RESEARCH NOTE

TWISTED SEX IN AN HERMAPHRODITE: MIRROR-IMAGE MATING BEHAVIOUR IS NOT LEARNED

Joris M. Koene[1,2] and J. Cosijn[1]

[1]*Ecological Science, Faculty of Earth and Life Sciences, VU University, Amsterdam, The Netherlands; and*
[2]*Terrestrial Zoology, Netherlands Centre for Biodiversity 'Naturalis', Leiden, The Netherlands*

Correspondence: J.M. Koene; e-mail: joris.koene@vu.nl

Many organisms display some degree of asymmetry, either morphological or behavioural, which is known as chirality or handedness, respectively (McManus, 2002; Palmer, 2004). The occurrence and distribution of discrete dextral (right-handed) and sinistral (left-handed) morphs within species and populations is generally explained by evolutionary and population-dynamic processes, especially frequency-dependent selection (e.g. Ueshima & Asami, 2003; Palmer, 2004) and sometimes by sexual selection (Jesson & Barrett, 2002; Schilthuizen *et al.*, 2007). In animals, chirality often interacts with other abilities and preferences of the organism. A case in point is hermaphroditic land snails, which display extreme chirality because a sinistral morph is anatomically a mirror image of a dextral morph including the coiling direction of the shell. Their mirror-image development, which is due to a single locus, is a maternally inherited trait that is expressed very early in development (Boycott & Diver, 1923; Sturtevant, 1923; Ueshima & Asami, 2003; Kuroda *et al.*, 2009; Utsuno *et al.*, 2011). Depending on mating position, sperm exchange can become more difficult or even impossible between different morphs, i.e. in interchiral matings, and can thus act as a premating mechanism for speciation (Ueshima & Asami, 2003; but see Davison *et al.*, 2005). For example, animals that need to position face-to-face to align their genital openings for reciprocal sperm donation generally do not manage to exchange sperm (Asami, Cowie & Ohbayashi, 1998; but see Schilthuizen *et al.*, 2007). However, when the mating position involves unilateral sperm donation by one individual that mounts the shell of the partner, interchiral mating is possible, although it requires an altered mating position by the sperm donor.

In the freshwater pond snail *Lymnaea stagnalis*, a model species for chirality research, sperm are donated unilaterally via shell mounting, and interchiral matings are possible (Davison *et al.*, 2009b). Interestingly, the shell mounting behaviour performed by the sperm donor has a directional component that seems to be determined by its own chiral morphology (Davison *et al.*, 2009b). To position itself on the partner's shell for insemination, the sperm donor crawls in a circular fashion around the shell of the mating partner (reviewed by Koene, 2010). Dextral snails do this anticlockwise, while sinistral snails do it clockwise (Davison *et al.*, 2009b). Previous behavioural studies on both pond snails and land snails have been performed with naïve, virgin snails that had never mated before (Asami *et al.*, 1998; Davison *et al.*, 2009b) and in many land snail species such animals do not manage to mate, despite attempts (e.g. Asami *et al.*, 1998). Given that virgin snails have no prior mating experience, it remains entirely possible that learning plays a role in determining mating success in such interchiral mating.

Interestingly, only in a few cases has the effect of learning been invoked as a potential driving force for the maintenance or elimination of chiral polymorphism (but see Neufeld & Palmer, 2011). In the studies that have addressed this, early learning experience and plasticity turned out to be determining factors for asymmetry (mice: Ribeiro *et al.*, 2010; parrots: Magat & Brown, 2009: cichlids: Van Dooren, Van Goor & Van Putten, 2010). Importantly, in pond snails the rare sinistral morph would have a big advantage if it could increase its male mating success with the common morph, for example, via learning how to orient itself on the mirror-image shell of a partner. Furthermore, these snails do learn and remember various tasks (e.g. Lukowiak *et al.*, 1996) and leaning in a mate-choice context is known to play an important role in other animal taxa (fish: Verzijden & Rosenthal, 2011; insects: Dukas 2006). Therefore, we tested the hypothesis that previous mating experience, with the same or the opposite chiral morph, affects mating ability and insemination success. With this approach, we have the unique opportunity to investigate experimentally the potential effect that learning has on mating success of chiral morphs. In addition, we can obtain some indications of whether an intrinsic difference may exist in mating ability and preference between the two different morphs, although the experiment was not designed to test these latter hypotheses.

For our mating trials we raised 80 sinistral and 80 dextral snails from the same juvenile age (5 weeks old). The sinistrals, which are extremely rare in nature, descended from the seven adult individuals collected from the only known population where this morph occurred in Germany (Hierck *et al.*, 2005; Asami, Gittenberger & Falkner, 2008; this strain has been used in all *Lymnaea* chirality studies). Dextrals are the common morph and originated, in this study, from many adults from one Dutch population. Both morphs have been bred and raised in our snail culturing facility for many years without addition of new stock from their respective field sites. For our experiment, juvenile snails were raised in three different pair

types: 20 pairs of sinistrals, 20 pairs of dextrals and 40 pairs consisting of one dextral and one sinistral. They were raised for 12 weeks in a large flow-through tank containing low-copper water at 20°C, with each pair housed in a 460-ml perforated jar and fed lettuce *ad libitum*. Once all animals were fully mature, i.e. producing egg masses and seen mating in both sexual roles, these focal snails were isolated in individual jars for 9 d to increase their willingness to mate (De Boer *et al.*, 1997) and labelled with nail polish for identification. At this point, shell lengths of the dextrals and sinistrals were, respectively, 26.9 ± 1.9 and 25.0 ± 2.7 mm. These focals were then offered one mating partner in an 8-h mating trial. For practical reasons these mating trials were equally divided over 2 sequential days. In each mating trial we tested all eight possible combinations of learning background and chiral morph. For example, a focal sinistral snail could have had a sinistral or dextral tutor and could be tested with either a sinistral or dextral standard partner (see grey combinations in Fig. 1). These same four combinations were tested with a dextral snail as the focal individual (see black combinations in Fig. 1). Standard partners were dextral or sinistral individuals of the same age that were raised in our breeding tanks and thus had ample opportunity to mate with their own morph (respective shell lengths: 25.8 ± 2.5 and 23.7 ± 2.0 mm). For each focal individual we observed mating behaviour and noted whether and how often the focal individual attempted to copulate as a male, whether intromission was reached and sperm were transferred. The latter was verified by sacrificing recipients and dissecting out the vaginal ducts to inspect whether an ejaculate was present. Right after mating, the relatively large ejaculate can be easily seen in the vaginal duct and is transported to the sperm-storage and sperm-digestion organs in the following hours (Koene *et al.*, 2009).

All focal individuals were allowed to inseminate their partner once and all data are from the complete mating sequence during which the focal was mounted on the recipient's shell. We recorded durations of courtship and copulation as well as body size of all individuals. Deaths of a few individuals during the learning phase led to exclusion of these pairs, leaving 149 pairs for observations. There were no qualitative differences between the mating behaviour recorded during the two consecutive trials, hence all data were pooled for further analysis. In all cases that we scored as a successful copulation behaviourally, a naive observer found sperm inside the recipient's vaginal duct.

Following the learning phase and subsequent mating trials, we analysed the occurrence of successful copulation, starting
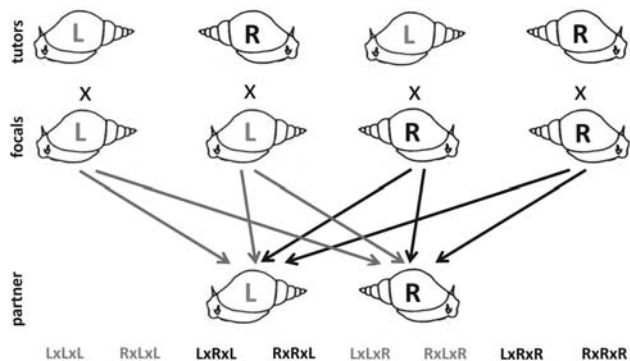
with a full-factorial nominal logistic fit (maximum likelihood) and removing all nonsignificant interactions. Thus, we tested for the effect of the factors focal chirality, tutor chirality and partner chirality (and all possible interactions) on the dependent variable occurrence of successful copulation. The resulting minimal model contained the factors: focal chirality ($\chi^2 = 9.88$, df $= 1$, $P = 0.0017$), tutor chirality ($\chi^2 = 0.080$, df $= 1$, $P = 0.78$; partner during learning phase), partner chirality ($\chi^2 = 6.66$, df $= 1$, $P = 0.0099$; partner during mating trial) and the interaction between focal and partner chiralities ($\chi^2 = 21.11$, df $= 1$, $P < 0.0001$). Hence, tutor chirality (i.e. learning background) did not influence male copulation success, thus showing that learning had no effect on either dextral or sinistral focals, which was the main focus of this study.

While bearing in mind that we are dealing with chiral morphs with different genetic backgrounds, it is nonetheless interesting to explore further the differences in mating patterns. For example, the data reveal that both focal and partner chirality were important for successful mating as a male and that these two factors interacted. This interaction occurred because sinistrals did successfully inseminate dextrals, while the opposite occurred rarely ($\chi^2 = 53.95$, df $= 3$, $P < 0.0001$; Fig. 2A). That dextrals did not succeed in inseminating sinistrals (regardless of learning background) was only partly explained by male mating attempts, because 47.4% of dextrals did attempt to mate. Compared to over 87% in the other three categories, this is significantly different ($\chi^2 = 32.76$, df $= 3$, $P < 0.0001$; Fig. 2B). Hence, the explanation for why dextrals did not manage to inseminate their sinistral partners despite attempts (while they clearly did succeed with dextral partners) must be sought in the details of their behaviour. It appears that the twisted position (consisting of a 180° turn of the body compared to normal mating) that animals had to assume to inseminate their partner rarely resulted in successful insemination by dextrals (rather, they kept attempting to reposition themselves, by resuming circling behaviour). On the contrary, sinistrals managed to assume this position successfully, thus inseminating their mirror-image partners (Fig. 2C and D; $\chi^2 = 27.86$, df $= 3$, $P < 0.0001$). That this position was also observed during normal courtship (i.e. intrachiral matings) is because it occurred when animals missed the correct position on the shell, by crawling too far along the shell's edge, and still attempted intromission (subsequently, they needed to resume circling/turning to reposition themselves, see also Van Duivenboden & Ter Maat, 1988). The 15 sinistrals that successfully inseminated dextrals did need more time to reach insemination compared to the 26 sinistral pairs and 26 dextral pairs (courtship durations $\pm$ SD, respectively, 157 ± 85, 73 ± 51 and 107 ± 51 min: ANOVA, $F_{2,64} = 2.49$, $P = 0.0002$; Tukey's test, $P < 0.05$), while copulation duration was not different. Regardless of learning background, sinistrals performed clockwise circling, while dextral performed this part of the courtship anticlockwise (consistent with previous work: Davison *et al.*, 2009b).

Thus, our study does not reveal any role for learning in the ability to mate with a mirror-image partner. Two other essential issues are highlighted by our findings, although it needs to be borne in mind that these might be confounded by the different genetic background and/or inbreeding of the different stock populations used (which does not apply to the learning results because neither of the morphs learned). First, dextrals made fewer attempts to inseminate sinistrals, therefore some sort of precopulatory mate choice may be involved. Fewer attempts were made, suggesting that this mating decision is taken early on in the mating interaction, possibly even prior to physical contact via pheromones released in the water or present in the partner's mucous trail. The importance of such chemical cues clearly requires more work, but would be consistent with previous findings related to cues relevant for sperm
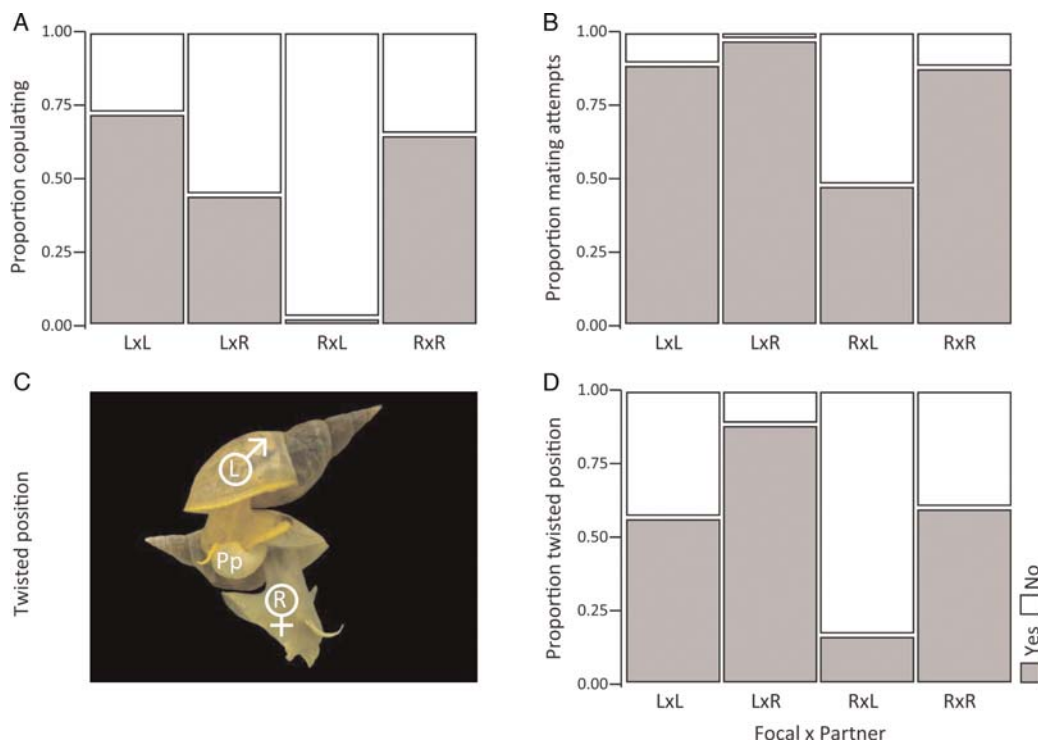

Figure 1. Schematic overview of the eight possible combinations of focal, tutor and test partner chirality of *Lymnaea stagnalis*. The scheme indicates the chirality of focals, tutors and test partners, resulting in eight different combinations. L indicates sinistral and R dextral, at the bottom of the figure, the combinations are listed as Tutor × Focal × Test partner.

Figure 2. Copulation behaviour of inter- and intrachiral pairs of *Lymnaea stagnalis*. Panels show proportions of successful inseminations (**A**) and male mating attempts (**B**), as well as the twisted position assumed by sperm donors in interchiral matings (**C**) and the occurrence of this position (**D**). For each chirality combination, focal is followed by partner (Focal × Partner). Sample sizes in **A** and **B** are, from left to right, 36, 35, 38 and 40 focals. Hence, these panels display the proportions of successful inseminations (copulations) and attempted inseminations (mating attempts) for all focals. Panel D only includes animals that attempted insemination ($N = 32$, 34, 18 and 35). Occurrences of behaviours are expressed in proportions of present (Yes, gray) or absent (No, white). The picture illustrating the twisted position shows the copulatory organ (Pp, preputium) and chirality of the sperm donor (L, sinistral) and recipient (R, dextral). This figure appears in colour in the online version of *Journal of Molluscan Studies*.

competition and sexual selection (Koene & Ter Maat, 2007). Because only one known population containing sinistrals was available and had a different genetic background than the dextral population that we used, our results cannot distinguish whether mate choice is based on cues related to chirality or inbreeding. To distinguish between the two, either other mixed-chirality populations need to be found to replicate the experiments with different populations, or the trait needs to be back-crossed to ensure the same genetic background. Second, sinistrals can successfully inseminate the opposite morph, while dextrals cannot. Hence, although these morphs are anatomical mirror images, including brain lateralization (Davison *et al.*, 2009b), this does not seem to extrapolate to completely mirrored behaviour. Using the abovementioned back-crosses it would be interesting to confirm these findings and investigate how successful these sinistrals are in sperm competition (using paternity analysis methods), although it seems unlikely that they can compensate their losses in female reproductive outcrossing (Davison, Barton & Clarke, 2009a) via their male reproductive success and selfing.

REFERENCES

ASAMI, T., COWIE, R.H. & OHBAYASHI, K. 1998. Evolution of mirror images by sexually asymmetric mating behavior in hermaphroditic snails. *American Naturalist*, **152**: 225–236.

ASAMI, T., GITTENBERGER, E. & FALKNER, G. 2008. Whole-body enantiomorphy and maternal inheritance of chiral reversal in the pond snail *Lymnaea stagnalis*. *Journal of Heredity*, **99**: 552–557.

BOYCOTT, A.E. & DIVER, C. 1923. On the inheritance of sinistrality in *Limnaea peregra*. *Proceedings of the Royal Society Series B: Biological Sciences*, **95**: 207–213.

DAVISON, A., BARTON, N. H. & CLARKE, B. 2009a. The effect of coil phenotypes and genotypes on the fecundity and viability of *Partula suturalis* and *Lymnaea stagnalis*: implications for the evolution of sinistral snails. *Journal of Evolutionary Biology*, **22**: 1624–1635.

DAVISON,, A., FREND, H.T., MORAY, C., WHEATLEY, H., SEARLE, L.J. & EICHHORN, M.P. 2009b. Mating behaviour in *Lymnaea stagnalis* pond snails is a maternally inherited, lateralized trait. *Biology Letters*, **5**: 20–22.

DAVISON, A., WADE, C.M., MORDAN, P.B. & CHIBA, S. 2005. Sex and darts in slugs and snails (Mollusca: Gastropoda: Stylommatophora). *Journal of Zoology*, **267**: 329–338.

DE BOER, P.A.C.M., JANSEN, R.F., KOENE, J.M. & TER MAAT, A. 1997. Nervous control of male sexual drive in the hermaphroditic snail *Lymnaea stagnalis*. *Journal of Experimental Biology*, **200**: 941–951.

DUKAS, R. 2006. Learning in the context of sexual behaviour in insects. *Animal Biology*, **56**: 125–141.

HIERCK, B.P., WITTE, B., POELMANN, R.E., GITTENBERGER-DE GROOT, A.C. & GITTENBERGER, E. 2005. Chirality in snails is determined by highly conserved asymmetry gen. *Journal of Molluscan Studies*, **71**: 192–195.

JESSON, L.K. & BARRETT, S.C.H. 2002. Solving the puzzle of mirror-image flowers. *Nature*, **417**: 707.

KOENE, J.M. 2010. Neuro-endocrine control of reproduction in hermaphroditic freshwater snails: mechanisms and evolution. *Frontiers in Behavioural Neuroscience*, **4**: 167.

KOENE, J.M., MONTAGNE-WAJER, K., ROELOFS, D. & TER MAAT, A. 2009. The fate of received sperm in the reproductive tract of a hermaphroditic snail and its implications for fertilisation. *Evolutionary Ecology*, **23**: 533–543.

KOENE, J.M. & TER MAAT, A. 2007. Coolidge effect in pond snails: male motivation in a simultaneous hermaphrodite. *BMC Evolutionary Biology*, **7**: 212.

KURODA, R., ENDO, B., ABE, M. & SHIMIZU, M. 2009. Chiral blastomere arrangement dictates zygotic left-right asymmetry pathway in snails. *Nature*, **462**: 790–794.

LUKOWIAK, K., RINGSEIS, E., SPENCER, G., WILDERING, W. & SYED, N. 1996. Operant conditioning of aerial respiratory behaviour in *Lymnaea stagnalis*. *Journal of Experimental Biology*, **199**: 683–691.

MAGAT, M. & BROWN, C. 2009. Laterality enhances cognition in Australian parrots. *Proceedings of the Royal Society B: Biological Sciences*, **276**: 4155–4162.

McMANUS, I.C. 2002. *Right hand left hand. The origins of asymmetry in brains, bodies, atoms and cultures*. Harvard University Press, Cambridge.

NEUFELD, C.J. & PALMER, A.R. 2011. Learning, developmental plasticity, and the rate of morphological evolution. In: *Epigenetics: linking genotype and phenotype in development and evolution.* (B. Hallgrímsson & B.K. Hall, eds), pp. 337–356. University of California Press, San Francisco.

PALMER, A.R. 2004. Symmetry breaking and the evolution of development. *Science*, **306**: 828–833.

RIBEIRO, A.S., LLOYD-PRICE, J., EALES, B.A. & BIDDLE, F.G. 2010. Dynamic agent-based model of hand-preference behavior patterns in the mouse. *Adaptive Behavior*, **18**: 116–131.

SCHILTHUIZEN, M., CRAZE, P.G., CABANBAN, A.S., DAVISON, A., STONE, J., GITTENBERGER, E. & SCOTT, B.J. 2007. Sexual selection maintains whole-body chiral dimorphism in snails. *Journal of Evolutionary Biology*, **20**: 1941–1949.

STURTEVANT, A.H. 1923. Inheritance of direction of coiling in *Limnaea*. *Science*, **58**: 269–270.

UESHIMA, R. & ASAMI, T. 2003. Single-gene speciation by left-right reversal—a land-snail species of polyphyletic origin results from chirality constraints on mating. *Nature*, **425**: 679.

UTSUNO, H., ASAMI, T., VAN DOOREN, T.J.M. & GITTENBERGER, E. 2011. Internal selection against the evolution of left-right reversal. *Evolution*, **65**: 2399–2411.

VAN DOOREN, T.J.M., VAN GOOR, H.A. & VAN PUTTEN, M. 2010. Handedness and asymmetry in scale-eating cichlids: anti-symmetry of different strength. *Evolution*, **64**: 2159–2165.

VAN DUIVENBODEN, Y.A. & TER MAAT, A. 1988. Mating behaviour of *Lymnaea stagnalis*. *Malacologia*, **28**: 23–64.

VERZIJDEN, M.N. & ROSENTHAL, G.G. 2011. Effects of sensory modality on learned mate preferences in female swordtails. *Animal Behaviour*, **82**: 557–562.