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**Sex change of primary males in a diandric labrid *Halichoeres trimaculatus*:
coexistence of protandry and protogyny within a species**

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Running headline: PROTANDRY AND PROTOGYNY IN DIANDRIC WRASSE

Two types of males, *i.e.* primary and secondary males, are known to coexist within a species of some protogynous fishes such as labrids and scarids. Secondary males are derived from females through sex change, *i.e.* they are protogynous hermaphrodites. Primary males have been considered to be non-sex-changing gonochores that are born as males. In the threespot wrasse *Halichoeres trimaculatus*, sex change of primary males was discovered by our field observations and aquarium experiments. In other words, protandry and protogyny coexisted within this species. Moreover, male-to-female-to-male sex change and female-to-male-to-female sex change were observed in aquarium experiments; *i.e.* reversed sex-change occurred in both protandrous and protogynous hermaphrodites. These results suggest that only the direction of sex differentiation before maturation may differ between the two sexual types that have been regarded as a primary male and a protogynous hermaphrodite.

Key words: bi-directional sex change; primary male; protandry; protogyny; Labridae.

INTRODUCTION

Two types of males, namely, primary males and secondary males are found to coexist in the diandric species of protogynous fish (Robertson & Warner, 1978; Warner & Robertson, 1978; Nakazono & Kuwamura, 1987; Nostro & Guerrero, 1996; Liu & Sadovy, 2004). Secondary males are derived from females through sex change, *i.e.* they are protogynous hermaphrodites. In contrast, primary males are considered to be non-sex-changing gonochores that are born as males. Primary and secondary males can be distinguished histologically because of distinct morphological differences between

the two types of testis in many diandric species such as wrasses (Labridae) (Reinboth, 1970; Warner & Robertson, 1978; Sadovy & Domeier, 2005).

In diandric labrid species, alternative male mating tactics have been reported in relation to change in body colour with growth (Reinboth, 1973; Warner *et al.*, 1975; Warner & Robertson, 1978; Nakazono, 1979; Colin & Bell, 1991; Kuwamura *et al.*, 2000). Females have pale drab colouration; this is the initial phase (IP). Later, they change sex to become secondary males that have bright colouration; this is the terminal phase (TP). Some of the TP males are primary males that have IP colouration when small. TP males, whether primary or secondary, establish mating territories and pair-spawn with visiting females. Small IP males cannot defend territories; however, they perform alternative mating tactics such as group spawning, streaking and sneaking.

Mechanisms of coexistence of the two sexual types, *i.e.* a protogynous hermaphrodite and a primary male have been studied in great detail in the bluehead wrasse *Thalassoma bifasciatum* (Bloch); it has been suggested that the sexual polymorphism is maintained by differential selection in relatively isolated local populations (Warner & Hoffman, 1980; Warner, 2001). The proportion of IP males increases with the local population size from zero to approximately 50%. On smaller reefs, large territorial TP males can successfully exclude small IP males from breeding; therefore, the protogynous hermaphrodites would be more fit than primary males. In larger local populations, the density of small IP males at the prime mating site is sufficiently high that the territories of large TP males are located elsewhere, and the majority of spawnings occur with the nonterritorial IP males. If the act of changing sex has a substantial reproductive cost, then primary males would be more fit than

hermaphrodites on larger reefs. This suggests that the overall frequency of the two sexual types should be a reflection of the underlying distribution of local population sizes. Warner and Hoffman (1980) suggested that some degree of habitat selection by the primary males might result in the different proportions of IP males based on the reef size. However, such ability of habitat selection in the larvae of primary males has never been examined.

During our field observation of alternative male mating tactics in the threespot wrasse *Halichoeres trimaculatus* (Quoy & Gaimard) (Suzuki *et al.*, in preparation), one of the tagged IP males was observed to change sex to female. Such protandrous sex change has never been theoretically predicted in diandric species, as mentioned above. Subsequently, aquarium experiments were conducted to examine the social conditions for the protandrous sex change. In this paper, the observed cases of sex change in IP males of *H. trimaculatus* in the field and aquarium are described, and the coexistence of protandry and protogyny within a diandric species is discussed.

MATERIALS AND METHODS

FIELD OBSERVATIONS

Mark-recapture research was conducted on the fringing reef of Sesoko Island (26° 38' N, 127° 52' E), Okinawa, southern Japan. In a study area of approximately 150 X 200 m in front of Sesoko Station of the University of the Ryukyus, almost all *H. trimaculatus* larger than 5 cm in total length were captured using screen nets and hand

nets during the breeding season from May to September 2002. Their total length was measured, and the sex was determined by the shape of the urogenital papillae and by pushing their abdomen to release eggs or sperms. Their body colour (IP or TP) was also recorded; IP males and females are white with a dark spot on the upper part of caudal peduncle, while TP males have a black spot centred on the fifth and sixth lateral line scales in addition to a black spot on the upper part of caudal peduncle. In order to distinguish the individuals, visible fluorescent Elastomer tag (Northwest Marine Technology Inc., Washington, USA) was injected subcutaneously and a small piece of plastic of a different colour and shape was attached with a nylon thread at the dorsal muscle. The tagged fish were released at their collection sites, and their mating behaviour was observed. Similar procedures of capture, measurement, release and observation of mating behaviour were conducted again in May 2003.

AQUARIUM EXPERIMENTS

Aquarium experiments were carried out at the Okinawa Churaumi Aquarium. The material *H. trimaculatus* was collected at Sesoko Island, Bise and Itoman of Okinawa Island during September 2003 and February 2005. The total length of the collected fish was measured; the body colour was recorded; and the sex was determined by the shape of the urogenital papillae and gamete release. In order to distinguish the individuals, the fish were marked with the fluorescent Elastomer tag. They were placed in a 500-l or 1,000-l tank with running seawater; the bottom of each tank was covered with sand and a number of plastic pipes and nets were set for shelter. The fish were fed with krill, shrimp, minced fish and squid and compound bait every morning.

Fourteen experiments were carried out to observe sex change (Table I). The individuals of the same sex (experiment #1–5) or individuals of both sexes (#6–14) were placed together in each tank (500-l for #1–12 and 1,000-l for #13–14). All fish survived until the end of each experiment (38 days to 13 months), except for three of 19 fish in experiment #12 and five of 31 fish in experiment #14 that were either dead or wounded and removed from the tank. The total length of the fish was measured; the sex was determined and the fish were photographed every 2–4 months and at the end of each experiment. The plankton net that was set at the end of the drainpipe of each tank was checked for eggs daily. When eggs were found, fertilization and development were confirmed microscopically. The water temperature ranged from 20.4 °C to 29.2 °C, and the spawning activities of the threespot wrasse decreased during winter.

RESULTS

FIELD OBSERVATIONS

A total of 354 individuals were captured and tagged during the breeding season of 2002. At their first capture, 10 were TP males (121–194 mm in total length); 51, IP males (51–138 mm); 280, females (46–162 mm); and 13, small IP fish of unknown sex (47–63 mm). The proportion of primary males in IP fish, excluding the fish of unknown sex, was 15% (51/331). Only seven (2%) of the tagged fish were recaptured in May 2003. Two were TP males (161–182 mm); one of these males had been a female in the previous year (*i.e.* protogynous sex change). The remaining five

were females (120–161 mm), of which, one had been an IP male in the previous year (*i.e.* protandrous sex change).

Detailed information regarding the protandrous sex change of the IP male is as follows. When the fish was caught for the first time in July 2002, it was an IP male that was 82 mm in total length and grew to 96 mm by September 2002. The male was observed to perform group spawning, streaking and sneaking several times during the summer. When it was recaptured in May of the next year, it was 136 mm in length, and its urogenital papilla shape had changed to that of a female. Subsequently, the fish was observed to pair-spawn with a TP male; a gamete cloud was visible at the spawning rush. Therefore, it was revealed that the fish had changed sex from a functional IP male to a functional female. Since the field observation and collection were not conducted during the nonbreeding season from November to March, the change in social status of the sex-changing fish during the winter could not be followed. In May 2003, not only the pair-spawned TP male but also a few females larger than the sex-changed fish were found within its home range; its social status appeared to be of a middle rank.

AQUARIUM EXPERIMENTS

Protandrous sex change occurred in experiments #11 and #14, and protogynous sex change occurred in experiments #1 and #10–14 (Tables I and II). In experiment #1, the largest of the four females changed sex within 38 days, *i.e.* when the fertilized eggs were collected, and it changed to TP male within 5 months. In experiments #2 and #3, the two IP males changed their body colour to become TP males

within 5 months. In experiment #4, none of the five IP males changed sex and colour until the end of the experiment for 7 months. In addition, in experiment #5, none of the four TP males changed sex and body colour until the end of the experiment for 8 months. Thus, in the present study, cohabitation of males without females did not induce sex change of males.

Both males and females did not change sex in experiments #6–9; the duration of these experiments was relatively short (38–42 days). In experiment #10, the female changed sex to become a TP male within 42 days; however, cohabiting IP and TP males did not change their sex. In experiment #11–13, the largest female and/or the second largest female changed sex within 7 months, and the smallest IP male (81 mm at the start of the experiment) changed sex to female in experiment #11 (Table I). In experiment #14, four IP males (48–104 mm at the start) changed sex to female, the largest one of which later changed back to IP male; four females (105–127 mm at the start) changed sex to male, the second largest one of which later changed back to female (Table II). Consequently, at the end of experiment #14, seven of the largest 10 individuals (132–170 mm) in the tank were males, and all the smaller 16 fish (92–132 mm) were females.

DISCUSSION

The field observations and aquarium experiments in the present study revealed sex change of IP males in the diandric wrasse *H. trimaculatus*. This is the first report on protandrous sex change of IP males in labrids, although it has long been

considered that in diandric species the IP males are non-sex-changing primary males that coexist with protogynous hermaphrodites (Reinboth, 1970; Warner & Robertson, 1978; Warner, 2001; Sadovy & Domeier, 2005). The social status of the sex-changed IP male in the field appeared to be of a middle rank, since there existed a TP male and a few females larger than the sex-changed fish within its home range. Similarly, sex change of IP males occurred only in the tanks that contained a few TP male(s) and females larger than them (#11 and #14). However, such protandrous sex change did not occur in other tanks with a similar social situation (#10, #12 and #13). Thus, the necessary social conditions for sex change of IP males remain to be elucidated.

The discovery of protandrous sex change in IP males may answer the long-time question of why the proportion of IP males in a population of diandric labrids is smaller on smaller reefs (Warner & Hoffman, 1980). The present study suggests that even if the larvae of primary males randomly settle on each reef without habitat selection, the proportion of IP males may change according to the frequency of their protandrous sex change; this would be controlled by TP males in smaller local populations. Moreover, IP males would get higher reproductive success by changing sex to female, if they are deprived of mating opportunities as males by larger TP males. In the present study area, the overall frequency of primary males during the breeding season was relatively low (15% of IP fish), and the mortality of the tagged fish was very high (2% survival until the next year). This suggested that the decrease in density at the mating site might have resulted in large territorial TP males' successfully excluding small IP males from breeding and subsequently caused sex change of the IP male.

The present study also confirmed that a secondary TP male that was derived

from female changed sex and body colour back to become an IP female. Such 'reversed sex-change' has been reported in other protogynous wrasse *Labroides dimidiatus* (Valenciennes) (Kuwamura *et al.*, 2002) and *Pseudolabrus sieboldi* Mabuchi & Nakabo (Ohta *et al.*, 2003) as well as other polygynous and protogynous fishes (*e.g.* goby, Sunobe and Nakazono, 1993; angelfish, Sakai *et al.*, 2003). Moreover, the present study also found that an *H. trimaculatus* secondary female that was derived from an IP male changed sex back to male (Table II). Similar bi-directional sex change (or both-ways sex change) has been reported in monogamous fishes (Kuwamura *et al.*, 1994; Nakashima *et al.*, 1995; Kuwamura & Nakashima, 1998; Munday, 2002), and their sex is determined by the social status in a monogamous or polygynous group. For example, in the cleaner wrasse *L. dimidiatus*, when a male dies, the largest female of his harem changes sex (Robertson, 1972), and when two unmated males approach each other, the smaller male changes sex to female (Kuwamura *et al.*, 2002). However, in the present study, change in social status of each individual was not followed when the reversed sex-change occurred in the tank (#14); in further studies, the social relationships between individuals need to be examined through detailed behavioural observations.

In conclusion, the present study confirmed not only the coexistence of protandry and protogyny within a diandric species but also reversed sex-change in both protandrous and protogynous hermaphrodites, *i.e.* male-to-female-to-male sex change and female-to-male-to-female sex change. In other words, bi-directional sex change can occur in both sexual types that have been regarded as a primary male and a protogynous hermaphrodite. This suggests that only the direction of sex differentiation before maturation might differ between the two sexual types; however, until now, we have no evidence to prove whether the difference is genetic or environmental. Sex change of

primary males has recently been observed in some other species of *Halichoeres*, *H. poecilopterus* (Temminck & Schlegel) and *H. tenuispinis* (Günther) (Yukio Fukui and Yoichi Sakai, personal communication). Long-term observations of tagged IP males in the field or aquarium would reveal the sex change of primary males in other diandric species.

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TABLE I. Aquarium experiments: number of cohabiting females, IP males and TP males and their total length (mm; range in parenthesis) at the start of each experiment, duration of the experiment and occurrence of sex change

#	Females	IP males	TP males	Duration	Sex changed
1	4 (96–120)			7 months	largest female
2		2 (101–126)		5 months	none
3		2 (103–128)		5 months	none
4		5 (85–108)		7 months	none
5			4 (125–146)	8 months	none
6	2 (81–111)	2 (98–128)		38 days	none
7	1 (97)	3 (113–127)		38 days	none
8	1 (98)		2 (121–135)	42 days	none
9	1 (113)	1 (114)	1 (121)	42 days	none
10	1 (109)	1 (100)	1 (124)	42 days	female
11	7 (84–130)	2 (81–117)	1 (155)	7 months	2nd largest female smallest IP male
12	15 (74–139)	4 (95–150)	1 (169)	7 months	largest female
13	19 (64–143)	4 (67–142)	1 (170)	7 months	largest female* 2nd largest female*
14	21 (48–127)	7 (48–122)	3 (108–159)	13 months	see Table II

* Shape of urogenital papillae changed to male type but sperm release was not observed; for the other sex-changed fish in #1, 10, 11 and 12, gamete release was confirmed both before and after sex change.

TABLE II. Change in size (total length, mm), body colour and sex of each individual that changed sex during experiment #14 from September 2004 to October 2005

<i>OS</i>	Sept.	Nov.	Jan.	May	July	Sept.	Oct.	<i>OE</i>	Sex change
2	127 IF	131 If	132 If	140 TM	140 TM	140 TM	143 IM	7	F to M
4	119 If	139 IM	142 IM	154 IM	159 IM	162 TM	169 TM	2	f to M
5	118 If	128 If	134 If	143 TM	146 If	145 IF	150 If	3	f to M to F
10	105 If	124 If	128 If	134 If	136 If	144 TM	147 TM	5	f to M
11	104 IM	128 Im	131 Im	135 IF	138 IF	141 IM	147 IM	4	M to F to M
18	66 IM	82 Im	84 If	94 If	103 If	107 If	112 IF	14	M to F
19	66 IM	81 If	83 Im	92 IF	95 If	95 If	98 If	22	M to F
31	48 IM	68 Im	72 If	87 IF	95 If	102 If	108 If	17	M to F

OS, size order at the start of experiment; *OE*, size order at the end of experiment; I, initial phase; T, terminal phase; f, female-type genital papilla without egg release; F, female-type genital papilla with egg release; m, male-type genital papilla without sperm release; M, male-type genital papilla with sperm release.