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REPORT

Growth acceleration, behaviour and otolith check marks associated with sex change in the wrasse *Halichoeres miniatus*

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Abstract In protogynous sex-changing fishes, females are expected to compete for the opportunity to change sex following the loss of a dominant male and may exhibit growth and behavioural traits that help them maintain their dominant status after sex change. A male removal experiment was used to examine changes in female growth and behaviour associated with sex change in the haremic wrasse *Halichoeres miniatus* and to test whether any changes in growth associated with sex change were recorded in otolith microstructure. Dominant females began displaying male-characteristic behaviour almost immediately after the harem male was removed. The frequency of interactions between females increased following male removal. In contrast, feeding frequency of females decreased. The largest one to three females in each social group changed sex following male removal and exhibited an increase in growth associated with sex change. Sex changers grew more than twice as fast as non-sex changers during the experimental period. This growth acceleration may enable new sex-changed males to rapidly reach a size where they can defend the remaining harem from other males. An optical discontinuity (check mark) was present in the otoliths of sex-changed fish, and otolith accretion rate increased significantly after the check mark, corresponding with the increased growth rate of sex-changing females. Wild caught males, but not females, exhibited an analogous check mark in their otoliths and similar increases in otolith increment widths after the check. This indicates that an increase in growth rate is a regular feature of sex-change dynamics of *H. miniatus*.

Keywords Coral reef fish · Sex change · Growth rate · Otolith · Check mark

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Introduction

Polygynous mating systems, where a small number of males monopolise breeding opportunities with many females, favour the evolution of large male size because large males have a competitive advantage over smaller males when competing for breeding partners (Emlen and Oring 1977; Keller and Reeve 1994; Shuster and Wade 2003). Polygynous mating systems also favour the evolution of protogynous (female to male) sex change, because the reproductive success of males in these mating systems tends to increase more steeply with size or age than does female reproductive success (size-advantage model of sex change, Ghiselin 1969; Warner 1975). Small males have very low reproductive success because they are excluded from breeding by larger males, whereas large males can have very high reproductive success if they become a harem master or breeding-territory holder (Warner 1984). Female reproductive success tends to increase more evenly with size; therefore, an individual will benefit by first reproducing as a female, and then changing sex to male when large enough to defend a group of females or a breeding site that females visit (Warner 1975; Charnov 1982).

Protogynous hermaphroditism among reef fishes is usually associated with a polygynous mating system (Warner 1984; Munday et al. 2006). Having a large body

size has two potential advantages for males in this mating and reproductive system. First, it enables them to defend a harem or breeding site from other males (Robertson 1981; Hoffman et al. 1985; Dugatkin and FitzGerald 1997; Kuwamura et al. 2000). Second, it might help them to maintain dominance over females and prevent them from gaining a size where they could change sex and challenge the male for his position as harem master or territory holder (Robertson 1972; Shapiro 1981; Sakai 1997; Sakai and Kohda 1997). Sex change typically occurs following the loss of a dominant male, and it is usually the largest female in a social group that changes sex to male (e.g. Fishelson 1970; Robertson 1972; Shapiro 1981; Hoffman 1983; Ross 1990; Warner and Swearer 1991; Walker and McCormick 2004; Black et al. 2005; Lorenzi et al. 2006). Any mechanism that enables a female to gain dominance over other females should increase her likelihood of sex change if an opportunity arises, and the likelihood she will be able to maintain her new position as the dominant male after sex change.

In species where a strict social hierarchy is enforced, such as strongly haremic species, female growth can be limited by interactions with the harem male and other females (Forrester 1990; Koebele 1985; Sakai and Kohda 1997; Wong et al. 2007). Agonistic interactions from dominant individuals can reduce subordinate growth by reducing the time spent foraging, increasing energy expenditure by avoiding aggressive interactions, or increasing physiological stress (Koebele 1985). In protogynous species, social control of subordinate growth helps maintain a dominance hierarchy where the largest female changes sex following the loss of the male (Robertson 1972; Kuwamura 1984; Sakai et al. 2002). Females in the process of sex change might therefore be expected to increase their growth rate at the time of sex change (Ross 1987; Garratt et al. 1993; Walker and McCormick 2004; Walker et al. 2007), because their growth is no longer limited by interactions with the male and because a rapid increase in size would help ensure that the dominant position is maintained after sex change.

In addition to physiological changes associated with sex reversal, there are also behavioural changes that occur. Male behaviour has been observed in females just a few minutes or hours after the removal of the dominant male (Reavis and Grober 1999; Nakashima et al. 2000; Sakai et al. 2003). This rapid transition of behaviour is most apparent in haremic species such as *Labroides dimidiatus* and *Halichoeres melanurus* (Nakashima et al. 2000; Sakai et al. 2002). Typically, the dominant female will expand her territory to encompass the territories of other females in the group and begin exhibiting male courtship behaviour before there is any evidence of sex change in the gonads (Sakai et al. 2003). Presumably, the dominant female assumes the male role almost immediately to prevent bachelor or territorial males outside the group from usurping the position, and so that she can assert dominance over other females within the group who might otherwise change sex and become the dominant male.

Most of what is known about the mechanisms controlling sex change in fishes, and the consequences of removing large individuals from the population, comes from a relatively small number of well-studied species. Many exploited species of reef fish exhibit protogynous sex change as a normal part of their life histories (Sadovy de Mitcheson and Liu 2008), but for most of these species, how they respond to removal of the largest individuals from their populations is not fully understood. Stock assessments for hermaphroditic species commonly fail to incorporate sex change, or simply assume that removing large males causes a simple one-to-one replacement of males by sex-changing females (reviewed by Alonzo et al. 2008). However, there is increasing evidence that the patterns and dynamics of sex change in reef fishes can be far more complex than commonly assumed (Munday et al. 2006), and that this complexity can have important implications for the reliability of stock assessments (Alonzo et al. 2008). Increasing the number of species for which the proximal mechanisms of sex change and consequences of removing large dominants are known will help determine if simple assumptions about sex change mechanisms are adequate for most management decisions, or if more complex models should be considered.

Otoliths are often used as a proxy record for the growth history and life history transitions of fishes (Thorrold and Hare 2002) and potentially provide a useful tool for comparing growth patterns in sex-changing fishes without the need for regular monitoring of a large number of individuals in the wild (Munday et al. 2004; Walker and McCormick 2004). The width of increments deposited periodically on the otoliths are frequently correlated with somatic growth (Campana 2001; Thorrold and Hare 2002), providing a record of the growth history of individual fish. Furthermore, some life history transitions are recorded as an optical discontinuity, or 'check mark', in the otolith. Several recent studies have identified a change in otolith structure associated with sex change in protogynous fishes (Walker and McCormick 2004, 2009; Walker et al. 2007). Such check marks could provide an important individual record of the time at sex change, thus allowing the complete reconstruction of hermaphroditic life histories without the need for laborious field surveys.

In this study, a male removal experiment was used to examine changes in female growth and behaviour associated with sex change in the circle-cheeked wrasse, *Halichoeres miniatus*. Changes in growth rate associated with sex change were tested by comparing the growth of

females that changed sex with females that did not change sex. Territory size and behavioural interactions of females were also monitored in one focal social group, before, during and after the removal of the dominant male to examine how quickly behaviour changed following the loss of the dominant male. Otoliths of sex-changed females and non sex-changed females were examined to determine whether daily otolith increments record changes in somatic growth are associated with sex change and to experimentally validate that sex change leaves a well-defined check mark in the otoliths of *H. miniatus*. Otoliths of wild caught fish were then examined for the presence of a sex-change check mark to compare the growth rates of individuals before and after sex change in the natural population.

Methods

Study site and species

This study was conducted at Lizard Island on the northern Great Barrier Reef (GBR, 14°40′S 145°28′E), Australia, between September and November 2005. The study species, *Halichoeres miniatus*, is a small, short-lived wrasse that inhabits the shallow macroalgal zone of reef flats throughout the Western Pacific (Randall et al. 1997). The species is sexually dimorphic, with males being larger and more brightly coloured than females. Dull-coloured females (initial phase (IP)) change sex to become brightly coloured males (terminal phase (TP)). No direct developing (primary) males have been detected in the population of *H. miniatus* at Lizard Island (Ryen 2007); therefore, this species appears to be a monandric protogynous hermaphrodite at this site. Social organization of this species can vary from haremic, with males aggressively defending a territory encompassing the territories of multiple site-attached females, to a more loosely organized system where male territories overlap and females move freely between territories (Ryen 2007). Harems were the only social system observed at Lizard Island.

Experimental induction of sex change

A male removal experiment was used to test the effect of sex change on female growth rate. Five social groups of *H. miniatus* occupying discrete coral rubble patches were identified. Patch size ranged from 10 to 30 m^2 and were at least 20 m apart. The male and the most likely sex-change candidates (three to seven of the largest females in each group) were captured using hand nets and an anaesthetic of dilute clove oil (Munday and Wilson 1997). Each fish was immediately measured using callipers (standard length (SL) to nearest 0.1 mm) and sexed according to its colour phase. Females were tagged with a unique elastomer tattoo (Northwest Marine Technologies) injected under the skin and returned to the exact location of capture once they had fully recovered from the anaesthetic. If the individual was male, it was brought back to the laboratory and its otoliths removed and stored dry for processing. Each social group was monitored daily to ensure that no transient males entered the area and took over the harem. After 44 days, all fish remaining in the social groups were recaptured, euthanized, and their length (SL), weight and colour phase (either IP or TP) recorded. Their otoliths were then removed and stored dry until processing. Gonads were removed and examined under a dissecting microscope to estimate the sex of each fish and then preserved in a solution of formaldehyde–acetic acid–calcium chloride (FAACC) for histological analysis. Once the sex of each fish was confirmed, the mean growth of sex changers and non-sex changers during the experimental period was compared using a *t*-test.

Behavioural observations

Focal behavioural sampling was carried out in one of the five groups described above to explore shifts in individual behaviour and social structure associated with male removal and sex change. Five days before the commencement of observations, a 15 × 25 m grid of nylon line at 5*5 m resolution was placed over the occupied habitat to aid in the relocation and tracking of individuals. The male and the four largest females (the most likely sex-change candidates) in the group were each observed for repeated 15-min periods over 3 days prior to male removal and 3 days after male removal to record the immediate effect of male removal on individual behaviour. Repeated 15-min observations were again made for each individual during the last 3 days of the experiment to examine the long-term effects of male removal on individual behaviour and social structure. All observations were made on snorkel, and sufficient time was allowed for the focal fish to become accustomed to the observer (≥5 min) before behaviour was recorded. Intraspecific interactions (chases, fin displays to another individual, physical contact, spawning) and feeding frequency (number of strikes to the substrate) were recorded, and location within the grid was plotted every 60 s on a scaled reference map. Individual territory sizes (the maximum polygon created from the furthest points travelled) were later measured from the digitally scanned composite maps using the spatial analysis program Optimus (v6.5, Media Cybernetics Inc.). The replicate estimates of territory size (m^2), interaction rate (per 15 min) and feeding rate (per 15 min) were averaged for each individual at each stage of the experiment and then compared using simple Repeated Measures (RM) ANOVA

as set out by Quinn and Keough (2002). In this analysis, individual fish were subjects, and stages of the experiment (before male removal, after male removal, end of experiment) were treatments. Average interaction rate was square-root transformed prior to analysis to improve the distribution of residuals. Due to time constraints, it was not possible to record behaviour for individuals in more than one group during the experimental period. Similarly, behaviour and growth could not be monitored among the many smaller sub-adult females in the focal groups (the ones unlikely to change sex) due to time constraints. However, the colouration of these individuals was noted as IP or TP throughout the experiment, so as to detect sex change should it occur.

Analysis of gonads

The developmental stage of the gonads from all presumed sex changers and an equal number of non sex-chargers was assessed by histological analysis. Gonads were embedded in paraffin-wax blocks, transversely sectioned at 5 μm and stained with Mayer's Haematoxylin and Young's Eosin-Erythrosin. Sections were viewed by high-power light microscopy and the proportion of male and female sex-cell types was estimated across a representative section of each gonad. The cell type under each major increment on an eyepiece micrometer was recorded at 400x magnification across the gonad section. Females gonadal tissue was categorized into: (a) pre-vitellogenic oocytes, (b) developing oocytes (cortical alveoli stage), (c) vitellogenic oocytes and (d) ripe oocytes (West 1990). Male gonadal tissue was categorized into: (a) spermatogonia, (b) spermatocytes, (c) spermatids and (d) spermatozoa (Nakamura et al. 1989). The presence of remnant ovarian features (e.g. degenerating oocytes, oocyte follicles and ovarian lumen) characteristic of female to male sex-change was also recorded.

Sex change by females in the male removal experiment (above) was confirmed where individuals initially recorded as female had TP colouration at the end of the experiment and their gonads contained male gonadal tissue, including spermatozoa. Since more than one female changed sex to male in some social groups, the frequency of occurrence of male sex cell types in the gonads was compared among all males using Chi-square test of independence to determine whether all males were at a similar stage of gonad development.

Analysis of otoliths

The sagittal otoliths from all experimental fishes were examined to determine the relationship between otolith growth and somatic growth, and to determine whether a distinct microstructural discontinuity (check mark) was deposited on the otolith during sex change. If sex change caused a distinctive check mark to be deposited in the otolith, then all sex changers should have exhibited an otolith check within 44 days from the otolith edge, while the check mark should be absent in the otoliths of non sex-changing females. Otoliths of all fish were sectioned along the nucleus following the procedures of Wilson and McCormick (1997) and viewed with a high-power light microscope. The timing of check mark deposition (if present) was estimated by counting the number of daily otolith increments between the check mark and the otolith edge. This final step was repeated on two separate occasions by independent observers to reduce error and observer bias. The mean of the two measurements was used in subsequent analysis.

Otolith increments in this species were validated daily following the protocol of Schmitt (1984). Briefly, 14 individuals were immersed in a tetracycline-sea water bath for 24 h. This results in the deposition of tetracycline into the otolith matrix, forming a fluorescent otolith marker. Following tetracycline immersion, individuals were kept alive and fed daily in aquaria with running sea water and a constant air supply. Individuals were sacrificed with an overdose of anaesthetic (clove oil) 35 d following the tetracycline immersion date. Daily increment deposition was then inferred by comparing the number of post-marker increments with the number of post-immersion days (35). The average number of increments observed between the fluorescent marker and the otolith margin was 34.64 ± 0.34 SE ($n = 14$).

If otolith increment width provides a reliable indicator of somatic growth, and females exhibit an increased somatic growth rate during sex change, then otolith increment widths should increase significantly in sex changers, but not in females that do not change sex. Daily otolith increment widths of all fish were estimated from digital images captured at 400x magnification using the spatial analysis program Optimus. Increment widths before and after male removal were compared using RM-ANOVA. For females that changed sex to male, otolith increments were centred on the sex-change check mark for each individual and the average width of daily otolith increments compared for 20 days before and 20 days after the check mark. A similar analysis was conducted for females that did not change sex by centering otolith increments on the day that coincided with the mean position of the sex-change check mark identified in the previous analysis.

Growth and sex change in the natural population

In order to investigate the patterns of sex change and growth in the natural, un-manipulated population, male and

female *H. miniatus* were collected from various locations in the Lizard Island lagoon using hand nets and a dilute solution of clove oil. The standard length (mm) and colour phase of each fish were recorded. Otoliths were removed for estimation of age and analysis of otolith increment width. Otoliths were prepared, sectioned and viewed as described above. The age of each fish was determined by counting the number of daily increments from the nucleus to the otolith edge. The sex of each fish was determined by macroscopic examination of the gonads under a dissecting microscope. Histological sections of gonads were also examined, as described above, to verify sex and to compare gonadal development of individuals in the sex-change experiment with those in the natural population.

The minimum age of sex change in the sample was estimated from the age frequency distribution of males and females in the sample. Within the estimated age range of sex change, otoliths of all fish were then examined for a check mark that might indicate the time of sex change. The age at sex change was estimated by counting the number of daily otolith increments between the check mark and the otolith nucleus. In order to determine whether growth rate increased following the presumed sex-change check mark, daily mean increment sizes were compared for 20 days before and 20 days after the check mark using RM-ANOVA. Average increment widths of sex changers (males) were also compared to those of non-sex changers (females) during the sex-change period using a one-way ANOVA. In this analysis, the width of otolith increments in females above the average age of sex change was compared to the width of otolith increments in males following the presumed sex-change check mark.

Table 1 The size (mm SL) of males and females in natural social groups before male removal and sizes of females that changed sex and those that remained as female 44 days after the removal of the resident male

Group	Before male removal		After male removal	
	♂ Size	♀ Size	♂ Size	♀ Size
1	67.5	53.4	62.7	←
		52.4		55.9
		50.2		52.7
		46.3		51.1
		46.3		–
		43.8		47.1
2	75.5	57.1	64.6	←
		56.7	61.2	←
		51.8		54.2
3	77.2	56.8	60.3	←
		49.4		53.8
		48.7		50.2
		44.6		47.9
4	87.2	57.2	68.6	←
		56.2	67.7	←
		50.3		55.0
5	79.4	61.7	65.1	←
		51.9	67.5	←
		51.4	61.3	←
		49.3		52.1

Fish were tagged for individual identification. ← indicates females that changed sex after male removal, – missing female

Results

Experimental induction of sex change

Natural social groups of *H. miniatus* contained a single dominant male and three to seven large females within the male's territory (Table 1). Between one and three of these females changed sex to male in each of the five social groups where the dominant male was removed (Table 1). In each group, it was the largest female(s) that changed sex. In three of the groups (groups 2, 4 and 5), more than one female changed sex. One female from group 1 was not recaptured and may have moved from the group or died. Numerous smaller females were observed within the territories of the larger females, but these were not captured and measured. All these small individuals retained initial phase colouration throughout the experiment.

All sex-changed fish had well-developed testes dominated by male tissue (Fig. 1a). Spermatozoa were present in peripheral sperm sinuses indicating that these were functional males. Most testes contain a remnant ovarian lumen and some contained degenerating oocytes or oocyte follicles. Gonads of all non-sex-changing females exhibited typical ovaries with all stages of oocyte development (Fig. 1b). In social groups where more than one individual changed sex to male, there was no difference in the proportion of male sex cell types present in the gonads of the smaller males compared to the largest male in all groups ($\chi^2 = 23.7$, d.f. $= 21$, $P = 0.3$), indicating that gonads of all males were at a similar stage of development.

There was a significant increase in the growth of females that changed sex to male compared to females that did not change sex ($t = 3.6$, $P = 0.005$). Females that changed sex grew over twice as much as females that did not change sex over the duration of the experiment ((sex-changer mean growth = 8.51 mm SL ± 1.40 SE; non-sex changer mean growth = 3.32 SL ± 1.37 SE). In all but one of the groups, the largest female remained the largest individual in the group after sex change. In group 5, however, the second

largest female grew faster than the initial largest female and became the largest member of the group after sex change (Table 1).

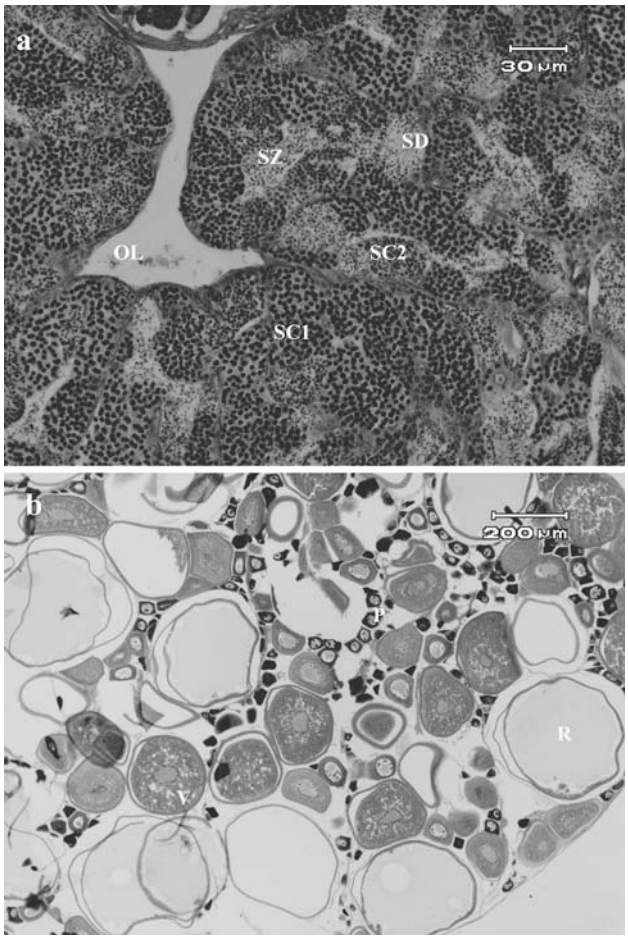

Fig. 1 Gonad structure of a female *Halichoeres miniatus* that changed sex to male (**a**) and a female that did not change sex (**b**), showing primary spermatocytes (SC1), secondary spermatocytes (SC2), spermatids (SD), spermatozoa (SZ) and remnant ovarian lumen (OL) in the testis and pre-vitellogenic oocytes (P), vitellogenic oocytes (V) and ripe oocytes (R) in the ovary

A check mark in the form of an optically dense discontinuity was apparent in the otoliths of all the females that changed sex ($n = 9$) in the male removal experiment (Fig. 2a, b). The check mark appeared as a growth increment with an unusually dark margin. The primary otolith growth axis (i.e. the axis through which increment width is greatest) also appeared to change direction in association with check-mark formation (Fig. 2a, b). Similar changes in otolith microstructure were not present in any of the individuals that remained female (Fig. 2c, d). The check mark in sex-changed females was between 26 and 44 days (mean = 40d) of the otolith margin, indicating that it was formed after the removal of the dominant male.

For those individuals that changed sex, the average daily otolith increment width for the 20 days immediately after the identified check was significantly larger than the average daily increment width for the 20 days immediately prior to the check (RM-ANOVA $F_{1,16} = 19.7$, $P < 0.001$, Fig. 3a). There was no difference in otolith increment widths at corresponding days in females that did not change sex (RM-ANOVA $F_{1,18} = 2.2$, $P = 0.155$, Fig. 3b). Increased otolith increment width in sex-changed fish is consistent with the observed increase in growth rate of females that changed sex compared to those that did not. There was a sharp increase in average increment width at the time of the check indicating acceleration in growth at the commencement of sex change.

Behavioural changes

The focal observation group (Group 5) displayed a haremic structure prior to the removal of the dominant male (Fig. 4a). Less formal observations of the other four experimental groups indicated a similar social structure in each. Males were observed to aggressively defend non-overlapping territories from other males and each male territory encompassed multiple, smaller, female territories. Males maintained their dominance through displays and

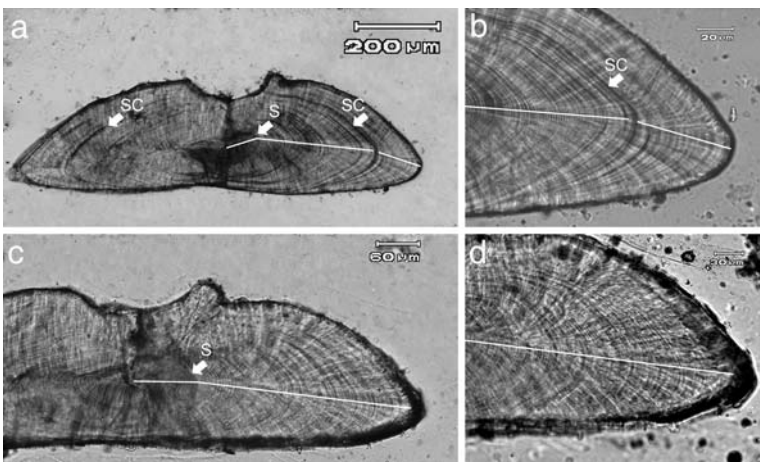

Fig. 2 Otoliths from a female *Halichoeres miniatus* that changed sex to male (**a**, **b**) showing the check mark (SC) associated with sex change. A similar check mark was absent in females that did not changed sex (**c**, **d**). A change in the axis of otolith accretion was evident following the check mark associated with larval settlement (S) and following the check mark associated with adult sex change (SC)

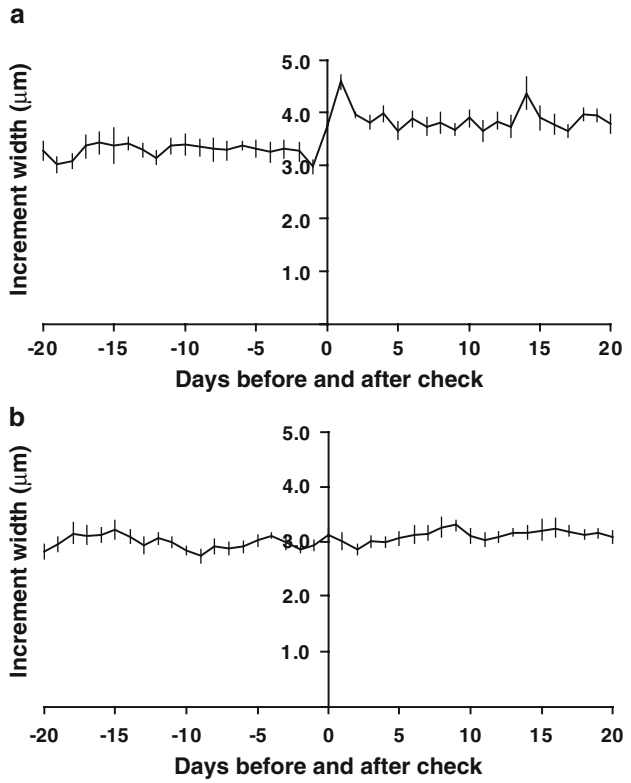

Fig. 3 Average (±SE) daily otolith increment widths of *Halichoeres miniatus* 20 days before and 20 days after the sex-change check mark identified in the otolith. **a** Female that changed sex to male ($n = 9$) and **b** females that did not change sex ($n = 10$)

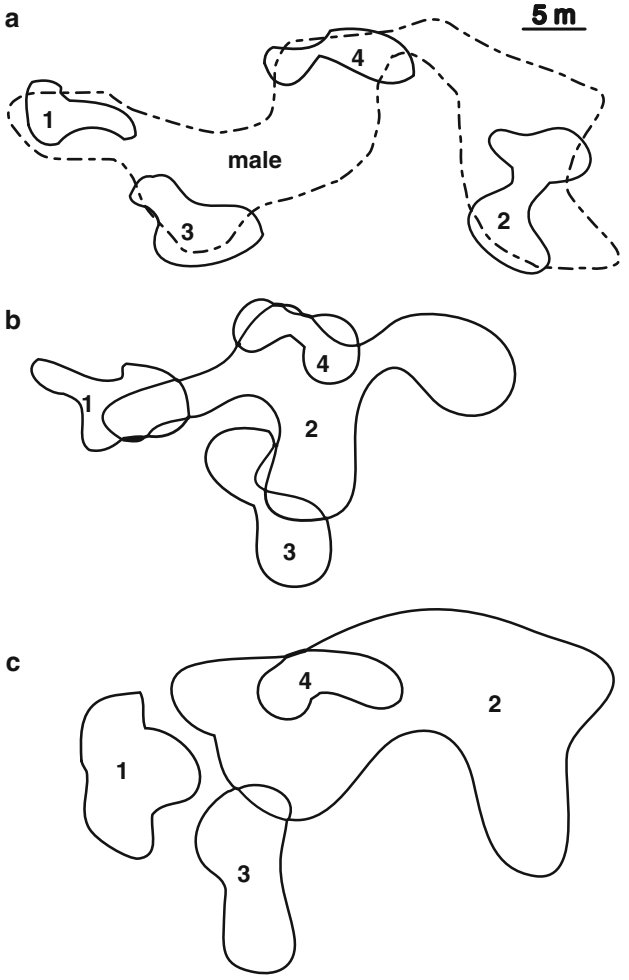

Fig. 4 The shape and relative size of the male and largest female territories in the focal study group (Group 5): **a** before male removal, **b** 22 days after male removal and **c** 44 days after male removal. Territory numbers match females in Fig. 4. Territory 1 belongs to the largest female and 4 to the smallest female. The dashed lines represent the original male's territory

chases towards the females within their territory. Females remained attached to the site and rarely moved outside their territory. Larger females displayed to and chased other large females that entered their territory, but largely ignored any small females already within their territory. Near spawning time males visited female territories more frequently until spawning took place.

The study area of the focal group encompassed the territory of the largest male in the area. The average territory size over 3 days for the dominant male (79.4 mm SL) was 120.48 m^2 (Fig. 4a). Three neighbouring males in the area had territories of 88.4, 87.52 and 19.64 m^2 (male SL: 74.2, 66.4, and 63.1 mm, respectively). The male interacted regularly with all the females within his territory. Before male removal, the four largest females had an average territory size of 13.25 m^2. After the dominant male was removed, female territories initially became indistinguishable and all the females roamed throughout the study area, often in a group. Territories were re-established within several days, at which time the second largest (and initially the most isolated) female began to assume the dominant male role and maintain the largest territory (Figs. 4b, 5a). By the end of the experimental period, this female and two other large females had changed sex to male. At the end of 44 days, the behaviourally dominant female had grown larger than the other two and controlled the largest territory, while the other two new males maintained territories similar in size to their original territories on the periphery of the dominant's territory (Figs. 4c, 5a). There was no significant increase in territory size between stages of the experiment (RM-ANOVA $F_{2,\ 6} = 2.07$, $P = 0.21$) because only the territory of the largest sex charger exhibited a sustained increase in size.

Before male removal, the largest females in the group generally encountered other females infrequently. Interaction rates increased after male removal for the three females that changed sex (Fig. 5b; RM-ANOVA $F_{2,\ 6} = 7.18$, $P = 0.025$), with the second largest female having the highest number of interactions. Interactions rates did not increase for the smallest female, which did not change sex (Fig. 5b). Feeding rates of all females decreased at the

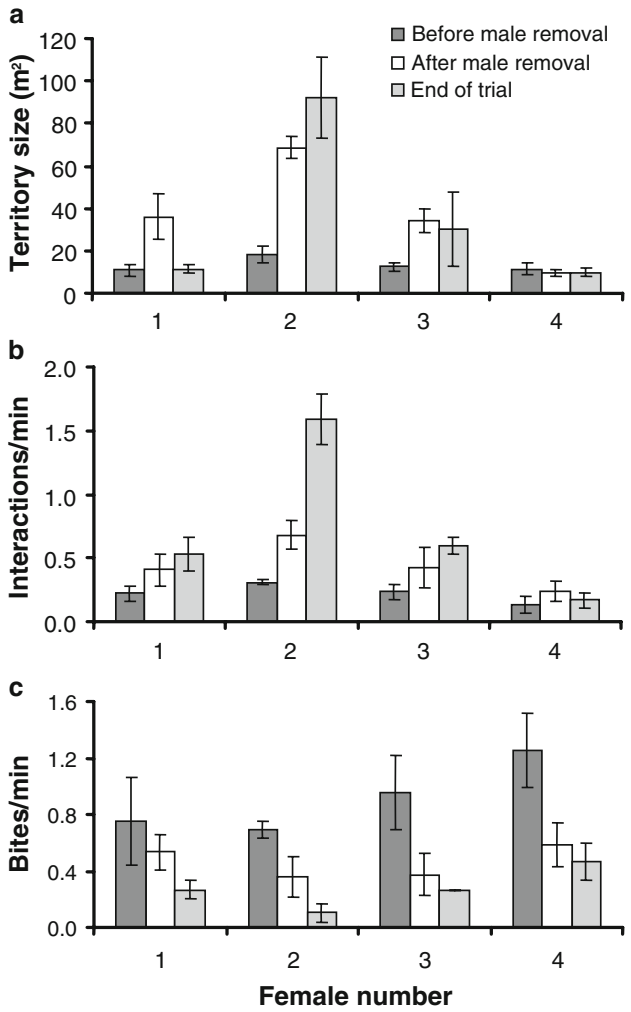

Fig. 5 Mean (±SE) territory size (**a**), frequency of female–female interactions (**b**) and feeding rates (**c**) before and after male removal for the four largest *Halichoeres miniatus* females in the focal observation group (Group 5). Females are ordered from largest to smallest. The first three females changed sex to male and the fourth remained female. Each mean is the average recorded over 3 consecutive days

same time (Fig. 5c; RM-ANOVA $F_{2,\ 6} = 22.58$, $P = 0.002$) suggesting that there was a shift in time budget from feeding to social interactions.

Before male removal, all the largest females in the focal group were observed to spawn with the male territory holder. Spawning behaviour within the group involving several different pairs of resident females was observed the same evening as the male was removed. After several days, the second largest female began to monopolise all spawning activity. Colour phase in the dominant female (second largest) began to change from IP to TP within a week of male removal. After 3 weeks, two other large females also began to change colour. At the end of the experiment (44 days), the three largest fish exhibited male colouration and histological analysis of the gonads confirmed that all three had become functional males.

Growth and sex change in the natural population

A total of 33 females and 16 males were collected from the natural population. All initial phase individuals were female, and no males were found in the smaller size classes; therefore, all males appear to be derived through sex change, as previously reported. The initial size and age distributions were representative of a protogynous species, with males only found in the larger size classes (Fig. 6a). There was more variation in the age distribution of males (Fig. 6b) compared to the size frequency distribution, indicating that females change sex to male at a wide range of ages. The youngest male was 197 days old, only marginally older than the youngest female in the sample (192 days).

The histological examination of the gonads confirmed that all terminal phase (brightly coloured) individuals were functional males. Testes contained only male sex cells, although most also exhibited a remnant ovarian lumen and some also contained degraded oocyte follicles. One individual had transitional colouration with only the beginnings of male facial markings. The gonads of this individual containing both proliferating testicular tissue and a large number of degenerating oocytes. In addition, the otolith of this individual contained a check mark located five daily increments away from the otolith edge.

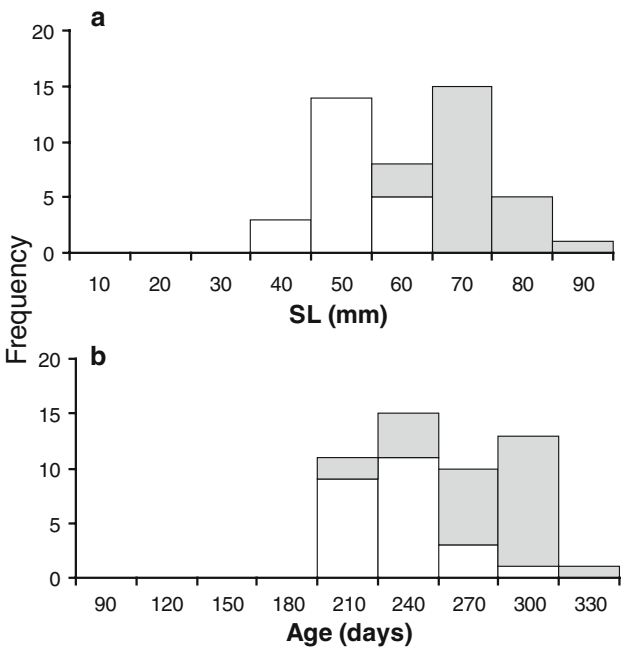

Fig. 6 Size (**a**) and age (**b**) frequency distributions of *Halichoeres miniatus* at Lizard Island, GBR. White represents females and grey is males

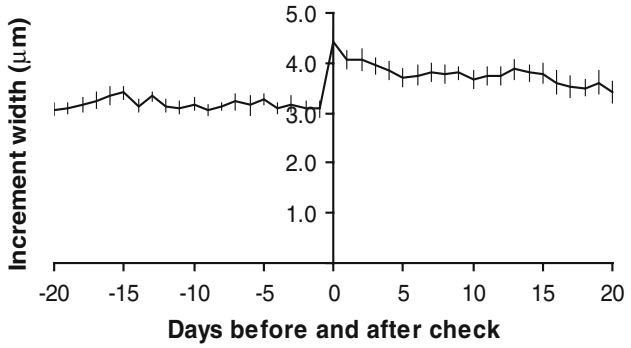

Fig. 7 Average daily otolith increment width (±SE) of *Halichoeres miniatus* individuals from 20 days before to 20 days after the identified check in the otoliths of wild caught individuals

A discernable optical discontinuity in the otolith, consistent with the sex-change check mark observed in experimental fish, was identified in 14 of 16 males from the wild population. A clear check was not evident in the otoliths of two males; however, a change in the otolith accretion rates was still distinguished in these fish. The estimated age at sex change based on the check mark was between 141 and 269 days (mean 209d). Daily otolith increment width was significantly larger after the check compared to before the check (RM-ANOVA: $F_{1,30} = 14.47$, $P = 0.001$, Fig. 7), indicating an increase in growth rate associated with sex change as observed in the experimental manipulation. Otolith increment widths of males 20 days after the sex-change check mark were significantly larger than otolith increment widths of females of the same age that had not changed sex ($F_{1,38} = 181.92$, $P < 0.001$).

Discussion

In this study, the largest one to three females in social groups of the wrasse *H. miniatus* changed sex following the loss of the dominant male. All sex changers exhibited an increase in growth rate associated with sex change. This rapid increase in size may improve the competitive ability of the new males to prevent harem takeovers by other males. It was also experimentally demonstrated that an optically apparent microstructural discontinuity (check mark) is formed in the otoliths during sex change and that the increase in somatic growth associated with sex change is reflected in otolith growth. A similar check mark and increase in otolith increment width was found in males, but not in females, from the natural population, indicating that increased growth associated with sex change is a common feature of *H. miniatus* growth dynamics.

Transitional growth acceleration has been reported in several other haremic sex-changing fishes (Walker and McCormick 2004; Walker et al. 2007). Increased size during and after sex change most likely benefits new males by increasing their ability to defend the harem against other males. Bachelor males or other territorial males frequently attempt to take over a harem after loss of the dominant male (Aldenhoven 1986; Warner 1991; Sakai 1997) and accelerated growth would increase the new males' ability to prevent such takeovers. Large size may also help the new male maintain a dominance hierarchy within the harem and prevent other females challenging for the dominant position (Robertson 1972; Hattori 1991; Sakai 1997; Sakai and Kohda 1997; Lorenzi et al. 2006; Wong et al. 2007).

In the focal group, behavioural changes in the largest females began almost immediately after the removal of the male. Similar behaviour has been observed in other species and is likely to be important in maintaining or establishing dominance relationships among prospective sex changers (Kuwamura et al. 2002; Sakai et al. 2003; Rodgers et al. 2005). Interaction rates and movement of females within the harem increased after removal of the harem male, whereas feeding rates declined. Importantly, sex-changing females exhibited an increase in growth at the same time that feeding decreased, suggesting that growth acceleration may be fuelled by energy stores rather than increased energy intake. Since only one focal group was observed for behavioural changes, the result from this part of the study must be interpreted with caution. Further studies on multiple groups will be necessary to determine whether increased growth in sex-changing females is usually supported by energy stores rather than increased foraging.

In social systems where sex change and growth are regulated by interactions between individuals, it might be expected that males would be replaced on a one-to-one basis by the largest dominant females (Robertson 1972; Ross 1990). In the male removal experiment, the largest female always changed sex following male removal, as has been observed in other haremic species. However, one or two of the next largest females also changed sex to male in three of the five groups. This suggests that the presence of the male, and not female dominance, is initially responsible for the timing of sex change in *H. miniatus*. This perspective is reinforced by the low level of interactions between females in the focal harem before the male was removed. After the removal of the male, female–female interactions increased, possibly due to the competition among females for control of the harem. Surprisingly, in one of the groups, the second largest female changed sex first, grew faster than the other sex-changing females, and became the dominant male. This observation is important because it illustrates that even though removal of the dominant male may be the cue for sex change in females, it may be dominance relationships among females that determine which one ultimately becomes the dominant

male following sex change. Although size is frequently a good predictor of dominance in fish societies (Forrester 1991; Buston 2003; Wong et al. 2007), other factors may also influence dominance hierarchies, and thus influence which individuals change sex (Rodgers et al. 2007), or the outcome of competition among sex-changing females.

In some situation where the largest female has a higher fecundity than the combined fecundity of other females in the group, the largest female may decline to change sex, presumably because her future reproductive success will be greater by remaining female (Munoz and Warner 2003, 2004). In this case, it would favour one of the smaller females changing sex to become the new harem male. This mechanism does not appear to have operated in our experiment because although some of the smaller females changed sex to males, the largest female in the group also changed sex in all instances. Therefore, the largest female did not decline sex change in any instance. Instead, it appears that the multiple females changed sex to compete for the dominant position within the harem.

Given the potential high pay-off associated with becoming a harem master, it might be advantageous for lower ranked females of sufficient size to change sex at the same time as the dominant female and challenge her for the male position. If the reproductive success of a harem master is much greater than the average reproductive success of females, challenger females need only succeed on a relatively small number of occasions for this to be an effective strategy (Rodgers 2003). Natural social groups of *H. miniatus* appeared to be haremic at this location, with only one male in each harem. Therefore, smaller females that change sex, but do not succeed in challenging the dominant sex-changing female, may become bachelor males and wait for an opportunity to takeover another harem (Aldenhoven 1986; Lutnesky 1994; Sakai 1997). Several new males entered the experimental area and were removed in the first few days after removal of harem males in the experiment. This, along with the sex change of multiple females, indicates that there might indeed be a small number of bachelor males in the population of *H. miniatus* and that these male will attempt to take over a harem if there is an opportunity to do so.

An alternative explanation for why more than one female changed sex in some groups is that new males were smaller than the resident male and, therefore, unable to defend a harem of the size of the original males. In the present system, as a female changed to male, she attempted to integrate the previous male's territory into her own. However, the territory size of the new dominant male did not reach the size of the initial male territory. The sex-changing female, being smaller than the original male, might not be able to maintain a territory of the same size as the original male. Reduced territory size of the new male and lower levels of interactions between peripheral females and the new male might stimulate peripheral females to change sex, resulting in the initial fragmentation of the original male's territory (Ross et al. 1983; Hoffman et al. 1985; Sakai 1997). Presumably, however, the new dominant male would expand his territory as he increases in size and ultimately exclude peripheral males. The result would be a dynamic of territory fragmentation and expansion following the loss of dominant males (Ross et al. 1990; Sakai 1997; Sakai and Kohda 1997). Once again, any other males that were ultimately excluded from the territory of the dominant male are likely to become bachelor males.

Finally, this study validated that an optical check mark in the otoliths develops during sex change in *H. miniatus* and that growth acceleration associated with sex change is recorded as an increase in the width of daily otolith increments. Comparisons to natural populations revealed a similar check in the otoliths as well as a similar increase in daily increment width. Although a distinctive check mark was not observed in all the wild caught fish, a change in the accretion rates was observed in all sex-changing individuals, suggesting that increased growth rates do indeed accompany sex change under natural conditions. It is likely that other hermaphroditic species may also acquire a check-mark in their otoliths indicating the time of sex change. Importantly, the presence of such a check mark would provide a unique and effective way to estimate the age of sex change and to investigate growth patterns associated with sex change without time consuming and logistically difficult individual tracking.

Many fishes are known to be hermaphroditic (Sadovy de Mitcheson and Liu 2008); however, the proximal mechanism of sex change has been experimentally tested in relatively few of these species. Only the largest female changes sex following male loss in some strongly haremic species (e.g. Robertson 1972; Walker and McCormick 2004), and it is often assumed that a similar mechanism operates in other sex-changing species. This study indicates that patterns of sex change can be more complicated than are commonly assumed. More than one female may change sex following the loss of a dominant male, and these females presumably compete for access to the position of harem male. Furthermore, it may not always be the initially largest female that secures the dominant male position if smaller females can rapidly increase their size, and overtake other females, when the opportunity for sex change arises. These results add to the increasing evidence for unrecognised diversity and complexity in sex change strategies by hermaphroditic animals. At the same time, they provide further evidence that otoliths provide a valuable store of information for exploring the complex life histories of sex-changing species.

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