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INTERNAL SELECTION AGAINST THE EVOLUTION OF LEFT–RIGHT REVERSAL

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Among metazoan species, left–right reversals in primary asymmetry have rarely gone to fixation. This suggests that a general mechanism suppresses the evolution of polarity reversal. Most metazoans appear externally symmetric and reproduce by external fertilization or copulation with genitalia located in the midline. Thus, reversal should generate little exogenous disadvantage when interacting with the external environment or in mating with the common wild-type. Accordingly, an endogenously caused fitness reduction may be responsible for the general absence of reversed species. However, how this selection operates is little understood. Phenotypic changes associated with reversal are usually inseparable from zygotic pleiotropy. By exploiting hermaphroditism and the maternal inheritance of left–right polarity, we generated dextral and sinistral snails that share the same zygotic genotype. Before hatching, these sinistrals developed lethal morphological anomalies more frequently than dextrals. Their shell shape at maturity differed from the mirror image of the dextral shell. These interchiral differences demonstrate pleiotropy in maternal effects of the polarity or linked genes. Variation in interchiral differences between parental crosses suggests the presence of epistatic variation in relative performance of sinistrals. Our results show that internal selection operates against polarity reversal, and we suggest that this is due to changes in blastomere configuration.

KEY WORDS: **Chirality, homochirality, maternal epistasis, maternal inheritance, maternal pleiotropy, *situs inversus*.**

Many Metazoa exhibit left–right asymmetry (chirality) in their internal structures, including the spatial arrangement and shapes of visceral organs such as the digestive tract (Okumura et al. 2008). This primary asymmetry is established early in development through breaking of extra- or intracellular symmetry across the mid-plane defined by anteroposterior and dorsoventral polarities (Martindale 2005; Nogi et al. 2005; Ball et al. 2010; Hashimoto and Hamada 2010; Vandenberg and Levin 2010b). In snails of the Lophotrochozoa (Spiralia) and nematodes of the Ecdysozoa, maternal substances in the egg cytoplasm predetermine the asymmetric geometry of the first few spiral cleavages (Freeman and Lundelius 1982; Wood 1998). In chordates except for mammals (Hirokawa et al. 2006), maternally expressed ion transporter proteins initiate embryonic asymmetry through the regulation of cell–cell junctions (Adams et al. 2006; Aw et al. 2010; Vandenberg and Levin 2010a). Such maternal inheritance

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systems can be expected if primary asymmetry develops when many substances in and around the developing embryo are still of maternal origin. They may be conserved in bilaterians (Oliverio et al. 2010).

The left–right polarity of primary asymmetry can be reversed by mutation, resulting in development of viable and complete left–right reversals throughout the body (Wood 1998; Sutcharit et al. 2007; Arenas-Mena 2007; Okumura et al. 2008). This phenomenon is called "situs inversus." However, rarely has genetic reversal of left–right polarity in development gone to fixation in metazoans. Some features of secondary asymmetry may be reversed between populations or species such as eye position in flounders (Hashimoto et al. 2007) and the claws of crabs (Shigemiya 2003), or between morphs within populations such as the mouth orientation of fish (Hori 1993; Seki et al. 2000; Nakajima et al. 2007; Van Dooren et al. 2010) and the abdomen of shrimps (Takeuchi et al. 2008). However, these reversed morphs retain the same polarity in visceral asymmetry and thus are not reciprocally situs inversus. This general absence of left–right reversed populations or species (homochirality) in primary asymmetry suggests that generally present mechanisms eliminate mutants with reversed polarity (Okumura et al. 2008). However, few studies have sought to answer why the left–right polarity of development is generally conserved (Wood 2005).

Most metazoans appear externally symmetric and reproduce by external fertilization or by copulation with genitalia in the midline. Thus, the left–right reversal of internal asymmetry would generate little exogenous disadvantage for their interactions with the external ecological environment or for mating with the prevalent phenotype of polarity. Accordingly, metazoan homochirality may be maintained by purifying selection resulting from an endogenous reduction in fitness, which is referred to as internal selection (Cheverud 1984; Arthur 1997, 2001; Fusco 2001; Schwenk and Wagner 2001), that is, selection acting early in development for reasons of internal coordination, relatively independent of ecological environmental variation.

Uniquely among metazoan animals, left–right reversed species have repeatedly evolved in gastropods through fixation of situs inversus (Vermeij 1975, 2002; Robertson 1993; Gittenberger 1988; Asami et al. 1998; Ueshima and Asami 2003; Wise et al. 2004; Sutcharit et al. 2007; Hoso et al. 2010). Thus, most of clockwise-coiled (dextral) and counterclockwise-coiled (sinistral) snails are reversed from each other in bilateral asymmetry of visceral structure as well as in coiling asymmetry. However, most species and groups are monomorphic for left–right polarity, with dextrality overwhelmingly predominant, except for a few groups of chirally dimorphic species (Okumura et al. 2008). In this sense, homochirality also prevails in gastropods.

In snails, copulation between dextral and sinistral morphs (enantiomorphs) is physically difficult due to mismatches in asymmetric courtship and copulation behaviors and in the lateral position of the genital orifice (Lipton and Murray 1979; Johnson 1982; Gittenberger 1988; Asami et al. 1998; Ueshima and Asami 2003). Thus, positive frequency-dependent selection caused by a mating disadvantage of the reversed individuals acts against situs inversus. In pulmonate snails, interchiral mating is more difficult in simultaneous reciprocal mating in globular to flat species than in non-reciprocal mating in tall species (Gittenberger 1988; Asami 1993; Asami et al. 1998; Davison and Mordan 2007; Jordaens et al. 2009). Thus, frequency-dependent selection against situs inversus should be weaker in tall species. As predicted, the rate of evolutionary reversal events (chiral speciation) was found to be significantly higher in groups of tall species (Gittenberger 1988; Asami et al. 1998). In Southeast Asia where snail-eating snakes specialize in preying on dextral snails (Hoso et al. 2007), the evolution of sinistral species by left–right reversal depends more on the presence of these snakes in flat snails than in tall snails (Hoso et al. 2010). These evolutionary patterns support the efficacy of positive frequency-dependent selection in the suppression of chiral speciation in gastropods with internal fertilization.

However, many marine gastropods reproduce by external fertilization, and thus should experience little difficulty in interchiral mating (Rolán-Álvarez and Rolán 1995; Asami et al. 1998; Okumura et al. 2008). Reversed species might therefore be expected to have evolved more frequently in externally fertilizing groups of gastropods than in internally fertilizing ones. However, the opposite situation is observed; polarity has been strictly conserved with few reversed species in externally fertilizing snails, whereas almost all reversed snail taxa are found in internally fertilizing groups (Vermeij 1975, 2002). Therefore, mechanisms other than positive frequency-dependent selection must be responsible for the pattern of snail homochirality in general, even while the difficulties of interchiral mating should suppress chiral speciation in internally fertilizing groups. According to their biogeography and fossil records, sinistral species of marine gastropods may have evolved in association with presumably relaxed predation, resource competition, or physical conditions such as wave strength, suggesting that reversed variants may be under purifying selection resulting from exogenous ecological disadvantages (Vermeij 1975, 2002).

At least in pulmonate gastropods, spiral cleavage proceeds in opposite direction between dextral and sinistral species from the first cell cycle (Crampton 1894; Camey and Verdonk 1970; Meshcheryakov and Beloussov 1975). As shown in four phylogenetically independent families (Wade et al. 2006; Okumura et al. 2008; Utsuno and Asami 2010), the left–right polarity of development in pulmonates is determined by a maternal effect of a single nuclear gene (Fig. 1, Sturtevant 1923; Boycott et al. 1930; Asami et al. 2008) and thus exhibits maternal inheritance (Toyama

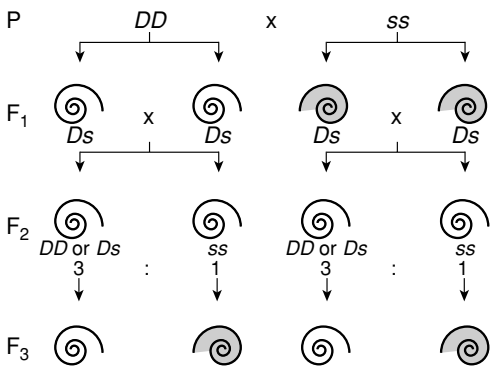


Figure 1. Maternal inheritance of left–right polarity. A maternal effect of a nuclear gene determines the polarity of spiral cleavage, depending on which of the visceral and coil asymmetries develop dextrally or sinistrally. In this example, the *Dextral* allele (*D*) is dominant to the *sinistral* allele (*s*). All the progeny of the maternal parent carrying *D* develop dextrally (open) while their genotype can be *DD*, *Ds,* or *ss*. Only the recessive homozygote *ss* produces the sinistral progeny (filled). When the *Dextral* allele (*D*) is dominant to the *sinistral* allele (*s*), all the progeny of the maternal parent that carries the *D* allele develop the dextral phenotype, but their genotypes can be *DD*, *Ds* or *ss*. The phenotypically dextral *ss* can mate with dextral *DD*, *Ds,* or *ss* with no physical difficulty because of their common polarity phenotype. By mating with dextrals, the *ss* dextrals produce only sinistral progeny. Consequently, coexisting enantiomorphs continue to share the same gene pool (genetic background) across generations, regardless of physical difficulties in interchiral copulation.

1913). When the left–right polarity of blastomere configuration is physically reversed by micromanipulation at the six-cell stage in the nematode *Caenorhabditis elegans* Maupas (Wood 1991) or at the eight-cell stage in the freshwater pulmonate *Lymnaea stagnalis* L. (Kuroda et al. 2009), embryos develop into the opposite enantiomorph regardless of the maternal polarity determinant. These cases indicate that cell–cell signaling and blastomere contact play a key role in the determination of left–right polarity in subsequent development. However, how subsequent left–right zygotic gene expression patterns become established is unknown. This may involve cell fate specification by segregation of mRNA molecules between sister blastomeres (Lambert and Nagy 2002; Kingsley et al. 2007) depending on the geometry of blastomere contact (Goulding 2003), although left–right asymmetry has not been detected in segregation patterns. The developmental system that generates a blastula with a determined cell fate through characteristic spiral cleavage has been conserved in the Spiralia (Henry and Martindale 1999; Henry et al. 2004; Lambert 2010), and maternal effects on left–right asymmetry may also be homologous among bilaterian animals (Oliverio et al. 2010).

In this inheritance system, the polarity genotype and phenotype do not match at the individual level (Fig. 1). Thus, when enantiomorphs coexist in sympatry, gene flow is expected to continue between polarity types, even if these cannot copulate with each other (Johnson et al. 1987, 1990). Thus, if traits differ between enantiomorphs within populations, this is not likely to be caused by diverged genetic backgrounds but rather by pleiotropy or epistatic interactions with the polarity determinant.

Gould et al. (1985) ascribed interchiral shape differences in sympatry to a developmental constraint; the reversed shell shape systematically deviates because polarity reversal in a fundamental body axis is not compatible with subsequent morphogenesis, which depends on the wild-type genetic background. Distortions resulting from this constraint may be detrimental to fitness, resulting in purifying selection against situs inversus (Maynard Smith et al. 1985; Arthur 2004). In the Polynesian tree snail *Partula suturalis* Pfeiffer, which is extinct in the wild (Murray et al. 1998), the dominant *sinistral* allele or linked genes widen the shell by zygotic expression regardless of the polarity phenotype (Johnson 1987) and maybe by maternal expression (Davison et al. 2009a). Also in the Southeast Asian tree snail genus *Amphidromus* (Sutcharit et al. 2006), shell shapes of enantiomorphs differ within populations (Nakadera et al. 2010; Schilthuizen and Haase 2010). Variability among populations in the direction of interchiral difference in *A. atricallosus* (Gould) indicates the presence of genetic variation in the pleiotropic effects of the polarity or linked genes on shape, which suggests epistasis between the polarity and other nuclear genes (Nakadera et al. 2010). These cases of pleiotropy by zygotic and maternal expression of nuclear genes are, hereafter, called zygotic and maternal pleiotropy, respectively (Nakadera et al. 2010).

We compared embryonic survival to hatching and shell morphology at maturity between enantiomorphs originating from a wild population of *L. stagnalis*, to investigate the presence of endogenous internal selection on life-history traits and phenotypic trait differences that might be involved in exogenous ecological selection. Here, we show that sinistral variants frequently develop lethal anomalies before hatching, despite the absence of genetic or environmental differences from the dextral wild-type except for the maternal products of the polarity or linked genes. Thus, internal selection operates against left–right reversal of development through the reduction of embryonic viability. The shell shape of sinistral survivors at maturity is different from the mirror image of dextrals. We exploited a particular breeding scheme to show that maternal pleiotropic effects of the polarity or linked genes, instead of zygotic pleiotropy, are responsible for these interchiral differences. Magnitudes of interchiral differences significantly varied among parental crosses, suggesting the presence of epistatic genetic variation between the polarity and other genes that affect the relative performance of the reversed phenotype compared to the wild-type development.

Materials and Methods

Maternal pleiotropic effects of a reversal-causing gene on morphogenesis and fitness are difficult to distinguish from its zygotic pleiotropic effects. We used a method that allows us to examine maternal and zygotic pleiotropic effects separately by a simple crossing scheme Fig. 2, Asami et al. 2007). This method uses two features of *L. stagnalis*, which is usually fixed for dextrality in the wild. First, it is a simultaneous hermaphrodite that easily reproduces by either outcrossing or self-fertilization. Second, its left–right polarity exhibits maternal inheritance at a single locus, where dextrality is dominant to sinistrality (Asami et al. 2008).

In this crossing scheme (Fig. 2), reciprocal crosses between the genotypes *DD* and *ss* provide F_1 heterozygotes (*Ds*) that develop opposite left–right polarity but do not differ systematically in nuclear genes between the morphs per cross, although individual segregation variation occurs within each cross and morph. In each parental cross, F_2 siblings are obtained by selfing F_1 sinistral *Ds*. Thus, each F_2 sibship shares maternal cytoplasmic substances from their maternal parent and develops only the dextral phenotype. Through recombination between maternal and paternal chromosomes in the F_1 generation and segregation among F_2 dextrals, genotypes at the polarity locus (*DD*, *Ds* and *ss*) are not associated with unlinked genes for other maternal substances. Thus, in each parental cross, the F_3 enantiomorphs receive "unlinked" maternal cytoplasmic substances and nuclear genes that originate from a common grandparent, independently of maternal products of the polarity and linked genes (Asami et al. 2007).

If only zygotic pleiotropic effects of the polarity or linked genes generate interchiral differences in these traits, F_1 enantiomorphs should not differ from each other, but F_2 dextrals should exhibit differences among the polarity genotypes. On the other hand, if the traits differ between the F_1 enantiomorphs, this may involve the effects of genetic imprinting or other maternal cytoplasmic substances, because F_1 dextrals and sinistrals are obtained by choosing a maternal or paternal contribution from each parent. However, if the F_3 enantiomorphs differ in those traits in the same manner as the F_1 enantiomorphs, the involvement of such factors other than the maternal products of the polarity or linked genes in the differences between enantiomorphs can be ruled out. Conversely, if the F_3 enantiomorphs do not differ in these traits, that rules out maternal effects of the polarity or linked genes on the differences between enantiomorphs.

BREEDING EXPERIMENTS

The present dextral and sinistral strains originated from a wild population of *L. stagnalis* in Neustadt an der Donau, Germany (Asami et al. 2008). We made a series of three parental crosses (1, 2, and 3, Fig. 2) between homozygotes for the *Dextral* (*D*) and *sinistral* (*s*) alleles. In the F_1 generation, we used only sinistrals and dextrals that were produced by outcrossing, which were identified by genotyping microsatellite loci (Asami et al. 2008) after they reproduced. We obtained F_2 and F_3 generations by selfing parental individuals. The breeding protocol was reported previously (Asami et al. 2008).

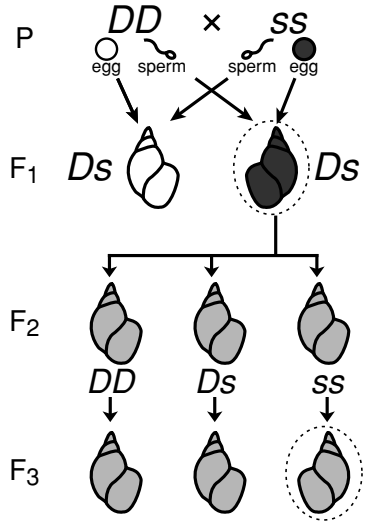


Figure 2. Mating scheme to examine the maternal and zygotic pleiotropic effects of the polarity gene. A maternal cytoplasmic factor produced by a nuclear gene determines the left–right polarity of spiral cleavage in *Lymnaea stagnalis*. Embryos develop dextrally or sinistrally depending on the polarity of early blastomere configuration. Thus, the individual phenotype is determined by the maternal genotype at this locus. The F_1 dextrals (open) and sinistrals (filled) were obtained by mating the parents reciprocally. The F_2 generation was obtained by selfing one F_1 sinistral (*Ds*) individual in each parental cross. Because the *Dextral* allele *D* is dominant to the *sinistral* allele *s*, all the progeny of these sinistrals develop into dextrals but are genotypically *DD*, *Ds,* or *ss*. The F_3 generation was obtained by selfing the F_2 dextrals. Dextral and sinistral embryos in the F_1 generation receive different maternal cytoplasmic substances from the different maternal parents. However, the F_2 siblings share maternal factors from the same maternal parent. Next to maternal products of the polarity and linked genes, the F_3 generation randomly receives maternal substances and nuclear genes that originate from a common grandparent. Thus, these cytoplasmic and genetic factors are not associated with the polarity phenotype.

ENDOGENOUS FITNESS TRAITS

To examine the development of embryos, we removed the jelly that covers the egg capsule and allowed each embryo to develop inside the intact translucent capsule, while submerged in dechlorinated tap water. We distinguished between dextral and sinistral embryos by examining the polarity of spiral cleavage in the eight-cell stage. We counted the morphologically abnormal progeny on the day of oviposition (day 0), and on days 2, 5, 9, and 13,

and among the hatchlings on day 13. We analyzed the effects of left–right polarity, parental cross, and interaction of polarity and parental cross on hatching success in the F_1 generation using a binomial generalized linear model and likelihood ratio tests (Crawley 2007). We investigated differences between polarity groups using posthoc simultaneous confidence intervals (Hothorn et al. 2008).

We measured the individual body weight of the F_1 enantiomorphs every five days from the 18th to the 63rd day after hatching, using 14, 10, and 14 dextrals and 15, 10, and 12 sinistrals obtained from parental crosses 1, 2, and 3, respectively. We investigated individual growth curves using nonlinear mixed models (Pinheiro and Bates 2000). We fitted a three-parameter logistic model to the data. The parameters represent an asymptotic size, the age at which 50% of the asymptote is reached (midpoint) and a scale parameter describing the slope of the growth curve at the midpoint. We investigated effects of parental cross and polarity on these parameters, while we allowed individual random variation in each of them. Effects of cross and polarity were tested using ANOVA methods for these nonlinear models (Pinheiro and Bates 2000).

We recorded the number of eggs produced by selfing F_1 individuals (11, 15, and 9 dextral and 9, 18, and 6 sinistral progeny of parental crosses 1, 2, and 3, respectively), during a 1-month period after the first oviposition. We examined the fecundity of F_1 enantiomorphs using a Poisson generalized linear model, with main effects of polarity phenotype, parental cross, and their interaction. We allowed for variation between parents by allowing overdispersion, and therefore we used a quasi-Poisson approach and *F*-tests.

Individuals in the F_2 generation are all of the same polarity, but differ in zygotic genotypes. An individual's genotype can be determined only by inspecting the polarity phenotypes of its offspring in the F_3 and F_4 generations. Individuals that do not hatch cannot be genotyped and therefore hatchabilities per genotype are not available in the F_2 generation. We limited our analysis on this generation to an overall comparison of proportions of sinistrals among the adults with offspring, and tested whether the proportions deviate from the Mendelian expectation based on random segregation of alleles in the *Ds* parents.

In the F_3 generation, we tested the main effects of polarity and parental cross, and interaction of polarity and parental cross on the hatching success of progeny (F_3) by using a binomial generalized model. We used a quasi-binomial distribution to accommodate heterogeneous hatching successes among F_2 parents. This test compared the hatchabilities of the dextral progeny produced by 3, 3, and 27 F_2 dextrals, which were accordingly *DD* or *Ds*, and the sinistral progeny produced by 4, 3, and 10 F_2 dextrals, which were *ss*, obtained from parental crosses 1, 2, and 3, respectively. The progeny from F_2 *DD* dextrals consists of *DD* dextrals only. The progeny from F_2 *Ds* dextrals contains *DD*, *Ds* and *ss* dextrals. Therefore, if the *s* allele has a zygotic effect on hatchability, the hatchability of F_3 dextrals becomes a mixture of the hatchabilities of the three genotypes, which the present experiment does not allow us to distinguish. Additionally, alleles segregating at other loci or variation in environmental conditions might turn hatchabilities into a more involved mixture, even among F_3 *ss* sinistrals. Thus, we used mixture analysis to check whether there are indications of zygotic pleiotropy when we compare the mixture components in the hatchabilities of F_3 dextrals and sinistrals. We compared models with different numbers of mixture components using information criteria (AIC, BIC, ICL, Leisch 2004), which were always in agreement. We carried out this analysis on the individuals from the parental cross with the largest sample size only, to exclude masking parental cross effects on the distribution of hatchabilities.

SHELL MORPHOMETRICS

We used the shells of 60- to 80-day old adults. In the F_1 generation, we used 38, 23, and 37 *Ds* dextrals, and 38, 23, and 38 *Ds* sinistrals, obtained from the parental crosses 1, 2, and 3, respectively. In the F_2 generation, we used 47 *DD* or *Ds* and 18 *ss* dextrals, 30 *DD* or *Ds* and nine *ss* dextrals, and 18 *DD* or *Ds* and five *ss* dextrals, obtained from the parental crosses 1, 2, and 3, respectively. By using two-dimensional digital images taken from a fixed angle orthogonal to the shell axis, we avoided possible morphometric biases that could result from interactions between the shells and vernier calipers, which are both left–right asymmetric and thus cannot be equivalently used for dextral and sinistral shells, and bias from the handedness of the observer. We bilaterally flipped the images of sinistrals to avoid possible measurement bias resulting from a priori knowledge of polarity.

We used a landmark-based geometric morphometric method (Dryden and Mardia 1998; Stone 1998; Ubukata 2003; Nakadera et al. 2010), which could detect slight differences in shape separately from size while preserving complete information about the relative spatial arrangements of landmarks (Zelditch et al. 2004; Ubukata et al. 2009). We recorded the two-dimensional coordinates of eight landmarks (Fig. 3), which were chosen based on the repeatability of their coordinates. As a size variable, we used centroid size, which is the square root of the summed squared distances from the centroid to the landmarks (Zelditch et al. 2004). We obtained a number of descriptive shape variables as follows. Per generation separately, nonshape properties (size, orientation and position) were removed by generalized least squares Procrustes superimposition (Gower 1975). Per shell, we then determined scores for a set of eight principal components of Procrustes tangent space coordinates (Dryden and Mardia 1998) that together explain more than 95% of shape variation per generation (Table 1). These principal component scores (PC) were used as

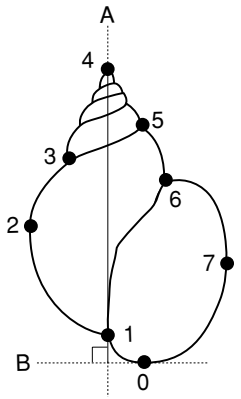


Figure 3. **Definitions of shell axis, height, and landmarks (LM0 to LM7). On a two-dimensional image taken at the above orientation, parallel to the shell axis for each shell, we plotted the following landmarks. Line A through the apex and LM1 (the anterior edge of the last whorl connecting to the inner edge of the basal lip) indicates the shell axis. Shell height is the distance between the apex and the perpendicular line B, which is tangential to the anterior edge of the basal lip. LM0, the point at which the line B touches the anterior edge of the basal lip. LM2, the point on the outermost edge of the last whorl in osculation with a line parallel to the axis. LM3, the anterior (left for a dextral shell and right for a sinistral shell) edge of the upper suture of the last whorl. LM4, the shell apex. LM5, the posterior edge (right for dextrals; left for sinistrals) of the upper suture of the last whorl. LM6, the junction of the posterior end of the lip and the last whorl. LM7, the outermost edge of the outer lip in osculation with a line parallel to the shell axis.**

shape variables in further analysis. They are uncorrelated in the pooled data per generation and are ordered according to the proportion of total shape variation that they explain. We tested the effects of morph, parental cross, and centroid size on shape variables (PC 1–8 per generation) by two-way multivariate analysis of covariance (MANCOVA) with centroid size as the covariate. We started from a model with all interactions and simplified the model by removing terms that were not significant according to an *F*-test (Pillai-Bartlett statistic). The tests reported are from direct comparisons between the selected model and a nested model where a term is dropped. To investigate whether particular significant MANCOVA effects occurred across all shape variables, or mainly in a restricted set that explained much or little of total shape variation, we also modeled each shape variable separately. Variation in centroid size was analyzed using a two-way ANOVA. To compare magnitudes of variances of shape variables between groups we used a multivariate Levene permutation test (Anderson 2006) on pairwise Procrustes shape distances between shells, followed by bootstrapped robust Levene tests (Lim and Loh 1996) on the individual shape variables.

Table 1. **Fractions of shell shape variance explained by 12 principal components of Procrustes coordinates in the F_1 and F_2 generations.**

PC	F_1 generation		F_2 generation	
	Explained %	Cumulative %	Explained %	Cumulative %
1	35.0	35.0	47.5	47.5
2	24.3	59.4	24.9	72.5
3	14.2	73.6	8.1	80.6
4	9.3	82.9	6.2	86.8
5	5.4	88.3	3.9	90.7
6	3.3	91.6	2.5	93.2
7	3.0	94.6	2.3	95.5
8	2.6	97.2	2.0	97.5
9	1.1	98.3	1.0	98.5
10	1.0	99.3	0.8	99.3
11	0.5	99.8	0.4	99.7
12	0.2	100.0	0.3	100.0

Results

ENDOGENOUS FITNESS TRAITS

Reciprocal crosses between *DD* and *ss* homozygotes generated F_1 dextral and sinistral *Ds* progeny (Fig. 2). These F_1 sinistral embryos developed morphological anomalies more frequently than their dextral siblings (Fig. 4A). The sinistral embryos frequently failed in gastrulation and became distorted, exhibiting, for example, dumbbell-shaped exogastrulae or hydropic gastrulae (Morrill 1982). Almost all of these morphologically anomalous embryos failed to hatch. The relative mortality of sinistrals compared with that of dextrals varied significantly among the parental crosses (i.e., interaction effect between the polarity phenotype and parental cross; $\chi^2 = 153.80$, df $= 2$, $P < 0.0001$). Overall, embryonic mortalities were significantly higher in sinistrals (confidence interval of the overall difference contrast on logit scale 1.47–1.82). Sinistral siblings hatched only 58.4% as successfully as dextral siblings, mean hatchabilities $\pm$ SE were 0.457 ± 0.057 and 0.783 ± 0.077, respectively (Fig. 4B).

We found no effects of parental cross or polarity on the asymptotic size ($\chi^2 = 10.38$, df $= 5$, $P = 0.07$) or midpoint ($\chi^2 = 5.27$, df $= 5$, $P = 0.39$). However, effects of parental cross ($F_{2,676} = 8.701$, $P = 0.00024$) and polarity ($F_{1,676} = 10.0008$, $P = 0.0016$) on the scale parameter were significant. The scale was slightly smaller for dextral snails, which means that their growth curve is a bit more steep at the midpoint than in sinistrals. The effect was barely discernible in graphs. Eventually, both polarity types are expected to reach the same sizes, because there are no differences in expected asymptotic sizes. Fecundity did not differ between enantiomorphs ($F_{1,66} = 1.613$, $P = 0.209$) whereas

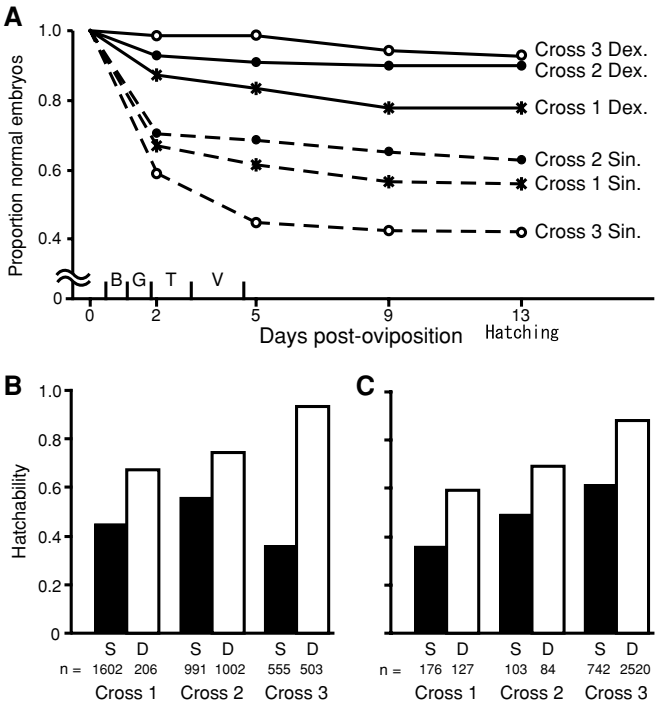


Figure 4. Differences in embryonic viability between sinistrals and dextrals. (A) Frequency of morphological anomalies in F_1 dextral *Ds* (D) and sinistral *Ds* (S). Crosses 1 to 3 represent the three parental crosses between *DD* and *ss* homozygotes to obtain the *Ds* enantiomorphs. B, G, T, and V indicate the blastula, gastrula, trochophore, and veliger stages, respectively. (B) Hatchability of F_1 *Ds* enantiomorphs. (C) Hatchability of F_3 enantiomorphs. *n* = number of siblings.

it differed between parental crosses ($F_{2,66} = 5.239$, $P = 0.0077$), and exhibited no interaction effect between the polarity phenotype and parental cross ($F_{2,63} = 0.54$, $P = 0.59$). Changes by indirect viability selection before hatching or the overall absence of maternal pleiotropic effects in these life-history traits can both explain the absence of interchiral differences after hatching.

In the F_2 generation obtained by selfing F_1 *Ds* sinistrals, all the individuals developed into dextrals as expected from the maternal effect of the *D* allele. By phenotyping their progeny, we found that 18 of 62, nine of 40, and five of 23 siblings in the F_2 generation were *ss* in parental crosses 1, 2, and 3, respectively. These proportions of enantiomorphs in adults did not significantly differ among the three crosses ($\chi^2 = 0.65$, df = 2, $P = 0.68$). We found no significant deviation from the Mendelian 1/4 ratio expected in the zygotes ($\chi^2 = 0.003$, df = 2, $P = 0.96$). Thus, there was no significant difference in survival to maturity between phenotypically dextral *ss* and other genotypes.

There was substantial overdispersion in the hatchabilities of F_3 (dispersion parameter 11.2). The F_3 dextrals and sinistrals were obtained by selfing F_2 individuals. Fewer sinistrals than dextrals hatched ($F_{1,46} = 30.37$, $P < 0.0001$), similarly to F_1 sinistrals (Fig. 4C). The mean hatchabilities ± SE across parental crosses were 0.472 ± 0.082 for sinistrals and 0.719 ± 0.084 for dextrals. This result excludes the involvement of genetic imprinting or unknown and unlinked maternal factors in the interchiral differences in survival before hatching. Hatchabilities differed among parental crosses ($F_{2,46} = 6.485$, $P = 0.0033$). The relative hatchability of sinistrals compared to dextrals, however, did not significantly differ among the parental crosses ($F_{2,44} = 0.719$, $P = 0.493$).

Mixture analysis indicated that the hatchabilities of sinistral *ss* offspring (F_3) were heterogeneous among *ss* F_2 maternal parents. The information criteria favored a model with a mixture of three components (AIC 2 components: 117.2; AIC 3: 90.04; AIC 4: 91.11), that is, there were three groups of *ss* parents that differed in progeny hatchability ($p_1 = 0.50$, $p_2 = 0.69$, $p_3 = 0.94$), despite no difference in progeny genotype. If zygotic expressions of the polarity or linked genes affect their hatchability, *DD* and *Ds* F_2 maternal parents would differ in progeny hatchability, because the former produce only *DD* offspring but the latter *DD*, *Ds*, and *ss* offspring. Thus, additional groups with different progeny hatchabilities, compared with the three groups in *ss* F_2 maternal parents, would be present among dextral *DD* and *Ds* F_2 maternal parents. However, there were only two groups with different progeny hatchabilities ($p_1 = 0.77$, $p_2 = 0.95$). That is, the number of component hatchabilities was not larger than three. There was, therefore, no indication of a zygotic pleiotropic effect of the *D* or *s* allele on hatchability.

SHELL MORPHOLOGY

In the F_1 generation, eight shape variables (PCs 1 to 8) explained 97.2% of total variance of shell shape at maturity (Table 1). The strength of dependence of shape on centroid size (allometry) differed between dextrals and sinistrals (Table 2), indicating that the shape differences between enantiomorphs also depend on shell size. This interaction was significant for relative warps 5 and 8, which together explain less than 10% of shape variation. The interaction between the effect of polarity phenotype and parental cross on shape was significant (Table 2), indicating that the shape differences between sinistrals and dextrals vary depending on parents. These interchiral differences cannot be explained by a zygotic pleiotropic effect of the polarity or linked gene, because they were both *Ds* in genotype. Our results also suggest that the degree and direction of morphological differences between the morphs are not constant but genetically variable.

To identify which component of shape significantly differed between F_1 dextral and sinistral shells, we analyzed each shape variable separately. The effects of polarity phenotype on the first variable ($F_{1,190} = 14.34$, $P = 0.0002$) and the fourth ($F_{1,192} = 37.73$, $P < 0.0001$) were significant. The effect of interaction between polarity and centroid size was significant for the fifth shape variable ($F_{1,192} = 6.46$, $P = 0.012$). For the last shape

Table 2. Results of two-way multivariate analysis of covariance with centroid size as a covariate, testing effects on the shape variables in the F_1 generation.

Factors	df	Pillai's trace	Approx. F	Numerator df	Denominator df	P
Polarity × centroid size	1	0.105	2.67	8	182	0.009
Polarity × parental cross	2	0.140	1.72	16	366	0.041

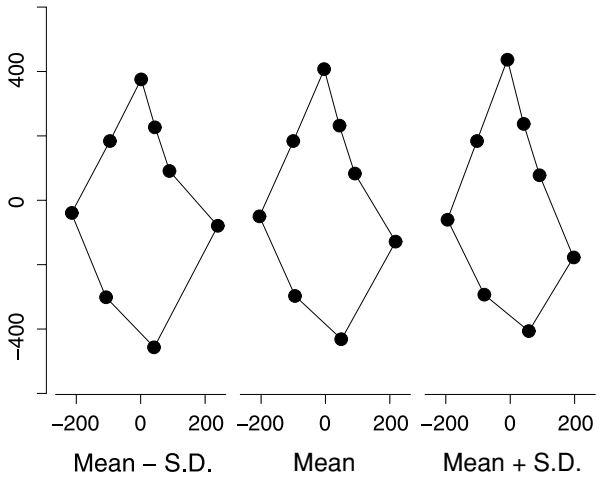


Figure 5. Shell shape variation caused by the first shape variable. This principal component of variation in Procrustes landmark coordinates explains 35% of shape variation. When the value of the shape variable (score) increases, it describes a change from a shell with a broader last whorl to a more slender shell with elongated first whorls. The difference between F_1 sinistrals and dextrals in this variable was significant, with sinistrals toward the left shape from the mean and dextrals more similar to the shape on the right. The difference between the shapes plotted in the left and right panels is two standard deviations. Between the F_1 morphs, it was 0.57 standard deviations, which is much less than shown here. The effect of left–right polarity was therefore inconspicuous when plotting Procrustes coordinates for the sinistral and dextral groups either separately per cross or pooled.

variable PC8, pairwise interactions between polarity and centroid size ($F_{1,187} = 13.07$, $P = 0.0003$) and those between polarity and parental cross ($F_{2,187} = 5.07$, $P = 0.007$) were both significant. Because the first shape variable explains the largest proportion of shape variation and apparently contributes most to the polarity effect in the MANCOVA, we focused on the shape differences between polarity phenotypes explained by that variable. Plotting the shape variation explained by it (Fig. 5), we found that dextrals achieve greater extension of the early whorls along the coiling axis than sinistrals, and that the last whorl of sinistrals was wider than that of dextrals. However, the effects were not conspicuous when simply inspecting overall shape differences between enantiomorphs and crosses.

The interaction between polarity phenotype and parental cross had a significant effect on centroid size ($F_{2,191} = 4.22$, $P = 0.016$), seemingly caused by the data for the third parental cross. However, when we did remove this relatively marginal interaction, main effects of cross ($F_{2,193} = 1.32$, $P = 0.27$) and polarity type ($F_{1,193} = 0.0043$, $P = 0.95$) on centroid size became nonsignificant. We therefore conclude that overall, there was no difference in centroid size between enantiomorphs.

In the F_2 generation, PCs 1 to 8 explained 97.5% of total variance of shell shape (Table 1). Among dextral individuals, genotypically *ss* shells were about 6% larger than *DD* and *Ds* shells in terms of centroid size ($F_{1,125} = 5.84$, $P = 0.017$), whereas parental cross had no effect on centroid size ($F_{2,122} = 1.23$, $P = 0.30$). This indicates a zygotic pleiotropic effect of the polarity gene on shell size. On the other hand, these dextrals showed no differences in shell shape between the *ss* and other polarity genotypes ($F_{8,115} = 1.40$, $P = 0.20$). Thus, the differences found in shell morphology between the F_1 enantiomorphs did not appear between the phenotypically dextral *ss* and individuals with one of the other genotypes (*DD* and *Ds*) in the F_2 generation. None of the tests comparing the variances of shape variables between enantiomorphs was significant. These results reinforce the conclusion from the shape analysis in the F_1 generation that a zygotic pleiotropic shape effect of the polarity or linked genes should be small, although our data do not rule out possible differences between dextral *DD* and *Ds* in shell shape. There were significant effects of centroid size and parental cross on shape in F_2 (Table 3), showing the presence of allometry similarly to the F_1 generation and the presence of effects of genetic background on shape.

Discussion

This study showed that sinistral variants develop morphological anomalies and die before hatching more frequently than the dextral wild type. The shell shape of sinistral survivors was not the mirror-image of dextral siblings. However, the two morphs exhibited no differences in expected asymptotic size or fecundity. These results indicate that internal selection operates against the *ss* maternal genotype through an endogenous disadvantage in the embryonic stages of the progeny.

The F_1 enantiomorphs per parental cross did not differ from each other in nuclear genes. Thus, zygotic pleiotropic effects of the polarity or linked genes are not responsible for interchiral differences detected in the F_1 generation, while maternal

Table 3. Results of two-way multivariate analysis of covariance with centroid size as a covariate, testing effects on shape variables in the F_2 generation.

Factors	df	Pillai's trace	Approx. *F*	Numerator df	Denominator df	*P*
Parental cross	2	0.406	3.72	16	234	< 0.001
Centroid size	1	0.425	10.70	8	116	< 0.001

pleiotropic effects of the *s* allele or linked loci on hatchability are demonstrated. The shape differences we found between enantiomorphs might be affected by the selection occurring before hatching, but it demonstrates a coupling between polarity and shape, therefore giving evidence of another maternal pleiotropic effect. In the F_2 generation, proportions of enantiomorphs suggest that survival from egg to adult did not differ between phenotypically dextral *ss* and other genotypes, giving no indication of a zygotic pleiotropic effect. Without knowing the hatchabilities or survival to maturity of the three genotypes (*DD*, *Ds,* and *ss*) separately, however, we cannot strictly rule out the possible presence of a zygotic pleiotropic survival effect, which has been shown in humans (Galis et al. 2006). Among these dextrals, the *ss* genotype was larger in shell size than the other genotypes. Thus, we did find a zygotic pleiotropic effect of the polarity gene on the size of the dextral phenotype. However, growth trajectories need to be collected for the different genotypes to judge whether size differences persist and might have fitness consequences. Our comparisons of shape variances and hatchability mixtures did not indicate any such effects to be strong. The F_3 morphs differed in hatching success similarly to the F_1 morphs. The F_3 morphs that originated from a common F_1 individual did not differ systematically in the genetic background or maternal cytoplasmic substances exclusive of the polarity or linked genes and of maternal products of those genes. That excludes potential contributions of other unlinked maternal products than the polarity and linked genes. Taking these results over the three generations together, interchiral differences in hatchability and adult shell morphology can be ascribed to the maternal pleiotropy of the polarity or linked genes.

We discovered significant variation in the hatchability of sinistrals relative to that of dextrals among parental crosses in the F_1 generation. This suggests that the polarity effect on embryonic viability is genetically variable. This variability was found even in our small sample of three parental pairs. Thus, there may be a substantial amount of genetic variation in the relative viability of sinistral embryos in the original wild population. Previous studies also suggested that polarity-dependent differences in embryonic viability are genetically variable in two species of the different land-snail families Bradybaenidae (Utsuno and Asami 2010) and Clausiliidae (Utsuno et al. 2010). Similarly to the embryonic viability analysis, we found that the degree of interchiral difference in adult-shell shape significantly depends on the parents, which suggest the presence of genetic variation in the degree of morphological differences between the morphs. These interactions are, however, only important for shape components that contribute relatively little to overall shape variation. The pleiotropic effect of the polarity gene on shell shape varies depending on populations also in *Amphidromus atricallosus* (Nakadera et al. 2010). All these results support that phenotypic differences between enantiomorphs may evolve quantitatively and exemplify a developmental bias (Arthur 2004) or relative constraint (Brakefield 2006), instead of evolutionarily inevitable byproducts (Gould and Lewontin 1979; Gould et al. 1985; Gould 1997).

Parental variation in the degree of interchiral differences investigated at the level of sibships provides important insights into the mechanism allowing the morphs to develop differently. The left–right polarity of development is a binary trait at the individual level (sinistral/dextral). On the other hand, the hatchability of sinistrals relative to that of dextrals varies quantitatively between sibships and significantly differs among parental crosses. For sinistrals to vary genetically in relative performance, allelic variation needs to be present in genes that depend functionally on the polarity phenotype or the maternal genotype for polarity. For this epistatic process, either or both maternally and zygotically expressed products of these genes ought to affect the development of the sinistral morph. Accordingly, parental variation in the degree of interchiral difference would represent epistatic genetic variation. This suggests that traits of early development, leading inter alia to binomial polarity, vary quantitatively between the morphs.

DEVELOPMENTAL MECHANISMS AND PHENOTYPES

Dextral and sinistral embryos of *L. stagnalis* were reported to achieve a mirror-image cell configuration through different cytoskeletal dynamics in the eight-cell stage (Shibazaki et al. 2004). In our observations of the same genetic lines (Asami et al. 2008), however, the blastomere configuration of sinistral embryos never reaches the mirror image of dextrals. Instead, throughout the third cell cycle and eight-cell stage, sinistrals consistently exhibit a quantitatively reduced asymmetry, resulting from reverse rotation of the first quartet of micromeres in a smaller angle around the animal-vegetal axis than dextrals exhibit (Utsuno, H., Asami, T. and Gittenberger, E. unpubl.

data). This rotation angle varies substantially among sinistral embryos. The quantitative reduction and variability of asymmetry in blastomere rotation may underlie the parent-dependent reduced viabilities of sinistral embryos resulting from morphological anomalies. Our results highlight the importance of further explicit studies of early cell dynamics in development.

Adult shell shapes differed between enantiomorphs. The shell gland appears in the trochophore larva and begins to secrete the larval shell (protoconch) in the veliger stage (Morrill 1982). Prior to these events, the maternal-zygotic transition of gene transcription starts in the eight- to 16-cell stages (Van den Biggelaar 1971; Edgar et al. 1994; Schier 2007). Thus, maternally expressed substances in egg cytoplasm would not persist long enough to affect the process of shell formation directly. Furthermore, the maternal product of the polarity gene is effective for polarity determination during only the first few spiral cleavages in a nematode (Wood 1991; Hutter and Schnabel 1995) and a snail (Kuroda et al. 2009). Afterward, the physical configuration of blastomeres is in turn responsible for the polarity of subsequent gene expression and morphogenesis (Hierck et al. 2005), which is supported by a comparison between distantly related dextral and sinistral gastropods (Grande and Patel 2009). Thus, direct maternal–zygotic epistasis between the maternal polarity substance and the products of zygotic gene expression may be restricted to a limited period in early development. Instead, the quantitative changes in early blastomere configuration may result from maternal epistasis through interactions between maternal products of the polarity gene and the other genes for the degree of asymmetry in blastomere cleavage. This process may be responsible for later interchiral differences in subsequent embryogenesis and adult shell morphology such that most epistasis might be due to the association between a phenotypic cell configuration and later gene expression.

PLEIOTROPY AND DOMINANCE

In this study, we examined the F_3 enantiomorphs to test whether the trait differences of F_1 enantiomorphs involved the effects of genetic imprinting or maternal substances produced by genes other than the polarity gene. In this system, the *Dextral* allele (*D*) produces the maternal determinant of dextrality in spiral cleavage, whereas the *sinistral* allele (*s*) is inactive with no production of maternal substance (Freeman and Lundelius 1982). Further, the genotypes *DD* and *Ds* can only be genotyped by phenotyping their descendants in the F_4 generation. For these reasons, we compared the zygotic and maternal pleiotropic effects of F_2 genotypes on the F_3 progeny between the *ss* and other grouped genotypes (*DD* and *Ds*), without distinguishing the latter two genotypes. By this comparison, we found that the centroid size of dextral *ss* is larger than that of dextral *DD* and *Ds*, showing the presence of a zygotic pleiotropic effect of the polarity genotype.

A pleiotropic effect of the polarity gene may not exhibit complete dominance similarly to polarity determination. For instance, the *Ds* genotype may generate a weaker effect on the quantitative asymmetry of blastomere configuration than the homozygotes. If so, the F_3 dextrals obtained from the F_2 *DD* and *Ds* may have exhibited larger phenotypic variation than the F_3 sinistrals obtained from the F_2 *ss*. However, the mixture analysis gave no indication of hatchability differences between the *DD* and *Ds* F_2 genotypes, or a difference in the variances of shell shape traits between the F_2 *ss* and other genotypes. Thus, possible differences in maternal or zygotic pleiotropic effect between *DD* and *Ds* yet remain to be tested, but the effects are anticipated to be small enough to go undetected in tests for comparing variances.

INTERACTIONS BETWEEN INTERNAL SELECTION AND OTHER TYPES OF SELECTION

Left–right reversal is known to be advantageous in some circumstances, for example, under specialized predation on dextral snails by snakes (Hoso et al. 2007, 2010), insects (Inoda et al. 2003) or crabs (Ng and Tan 1985; Dietl and Hendricks 2006), or in secondary contact where reversed snails can avoid detrimental species hybridization (Johnson et al. 1993; Uit de Weerd et al. 2006). As long as the reversed morph persists under these exogenous advantages, and the relative viability of sinistrals compared with the viability of dextrals is genetically variable as this study suggests, viability selection in embryos should favor reversed variants that hatch as successfully as the wild type. Thus, internal selection against reversal continues to be weakened.

Moreover, in internally fertilizing snails, a reversed morph gains a mating advantage over the wild type, once it exceeds 50% of the population by an exogenous advantage or through genetic drift (Johnson 1982; Orr 1991; Van Batenburg and Gittenberger 1996; Stone and Björklund 2002; Ueshima and Asami 2003; Hoso et al. 2010). Population fixation for polarity reversal instantaneously completes premating reproductive isolation from other nonreversed populations, where the polarity gene acts as a speciation gene (Gittenberger 1988; Ueshima and Asami 2003; Hoso et al. 2010). Viability selection also continues throughout. This process should be unique to animals with difficulties of interchiral mating and may explain the contrastingly frequent and rare evolutions of species fixed for situs inversus in internally and externally fertilizing groups, respectively. One must, however, still take into account the possibility of species-level selection after population fixation for reversal, which may not necessarily be equivalent between internally and externally fertilizing snails.

Populations of the dextral morph in *P. suturalis* could probably have benefited from such reproductive character displacement that reduces detrimental hybridization with closely re-

lated sinistral species, while populations of the original sinistral morph remained in allopatry from those sinistral species (Johnson et al. 1993). The two morphs were separately maintained in monomorphic populations through positive frequency-dependent selection (Johnson 1982). In *A. inversus* (Müller), disassortative mating between enantiomorphs may underlie their coexistence (Schilthuizen et al. 2007). Morph frequencies, however, strongly deviate from 0.5 in many enantiomorphic populations in the genus (Sutcharit et al. 2007; Nakadera et al. 2010). Shape differences between the morphs in *P. suturalis* and *Amphidromus* probably accumulated by selective forces while the morphs co-occurred within populations, and otherwise by drift.

In contrast, for example, in *L. stagnalis*, sinistral variants have appeared and disappeared apparently independently in at least three distant places (Vinarski 2007; Asami et al. 2008). Only six sinistral specimens have been found by searching museum collections in the two dextral species of *Cerion* (Gould et al. 1985). These cases as well as records in many other groups (Pelseneer 1920; Gittenberger 1988; Rolán-Álvarez and Rolán 1995; Páll-Gergely 2009) represent a repeated transient appearance of situs inversus. The genetic or evolutionary mechanisms responsible for trait differences between wild type and rarely found reversed variants in these examples may not be directly comparable to those in *Partula* or *Amphidromus* that have maintained enantiomorphs for a long time (Davison et al. 2009b), but might give better indications of general aspects of selection against new reversals and the conservation of homochirality in animal populations.

HOMOCHIRALITY

Our results have important implications for understanding the general homochirality in the primary asymmetry of animals, which may be developmentally established by homologous maternal genes in many bilaterians (Oliverio et al. 2010). Most metazoans including spiralians are markedly different from coiled gastropods by being basically symmetric in their external body plan, and so their left–right reversal would not significantly affect their external functions. Furthermore, reversed variants mate with the wild type with little difficulty by external fertilization or copulation with genitalia located close to the midline. Accordingly, unlike those in internally fertilizing snails, the reversed variants in many metazoans should experience little exogenous advantage or disadvantage for surviving predation or mating, regardless of their frequency. Therefore, they may only be eliminated by internal selection as shown here, although the endogenous mechanisms responsible for selection may differ from the present case owing to the diversity in developmental systems. General presence of negative internal selection and absence of positive ecological selection may explain why so few metazoan populations have been fixed for situs inversus, compared with snails that need to copulate for reproduction.

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