

# A snail with unbiased population sex ratios but highly biased brood sex ratios

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Extraordinary sex ratio patterns and the underlying sex-determining mechanisms in various organisms are worth investigating, particularly because they shed light on adaptive sex-ratio adjustment. Here, we report an extremely large variation in the brood sex ratio in the freshwater snail, *Pomacea canaliculata*. In eight rearing series originating from three wild populations, sex ratios were highly variable among broods, ranging continuously from almost exclusively males to almost exclusively females. However, sex ratios were similar between broods from the same mating pair, indicating that sex ratio is a family trait. Irrespective of the large variations, the average sex ratios in all rearing series were not significantly different from 0.5. We argue that Fisher's adaptive sex-ratio theory can explain the equal average sex ratios, and the results, in turn, directly support Fisher's theory. Polyfactorial sex determination (in which sex is determined by three or more genetic factors) is suggested as the most likely mechanism producing the variable brood sex ratio.

**Keywords:** sex ratio; variation; polygene; *Pomacea canaliculata*

## 1. INTRODUCTION

Adaptive sex-ratio adjustment has been paid serious theoretical and empirical attention in evolutionary biology since Fisher's classic work (Fisher 1930). Fisher suggested that population sex ratios should become 0.5 (proportion of males) under random mating because frequency-dependent selection promotes equal parental expenditure on sons and daughters. Fisher's theory has traditionally been tested against organisms in which its premises do not hold (Hamilton 1967). For instance, sex ratios should be female-biased under local mate competition between males in some insects and mites (Hamilton 1967) or male-biased under local resource competition between females in some mammals (Clarke 1978).

In contrast to the adaptive significance of sex-ratio adjustment, little attention has been paid to sex-ratio patterns and their underlying sex-determining mechanisms in organisms other than vertebrates and arthropods. However, such information is important for a better understanding of sex itself (see Bull 1983) and especially of adaptive sex-ratio adjustment (Cook 2002). For instance, one might think that the occurrence of a 0.5 sex ratio in an organism with random mating would directly support Fisher's theory. However, this is problematic, because, in most organisms with separate sexes, sex is determined by a pair of sex chromosomes (heterogamety) and a binomial distribution of sex ratios *ca.* 0.5 is expected under this mechanism without any adaptive reasons (Williams 1979). The widespread heterogamety among different organisms may, itself, be a result of natural selection towards equal sex ratios, but the occurrence of a 0.5 sex ratio in an organism with sex-determining mechanisms allowing variable sex ratios would directly support Fisher's theory. To

date, there has been little direct support for it (Conover & Van Voorhees 1990).

The apple snail, *Pomacea canaliculata* (Lamarck), is a freshwater ampullariid gastropod originating from South America. It was deliberately introduced into many Asian countries for human food, and has become a major pest of rice (Yusa & Wada 1999). In Japanese paddy fields, the snails actively mate and spawn from July to September (Tanaka *et al.* 1999). Egg masses, typically containing 50–300 eggs, are laid on plants above water, and hatching occurs within two weeks. After hatching, young snails grow until October, when the fields are drained (Tanaka *et al.* 1999). The snails bury themselves in the soil, and some young snails survive over winter (while most adults die; Oya *et al.* 1987). After the fields are flooded to transplant rice seedlings in June, the young snails grow rapidly to reach sexual maturity.

In the course of studying the reproductive ecology of the snails, we found an extremely large variation in their brood sex ratios. We report the pattern of sex-ratio variations, discuss the adaptive significance of this phenomenon and suggest possible underlying mechanisms.

## 2. MATERIAL AND METHODS

### (a) *Field-collected spawn*

Spawn of *P. canaliculata* were collected in a rice field where no pesticides had been used in Shichijo Town, Kumamoto Prefecture, southwest Japan (32°57' N; 130°45' E). Between 25 and 30 spawn laid on stems of rice plants or weeds were collected each month from July to September 1999. After collection, each spawn was weighed, kept in a Petri dish (9 cm in diameter and 2 cm in height), and placed in a screen-house under natural temperature and light conditions but sheltered from direct sunlight and rainfall. When eggs hatched, up to 40 hatchlings (average of 35.2) were randomly collected from each spawn and reared in 2 l aquaria. After a further few days, the numbers of hatched and unhatched eggs were counted to calculate hatchability, egg number per spawn and egg weight. The

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aquaria were covered with 1 mm mesh to allow water exchange and to prevent snail escape. They were placed in three large outdoor tanks (100 cm × 200 cm × 10 cm) with a continuous water flow. Snails were fed four to six times per week with commercial food pellets formulated for carp ('Hikari', Kyorin Co., Himeji, Japan) and the amount fed depended on their density and size. A small amount of powdered oyster shell was occasionally added to provide calcium. After at least 50 days of rearing, snails with a shell height of 14 mm or more were dissected to identify their sex, determined by the presence or absence of testes near the apex. Using albino males, whose testes are visible through their shells without dissection, it was shown that all males develop testes by this size and do not subsequently change sex. Smaller snails were reared until they reached the 14 mm shell size before dissection.

Snails from spawn collected in September did not grow well because of the decreasing water temperature. The tanks were therefore heated to 18–21 °C from December until the end of the experiment in February. Laboratory experiments show that water temperature does not affect sex ratios in this snail (Y. Yusa, unpublished data).

### (b) Pairing experiment

Thirty mating pairs were collected in a rice field in Shichijo Town (3 km from the field where the spawn were collected) in September 1998. Each pair was reared in a 2 l aquarium (without mesh) under outdoor conditions. They were fed carp food pellets, and the water was changed at least once a week. Spawning of females was observed daily. The second and fourth spawn of each pair were collected, kept in Petri dishes, weighed 3–6 days after spawning, and allowed to hatch under laboratory conditions (25 °C, 14 L : 10 D). A maximum of 80 hatchlings (average of 61.4) were randomly collected from each spawn and reared in a 20 l aquarium, with aeration, in the laboratory. They were fed carp food pellets, and the water was changed once in two weeks. Snails were sexed as previously described.

In 1999, offspring of snails used in 1998 were used in our experiments. To assure paternity, immature snails with a shell height of 14–18 mm were sexed by slightly opening the operculum to check the presence or absence of a penis sheath, and then paired. Pairs were reared in 2 l aquaria under laboratory conditions. The second spawn of each pair were collected, as well as the fourth of nine randomly selected pairs. A maximum of 40 hatched snails per spawn (average of 36.8) were reared and sexed in the same way as in 1998.

In April 2001, snails were collected in lotus fields in Kumamoto City (ca. 25 km from both collection sites in Shichijo Town). They were brought to the laboratory, reared in pairs, and allowed to spawn as in 1999. Most were immature when collected, but females with a shell height greater than 18 mm were reared with male partners for at least two months before spawn were collected to assure paternity (females store sperm for up to 41 days; Estebenet & Cazzaniga 1993). Forty hatchlings per spawn were reared in the same way as the field-collected spawn.

### (c) Statistical analyses

Because of the large variations in the sex ratio, non-parametric statistics were used, except for descriptive purposes. All the statistical analyses were conducted with STATVIEW 5.0.1 (SAS Institute Inc. 1998).

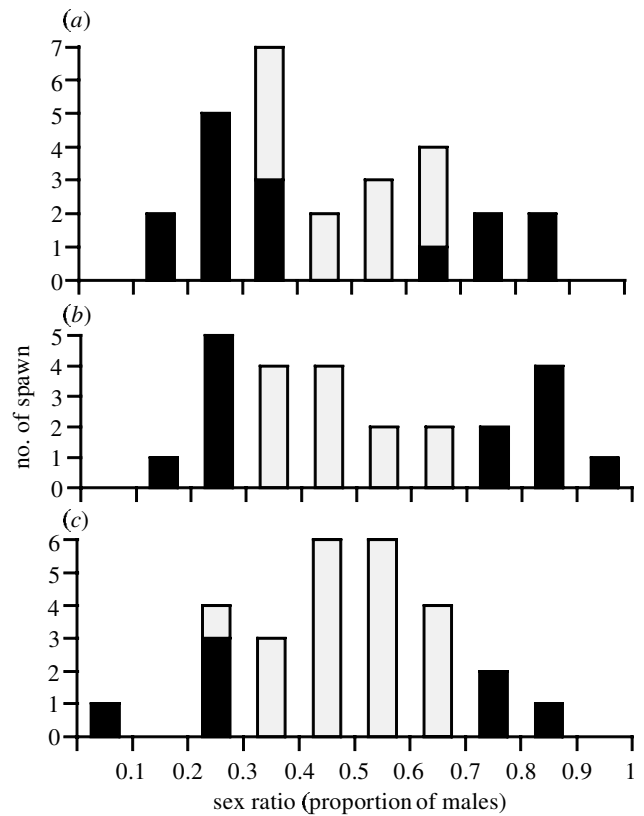


Figure 1. Brood sex ratio (proportion of males) of field-collected spawn. Black areas denote spawn whose sex ratios are significantly different from 0.5 (binomial test). (a) July; (b) August; (c) September.

## 3. RESULTS

### (a) Field-collected spawn

Most hatchlings (average of 97–98%) survived during rearing for at least 50 days, after which their sexes were identified. The sex ratio, defined as the proportion of males among all snails sexed (maximum of 40 individuals) in a brood, ranged continuously from almost exclusively males to almost exclusively females in all sampling months (figure 1). The highest brood sex ratio (0.95) occurred in the August sampling (38 males : 2 females), and the lowest (0.00) in September (0 males : 22 females).

In the July sampling, the sex ratio was significantly different ( $p < 0.05$  by binomial test) from 0.5 in 15 out of 27 spawn. This proportion is much higher than the proportion expected by chance (only one or two out of 27 spawn are expected to be statistically significant at  $p = 0.05$ ). Likewise, the sex ratio was significantly biased in 13 out of 25 spawn in the August sampling, and in 7 out of 27 in the September sampling. Irrespective of the large variation among spawn, the average sex ratio was very close to 0.5 in all three sampling months (mean  $\pm$  s.d. =  $0.47 \pm 0.20$  in July;  $0.53 \pm 0.25$  in August;  $0.47 \pm 0.18$  in September). The numbers of spawn with sex ratios lower than 0.5 and those higher were not statistically different (16 : 11 in July; 14 : 11 in August; 14 : 13 in September; all  $p > 0.4$  by binomial test;  $p > 0.3$  when the data were combined).

Among the possible factors affecting the sex ratio (table 1), a significantly negative correlation was only found between the sex ratio and the weight of an egg in the Sep-

Table 1. Kendall's rank coefficient ( $\tau$ ) between life-history traits and brood sex ratio (proportion of males). The weight of the mother or father was measured when the first spawn was observed in the whole culture.

	field (July)	field (Aug.)	field (Sept.)	Shichijo (1998)		Shichijo (1999)		Kumamoto
				2nd spawn	4th spawn	2nd spawn	4th spawn	
weight of mother	—	—	—	-0.03	0.01	-0.29*	-0.28	0.14
weight of father	—	—	—	-0.18	-0.06	0.11	-0.17	0.01
weight of an egg	-0.19	-0.12	-0.36**	-0.36*	-0.45**	-0.15	-0.17	-0.20
no. of eggs/spawn	0.12	-0.11	0.11	0.15	0.52**	0.15	0.28	0.03
hatchability	0.13	-0.18	-0.12	0.15	0.22	0.01	0.11	0.09
survival rate	0.04	-0.02	-0.14	0.38*	-0.07	0.02	-0.06	-0.27
no. of spawn studied	27	25	27	23	18	25	9	15

\*  $p < 0.05$ ; \*\*  $p < 0.01$ .

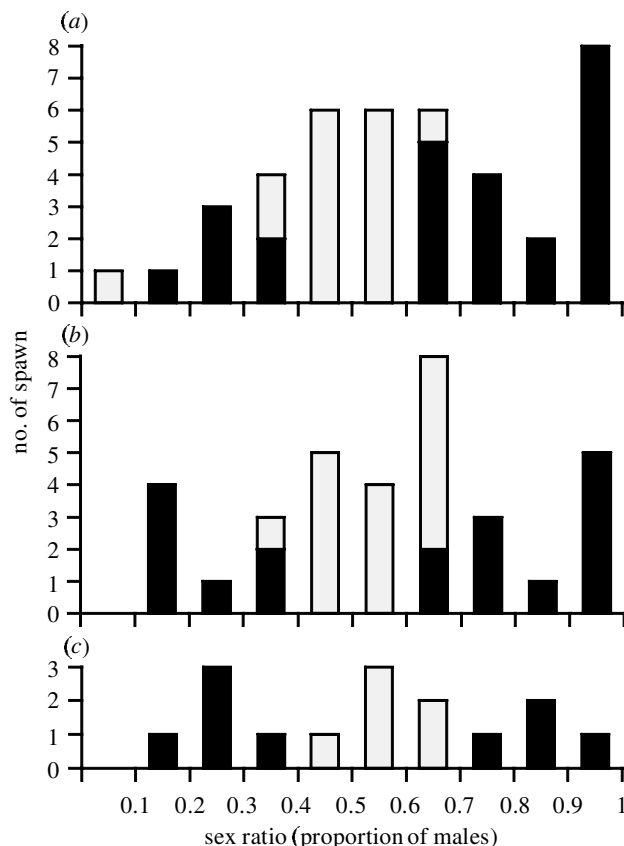


Figure 2. Brood sex ratio (proportion of males) of laboratory-laid spawn. The second and fourth spawn of each pair were collected in 1998 and 1999. Black areas denote spawn whose sex ratios are significantly different from 0.5 (binomial test). (a) 1998; (b) 1999; (c) 2001.

tember sampling. The number of eggs per spawn, hatchability and survival rate were not correlated with the sex ratio in any month.

### (b) Pairing experiment

The average survival rate during rearing was 98% in 1998, 94% in 1999 and 98% in 2001. As in the field-collected spawn, large variations in sex ratios were found in spawn in all rearing series (figure 2). The highest brood sex ratio (1.00) occurred in 1998 (80 males : 0 females), and the lowest (0.00) also in 1998, but in this case only four hatchlings were available (0 males : 4 females). The second lowest sex ratio (0.13) occurred in 2001 (5 males : 35 females).

Sex ratios of 25 : 41 spawn were significantly different ( $p < 0.05$  in binomial test) from 0.5 in 1998, in 18 : 34 spawn in 1999, and in 9 : 15 in 2001. These proportions were all much higher than expected by chance. Sex ratios of the spawn appeared to be slightly male biased in 1998 (mean  $\pm$  s.d. in the second spawn,  $0.61 \pm 0.23$ ,  $n = 23$ ; in the fourth spawn,  $0.57 \pm 0.28$ ,  $n = 18$ ) and in 1999 (in the second spawn,  $0.56 \pm 0.24$ ,  $n = 25$ ; in the fourth spawn,  $0.59 \pm 0.27$ ,  $n = 9$ ), but almost equal in 2001 ( $0.52 \pm 0.25$ ,  $n = 15$ ). The number of spawn with sex ratios lower than 0.5 and those higher were not statistically different in 1998 (8 : 15 in the second spawn; 8 : 10 in the fourth spawn), in 1999 (10 : 15 in the second spawn; 3 : 6 in the fourth spawn), or in 2001 (7 : 8; all  $p > 0.2$  by binomial test;  $p > 0.05$  when all the data were combined).

Among the possible reproductive or growth characteristics related to the sex ratio (table 1), the weight of an egg had a significantly negative correlation with the sex ratio in both the second and fourth spawn in 1998. Other characteristics, such as the egg number per spawn in the fourth spawn in 1998, had a significant relationship with the sex ratio in one of the rearing series (table 1). However, they did not have a consistent relationship in the other rearing series.

To test the effect of food availability on the sex ratio, snails reared from each spawn were divided at sex identification in 1998 into two equal groups of small and large shells. The sex ratio was then calculated separately for each group. There was a positive correlation between sex

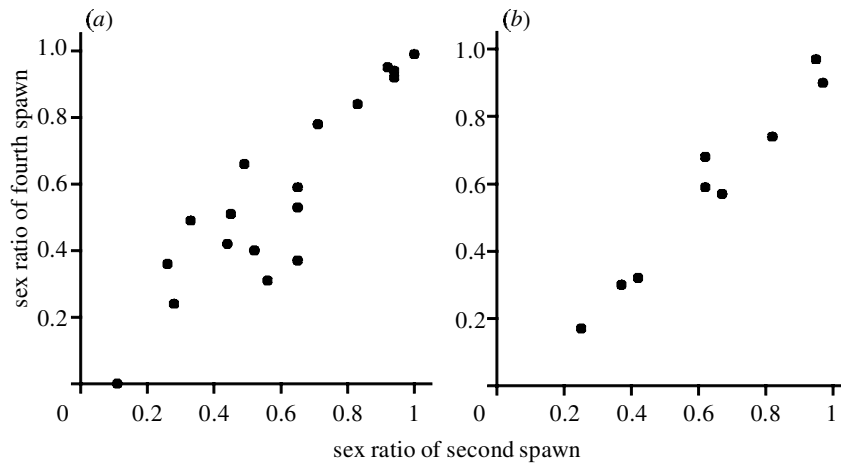


Figure 3. Relationship between sex ratios (proportion of males) of the second and fourth spawn from the same pairs. (a) 1998; (b) 1999.

ratios of the larger and smaller groups of snails from the same spawn ( $n = 23$ ,  $\tau = 0.70$ ,  $p < 0.001$  in the second spawn;  $n = 18$ ,  $\tau = 0.61$ ,  $p < 0.001$  in the fourth; Kendall's rank correlation). Sex ratios were not significantly different between larger and smaller groups from the same spawn ( $p > 0.4$  in the second spawn;  $p > 0.8$  in the fourth; Wilcoxon's signed-ranks test).

There was a highly positive correlation between sex ratios of the second and fourth spawn from the same pair in 1998 and 1999 (figure 3;  $n = 18$ ,  $\tau = 0.70$ ,  $p < 0.001$  in 1998;  $n = 9$ ,  $\tau = 0.81$ ,  $p < 0.01$  in 1999; Kendall's rank correlation).

#### 4. DISCUSSION

The brood sex ratio of *P. canaliculata* varied greatly, from almost exclusively sons to almost exclusively daughters in all rearing series. However, sex ratios of different spawn from the same mating pair were similar, indicating that the brood sex ratio is a family trait. To the best of our knowledge, such a large sex-ratio variation among families has not been reported before in gastropods. It has been seen in few other animals, such as some poeciliid fish (reviewed in Bull 1983), a copepod, *Tisbe gracilis* (Battaglia 1958), a wasp, *Nasonia vitripennis* (Werren *et al.* 1981), an oyster, *Crassostrea virginica* (Haley 1977) and a polychaete, *Ophryotrocha labronica* (Akesson 1970, 1972; Premoli *et al.* 1996).

In contrast to the great variation in brood sex ratios, average sex ratios in populations reared under different conditions were all close to 0.5. Moreover, differential values of hatchability and mortality are rejected as the main causes of the sex-ratio variation, since they were not correlated with the sex ratio in most cases. Thus, *P. canaliculata* is probably the only known animal clearly showing both highly variable brood sex ratios and equal population sex ratios. In the polychaete worms, the population sex ratio ranges from 0.5 (Premoli *et al.* 1996) to female-biased (Akesson 1970, 1972). However, sex ratio appears to be male-biased in the oyster (Haley 1977), although in these cases hatchability, mortality or later sex change might have modified the primary sex ratio.

Although the precise sex-determining mechanism in *P. canaliculata* remains unknown (see below), the large vari-

ation of sex ratio in this snail is not explicable solely by heterogamy. Thus, the equal population sex ratios are not the consequences of heterogamy. Instead, they can be explained by frequency-dependent selection promoting equal sex ratios (Fisher 1930).

Therefore, the results in this study directly support Fisher's sex ratio theory. Ideally, support for Fisher's theory would require evidence for the following: (i) variable brood sex ratios that warrant evolutionary shifts; (ii) occurrences of 0.5 population sex ratios; and (iii) operation of frequency-dependent selection towards the unbiased sex ratios. The first criterion, variable sex ratios, clearly holds in this snail. Second, unbiased population sex ratios actually occurred in different rearing series originating from three wild populations. The three populations were at least 3 km apart from each other and were separated by hills or rivers. Hence, they can be regarded as independent populations in the short term. The third criterion, operation of frequency-dependent selection, was not demonstrated in this study. As far as we are aware, it has only been demonstrated in laboratory populations of *Menidia menidia* (Conover & Van Voorhees 1990), by monitoring changes in sex ratios for some generations. However, *P. canaliculata* may give an ideal opportunity to test this in the field, because its distribution is still expanding (Yusa & Wada 1999). Thus, observations on sex ratios in newly invaded populations and their subsequent changes would be very interesting.

Among life-history traits possibly related to the sex ratio, only egg weight had a significantly negative relationship in more than one rearing series. The negative relationship means that female-producing eggs tend to be heavier than male-producing eggs, and suggests an underlying adaptive significance, such as the greater advantage to females of attaining a large size. The relationship between egg weight and the sex ratio would also mean that actual parental expenditure is more female biased than the observed sex ratios in number. However, we think that equal expenditure on male and female offspring holds at the population level, because average sex ratios were almost 0.5, even in the rearing series where the relationship between egg weight and the sex ratio was not significant. In turn, the relationship might be a possible reason for the slightly (but not significantly) male-biased sex

ratios observed in some rearing series. In fact, the highest (0.61) and third highest (0.57) population sex ratios occurred in 1998, when the negative correlation coefficient between sex ratio and egg weight was second highest ( $-0.36$ ) and the highest ( $-0.45$ ), respectively.

When the population sex ratio is at equilibrium, parents with a wide range of brood sex ratios have almost equal fitness (Williams 1979). This condition allows sex-ratio variation in *P. canaliculata*. However, why sex ratios should vary in this snail, and not in most other organisms is a different problem. Unfortunately, we cannot, as yet, identify the adaptive reason for the sex-ratio variation (Hardy 1992). A possible reason, although non-adaptive, might be that mixing of populations with different sex-determining systems caused the variable sex ratios. In fact, the snails were introduced to Japan by different routes (Yusa & Wada 1999) and possibly had different origins. To test the theory, sex-ratio variation of populations should be studied in their original habitats.

Concerning the possible mechanism producing the large sex-ratio variation, polyfactorial sex determination (i.e. three or more genetic factors determine sex; Bull 1983) appears to be the most probable. In fact, a between-family sex-ratio variation is one of the three diagnoses of polyfactorial sex determination (Bull 1983). The other two diagnoses are parental effects on the sex ratio and a response of the sex ratio to selection. Studies investigating parental effects on the sex ratio are now progressing; the results suggest the presence of such effects (Y. Yusa, unpublished data). Moreover, polyfactorial sex determination agrees with the hypothesis that variable sex ratios in this snail occurred by mixing of populations with different sex-determining factors.

An example of polyfactorial sex determination is found in a poeciliid fish, *Xiphophorus maculatus*. In this fish, individual sex is determined by the combination of three genetic factors, X, Y and W (Bull 1983). In this case, the family sex ratio is variable among pairs: 0.5 in four of the six types of mating, 1.0 in another type of mating and 0.25 in the remaining one. In *P. canaliculata*, the variation in brood sex ratio is more continuous, suggesting a more complicated polyfactorial genetic system.

However, polyfactorial sex determination is not the only possible cause of the large between-family sex-ratio variation. For instance, different proportions of X-carrying and Y-carrying sperm used for fertilization will lead to the variable sex ratios even under heterogamety, as occurs in bulls and boars (Chandler *et al.* 1998). Cytological evidence has given contradictory results concerning heterogamety in *P. canaliculata*: von Brand *et al.* (1990) suggested the presence of X and Y chromosomes, whereas Mercado Laczkó & Lopretto (1998) reported their absence. It is possible that these snails have different sex-determining systems. However, a further study, using a banding technique, is required to confirm this possibility, as chromosomes of *P. canaliculata* are relatively small and numerous ( $2n = 28$ ) and difficult to distinguish from each other.

Environmental sex determination, where sex is determined by such factors as temperature, day length or food availability (Bull 1983; Cook 2002), also causes sex-ratio variation. However, in this study, the average sex ratios of snails reared under outdoor conditions were almost identical

among different sampling months, suggesting that day length and water temperature did not affect the sex ratio. Sex ratios of larger and smaller snails within the same brood were not significantly different. Thus, food availability probably did not affect sex determination. A more detailed study of the effects of various environmental factors on the sex ratio also confirms the absence of such effects (Y. Yusa, unpublished data).

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