Insights into sexual size and shape dimorphism in *Semisulcospira niponica* (Gastropoda, Semisulcospiridae)

Running title: Sexual dimorphism in *Semisulcospira niponica*

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ABSTRACT

Considerable intraspecific variation has been known in the shell morphology of the freshwater snail genus *Semisulcospira*. However, sexual dimorphism and allometric growth have not been elucidated in the genus, although these factors contribute to generating intraspecific variation. We used a combination of geometric (GMM) and traditional morphometrics methods (TMM) in a single population of *S. niponica* to assess sexual differences in shell size and shape at maturity. The results of a generalised Procrustes analysis of variance revealed significant differences in shell size and shape between males and females. A principal component analysis showed allometric differences between males and females; PC1 values and their overlap between the sexes decreased with size. PC1 explained 35.5% of the total variance, which corresponded to vertical elongation of the shell spire and aperture, and broadening of the shell. The results of a canonical variate analysis using the ten largest specimens of each sex showed that females have less elongate shells with rounder apertures and have broader body and penultimate whorls than males. TMM using nine morphological characters supported sexual shell morphological differences and correlations between shell size and shape associated with different growth stages. However, GMM was more sensitive for detecting shape differences than TMM. For example, TMM explained the observed increase in shell diameter primarily as a function of size, whereas GMM detected sexual differences in shell diameter as shape differences between the sexes. Furthermore, dimorphism and allometry in shell sculpture traits could be explored only by TMM. For accurate evaluation of shell morphology in *Semisulcospira*, it is important to use larger specimens after separating males and females since the present results revealed sexual differences in size and shape, some of which become more evident with age. It is also essential to employ both GMM and TMM because the two methods capture different aspects of morphological variation in shell outline and sculpture.

INTRODUCTION

Sexual dimorphism and allometric growth contribute to generating shell morphological variation in molluscs. Sexual dimorphism refers to differences in phenotypic traits between males and females within the same species (Shine, 1989) and has been demonstrated in many gastropods and bivalves (*e.g.* Joaquino *et al.*, 2017; Sawangproh *et al.*, 2021).

Information on sexual dimorphism in freshwater snails has been elucidated in the family Viviparidae with traditional (TMM) and geometric (GMM) morphometric methods; the former measures a linear distance between two homologous points on the shell, whereas the latter permits the analysis of homologous points together with shape information using landmarks and semi-landmarks. It has been indicated that GMM has an advantage over earlier methods in capturing shape variation in the family (Minton & Wang, 2011; Moneva *et al.*, 2012). Sexual size differences in viviparid snails have been identified, with females possessing larger shells than males (Jokinen, Guerette & Kortmann, 1982; Brown, Varza & Richardson, 1989; Sawangproh *et al.*, 2021). Shape differences were also revealed, with females having a more globose shell and a wider aperture and body whorl (Moneva *et al.*, 2012; Uvayeva *et al.*, 2021), suggesting the differences allow females to brood embryos more successfully (Minton & Wang, 2011). Similar sexual size and/or shape differences have been observed in other freshwater snails: *Elimia* Adams & Adams, 1854 (Branson, 1971; Richardson & Scheiring, 1994); *Melanoides* Olivier, 1804 (Brande *et al.*, 1996); *Pomacea* Perry, 1810 (Cabuga *et al.*, 2017; Tamburi, Seuffert & Martín, 2018).

Differences in shell growth rate also are a potential source of morphological variation in gastropods and have been observed in many gastropod taxa (Urdy *et al.*, 2010). Different allometric patterns result in different shell morphology among groups such as sexes and can also contribute to sexual dimorphism in adult gastropod shells (Stamps, 1993). As with sexual dimorphism, allometric growth in freshwater snails has been well studied in Viviparidae, and different allometric trajectories between the sexes have been observed to generate sexual dimorphism in shell shape among members of *Viviparus* Montfort, 1810 (Berezkina & Zotin, 2013; Uvayeva *et al.*, 2021). In addition, Minton &

Wang (2011) reported that the dimorphism was caused by inherent shape differences between males and females in the viviparid *Callinina subpurpurea* (Say, 1829).

The genus *Semisulcospira* Boettger, 1886 is widely distributed in Japan, Korea, China, and Taiwan and is distinguished by its unique viviparous reproductive mode among the four genera of Semisulcospiridae Morrison, 1952 (Strong & Köhler, 2009; Du, Guo-Hua & Jun-xing, 2019). Lake Biwa, the largest lake in Japan, has harboured diverse members of *Semisulcospira,* and to date, 16 extant and 11 fossil species are known to be endemic to the lake (Matsuoka & Miura, 2019; Sawada & Nakano, 2021). Shell morphology of Lake Biwa species has been studied comprehensively, and significant intraspecific variation has been revealed and is reflected in their taxonomic diagnoses (*e.g*. Davis, 1969; Watanabe & Nishino, 1995; Sawada & Nakano, 2021). To our knowledge, however, sexual dimorphism and allometric growth have not been documented in the genus. Nonetheless, a positive correlation between the size of embryonic shells and that of their mother has been shown in *S. nakasekoae* Kuroda, 1929 (Takami, 1994).

We investigated sexual dimorphism and correlation between growth stages and shell morphology of sexually mature individuals of *Semisulcospira niponica* (Smith, 1876) within a single population using GMM. We also evaluated the contributions of sexual size and shape differences to the measurements of morphological characters used in TMM.

MATERIAL AND METHODS

Material examined

In total, 136 specimens of *S. niponica* were used for morphological analyses: 129 sexually mature snails (54 males and 75 females) and seven juveniles. At Otsu on Lake Biwa on June 26, 2020, snails were randomly collected by hand irrespective of sex and shell size. Although a phylogenetic study showed that *S. niponica* consists of several lineages, the snails from the southern part of the lake, including the study area, form a monophyletic group (Miura *et al.*, 2019). Accordingly, we consider the population examined in this study to be monospecific.

Specimens were separated into shells and soft parts after being boiled in hot water at 95°C for 30 seconds. After dividing shells and soft bodies, snails were identified as sexually mature males or females, or as juveniles according to the midgut colour and the reproductive organ shape (prostate/brood pouch), following Itagaki (1960) and Nakano & Nishiwaki (1989). Brood pouches of adult females were dissected, and embryos were treated with 12% sodium hypochlorite at 26°C for one day to remove soft parts. The examined specimens have been deposited in the Zoological Collection of Kyoto University (males, KUZ Z3959; females, KUZ Z3766; juveniles, KUZ Z3960).

Geometric morphometrics

A landmark-based two-dimensional geometric morphometric approach was applied to assess shell size and shape variation between sexes and among growth stages. All specimens were first fixed horizontally and photographed using a Nikon D7100 camera with a Tamron SP 90 mm f/2.8 1:1 macro lens for Nikon. After setting scale factors, ten landmarks and 21 semi-landmarks were selected along the sutures (landmark [lm] 1, 3, 5, 8, 26, 29, 31), periphery (lm 2, 4, 6, 7, 19–21, 23–25, 27, 28, 30), and aperture (lm 9–18) (Table S1; Fig. 1) using tpsDig2 software (Rohlf, 2018).

A generalised Procrustes analysis (GPA) was conducted to eliminate differences due to displacement, rotation, and scaling (Rohlf & Slice, 1990). In a GPA, semi-landmarks are slid along a tangential direction with minimised bending energy toward a mean reference shape to remove tangential variation (Perez, Bernal & Gonzalez, 2006). Next, a principal components analysis (PCA) was conducted for the Procrustes coordinates of all specimens to visualise shape variation. Sexual differences in shell size and shape, and correlations between size and shape, were tested by a Procrustes analysis of variance (ANOVA) using log-transformed centroid size (logCS) and Procrustes coordinates. Differences were considered significant at $p < 0.05$.

The PCA revealed that sexual shape differences were more prominent in larger individuals, although a significant difference between regression slopes of the two sexes was not detected in the

Procrustes ANOVA ($F = 0.46$, $p = 0.324$). Sexual shape variation was explored using ten individuals with the largest centroid size (CS) of each sex, considering that previous studies observed a considerable intraspecific variation with a larger number of specimens (Davis, 1969, the largest 10% of 300–1000 individuals; Watanabe & Nishino, 1995, the largest 30 specimens). Allometric shape variation in shell morphology was removed with a multivariate regression by calculating the effect of within-group variation. Shape variances were visualised by carrying out a canonical variate analysis (CVA) for the regression residuals because CVA maximises between-group differences relative to within-group variation (Zelditch *et al.*, 2004). The GPA, PCA, and Procrustes ANOVA were conducted with the ‘geomorph’ package (Adams *et al.*, 2021) for R ver. 3.6.1 (R Development Core Team, 2019). The CVA, and visualisation of PCA results were performed using MorphoJ ver. 1.07 (Klingenberg, 2011).

Traditional morphometrics

Morphological characters of adult specimens were measured and counted following previous studies (Watanabe & Nishino, 1995, fig. 2; Sawada & Nakano, 2021, fig. 2). Body whorl length has been used as a standard proxy for shell size and was measured in this study at the anterior tip of the body whorl. Fourth whorl length was measured as the width of the fourth whorl above the tip of the body whorl. Because the shell apex of *Semisulcospira* snails is often eroded, the fourth whorl is the most posterior whorl which is consistently preserved in many individuals. Accordingly, comparison of body whorl length with fourth whorl length enables repeatable evaluation of the elongation rate. Aperture length was measured as the longest diameter of the aperture, and aperture width as the maximum distance perpendicular to aperture length. Spire angle was measured as the angle between the lines connecting the two most prominent points along the periphery from the body to third whorls. Measurements were obtained from the same digital images used for GMM with ImageJ 1.51k (Schneider, Rasband & Eliceiri, 2012).

The abbreviations of the morphological characters examined are as follows: Adult shell: ASR, aperture slenderness ratio (the proportion of aperture length to aperture width); BCN, basal cord number; BWL, body whorl length; RN, rib number on penultimate whorl; SA, spire angle; SCN, spiral cord number on penultimate whorl; SH, shell height; SW, shell width; WER, whorl elongation ratio (proportion of aperture height to fourth whorl length). Embryonic shell: EN, number of embryos; SHE, shell height of the largest embryo.

Measurements of ten individuals with the largest CS of each sex were compared to examine the contribution of sexual size and shape differences to traditional morphometrics. Correlations between eight characters and BWL in males and females were tested using two-sided Kendall rank correlations because significant normality for BWL and other characters was not shown using the Shapiro-Wilk test (see Results). Differences were considered significant at $p < 0.05$. All traditional statistical analyses were conducted using R ver. 3.6.1.

RESULTS

Geometric morphometrics

Sixty PCs were generated from the PCA conducted for the Procrustes coordinates. The first PC explained 35.5% of the total variance, 68.1% of which was explained by the first five PCs. PC1 mainly explained the vertical elongation of the shell spire (lm 1–3, 31) and aperture (lm 9, 14, 18) and the broadening of the shell (lm 8, 10–12, 21–26). PC2–5 explained variation in spire elongation (lm 4–6, 19–23, 26–29), shape and relative position of aperture (lm 9–18), and the broadening of body to third whorls (lm 1–6, 23–28) (Fig. S1).

The scatter plot of CS against PC1 showed an overall decrease of PC1 and a reduced overlap of PC1 between sexes as CS increased (Fig. 2). The minimum size at sexual maturity was similar in males and females (male, 2.66 CS; female, 2.67 CS), but the largest specimen was female (male, 4.68 CS; female, 5.56 CS). The size at sexual maturity varied among individuals because CS of some juveniles exceeded those of minimally mature snails.

A significant sexual size difference was detected by Procrustes ANOVA for logCS ($F = 2.28$, $p = 0.005$). The Procrustes ANOVA performed for the Procrustes coordinates detected significant sexual shape differences ($F = 3.57$, $p = 0.001$). Correlations between logCS and Procrustes coordinates were also shown ($F = 5.25$, $p = 0.001$), whereas the regression slopes were not significantly different between sexes ($F = 0.46$, $p = 0.324$).

The multivariate regression analysis predicted 29.7% of the total variation. CV1, which explained 100% of the total variance, was generated using the ten snails with the largest CS of each sex. The wireframe graph illustrating shape variation along CV1 indicated that females possess shells with a rounder aperture (lm 12–15) that are less elongate (lm 1–5, 12–15, 31) and with broader body and penultimate whorls (lm 6, 7, 21–29) than males (Fig. 3).

Traditional morphometrics

Morphometric characters of ten individuals with the largest CS in males and females are shown in Table 1. Except for the minimum RN value, all the mean, minimum, and maximum values of BWL, RN, SA, SCN, SH, and SW were larger in females than in males. By contrast, males had higher mean and maximum values of ASR and WER.

The Shapiro-Wilk test rejected normality of BWL for females ($W = 0.97$, $p = 0.04$) and of other characters for males (BC, $W = 0.81$, $p <.001$; RN, $W = 092$, $p <.001$; SC, $W = 0.81$, $p <.001$). The correlation coefficients indicated a significant correlation between BWL and the other eight characters in both sexes (Table 2). Except for BCN and SA in males, all characters were significantly correlated with BWL in males and females. ASR, WER, and SA values decreased as individuals grew in both sexes. By contrast, measurements and counts of the other adult and embryonic shell characters increased with larger BWL.

DISCUSSION

Sexual dimorphism in Semisulcospira niponica

The present results of GMM and TMM elucidated previously unknown sexual size and shape dimorphism in Semisulcospiridae. As in some other gastropods (Minton & Wang, 2011; Terence *et al.*, 2019), the results of the Procrustes ANOVA for logCS revealed that the examined females of *S. niponica* have significantly larger shells than males. The minimum size of sexually mature individuals was similar in both sexes, whereas the maximum size was larger in females, indicating that females grow larger than males after reaching sexual maturity. A size difference between males and females was also evident in the measurements of BWL (Table 1).

Significant sexual shape differences also were revealed by Procrustes ANOVA. CVA results with the ten largest individuals of each sex showed that females of *S. niponica* possess a less elongate shell with a rounder aperture and broader body and penultimate whorls than males. The measurements of ASR, WER, SA, and SW supported the results of GMM. The body and penultimate whorls, where sexual differences in shell shape were more prominent, corresponds to the location of the brood pouch on the lateral to the ventral side of the body whorl in females (Itagaki, 1960).

Correlation between shell size and shape of Semisulcospira niponica

Significant correlations between shell size and shape were shown in *S. niponica* by Procrustes ANOVA. Significant increases or decreases in measurements related to BWL were revealed in four characters for both sexes (ASR, RN, SCN, WER), and in four characters only for females (BCN, EN, SA, SHE) (Table 2). Although the correlation patterns were not significantly different between males and females, PC1 values decreased more in males than females with increasing CS (Fig. 2). This is also the case in *Viviparus* and *Pomacea*, where sexual shell shape differences increase with size (Tamburi *et al.*, 2018; Uvayeva *et al.*, 2021). In addition to these allometric factors, sexual variation in maximum shell size contributed to the variation in ASR, SA, SW, and WER measurements.

Shell shape change associated with shell size has not been previously revealed in semisulcospirids. In addition to our results, preceding studies have observed allometric changes in shell shape in diverse groups of freshwater snails (Chiu *et al*., 2002; Estebenet & Martin, 2003; Tamburi *et al*., 2018). Therefore, these shifts seem not to be rare in freshwater gastropods.

Correlations between shell size and sculpture (BCN, RN, SCN) were explored by traditional statistics. In several species of *Semisulcospira*, prominent sculpture on earlier whorls becomes indistinct on later whorls in the same individual (*e.g.* Kajiyama & Habe, 1961; Urabe, 1992). Although shifts in shell sculpture with shell growth had not been observed in other freshwater snails to our knowledge, it has been shown in Pleuroceridae that distinct carinae may disappear as an individual grows (Dillon & Ahlstedt, 1997; Whelan, Johnson & Harris, 2012). Accordingly, the increase in sculpture number and the loss of sculpture related to shell growth may be more common in freshwater gastropods than documented.

The present correlations between adult shell size and embryonic shell number and size corroborate those obtained in a previous study (Takami, 1994). It has been suggested that the increase in embryo number is caused by the increased capacity of the brood pouch that comes with increased body size (Takami, 1994, 1998). In Viviparidae, it has been shown that constructive differences in reproductive organs between males and females may lead to sexual differences in shell morphology (Van Bocxlaer & Strong, 2016). Considering that sexual shell dimorphism is prominent in the brood pouch location in *S. niponica*, the increase in brood pouch capacity is likely to bring about an increase in embryo number, and at the same time, may contribute to expression of sexual size and shape dimorphism.

Implications for morphological examination of Semisulcospira *species*

As indicated previously (Minton & Wang, 2011; Moneva *et al*., 2012), GMM was more sensitive for detecting shape differences than traditional approaches in the present analysis. For example, the increase in shell diameter was explained mainly by the increase in size with TMM, whereas with GMM, it was shown to be a function of shape caused by a broader body whorl in females. Shell elongation differences (lm 1–5, 12–15, 31) explored by GMM were revealed to contribute to the males' higher values of WER in the traditional measurements.

Shell sculpture traits could be analysed only by TMM in this study and were also sexually dimorphic. Because GMM cannot evaluate variation in shell sculpture, it is essential to employ both approaches in taxa such as *Semisulcospira*, for which shell surface sculpture is used in taxonomic diagnoses (Watanabe & Nishino, 1995; Sawada & Nakano, 2021).

In morphological comparisons between species, it is important to eliminate intraspecific sources of variation to correctly assess interspecific variation. In this study, examination of fully mature individuals decreased shell morphological variation attributed to sexual dimorphism and allometric growth, although explicit criteria for the mature condition and the appropriate sample size could not be defined. Accordingly, future morphological studies of *Semisulcospira* should examine larger specimens of each sex after separating males and females.

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Table 1. Adult shell characters of ten individuals with the largest centroid size in males (KUZ Z3959) and females (Z3766) of *Semisulcospira niponica*: minimum–maximum value (mean ± SD).

Character	male	female
Aperture slenderness ratio (ASR)	1.56–1.81 (1.70 ± 0.07)	1.45–1.74 (1.61 ± 0.11)
Basal cord number (BCN)	2–5 (3.7 ± 0.8)	3–4 (3.7 ± 0.5)
Body whorl length (BWL)	13.5–15.6 (14.6 ± 0.6)	17.0–19.3 (18.2 ± 0.7)
Rib number on penultimate whorl (RN)	10–15 (12.2 ± 1.9)	10–17 (13.4 ± 2.0)
Spire angle (SA)	18.7–26.7 (21.5 ± 2.5)	19.9–27.0 (22.8 ± 2.0)
Spiral cord number on penultimate whorl (SCN)	3–5 (4.6 ± 0.7)	4–6 (4.8 ± 0.9)
Shell height (SH)	22.1–26.2 (23.9 ± 1.6)	26.9–30.0 (28.7 ± 1.1)
Shell width (SW)	9.7–11.2 (10.4 ± 0.4)	12.4–14.8 (13.3 ± 0.7)
Whorl elongation ratio (WER)	2.71–3.39 (3.01 ± 0.21)	2.42–3.24 (2.71 ± 0.25)

Table 2. Results of Kendall correlation tests between body whorl length (BWL) and other shell characters of *Semisulcospira niponica*. The symbols “*” and “**” indicate *p* values of ≤0.05 and ≤0.01, respectively. Abbreviations: ASR, aperture slenderness ratio (the proportion of aperture length to aperture width); BCN, basal cord number; EN, number of embryos; RN, rib number on penultimate whorl; SA, spire angle; SCN, spiral cord number on penultimate whorl; SHE, shell height of the largest embryo; WER, whorl elongation ratio (proportion of aperture height to fourth whorl length).

Character	Sex	tau value	*p* value	Correlation pattern
ASR	male	-0.276	<.001**	decrease
	female	-0.185	0.018*	decrease
BCN	male	0.205	0.054	increase
	female	0.327	<.001**	increase
EN	female	0.486	<.001**	increase
RN	male	0.535	<.001**	increase
	female	0.431	<.001**	increase
SA	male	-0.117	0.213	decrease
	female	-0.345	<.001**	decrease
SCN	male	0.249	0.022*	increase
	female	0.433	<.001**	increase
SHE	female	0.358	<.001**	increase
WER	male	-0.391	<.001**	decrease
	female	-0.362	<.001**	decrease

Figure Legends

Figure 1. Positions of ten landmarks (black dots) and 21 semi-landmarks (white dots) used in geometric morphometrics of *Semisulcospira niponica*.

Figure 2. Scatter plots of PC1 *versus* centroid size of males (KUZ Z3959), females (Z3766), and juveniles (Z3960) *Semisulcospira niponica*.

Figure 3. Differences in mean shell shape of ten individuals with the largest centroid size (CS) in males (grey) and females (black) of *Semisulcospira niponica*.

Figure S1. Thin-plate spline transformation grids of PC1–5. Scale factors of each PC are set to 0.07 to visualise variation.

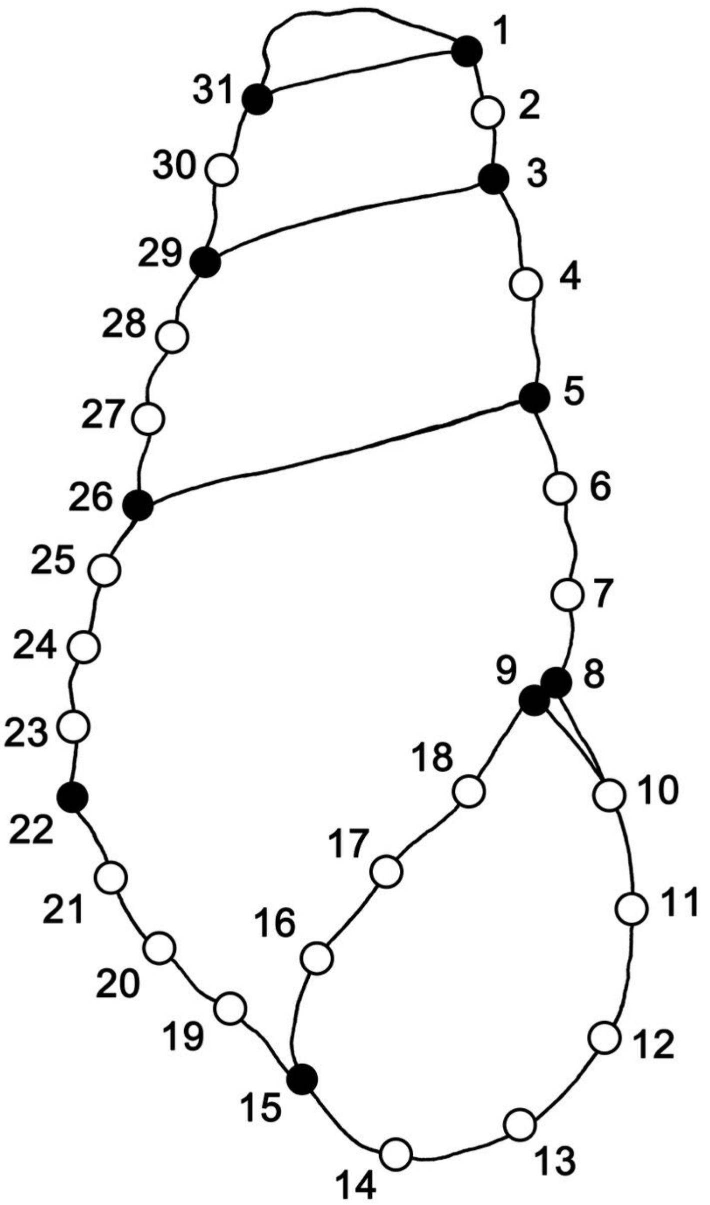
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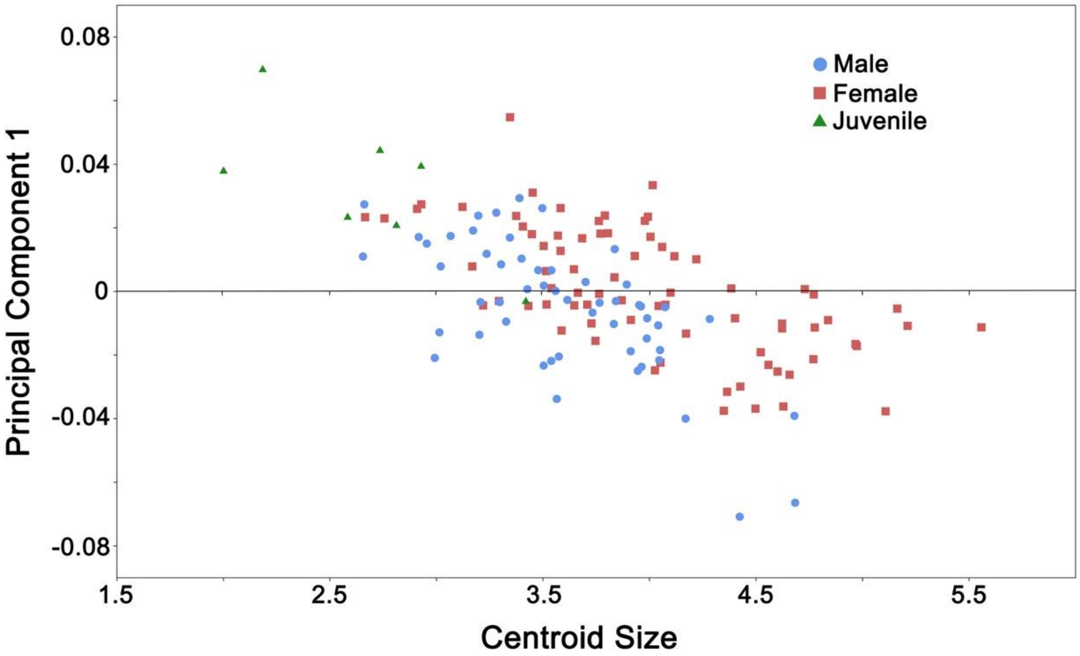
Fig. 1.



Principal Component 1
0.08
0.04
0
-0.04
-0.08
● Male
■ Female
▲ Juvenile
1.5
2.5
3.5
4.5
5.5
Centroid Size

Fig. 2.

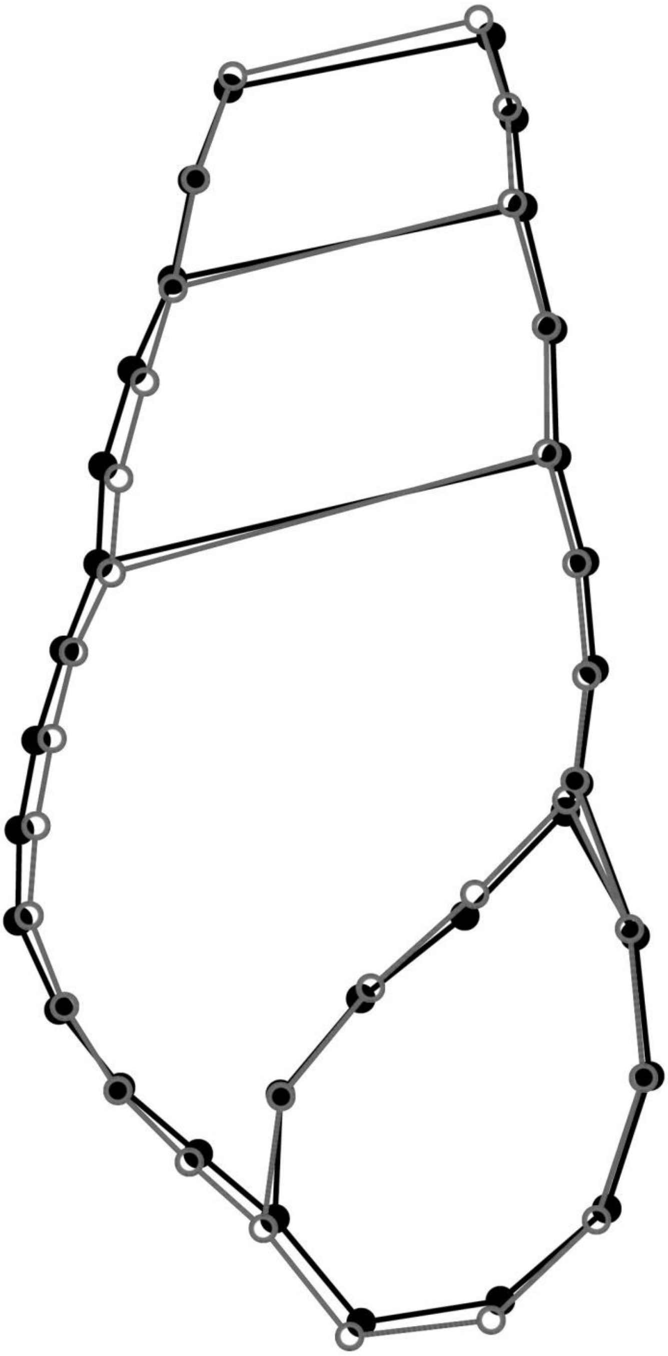


Fig. 3.

Supplementary Materials

Table S1. Definition of 10 landmarks and 21 semi-landmarks on the shells of *Semisulcospira niponica* examined in this study.

Landmark No.	Definition
1	Most depressed point on right edge between fourth and fifth whorls
2	Equidistant between landmarks 1 and 3, following the shell contour
3	Most depressed point on right edge between third and fourth whorls
4	Equidistant between landmarks 3 and 5, following the shell contour
5	Most depressed point on right edge between penultimate and third whorls
6	Equidistant between landmarks 5 and 8, following the shell contour
7	Equidistant between landmarks 5 and 8, following the shell contour
8	Most depressed point on right edge between body and penultimate whorls
9	Most posterior point of aperture
10	Equidistant between landmarks 9 and 15, following the outer lip of aperture
11	Equidistant between landmarks 9 and 15, following the outer lip of aperture
12	Equidistant between landmarks 9 and 15, following the outer lip of aperture
13	Equidistant between landmarks 9 and 15, following the outer lip of aperture
14	Equidistant between landmarks 9 and 15, following the outer lip of aperture
15	Most anterior point of the boundary between aperture and body whorl
16	Equidistant between landmarks 9 and 15, following the inner lip of aperture
17	Equidistant between landmarks 9 and 15, following the inner lip of aperture
18	Equidistant between landmarks 9 and 15, following the inner lip of aperture
19	Equidistant between landmarks 15 and 22, following the shell contour
20	Equidistant between landmarks 15 and 22, following the shell contour
21	Equidistant between landmarks 15 and 22, following the shell contour
22	Most projecting point on left edge of body whorl
23	Equidistant between landmarks 22 and 26, following the shell contour
24	Equidistant between landmarks 22 and 26, following the shell contour
25	Equidistant between landmarks 22 and 26, following the shell contour

26	Most depressed point on left edge between penultimate and third whorls
27	Equidistant between landmarks 26 and 29, following the shell contour
28	Equidistant between landmarks 26 and 29, following the shell contour
29	Most depressed point on left edge between third and fourth whorls
30	Equidistant between landmarks 29 and 31, following the shell contour
31	Most depressed point on left edge between fourth and fifth whorls

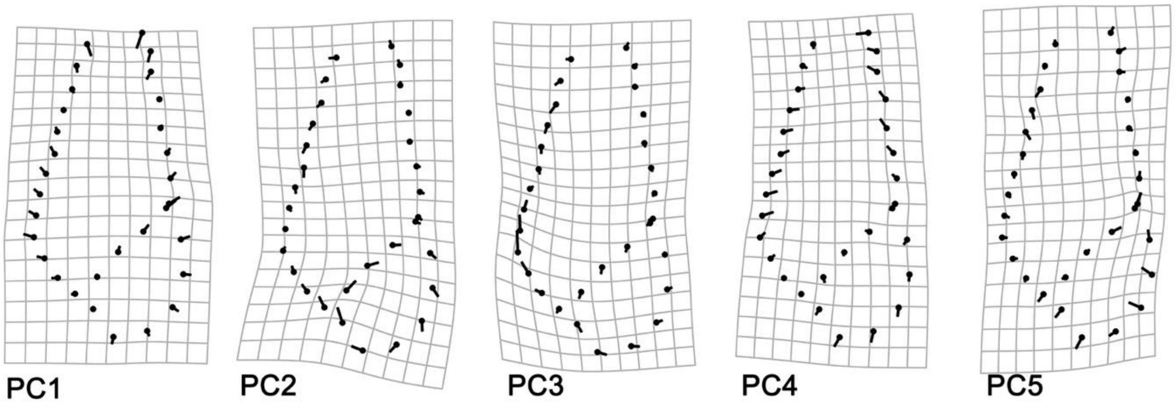


Fig. S1.