slower gonadal and associated gonadal hormone changes in producing these behavioural changes. However, as fast, non-genomic actions of steroid hormones on neural tissue are well known (Nakebura *et al.* 1986; Schumacher *et al.* 1990; Orchinik *et al.* 1991) and because steroidogenic cells in the gonads of many vertebrates are directly innervated and their activity could therefore be rapidly modulated (Gresik 1973), gonadal influences on behavioural changes during natural sex change cannot be excluded. Here, we report that gonad removal does not block the rapid assumption of male-typical territorial defence, courtship, and spawning behaviours that normally accompanies the attainment of dominance status and natural female-to-male sex change in the bluehead wrasse, *Thalassoma bifasciatum*.

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2. METHODS

(a) Study species

The bluehead wrasse exhibits female-to-male sex change and has two alternative male mating morphs (a diandrous protogynous hermaphrodite, Warner *et al.* 1975). Females and one adult male morph are light yellow with a dark dorsal stripe (initial phase or IP colouration) and are not territorial or aggressive. Terminal phase males (TP males) are brightly coloured with blue heads, green over most of the body, and a vertical white bar bordered by black bars just posterior to pectoral fin insertion. TP males exhibit territorial aggression in defence of spawning sites, as well as distinctive courtship behaviours. TP colours show temporary colour changes (within seconds) during the spawning period including a fading of the blue and green colours ('opalescent') correlated with courtship and a deepening of these hues correlated with aggression (Dawkins & Guilford 1993). During an afternoon spawning period, females travel to spawning sites and either: (i) are courted by and pair spawn with a TP male at a defended site; or (ii) mate with a group of IP males at undefended group-spawn sites. Mating consists of a rapid ascent approximately 1 m into the water column in a 'spawning rush'; eggs are released at the apex of this rush, and the fish quickly return to the bottom. Females spawn approximately two out of every 3 d, whereas successful TP males spawn 30–50 times per day on average (Warner 1984).

Both females and IP males can undergo role and colour change to become TP males (this also entails sex change for females). Sex change is primarily under social control and can be induced experimentally by removal of dominant terminal phase (TP) males from small reef populations (Warner & Swearer 1991). Complete gonadal sex change takes approximately 7–10 d.

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Table 1. *Assumption of behaviours characteristic of terminal phase males by ovariectomized female bluehead wrasses*

behavioural sampling	number of individuals	full courtship and temporary colour changes	male-role spawning initiated on 1st day	male-role spawning initiated on 2nd day
observed from first day of dominance	13	13	11	2
first observed 2 d after becoming dominant	2	2	unknown	2
not observed in dominant status	1	0	0	0
totals	16	15	11/13	2/2

(b) Study location

The experimental reefs used were numbers 1, 4, 9a, 9b, 10, 12, 16b, 18 in Tague Bay (described in Gladfelter & Gladfelter 1987), and one small reef in Boiler Bay, St. Croix, U.S. Virgin Islands (17°46′ N, 64°36′ W). These small patch reefs supported total bluehead wrasse populations of approximately 50–200 individuals of which two to nine were TP or intermediate stage males.

(c) Experimental procedures

To address experimentally the necessity of the gonads for behavioural sex change, we surgically ovariectomized or did sham operations ('shams') on the largest resident females from small patch reefs and then made them socially dominant through removal of TP males (Warner & Swearer 1991). All large adult bluehead wrasses (more than 45 mm standard length) were captured from a reef over a 30–45 min period during the morning hours by lift netting. TP males were immediately released back on the reef. IP individuals were held in floating enclosures until the early afternoon. Holding the fish until early afternoon allows egg hydration and ovulation, which facilitates sexing (confirmation by gamete extrusion) and reliably indicates non-sex changing, normal female reproductive function. After sexing and egg stripping, the largest two to seven females from a reef were surgically ovariectomized or sham-operated as follows: after being deeply anaesthetized in an aerated seawater solution of 85 mg l⁻¹ MS-222 for 6–8 min, an approximate 1 cm mid-ventral incision was made midway between the pelvic fin insertions and cloaca, the ovaries were removed, and the incision was closed with 6–0 surgical silk. The gills were periodically irrigated with fresh seawater during surgery. Recovery from surgery was rapid and females were returned to their home reefs on the day of capture between 17h00 and 18h30 or, for one reef, at 09h00 the next morning. IP males from experimental reefs were released at a distant site to prevent them from contesting dominance status with experimental females later in the experiments. Following 2 d recovery on the reef for experimental females, TP males were removed after the spawning period to create social conditions which induce sex change (Warner & Swearer 1991).

An additional longer term ovariectomy treatment consisted of six ovariectomized females released on their home reefs (n = 2 reefs) and observed in the presence of resident TP males during spawning periods at 2, 5 and 7 d after surgery. This treatment tested whether ovariectomized females would show TP male behaviour from 3 to 7 d post surgery in the presence of resident TP males and served as a control for the possibility that ovariectomy and not TP male removal stimulated behavioural change. After 7 d, TP males were removed to determine whether the increased time as ovariectomy would block the initiation of male behaviour.

We examined excised ovaries histologically to confirm the presence of oocytes in various developmental stages and a lack of sex change indicators (oocyte atresia, spermatogenesis). Experimental females for which data are presented were 56–67 mm in standard length (ovariectomized: 60.58 mm ± 0.54 (n = 17), shams: 62.55 mm ± 0.58 (n = 8); mean ± s.e.).

Experiments on these same reefs in 1993 and 1994 demonstrated that neither sham operations (n = 4) or severing the blood and nervous connection at the rostral apex of the ovarian lobe blocks gonadal sex change in the intact or rostrally denervated lobes (unilateral denervation: n = 7, bilateral denervation: n = 2). Regardless of treatment, both degeneration of ovarian tissue and proliferation of spermatogenic tissue were evident at 7–14 d after an individual had been made socially dominant. We did not examine females in subordinate social status with bilateral rostral denervation to determine whether this would stimulate gonadal change, but no indications of gonadal change were seen in the manipulated or intact lobes of one female who remained subordinate, but had the rostral vascular and neural connections to one gonad severed.

(d) Marking and behavioural observations

Experimental fish were individually marked with unique patterns of Alcian blue dye spots injected subcutaneously. Behavioural observations consisted of 10-minute focal individual samples during the spawning period and were recorded on slates. Observations focused on the sexual and aggressive behaviours described in figure 1.

(e) Statistical analysis

All statistical analyses were performed using Systat 5.2.1 for the Apple MacIntosh (Systat, Inc., Evanston, Illinois). Some behavioural samples in experimental female groups were repeated measures (various time points on individuals). This prevented a simultaneous comparison of all the female groups and TP males. We instead did various within-individual and between-group tests to examine behavioural differences and changes (described in figure 1 legend). Behavioural frequency data were square-root transformed $(X' = (X+0.5)^{1/2})$ for parametric analyses by ANOVA and Tukey HSD post hoc tests. Tests were two-tailed unless otherwise noted.

3. RESULTS AND DISCUSSION

Gonad removal in ovariectomized fish did not block rapid behavioural sex change following removal of resident TP males (see table 1). We observed 15 of 16 ovariectomized fish on six separate reefs occupy spawning sites, exhibit all the courtship and spawning behaviours typical of TP males, and display temporary
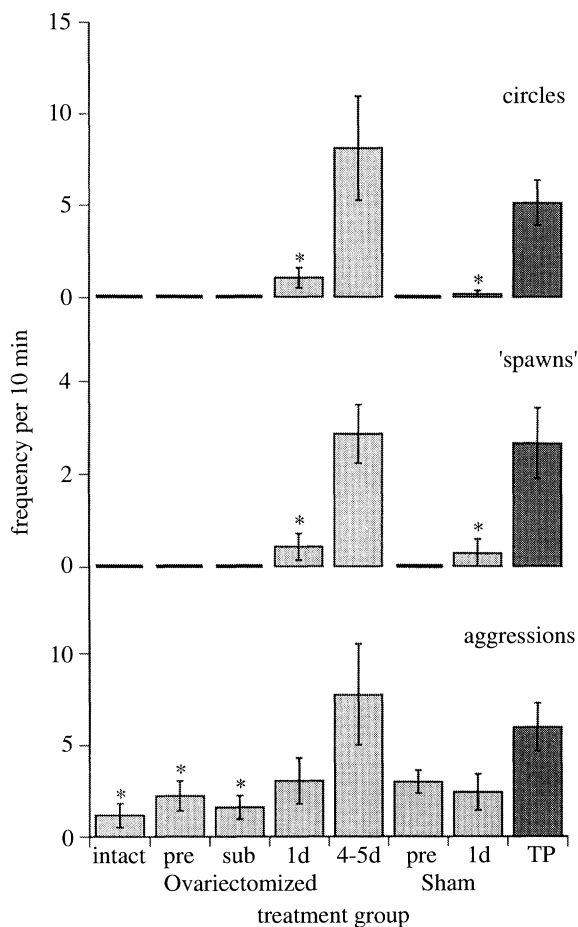
Figure 1. Behavioural frequencies per 10-minute focal individual observation during the spawning period (mean ± s.e.). Asterisks indicate means significantly different from those for TP males. Bars at base indicate group behavioural frequencies of zero (behaviours not exhibited). 'Circles', the most common male courtship behaviour, are performed above the spawning site and involve tight circular swimming coupled with rapid fluttering of the pectoral fins. 'Spawns' are upward spawning rushes in which the focal individual participates in the male spawning role. 'Aggressions' are fast, directed swimming movements at conspecifics. Groups include: intact, unmanipulated females ($n = 6$ individuals); subordinate ovariectomized fish in the presence of either TP males ('pre', $n = 16$) or dominant ovariectomized fish ('sub', $n = 5$); dominant ovariectomized fish at 1 d ($n = 14$) and 4–5 d ($n = 5$) after becoming dominant; sham fish before ($n = 6$) and at 1 d ($n = 5$) after becoming dominant; and successful established TP males on small reefs ($n = 6$). Individual values were calculated as the average of two 10-minute focal-individual observations per spawning period per individual for all groups except intact females (one focal per individual).

Statistical comparisons: groups enclosed by square brackets are not significantly different. Alpha level is 0.05 unless otherwise noted.

Within-individual ovariectomized ('ovx') comparisons: circles – 1-day > pre: $p < 0.005$, $n = 14$, signed rank test; complete ovx only, $p < 0.05$, $n = 6$; 4–5 d > 1 d: paired-$t = 2.367$, d.f. = 4; 'Spawns'- 4–5 d > 1 d: paired-$t = 2.982$, d.f. = 4; aggressions – 4–5 d > (pre, 1 d): repeated measures ANOVA, $F_{2,8} = 5.045$, $n = 5$, one-tailed contrasts.

Treatment group comparisons: circles – TPs > (1 d ovx, 1 d sham): $F_{2,22} = 13.527$, $p < 0.001$, multiple comparisons –$p < 0.002$; TPs = 4–5 d ovx: $t = 0.878$, d.f. = 9, $p = 0.403$; 'spawns' – TPs > (1 d ovx, 1 d sham): $F_{2,22} = 10.062$,

$p < 0.002$, multiple comparisons –$p < 0.005$; TPs = 4–5 d ovx: $t = 0.261$, d.f. = 9, $p = 0.800$; aggressions – TPs > (intact females, pre-ovx), TPs = pre-shams: $F_{3,29} = 3.732$, $p < 0.05$, significant multiple comparisons –$p < 0.05$; TPs > sub-ovx: $t = 2.871$, d.f. = 9; TPs = (1 d ovx, 1 d sham): $F_{2,22} = 1.296$, $p = 0.294$; TPs = 4–5 d ovx: $p = 0.544$, d.f. = 9, $p = 0.600$.

spawning colouration within 2 d of becoming socially dominant (behaviours defined in figure 1). Temporary spawning colouration consists of a bluish colouring of the head, darkening of the body coupled with the appearance of several white vertical bars, and darkening of the pectoral fin tips. The temporary colour change mechanism may be the same as that in TP males. Most of these ovariectomized fish showed all elements of male courtship and 'spawned' in the male role by the end of the first day they were observed in dominant status. The smallest ovariectomized fish in these experiments (56 mm SL) was apparently prevented from becoming socially dominant by immigration of slightly larger individuals from a large nearby reef (usually visible from the experimental reef).

The display of TP-male behaviour was dependent solely on attaining social dominance. Dominance in this species is dependent on relative body size. Gonad removal alone did not produce behavioural changes: no courtship or male spawning behaviours were shown by ovariectomized fish in the presence of either TP males or of more dominant ovariectomized fish following removal of TP males (see figure 1). Sequential removals of dominant ovariectomized fish clearly demonstrated the critical role of social dominance in behavioural change (see figure 2). The small experimental reefs used typically had only one or two successful spawning sites where behaviours typical of TP males could be exhibited in a given spawning period. Sequential removals of dominant ovariectomized fish exhibiting male behaviour resulted in immediate assumptions of dominance status and the associated display of male behaviours by other smaller ovariectomized fish on these reefs.

The male-typical spawning rushes by both ovariectomized and sham fish appeared identical to those observed in TP males and intact sex-changing individuals. Moreover, the intact female blueheads who participated in these spawning rushes apparently also regarded the behaviour of dominant ovariectomized females as 'male' because their behaviour was indistinguishable from that of females in spawning rushes with natural TP males. Most convincingly, these females released their eggs. Egg release was indicated by: (i) the consistent loss of the prominent abdominal bulge in females (created by ovulated eggs) following these spawns; (ii) visible gamete clouds; and (iii) confirmed by capture and inspection of large numbers of unfertilized eggs (ten male-role 'spawns' captured from ten females spawning with five different ovariectomized fish, 25–224 eggs inspected per spawn, none were fertilized, methods in Shapiro *et al.* 1994).

To confirm whether ovariectomies were complete, we recaptured 12 of the ovariectomized fish, either
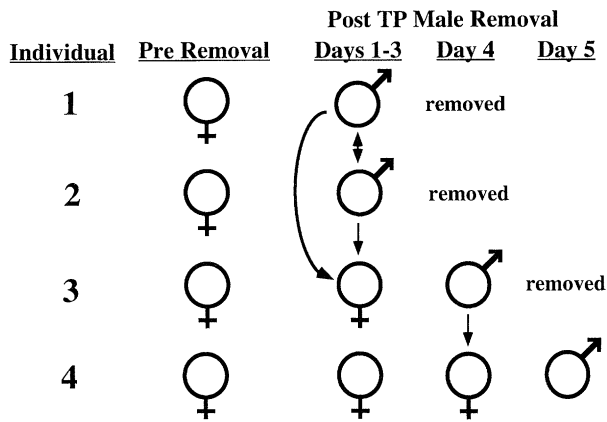
Figure 2. Behavioural sex changes on reef 21. Female symbols indicate no TP male-typical behaviour shown by that individual on the observation day. Male symbols indicate full male courtship and 'spawning' in the male role. Arrows indicate initiators and recipients of aggression. Dominant individuals were removed during the afternoon following the spawning period. We also observed this pattern of sequential dominance assumption by ovariectomized females on two other reefs.

immediately after spawning in the male role ($n = 6$) or a number of days later (1–9 d, $n = 6$). A total of six showed no trace of gonadal tissue, while six others had small remnant gonadal fragments consisting of regressed ovarian tissue, but no discernible spermatogenic tissue. A total of four of these fish had remnant fragments weighing less than 1 mg (wet weight) while two others had fragments weighing 2.5 and 13 mg respectively. The intact ovary wet weight from females of the size used here is approximately 190 mg. Because the timing and nature of male behaviour initiation did not differ between successfully ovariectomized females and those with small remnant gonadal fragments, these groups are considered together below in the discussion of behavioural frequencies.

Frequencies of courtship and 'spawning' behaviour typical of TP males did not differ significantly between ovariectomized females and shams on the day they assumed social dominance, but these levels were lower than in established TP males (table 2, figure 1). By 4–5 d after becoming dominant, ovariectomized fish showed courtship and male-role 'spawn' frequencies that were significantly greater than frequencies observed on the first day and not different from those of established TP males. Aggressive behaviour levels in ovariectomized females on the first day of social dominance were not significantly higher than in ovariectomized females in subordinate status. However, by 4–5 d after assuming dominance, aggressive acts were more frequent than on the first day of social dominance. Importantly, changes in aggression were caused by territorial behaviour in a sexual context (defence of spawning sites) rather than the less common aggression over food items shown by non-changing females. Feeding and territorial aggression were not recorded separately during behavioural observations, but non-changing females showed no territorial aggression and feeding-related aggression by fish undergoing behavioural change was exceedingly rare during

spawning periods. Observations at 4–5 d post-TP male removal were not obtained for the sham fish as large individuals immigrated onto one reef and displaced the sham fish as dominants and a dominant sham fish disappeared from another reef after being observed in dominant status 1 d after TP male removal.

Gonad removal does appear to block permanent colour changes. The teleost androgen 11-ketotestosterone (11-KT) has been implicated in producing male secondary morphological characters in a variety of fishes (reviews in Liley & Stacey 1983; Brantley *et al.* 1993). In a close relative of *T. bifasciatum*, the saddleback wrasse *Thalassoma duperrey*, both plasma levels and *in vitro* gonadal production of 11-KT are higher in TP males than in females or IP males (Nakamura *et al.* 1989; Hourigan *et al.* 1991). Similar differences in plasma 11-KT levels are seen between sexual phases in the stoplight parrotfish *Sparisoma viride* (Cardwell & Liley 1991). Intact, dominant sex-changing females on these same reefs show both testicular development and the beginnings of permanent colour change within 5 d of TP male removal (Warner & Swearer 1991). In contrast, four ovariectomized females left as dominants on different reefs maintained courtship and spawning behaviour characteristic of TP males, but did not show any permanent colour changes over 9–25 d. Dominant ovariectomized females were last observed at 9, 15, 22, and 25 d. On two reefs, we removed smaller intact individuals showing the beginnings of permanent colour change at 7–8 d after TP male removal, and found that two more smaller intact females had initiated permanent colour change by 15 d (7–8 d later). A total of three sham-operated fish on another reef did show permanent colour changes by 7–10 d after TP male removal. The lack of colour change in ovariectomized females therefore indicates that an important source of steroid hormones for at least one component of sexual phenotype change has been removed.

Neither the gonads or, by implication, changes in gonadal activity are necessary for behavioural sex change while it is occurring. We cannot say that the gonads play no role in natural behavioural sex change, but the similar behavioural profiles of ovariectomized females and shams and the similar responses of their female spawning partners argue against a significant current gonadal role. However, we have not refuted two other possibilities for steroid hormones influencing male-typical behavioural development. First, steroid hormones from non-gonadal sources could stimulate behavioural change. Social status and interactions influence corticosteroid levels in a variety of species (Greenberg *et al.* 1984; Sapolsky 1993; Knapp & Moore 1995) and corticosteroids can act at neuronal membranes to affect sexual behaviour, although the known actions are inhibitory (Orchinik *et al.* 1991; Rose *et al.* 1993). The main source of corticosteroids in teleosts, the interrenal gland, can also produce androgens (Schreck *et al.* 1989). The lack of colour changes in ovariectomized females argues against significant interrenal androgen production, but the levels necessary to induce behavioural and morphological changes could differ. Alternatively, significant

interrenal testosterone production, but not 11-KT production, could cause behavioural change without colour change. The second possible role for steroid hormones is that the neural substrates which subserve male-typical behaviour develop during the female phase under the influence of ovarian androgens, but the display of this behaviour is socially inhibited. There are two observations that argue against this possibility. Terminal phase males have greater numbers of gonadotropin-releasing hormone cells than either females or IP males in the preoptic area of the hypothalamus, a critical integrative area for male sexual behaviour (Grober & Bass 1991; Grober *et al.* 1991). More recently, we found that *in situ* hybridization with a probe directed at arginine vasotocin messenger RNA indicates higher levels of this hormone in the preoptic area of TP males than females, and that levels rapidly increase during sex change (J. Godwin, R. Sawby, R. R. Warner, D. Crews & M. S. Grober, unpublished data). Another fish, the goby *Trimma okinawae*, that exhibits serial female-to-male and male-to-female sex changes also shows changes in preoptic area AVT cells that occur concurrently with sex change (Grober & Sunobe 1996). Arginine vasotocin influences sexual behaviour in a variety of vertebrates (Moore 1992). Whether or not these intra- and intersexual dimorphisms underlie the differing displays of behaviour typical of TP males, they do suggest that the dominant male neural phenotype develops when dominant status is assumed rather than before. The hypothesis of male behavioural organization by recent ovarian androgen exposure would be supported if behaviour typical of TP males could develop, but not persist in the absence of gonads. Our observations only lasted from 9 to 25 d after females assumed dominance and we do not have detailed behavioural observations for these later periods, but there was no obvious decrease in the display of behaviour typical of TP males by these females. These fish were all showing strong male courtship, spawning, and aggressive defence of spawning sites when last observed.

The full development and expression of dominant male behaviour in the absence of a gonad is a departure from known patterns of behavioural control in vertebrates. While male behaviour in the absence of a testis is known from other sexually reproducing vertebrates (Liley & Stacey 1983; Moore & Kranz 1983; Crews 1991), cases where development of this behaviour has been studied show a critical role for earlier testicular influences. In contrast, female bluehead wrasses can rapidly develop a male behavioural phenotype without current or previous exposure to a testis. We interpret this apparent independence of behavioural change from at least current gonadal influences as a response to the need for rapid assertion of behavioural dominance in a mating system characterized by strong mate competition.

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APPENDIX

Definitions of statistical abbreviations

$F_{2,22}$: ANOVA F values with subscript numbers indicating numerator and denominator degrees of freedom respectively

p: probability value

t: t-value from Student's t-distribution

d.f.: degrees of freedom in statistical test