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Life-history pathways in relation to gonadal sex differentiation in the anemonefish, *Amphiprion clarkii*, in temperate waters of Japan

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Synopsis

The process of gonadal sex differentiation in *Amphiprion clarkii* was investigated for 2 years at Murote Beach, Shikoku Island, Japan. Six color phases were discriminated on the basis of the caudal fin coloration, which corresponded well to six gonadal phases. From changes of the color phases with growth, three life history pathways were detected: (1) subadult male → subadult female → adult female, (2) subadult male → adult male → adult female, (3) subadult male → adult male. Different pathways were due to the difference of timing among individuals in the development of ovarian tissues of the hermaphroditic gonads involving the atrophy of testicular tissues. Irreversible differentiation of ovarian tissues of the gonads occurred more frequently among nonbreeders (10 cases) than among breeders (4 cases). The second pathway, which has been thought the norm of tropical anemonefishes, was therefore not primary in this population. This can be attributed to ecological conditions: fish are able to move between host sea anemones and nonbreeders can escape from social suppression by adult pairs because of high population density of hosts.

Introduction

Anemonefishes of the genus *Amphiprion* are protandrous with a monogamous mating system (Fricke & Fricke 1977, Moyer & Nakazono 1978, Ross 1978a, Fricke 1979, and reviews by Keenleyside 1979, Thresher 1984). Their gonads consist of ovarian tissue with only immature oocytes and testicular tissue in both the nonbreeder and functional male states, and only ovarian tissue in the functional female state (Fricke & Fricke 1977, Moyer & Nakazono 1978). The social unit generally consists of a male-female breeding pair and a varying number of nonbreeders (Allen 1972, Fricke 1974, 1979, Fricke & Fricke 1977, Moyer & Nakazono 1978, Ross 1978b). Sex change and maturation are socially controlled: a male changes sex after the disappearance of a female from the social unit, and one of the nonbreeders becomes a functional male after the disappearance or sex change of the resident male (Fricke & Fricke 1977, Moyer & Nakazono 1978, Ross 1978a, Fricke 1979, 1983).

Recently, Ochi & Yanagisawa (1987) and Ochi (1989a) found some nonbreeders of *Amphiprion clarkii* becoming females without passing through a functional male state at Murote Beach in temperate waters of Japan. These may be either gonochoristic females or 'prematurational sex changers' (for definition see Warner & Robertson 1978); their actual state should be determined by detailed observations on the gonadal change with development.

The aim of this study is to know the actual state of these individuals and the significance of becoming female without experiencing a functional male state. In our preliminary study, several color phases were recognized in the caudal fin and the correspondence of these phases to gonad phases was suggested. We then observed color changes of individually marked fish in the field and examined the exact relationships between the color and the gonad phases. This paper indicates that the individuals of the anemonefish take one of three life-history pathways, which arise from the difference of timing among individuals in the differentiation of the hermaphroditic gonads into the female state.

Materials and methods

The field study was conducted at Murote Beach (33°00' N, 123°30' E), on the west coast of Shikoku Island, Japan, where the only anemonefish species occurring is *A. clarkii*. *A. clarkii* uses one species of sea anemone (*Parasicyonis maxima*) for shelter or spawning sites (Ochi 1985). This fish was monitored in two 40 × 50 m quadrats (Area 1 and Area 2), which were 4–10 m in depth and about 50 m apart from each other. Specimens for the examination of gonad conditions were collected in Area 1.

Locations of sea anemones were plotted on a map in both study sites. The long and short axial lengths of sea anemones were measured twice in Area 2. The maximum value of an area that tentacles of a sea anemone covered (long axial length × short axial length × 3.14/4) was used as an index of its size.

All fish larger than 50 mm in standard length (SL) and a few fish smaller than 50 mm SL in Area 1 and all fish larger than 40 mm SL in Area 2 were marked individually by injecting acrylic paint under the skin (see Thresher & Gronell 1978). At the same time, standard length of these fish was measured and coloration of their caudal fins was checked. Locations of each marked fish were plotted on the map at intervals of 10 sec for 15 min once in September 1985 and May 1986 in Area 1 and in May and September 1986, and May and September 1987 in Area 2, and a line encircling all plotted points in each month was regarded as the boundary of its territory or home range (see Ochi 1986).

Breeders and nonbreeders were defined as fish which had reproduced or not. To investigate the occurrence of reproduction, we patrolled Area 1 and Area 2 every four days during May–October, in 1985 and 1986 and in 1986 and 1987, respectively, and checked the presence or absence of an egg mass in the vicinity of sea anemones. Since the breeding season of this fish was from early June to early October and the period between spawning and hatching was more than 6 days even in the warmest season (Ochi 1985), we assumed that all reproduction by every fish could be detected from these censuses.

Changes of the coloration in the caudal fin, shifts of home ranges, and formation and separation of pairs were also checked every 4 days in the breeding season and irregularly in the nonbreeding season. If any indication of color change or shift of the home range was noticed in the nonbreeding season, observations were carried out on successive days. The date when such a change or shift was confirmed was regarded, for convenience, as the day of its occurrence. The term pair is used in this paper for an association of two fish whose home ranges almost completely overlapped for more than 1 week.

In Area 2, agonistic interactions of some selected nonbreeders with other individuals were observed for 15 min several times. These interactions included rush, dorsal-leaning, ventral-leaning and appeasement behavior such as head-standing, head-shaking and substrate-biting (Yanagisawa & Ochi 1986).

In Area 1, all nonbreeders larger than 50 mm SL (33 fish) and some of breeders (16) and nonbreeders smaller than 50 mm (7) were collected in May, August and October 1986. The specimens were fixed in Bouin's solution for 48 h and preserved in 70% ethanol. The gonads were removed and embedded in paraffin blocks. Serial cross sections (5 μm thick) of two or three parts of a gonad were stained with haematoxylin and eosin and were examined under a microscope.

Results

Six color phases

Six color phases were discriminated in *A. clarkii* on the basis of the coloration of the caudal fin: three phases in nonbreeders and three in breeders (Fig. 1). All nonbreeders smaller than 58 mm SL and some nonbreeders smaller than 67 mm SL had transparent caudal fins (juvenile) (Table 1). Larger nonbreeders had caudal fins with orange borders (A-nonbreeder) or creamy white fins (B-nonbreeder). A-nonbreeder phase could be divided into two subphases according to the width of the orange borders (A1, narrow: A2 broad), though the division was somewhat arbitrary. Among breeders, males and females had orange caudal fins and white caudal fins, respectively, and sex-changing fish caudal fins faintly orange basally and whitish distally, as already reported from temperate waters of Japan (Moyer 1976, Moyer & Nakazono 1978, Ochi 1989a).

A1-nonbreeders were smaller than A2-nonbreeders (Table 1, t-test, $t_s = 7.39$, $df = 56$, $p < 0.01$). A2-nonbreeders were slightly smaller than B-nonbreeders ($t_s = 3.09$, $df = 49$, $p < 0.01$). Size ranges of males and females overlapped widely, although females were significantly larger ($t_s = 6.08$, $df = 163$, $p < 0.01$). The mean size of five sex-changing fish was similar to that of males. However, three sex-changing fish found by Ochi (1989a) in this study site between 1983 and 1986 all originated from males below the average size of males.

Social structure in relation to color phases

The details of social structure and reproduction of *A. clarkii* at Murote Beach were given by Ochi (1985, 1986, 1989a, b), although he did not distinguish between the color phases of A- and B-nonbreeders. We paid special attention to the behavior of nonbreeders in relation to their color phases.

(1) Territory and home range

One hundred and one sea anemones occurred in Area 1 (Fig. 2) and 107 sea anemones occurred in Area 2 (Fig. 3). The average density of sea anemones was 5.7 individuals per 100 m^2. The average size of sea anemones was 1032 cm^2 ± 567.7 SD (N = 248, range = 27 to 2765).

Pairs of breeders held territories including one to ten sea anemones (Fig. 2, $\bar{x} = 3.5 \pm 1.8$ SD, range = 1 to 9, mode = 3, N = 21, for Area 1, and Fig. 3, $\bar{x} = 3.4 \pm 1.6$ SD, range = 1 to 10, mode = 3, N = 62, data of two years combined, for Area 2). Of all sea anemones, 58.5% in Area 1 (N = 117) and 85.9% in Area 2 (N = 248) were controlled by breeding pairs (see also Ochi 1985, 1989a, b).

Nonbreeders had their home ranges in the outskirts of and interstices between territories of breeding pairs. Home ranges of A2-nonbreeders did not overlap with each other, and neither did home ranges of B-nonbreeders (Fig. 2a, 3a). In some cases, home ranges of a B-nonbreeder and an A2-nonbreeder almost completely overlapped; these nonbreeders were regarded as pairs. Home ranges of A2-nonbreeders often overlapped with those of juveniles and A1-nonbreeders, as did home ranges of juveniles and A1-nonbreeders with one another (Fig. 2b, 3b). The mean size of hosts in home ranges of nonbreeders ($\bar{x} = 730$ $cm^2 \pm 430$ SD, N = 73) was smaller than that in breeders' territories ($\bar{x} = 1032$ $cm^2 \pm 566$ SD, N = 213, t-test, $t_s = 5.02$, $df = 284$, $p < 0.01$).

(2) Reproduction

The number of breeding pairs ranged from 21 to 22 in Area 1 and from 29 to 33 in Area 2. Standard lengths of females and males in pairs were positively correlated ($r = 0.62$, $p < 0.01$, N = 62). Females were usually larger than their mates but in 8 of 62 pairs (12.9%) males were equal to or larger than females.

Females produced one to nine clutches in one breeding season ($\bar{x} = 4.9 \pm 1.76$ SD, N = 62). Of females which spawned in two breeding seasons, 96.2% used the same hosts for spawning. The average size of sea anemones used for spawning was 1545 cm^2 ± 514.3 SD (N = 70, range = 542 to

2765). Of these sea anemones, 95.7% were larger than 800 cm^2.

Five pairs of A2- and B-nonbreeders were seen in Area 2 in September 1986. Among them, four individuals which occupied hosts larger than 800 cm^2 spawned by the next September, but one individual which included only hosts smaller than 800 cm^2 never spawned during the study period.

(3) Re-formation of pairs and mate acquisition of nonbreeders

During the study period, 14 pairs separated or disappeared and 25 pairs were newly formed. Of the 25 new pairs, seven were formed between breeders (Table 2) and eight between breeders and nonbreeders (Table 3). The remaining 10 were pairs of nonbreeders: six were between A2- and B-nonbreeders and four between A2-nonbreeders (Table 4). Pairs including at least one breeder usually started spawning within 2 months after pair formation, if they were formed by the middle of breeding season (Table 2, 3). Among pairs of nonbreeders, those between A2- and B-nonbreeders tended to spawn sooner than those between A2-nonbreeders (Table 4).

In 2 years, 20 A2-nonbreeders and seven B-nonbreeders obtained mates (Table 5). The ratio of solitary A2-nonbreeders to solitary B-nonbreeders was about 3 : 1 (22 : 5 in breeding season of 1986, 12 : 5 in nonbreeding season of 1986, 11 : 4 in breeding season of 1987). Per capita rate of mate acquisition did not differ significantly between A2- and B-nonbreeders (Fisher's exact probability test, $p = 0.26$). The frequency of mate acquisition was not different between the breeding and nonbreeding seasons.

Change of color phase

Of 106 individuals which were observed for more than 2 months, 47 changed their color phases; 19 of them changed more than once. All juveniles which changed coloration became A1-nonbreeders and all A1-nonbreeders which changed coloration became A2-nonbreeders (Table 6). Among A2-nonbreeders which changed coloration, about a half

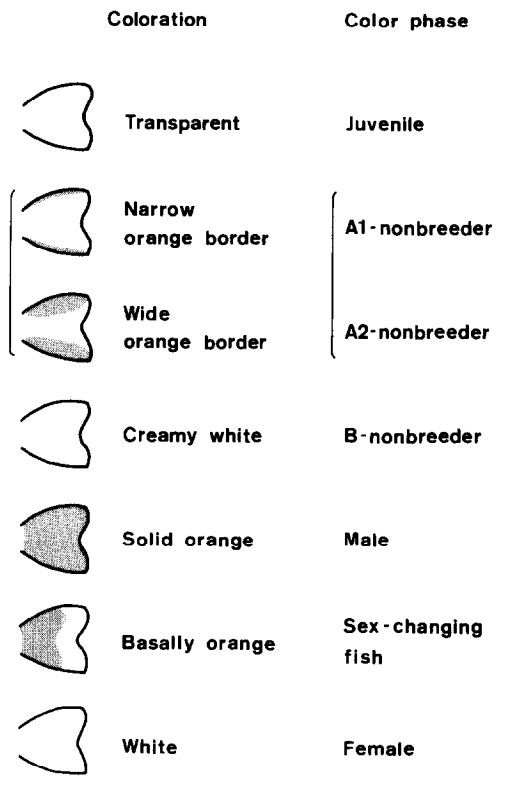

Fig. 1. Six color phases of the anemonefish at Murote Beach based on coloration of the caudal fin.

Table 1. Standard length of the anemonefish of six color phases. Data from May 1986 and from September 1986 and 1987 were used for Area 1 and Area 2, respectively.

Color phase	Standard length (mm)			
	N	Mean	SD	Range
<Area 1>				
Juvenile	6	55.3	3.0	51–60
A1-nonbreeder	10	65.0	3.1	61–68
A2-nonbreeder	9	69.2	3.0	66–75
B-nonbreeder	5	72.0	5.0	65–77
Sex-changing fish	2	84.0	0	84
Male	22	84.2	3.8	76–90
Female	22	87.1	3.9	79–95
<Area 2>				
Juvenile	50	53.2	6.9	41–67
A1-nonbreeder	15	63.7	3.3	58–69
A2-nonbreeder	24	72.8	4.0	69–81
B-nonbreeder	13	77.4	4.1	71–83
Sex-changing fish	3	83.0	2.5	80–86
Male	62	83.0	4.3	73–89
Female	59	87.2	3.6	79–94

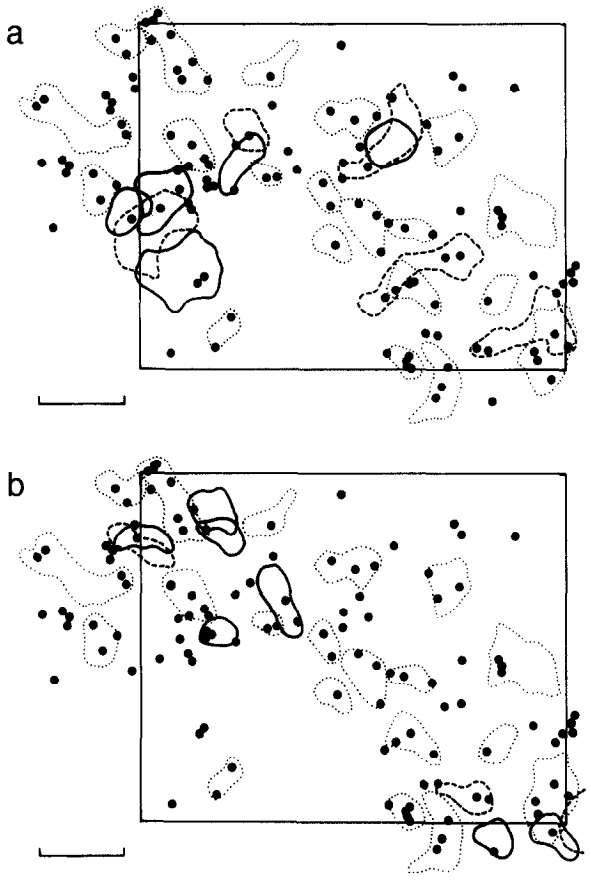

Fig. 2. Home ranges of the anemonefish at Area 1 in May 1986. Areas encircled with a dotted line are territories of breeding pairs. Solid circles indicate locations of sea anemones. Scales indicate 10 m. a- Home ranges of B-nonbreeders (solid line) and A2-nonbreeders (broken line). b- Home ranges of A1-nonbreeders (solid line) and juveniles (broken line).

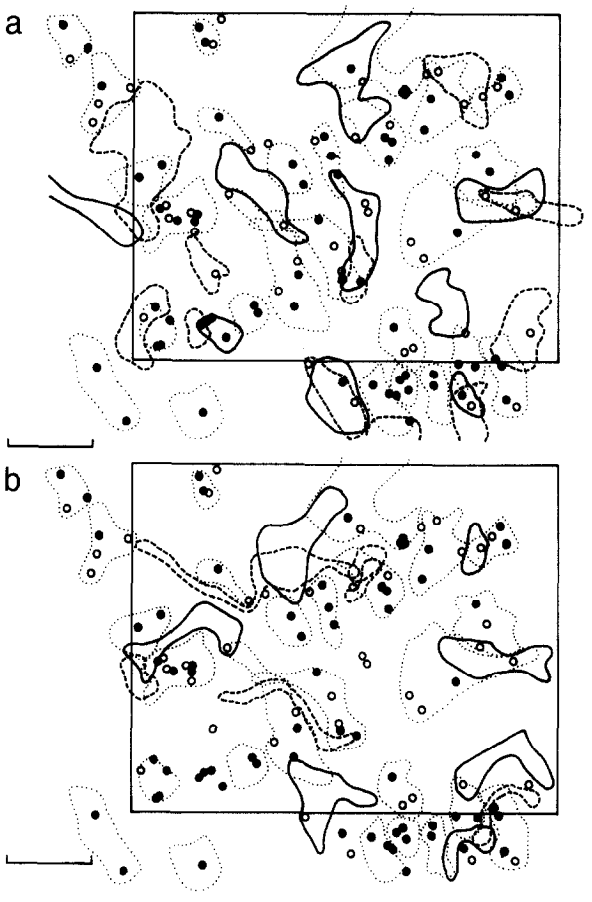

Fig. 3. Home ranges of the anemonefish at Area 2 in September 1986. Areas encircled with a dotted line are territories of breeding pairs. Solid and open circles indicate locations of sea anemones larger and smaller than 800 cm^2, respectively. Scales indicate 10 m. a- Home ranges of B-nonbreeders (solid line) and A2-nonbreeders (broken line). b- Home ranges of A1-nonbreeders (solid line) and juveniles (broken line).

(10/21) became B-nonbreeders and the rest became males. There was no significant size difference between these two groups of A2-nonbreeders (Table 6, t-test, $t_s = 1.82$, $df = 19$, $p > 0.05$). B-nonbreeders changed only into females and males only into females. Changes from males to females (= sex change) occurred on fewer occasions than changes from B-nonbreeders to females (4 vs. 11). Females never changed into other color phases. The time spent on the change of color phases varied a great deal from individual to individual.

Of 10 A2-nonbreeders which changed into B-nonbreeders, six were solitary and four were paired with other A2-nonbreeders when they started their color changes (Table 4). The latter four were all larger than their mates (76.5 mm ± 1.2 SD and 72.5 mm ± 1.1 SD, N = 4). The time spent in the color change was not significantly different between the solitary and paired fish ($\bar{x}$ = 44.2 days ± 13.7 SD, N = 6 and $\bar{x}$ = 41.0 days ± 6.7 SD, N = 4). Among solitary A2-nonbreeders, those which were changing into B-nonbreeders usually stayed more apart from females and were accordingly less frequently attacked by them than those which were not changing (Table 7). Among paired A2-nonbreeders, those which were changing into B-nonbreeders were less frequently attacked by

Table 2. Mate acquisition of breeders by pairing with other breeders and their first breeding.

Size (mm SL) and sex of immigrant fish	Size (mm SL) and sex of resident mate	Date of pair formation (A)	Date of breeding (B)	Interval (days) between (A) and (B)
86 Male	82 Female	22.6.1986	26.6.1986	4
83 Male	92 Female	2.6.1986	20.6.1986	18
74 Male	83 Female	16.6.1986	15.7.1986	29
86 Male	91 Female*	21.5.1986	14.7.1986	54
86 Male**	88 Male	6.9.1986	9.6.1987	273
84 Female	87 Male	19.5.1986	30.6.1986	42
89 Female	87 Male	before Apr 1987	This female was diandric and did not spawn with this male.	

* = This female was also an immigrant.
** = This male changed sex after pairing.

their mates and more frequently attacked their mates than those which were not changing (Table 8). Solitary B-nonbreeders never became females without pairing with other fish. Color changes from B-nonbreeders to females were so vague that an intermediate phase between B-nonbreeders and females could not be recognized. Paired B-nonbreeders were less frequently attacked by females and males than solitary B-nonbreeders (Table 9).

Color phase change of males (= sex change) occurred while they were paired with A2-nonbreeders (2 cases) or a male (1 case) (see Tables 2, 3). Two males which were paired with A2-nonbreeders were larger than their mates (83 vs. 78 and 80 vs. 75 mm SL). One male which was paired with another male was, however, a little smaller than his mate (86 vs. 88 mm SL); this male had been solitary and had started changing coloration before the pair formation.

Six gonad phases

The gonad structure of *A. clarkii* was categorized into six phases based on the state of spermatogenesis, oocyte development (see Selman & Wallace 1986), and the presence or absence of spermatocyte cysts, an ovarian cavity and ovigerous lamellae (Table 10, Fig. 4). Oogonia and spermatogonia could not be discriminated in this study.

(1) Ripe female phase – the gonad had an ovarian cavity and ovigerous lamellae with various developmental stages of oocytes, but did not have any spermatocytes, spermatids or sperm (Fig. 4a). This phase was further divided into two

Table 3. Mate acquisition of nonbreeders by pairing with breeders and their first breeding.

Size (mm SL) and color phase of immigrant fish	Size (mm SL) and sex of its mate	Date of pair formation (A)	Date of breeding (B)	Interval (days) between (A) and (B)
78 A2-nonbreeder	83 Male*	7.9.1987	No spawning	>23
75 A2-nonbreeder	80 Male*	23.9.1987	No spawning	> 7
70 A2-nonbreeder	83 Female	14.7.1986	16.7.1986	2
67 A2-nonbreeder	85 Female	4.6.1986	20.6.1986	16
67 A2-nonbreeder	87 Female	4.6.1986	25.6.1986	21
70 A2-nonbreeder	83 Female	8.6.1986	20.7.1986	42
75 A2-nonbreeder	92 Female	24.9.1986	9.6.1987	254
83 B-nonbreeder	81 Male	Aug 1987	No spawning	>40

* = These males changed sex after pairing.

Table 4. Mate acquisition of nonbreeders by pairing with other nonbreeders and their first breeding.

Size (mm SL) and color phase of nonbreeders which paired		Date of pair formation (A)	Date of breeding (B)	Interval (days) between (A) and (B)
75 B-nonbreeder	71 A2-nonbreeder	4.6.1987	24.6.1987	20
79 B-nonbreeder	74 A2-nonbreeder	6.6.1987	3.7.1987	27
76 B-nonbreeder	73 A2-nonbreeder	20.4.1987	23.6.1987	64
80 B-nonbreeder	72 A2-nonbreeder	9.6.1987	No spawning	>113
78 B-nonbreeder	72 A2-nonbreeder	23.5.1987	No spawning	>131
80 B-nonbreeder	68 A2-nonbreeder	18.7.1986	Disappeared by Apr 1987	
72 A2-nonbreeder*	64 A2-nonbreeder	23.5.1987	11.9.1987	111
73 A2-nonbreeder*	72 A2-nonbreeder	4.6.1986	17.7.1987	408
74 A2-nonbreeder*	68 A2-nonbreeder	10.7.1986	29.9.1987	354
78 A2-nonbreeder*	67 A2-nonbreeder	3.8.1986	Disappeared by Jan 1987	

* = These A2-nonbreeders became B-nonbreeders after pair formation.

subphases (I and II) according to the presence or absence of vitellogenic oocytes (Table 10).

(2) Pre-ripe female phase – the gonad had an ovarian cavity and ovigerous lamellae with only perinucleolus oocytes, but did not have any spermatocytes, spermatids or sperm (Fig. 4b).

(3) Transitional phase – the gonad had perinucleolus oocytes, spermatids and sperm, occasionally together with a few spermatocytes, but did not have spermatocyte cysts, an ovarian cavity and ovigerous lamellae (Fig. 4c, d).

(4) Ripe male phase – the gonad had a complex structure consisting of many spermatocyte cysts at various stages of spermatogenesis, spermatids, sperm and perinucleolus oocytes, but did not have an ovarian cavity and ovigerous lamellae (Fig. 4e).

(5) Pre-ripe male phase – the gonad had a few spermatocyte cysts, spermatids and sperm and perinucleolus oocytes, but did not have an ovarian cavity, ovigerous lamellae, and complex structure consisting of many spermatocyte cysts (Fig. 4f).

(6) Immature phase – the gonad included perinucleolus oocytes but did not have any spermatocytes, spermatids, sperm, or an ovarian cavity or ovigerous lamellae (Fig. 4g).

Gonads in the ripe female phase and pre-ripe female phase had only an ovarian structure. Gonads in the ripe male phase and pre-ripe male phase had both ovarian tissue and testicular tissue; the ovarian tissue, however, contained only immature oocytes at the perinucleolus stage. Immature phase gonads had no spermatocytes, spermatids and sperm but may include some spermatogonia. Gonads of transitional phase had spermatids or sperm

Table 5. Mate acquisition of the anemonefish in breeding season and nonbreeding season. The numbers of fish which changed into B-nonbreeders or females are in parentheses.

	1985 Non-breeding season	1986 Breeding season	1986 Non-breeding season	1987 Breeding season	Total
Fish which gained their mates	9	14	9	5	37
A1-nonbreeder	0	0	0	0	0
A2-nonbreeder	5 (1)	9 (3)	4	2	20 (4)
B-nonbreeder	1	1	4	1	7
Male	3 (1)	3 (1)	0	2 (2)	8 (4)
Female	0	1	1	0	2

Table 6. Changes of color phases in the anemonefish observed in Area 2 during May 1986 and September 1987.

Changes of color phase	Size of fish before change (mm SL)				Time spent in change (days)		
	N	Mean	SD	Range	Mean	SD	Range
J → A1	14	58.3	4.2	49–64	99	74	28–255
A1 → A2	19	64.1	4.2	54–70	68	31	17–133
A2 → M	11	70.3	3.4	65–76	73	49	26–166
A2 → B	10	73.0	3.4	67–78	32	12	26– 68
M → F	4[+]	83.3	2.5	80–86	52	33	27– 98
B → F[++]	11	–	–	–	–	–	–

J = Juvenile, A1 = A1-nonbreeder, A2 = A2-nonbreeder, B = B-nonbreeder, M = Male, F = Female, [+] = one was omitted from calculation, [++] = This color change was so vague that the exact dates of its beginning and completion could not be determined.

but did not have any spermatocyte cysts which precede spermatids and sperm. This means that these gonads are not at the intermediate state between the immature phase and the pre-ripe male phase, but between the pre-ripe male or male phase and the pre-ripe female or ripe female phase.

Relationship between color and gonad phases

All females and males had the gonads of ripe female phase and ripe male phase, respectively (Table 11). Of two sex-changing fish examined, one (which had started changing coloration 22 days before being sampled) had the transitional phase gonads and the other (which had started changing coloration 114 days before being sampled) had the pre-ripe female phase gonads. There were no substantial differences in the gonad structure between females which originated from males (N = 3) and those which originated from B-nonbreeders (N = 3). One male (93 mm SL) which did not change sex after disappearances of his mate and remained solitary for a year had the gonad of ripe male phase.

Table 7. Aggressive interactions of solitary A2-nonbreeders (per 15 min) with other conspecifics.

Color phase	Not changing into B-nonbreeder			Changing into B-nonbreeder			U-test
	Mean	SD	Range	Mean	SD	Range	
Attacked by							
Female	1.96	3.71	0–22	0.15	0.48	0–2	**
Male	0.52	1.22	0–7	0.50	1.02	0–3	NS
B-nonbreeder	0.19	0.75	0–6	0	0	0	–
A-nonbreeder	0.08	0.47	0–4	0	0	0	–
Juvenile	0.01	0.10	0–1	0	0	0	–
Attacked against							
Female	0.31	0.87	0–5	0.05	0.22	0–1	NS
Male	0.26	0.84	0–5	0.55	1.40	0–6	NS
B-nonbreeder	0.10	0.53	0–3	0	0	0	–
A-nonbreeder	0.35	1.10	0–7	0.45	1.96	0–9	NS
Juvenile	0.30	1.00	0–6	0.80	3.25	0–20	NS
Number of fish		29			20		
Observation time		1440 min			300 min		

** = $p < 0.01$, NS = not significant.

Table 8. Aggressive interactions of paired A2-nonbreeders (15 min) with other conspecifics.

Color phase	Not changing into B-nonbreeder			Changing into B-nonbreeder			U-test
	Mean	SD	Range	Mean	SD	Range	
Attacked by							
Mate	2.76	4.05	0–18	0.17	0.55	0–2	**
Female	0.68	2.12	0–11	0.33	0.62	0–2	NS
Male	0.38	1.28	0–8	0.25	0.60	0–2	NS
B-nonbreeder	0.06	0.42	0–3	0	0	0	NS
A-nonbreeder	0.06	0.31	0–2	0.08	0.28	0–1	NS
Juvenile	0	0	0	0	0	0	–
Attacked against							
Mate	0.74	1.57	0–9	2.08	3.66	0–13	**
Female	0.12	0.32	0–1	0.42	0.86	0–3	NS
Male	0.14	0.45	0–2	0.33	0.75	0–2	NS
B-nonbreeder	0.06	0.31	0–2	0.17	0.55	0–2	NS
A-nonbreeder	0.36	1.05	0–5	0	0	0	–
Juvenile	0.80	3.25	0–20	0.75	1.69	0–6	NS
Number of fish		13			4		
Observation time		750 min			180 min		

** = $p < 0.01$, NS = not significant.

Table 9. Aggressive interactions of B-nonbreeders (15 min) with other conspecifics.

Color phase	Solitary			Paired			U-test
	Mean	SD	Range	Mean	SD	Range	
Attacked by							
Mate	–	–	–	0.93	2.10	0–10	–
Female	1.88	4.26	0–22	0.22	0.73	0–3	**
Male	0.55	2.28	0–13	0.02	0.15	0–1	**
B-nonbreeder	0	0	0	0	0	0	–
A-nonbreeder	0	0	0	0.02	0.15	0–2	–
Juvenile	0	0	0	0	0	0	–
Attacked against							
Mate	–	–	–	3.51	5.12	0–16	–
Female	0.33	0.84	0–4	0.38	1.16	0–6	NS
Male	0.64	1.53	0–7	0.22	0.84	0–4	NS
B-nonbreeder	0.09	0.51	0–3	0.09	0.46	0–3	NS
A-nonbreeder	0.91	3.65	0–21	0.31	0.89	0–5	NS
Juvenile	0.45	1.13	0–4	0.56	1.29	0–5	NS
Number of fish		9			10		
Observation time		495 min			675 min		

** = $p < 0.01$, NS = not significant.

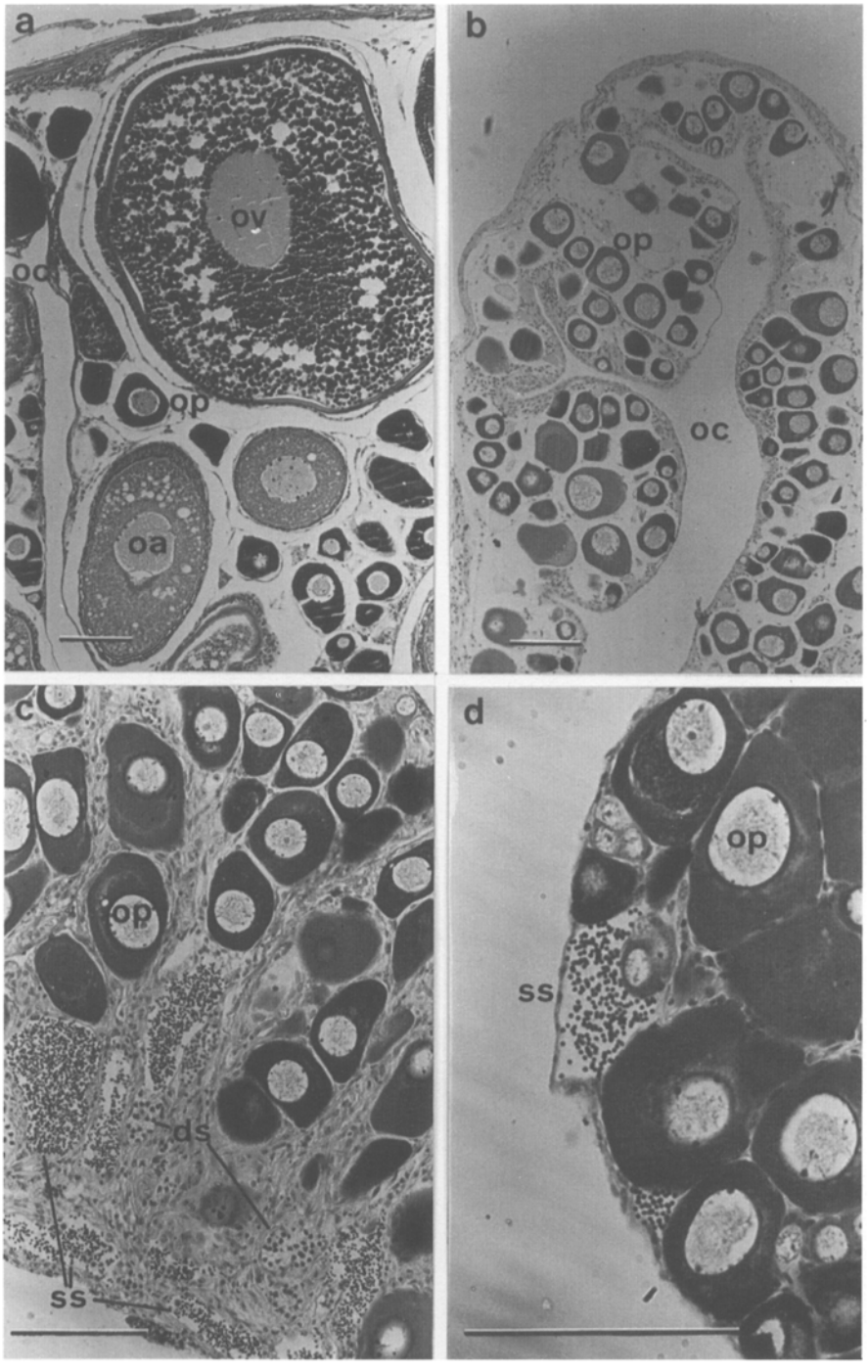

Fig. 4a–d. Gonad phases of the anemonefish. Scales indicate 100 μm. a- Ripe female gonad (90 mm SL, female), showing an ovarian cavity (oc) and oocytes in the perinucleolus state (op), cortical alveolus stage (oa) and vitellogenesis stage (ov). b- Pre-ripe female gonad (67 mm SL, B-nonbreeder), showing an ovarian cavity (oc) and oocytes in the perinucleolus stage (op). c- Transitional gonad (84 mm SL, sex-changing fish, 22 day after onset of sex change), showing spermatids and/or sperm (ss), degenerating spermatocyte cysts (ds) and oocytes in the perinucleolus stage (op). d- Transitional gonad (67 mm SL, B-nonbreeder), showing a few spermatid and/or sperm (ss) and oocytes in the perinucleolus stage (op). Neither spermatocyte cysts nor denuded spermatocytes are seen.

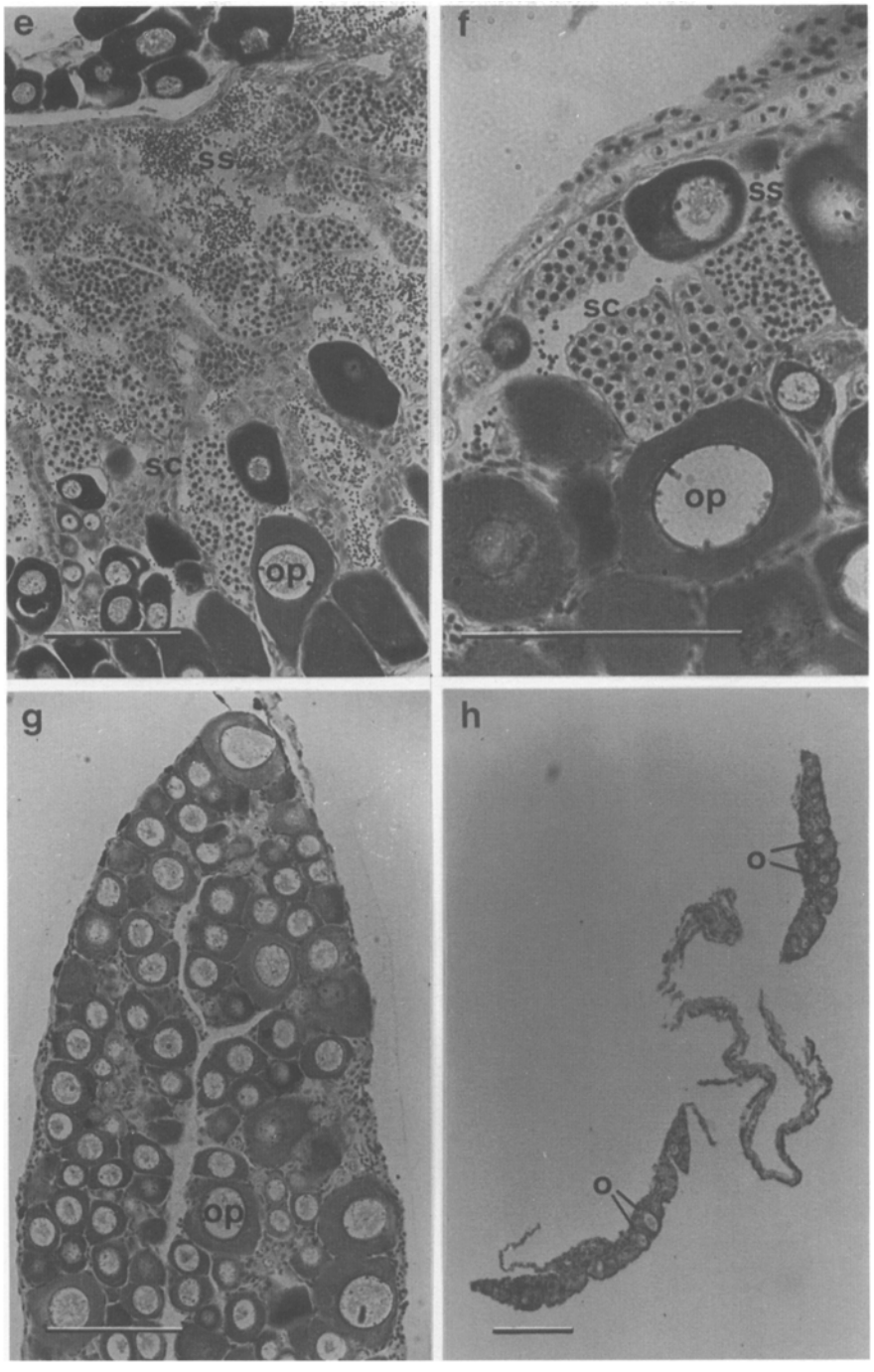

Fig. 4e–h. e- Ripe male gonad (89 mm SL, male), showing many spermatocyte cysts (sc) and spermatids and/or sperm (ss). Oocytes in the perinucleolus stage are also seen (op). f- Pre-ripe male gonad (68 mm SL, A2-nonbreeder), showing many occytes in the perinucleolus stage (op), a few spermatocyte cysts (sc) and spermatid and/or sperm (ss). g- Immature gonad (54 mm SL, Juvenile) which had only oocytes in the perinucleolus stage (op). h- Gonad of small juvenile (24 mm SL), showing oocytes before the perinucleolus stage (o).

Table 10. Gonad phases of the anemonefish determined by gonad features.

Features of gonad Gonad phase	ov	oa	oc	l	op	sc	sp	ss	cs
Immature	−	−	−	−	+	−	−	−	−
Pre-ripe male	−	−	−	−	+	+	+	+	−
Ripe male	−	−	−	−	+	+	+	+	+
Transitional	−	−	−	−	+	−	+/−	+	−
Pre-ripe female	−	−	+	+	+	−	−	−	−
Ripe female I	−	+	+	+	+	−	−	−	−
Ripe female II	+	+	+	+	+	−	−	−	−

ov = oocytes in the vitellogenesis stage, oa = oocytes in the cortical alveolus stage, oc = ovarian cavity, l = ovigerous lamellae, op = oocytes in the perinucleolus stage, sc = spermatocyte cysts, sp = spermatocytes, ss = spermatids and/or sperm, cs = complex structure consisted of many cysts, + = present, − = absent, +/− = few or absent.

Larger B-nonbreeders had the gonads of pre-ripe female phase or ripe female phase I, but the smallest three had the transitional or immature phase gonads (Fig. 5). Judging from body size, B-nonbreeders at the transitional or immature phase may have been regressing testicular tissue before differentiating into the pre-ripe female phase.

A-nonbreeders had the pre-ripe male phase or transitional phase gonads. Three A-nonbreeders at the transitional phase may have been at an intermediate state between the pre-ripe male phase and the pre-ripe female phase. There was no essential difference in the gonad structure between A1- and A2-nonbreeders, although A1-nonbreeders had a smaller number of spermatocyte cysts in the gonads.

Most of juveniles had the immature phase gonads (Table 11). Three relatively large juveniles had either pre-ripe male phase or transitional phase gonads (Fig. 5). Gonads of two of these juveniles may have started changing from the immature phase to the pre-ripe male phase. Gonads of the other one may have started changing from the immature phase to the pre-ripe female phase. Two juveniles less than 40 mm (24 and 37 mm SL) had immature gonads with young oocytes developing into the perinucleolus stage but had no spermatocytes (Fig. 4h).

Discussion

Life history pathways

In some sequentially hermaphroditic fishes, the change of body coloration accompanies an alternation of gonad structure resulting in sex change

Table 11. Correspondence between color phases and gonad phases in the anemonefish collected at Area 1.

Color phases Gonad phases	Juvenile	A-nonbreeder	Male	Sex-changing	B-nonbreeder	Female
Immature	11				2	
Pre-ripe male	2	14				
Ripe male			5			
Transitional	1	3		1	1	
Pre-ripe female				1	3	
Ripe female I					3	2
Ripe female II						7
Total	14	17	5	2	9	9

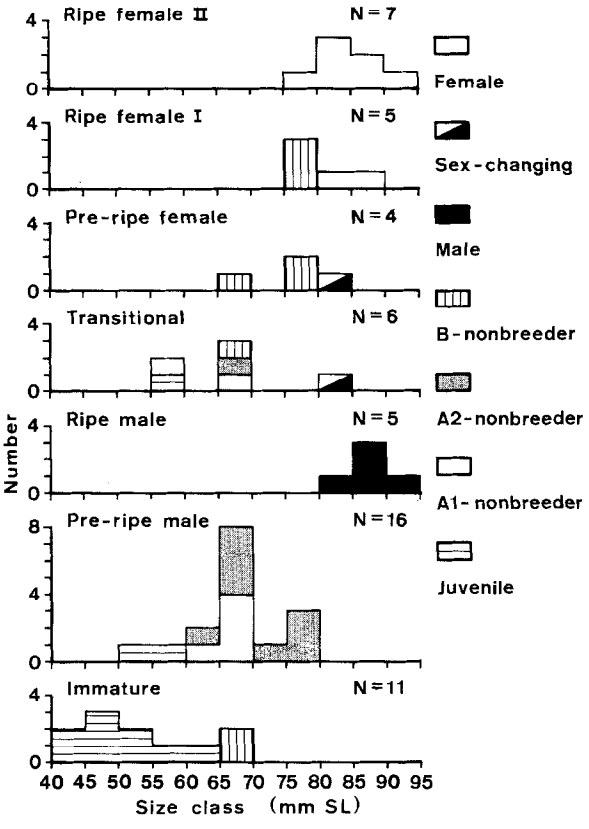

Fig. 5. Relationships of gonad phases to body size and color phases.

(Fishelson 1975, Robertson & Warner 1978, Warner & Robertson 1978, Ross et al. 1983). In the present study, caudal fins of *A. clarkii* changed coloration, not only when they changed sex but also when they were nonbreeders. Color phases corresponded approximately to the gonad phases; color phases of males and females corresponded to ripe male phase and ripe female phase, respectively, and color phases of juveniles, A-nonbreeders and B-nonbreeders to immature phase, pre-ripe male phase and pre-ripe female phase, respectively. One exception is the transitional phase of gonads. This gonad phase was included in four color phases, juveniles, A-nonbreeders, B-nonbreeders and sex-changing fish. Some discordances between the color phases and the gonad phases may be explained by a small time-lag between the change of coloration and that of gonadal conditions.

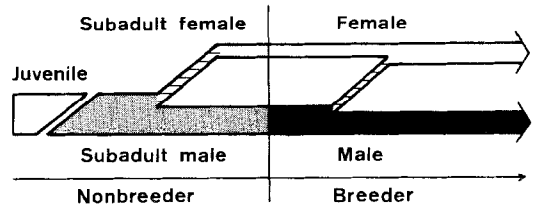

Fig. 6. Scheme of three life history pathways of the anemonefish at Murote Beach. Width of each line indicates relative frequency of each pathway.

Because of the fairly good correspondence of color phases to gonad phases, five life states can be distinguished on the basis of caudal fin coloration if the sex-changing color phase is not regarded as an independent life state. Fish of male and female color phases can be referred to as adult males and adult females, respectively, and fish of juvenile, A- and B-nonbreeder color phases as juveniles, subadult males and subadult females, respectively.

Observed patterns of color phase changes (Table 6) indicate that at least two life history pathways exist after the juvenile period (Fig. 6): (1) subadult male → subadult female → adult female, (2) subadult male → adult male → adult female. Males, especially larger ones, rarely changed sex and many males finished their careers as males (also see Ochi 1989a). Therefore, the third pathway, (3) subadult male → adult male, should be regarded as a distinct one (Fig. 6). Another life history pathway, juvenile → subadult female → adult female, can not be denied, since three subadult females (= B-nonbreeders) had immature phase or transitional phase gonads and one juvenile had transitional phase gonads (Table 11). However, this pathway was not confirmed by the observation of changes in the color phase (Table 6).

Femininity differentiation in hermaphroditic gonad

Warner & Robertson (1978) defined 'prematurational sex change' of protogynous hermaphrodites as sex change which occurs before the female ever functions as an adult, and 'postmaturational sex change' as sex change after a functional female state. However, the term 'prematurational sex

change', in reality, only means that the male or female parts of a hermaphroditic gonad have irreversibly differentiated before the breeder state. Strictly speaking, their wording is not appropriate, because sex change is a change from one functional sex to the other. The essential quality of hermaphrodites, especially in the nonbreeder state, is that they have the ability to select either sex rather than to change sex. Instead of 'prematurational sex change', we propose here the term 'femininity (or masculinity) differentiation in nonbreeder state' for protandrous (or protogynous) hermaphrodites.

Subadult females in *A. clarkii* can be regarded as having undergone femininity differentiation in the nonbreeder state, because (1) subadult females were derived from subadult males, and (2) subadult females matured only as females and never became functional males. The three life-history pathways found in this study are obviously due to the difference in the timing of femininity differentiation: it occurs in the nonbreeder state in the first pathway and in the breeder state (= sex change) in the second, but it never occurs in the lifetime in the third.

Ochi's (1989a) and our field observations suggest that the timing of femininity differentiation is not genetically determined but socially regulated, as in the sex change of tropical anemonefishes (Fricke & Fricke 1977, Ross 1978a, Fricke 1979, 1983). Femininity differentiation in the nonbreeder state occurred in paired subadult males whose mates were smaller than they (Table 4) and in solitary subadult males which had received only few attacks from females (Table 7). Femininity differentiation in the breeder state (= sex change) was mainly seen in males which had lost their mates and then re-paired with smaller males or subadult males (Table 2, 3, also see Ochi 1989a). Femininity differentiation did not occur in large males: they managed to pair with females after they lost their mates (see Ochi 1989a).

Most females of anemonefishes so far studied in the tropical waters are believed to have functioned as males (Fricke & Fricke 1977, Moyer & Nakazono 1978, Ross 1978a, Fricke 1979, 1983). In *A. clarkii* at Murote Beach, in contrast, the second pathway, which involves sex change, is not primary (Fig. 6). In this study, only four males changed sex, while 10 subadult males changed into subadult females and 11 subadult females became females. In Ochi's (1989a, b) study at Murote Beach, only three males changed sex, while 8 of 31 nonbreeders (probably most are subadults, but a few may have been solitary males) which gained breeding posts became directly females. These facts indicate that femininity differentiation in the nonbreeder state is common and most females are recruited directly from subadults.

Alternative life-history pathways, seen in *A. clarkii*, have been reported in many sequentially hermaphroditic fishes (reviewed by Charnov 1982, Shapiro 1984, Warner 1984, Sadovy & Shapiro 1987). Among protandrous fishes, individuals which function only as females in their lives are known in some gonostomatids (Fisher 1983, Badcock 1986) and some porgies (Lissa-Frau et al. 1976). They are referred to as 'prematurational sex changers' or 'primary females'. In protogynous fishes, 'primary males' (defined by Reinboth 1970) have been reported (Reinboth 1970, Warner et al. 1975, Robertson & Warner 1978, Warner & Robertson 1978, Warner & Hoffman 1980, Thresher 1984, Warner 1984) and 'prematurational sex changers' have been found to appear under some particular environmental or social conditions (Robertson & Warner 1978, Warner & Robertson 1978, Robertson et al. 1982, Hoffman 1983, 1985).

Some authors assume that primary males are genetical gonochorists, since their gonad structure is different from secondary males which have been derived from functional females (Reinboth 1970, Warner et al. 1975, Warner & Robertson 1978, Thresher 1984, Sadovy & Shapiro 1987). However, evidence of genetical control of sex determination in hermaphroditic fishes is poor (Yamamoto 1969, Warner 1978, Shapiro 1984, 1989). The process of sex differentiation in primary males may be conditional the same way as in *A. clarkii*. The difference of gonad structure between primary and secondary males may be simply due to the nature that matured female tissues tend to remain after they have regressed (Sadovy & Shapiro 1987, Cole & Robertson 1988). It seems that alternative life-history pathways so far found in sequentially hermaphro-

ditic fishes have resulted from flexible sex differentiation which may be triggered by the environmental and social conditions.

Significance of femininity differentiation in nonbreeder state

Since anemonefishes depend on host anemones, the distribution pattern of hosts strongly influences their social and mating systems (Allen 1972, Moyer & Sawyers 1973, Keenleyside 1979, Moyer 1980, Thresher 1984, Ochi & Yanagisawa 1987, Ochi 1986, 1989a, b). Protandry of anemonefishes has been regarded as an adaptation to low population density and unpredictable distribution of hosts and high predation pressures outside of hosts in tropical waters, which are the main habitat of anemonefishes (Fricke & Fricke 1977, Moyer & Nakazono 1978, Fricke 1979). There, one social unit of anemonefishes usually consists of a male-female breeding pair and a varying number of nonbreeders (Allen 1972, Fricke & Fricke 1977, Moyer & Nakazono 1978, Ross 1978b, Fricke 1979). A male changes sex after the disappearance of a female from the social unit, and one of the nonbreeders becomes a functional male after the disappearance or sex change of the resident male (Fricke & Fricke 1977, Moyer & Nakazono 1978, Ross 1978a, Fricke 1979, 1983).

In Murote Beach, however, host sea anemones usually occur in dense populations compared to tropical waters (Ochi 1986, 1989a, b, Yanagisawa & Ochi 1986, Ochi & Yanagisawa 1987). Under such conditions, *A. clarkii* moves between hosts and has no social unit typical of the tropical anemonefishes (Moyer & Sawyer 1973, Moyer & Nakazono 1978, Moyer 1980, Ochi 1986, 1989a, b, Yanagisawa & Ochi 1986, Ochi & Yanagisawa 1987). Subadults have home ranges outside of breeding pairs' territories. Solitary subadult males which were changing into subadult females received fewer aggressions from adult females than did solitary subadult males which were not changing into subadult females (Table 7). Pairs of subadults were formed in the interstices of breeding pairs' territories and either mate subsequently progressed femininity differentiation. These facts suggest that the presence of an adult female suppresses the femininity differentiation of subadults.

Per capita rate of mate acquisition by subadult females was not different from that of subadult males (Table 5). Subadult females also usually started spawning sooner than subadult males did, after they paired with subadult males (Table 4). These results suggest that, for solitary subadult males, femininity differentiation is not less successful than to remain at the pre-ripe male phase until they obtain the mates. Success of the two choices may depend on the number of adjacent subadult males which adopt the same choice.

Allen (1972) suggested that *A. clarkii*, inhabiting both tropical and temperate waters, is less dependent on host sea anemones and a more efficient swimmer than other anemonefishes. This may mean that this species has no typical social unit even in tropical waters. Actually, our preliminary field study (Hattori unpublished) had revealed that this species has no typical social unit and some nonbreeders dwell outside of the breeding pairs' territories in the tropical reefs of the Ryukyu Islands, Japan. We suspect that nonbreeders of *A. clarkii* in tropical waters can also become females without functioning as males if an opportunity arises. This possibility has also been suggested for other tropical anemonefishes (Fricke & Fricke 1977, Fricke 1979, Thresher 1984). At present, it is not known whether femininity differentiation in the nonbreeder state is characteristic of *A. clarkii* in temperate waters or in both temperate and tropical waters, and whether it is common to some groups of anemonefishes. This problem is currently under investigation to assess the significance of femininity differentiation in the nonbreeder state.

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