

# An unexpected twist: Sperm cells coil to the right in land snails and to the left in song birds

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**Keywords:** Gastropoda, Passeriformes, spermatozoa, chirality, dextral, sinistral

## Abstract

In animals, cell polarity may initiate symmetry breaking very early in development, ultimately leading to whole-body asymmetry. Helical sperm cells, which occur in a variety of animal clades, are one class of cells that show clearly visible bilateral asymmetry. We used scanning-electron microscopy to study coiling direction in helical sperm cells in two groups of animals that have figured prominently in the sperm morphology literature, namely land snails, Stylommatophora (514 spermatozoa, from 27 individuals, belonging to 8 species and 4 families) and songbirds, Passeriformes (486 spermatozoa, from 26 individuals, belonging to 18 species and 8 families). We found that the snail sperm cells were consistently dextral (clockwise), whereas the bird sperm cells were consistently sinistral (counterclockwise). We discuss reasons why this apparent evolutionary conservatism of sperm cell chirality may or may not be related to whole-body asymmetry.

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## Introduction

When three-dimensional structures are bilaterally asymmetric, they become chiral, meaning that the two mirror-images cannot be superimposed. In animal body forms, chirality, either external, internal, or both,

is a near-ubiquitous feature (reviewed in, for example, Ludwig, 1932; McManus, 2004). Well-known examples are coiled snails, claw asymmetry in crabs, eye torsion in flatfish, and vertebrate internal visceral organisation. However, the evolutionary developmental biology of symmetry breaking remains poorly understood (Palmer, 2004; Schilthuizen 2013; Davison *et al.*, 2016).

Some symmetry-breaking developmental cascades have been partially resolved (Grande and Patel, 2009; Basu and Brueckner, 2008; Frasnelli *et al.*, 2012). However, most of these occur at a relatively advanced stage of development, and evidence is mounting that the actual onset of symmetry breaking takes place much earlier in development and involves chiral molecules involved in the cytoskeleton (Brown and Wolpert, 1990; Vandenberg and Levin, 2010). For example, Davison *et al.* (2016) showed that, in the pond snail, *Lymnaea stagnalis* (Linnaeus, 1758), and the frog, *Xenopus laevis* Daudin, 1802, the upstream action of formins, proteins that regulate the polymerization of cytoskeletal actin filaments (Evangelista *et al.*, 2003), and, hence, cell polarity (Nelson, 2003), translated downstream into reversed or randomized whole-body chirality. These observations support the “F-molecule” hypothesis of Brown and Wolpert (1990), which suggests that a chiral molecule in the zygote sets in motion directionally asymmetric development during ontogeny, and that such a mechanism may be acting in animals generally (Oliverio *et al.*, 2010). In gastropods, this would agree with the single-gene, delayed inheritance, in which the offspring’s coiling is determined by the mother’s, not by the offspring’s genotype (Sturtevant, 1923; Schilthuizen and Davison, 2005).

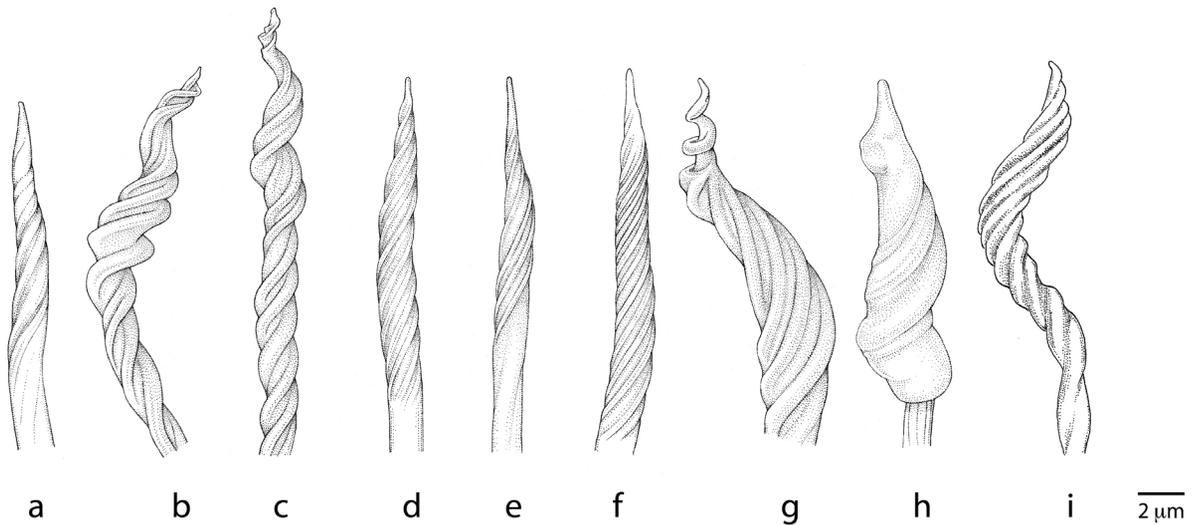


Figure 1. Drawings of sperm cell heads of nine species of Pulmonata: a, *Arianta arbustorum*; b, *Arion rufus*; c, *Arion subfuscus*; d, *Cepaea nemoralis*; e, *Cornu aspersum*; f, *Helix pomatia*; g, *Limax maximus*; h, *Succinea putris*; i, *Amphidromus inversus* (a–h originals, drawn by Erik-Jan Bosch; i after Schilthuizen and van Heuven, 2011).

Evaluating if and how molecular, intracellular chirality may be connected to organismal chirality is laborious and time-consuming, especially if this is to be done across multiple species. Methods for rapid assessment of cell polarity would facilitate this work. Recently, techniques to visualise cellular chirality by allowing cell populations to grow on ring-shaped micropatterns (Wan *et al.*, 2011) have become available. In some taxa, the striking morphology of mature sperm cells may also help. In many Mollusca, Gastrotricha, Kinorhyncha, Tardigrada, and in several groups of arthropods and vertebrates, spermatozoa have a helical structure (Ludwig, 1932; Baccetti and Afzelius, 1976; Jamieson, 2007), and cell polarity may be readily determined by inspection of their chirality. In helical asymmetry, the two mirror-image forms are called dextral, right-handed, or clockwise coiled (D) and sinistral, left-handed, or anti-clockwise coiled (L), where helix coiling direction is determined by the rotation (clockwise or anti-clockwise) as the helix is traced starting at the end nearest the observer when viewed end-on. Systematic analyses of spermatozoon dextrality and/or sinistrality may provide further insights into connections between cytoskeleton chirality and asymmetric development.

During spermiogenesis of helical sperm cells, a sheath of microtubules forms, which subsequently develops into a microtubular helix that envelops the spiral acrosome and nucleus, and, in some cases, mid-piece, and is then shed (Healy, 2001; Aire, 2014). It has

been a matter of contention which molecular cell component drives the formation of this helical structure. Some authors have implicated the microtubular helix (*e.g.*, Kondo *et al.*, 1988). However, because the contact between the microtubuli and the spiral nucleus is minimal and transient, this is no longer considered tenable, and, instead, the crystalline conformation of DNA-histone molecules in the nucleus and/or the contents of the acrosome are currently thought to organise in a helical arrangement, with the microtubular helix secondary to that (Fawcett *et al.*, 1971; Threadgold, 2017).

Although numerous studies have documented helical shape of sperm cells, few pay explicit attention to the coiling direction of these helices. In fact, as Ludwig (1932) lamented, a considerable number of authors seem to have been unconcerned about the true coiling direction of the sperm cells in their study organism, and have depicted either a left- or right-handed spiral based on personal preference rather than biological reality. Photographic images also do not often give a reliable indication of coiling direction. By their nature, transmission light or electron microscope images do not reveal whether a gyre is clockwise or anticlockwise, whereas scanning electron microscope images are sometimes mirror-imaged during the publication process (see Discussion). Consequently, very little reliable information is available on the degree of intra-individual, interspecific, and intraspecific variability in sperm cell coiling direction.

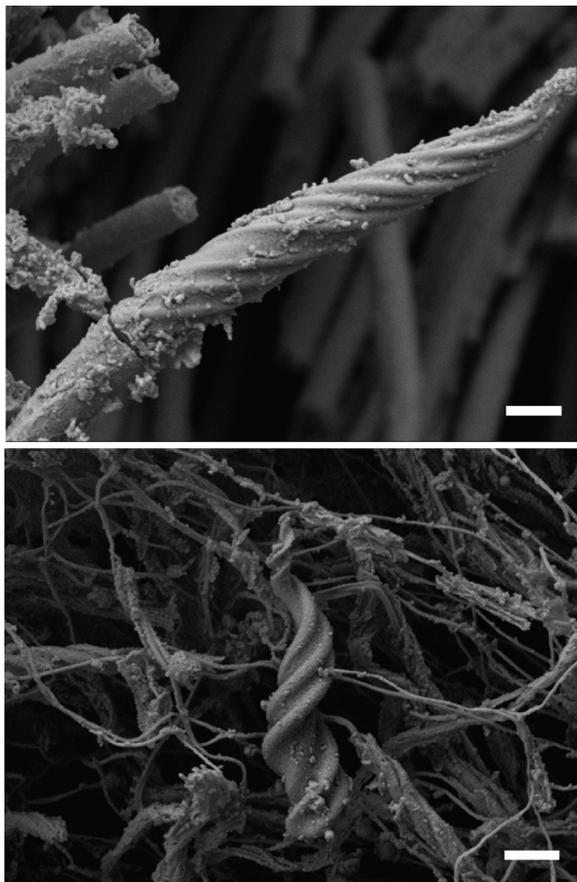


Figure 2. Scanning electron-micrographs of a sperm cell head among other sperm cells within a spermatophore for *Cepaea nemoralis* (top) and *Arion subfuscus* (bottom). Scale bar = 1  $\mu$ m.

We therefore used scanning electron microscopy on relatively large numbers of mature sperm cells from multiple species of two taxa with helical sperm cells that have figured prominently in the sperm morphology literature, namely land snails and slugs (Stylommatophora) and songbirds (Passeriformes). With some provisos, we confirm Ludwig's (1932) suspicion that in land snails, spermatozoa appear to be consistently dextral, whereas in songbirds, they are sinistral.

## Materials and Methods

### *Snails and slugs: materials*

Stylommatophoran sperm cells are most easily obtained from the spermatophores that these hermaphroditic animals produce and transfer during mating. Therefore, adult, large-bodied snails and slugs were

collected in several locations in the Dutch provinces of Zuid-Holland, Noord-Holland, and Flevoland during spring and early summer (when mating takes place) of 2014 and 2015. We collected animals that were found in copula, but also mature animals that may have recently mated and therefore contain fresh sperm in the spermatophore-receiving organ. All specimens were stored in 70% ethanol in the field. The animals were identified, dissected, and, when spermatophores were encountered, these were carefully removed and cut or broken lengthwise. The exposed halves were then dehydrated in an automated Leica EM CPD300 critical-point drier and affixed on stubs for scanning electron microscopy.

### *Birds: materials*

We used Passeriformes sperm samples that had been preserved in 5% formalin at the Department of Animal and Plant Sciences, University of Sheffield. These samples had been obtained for previous studies, using a range of techniques (as detailed in Birkhead *et al.*, 1993; Immler and Birkhead, 2005, 2007). First, samples were cooled at 5°C overnight. Then, 10  $\mu$ l of the pellet of the sample was placed directly on SEM stubs and air-dried for scanning electron microscopy.

### *Scanning electron microscopy*

Sperm samples were coated with platinum/palladium (2 nm and 20 nm in the case of bird and snail sperm cells, respectively). Then, they were viewed in a JEOL JSM-7600F FEG-SEM, at magnifications up to 20,000 $\times$ . For each sample, we attempted to view and photograph at least 20 sperm cell heads. Coiling direction was determined by eye for each sperm cell. Using the letters embossed on the stub holders, it was confirmed that the image is not mirrored at any stage during the imaging process.

## Results

For the Stylommatophora, we obtained photographs of 514 sperm cells, from 27 individuals, belonging to 8 species, and 4 families (SI1). Although sperm morphology varied considerably (Figs. 1, 2), all cells, without exception coiled dextrally (SI2). For the Passeriformes, we obtained photographs of 486 sperm cells, from 26 individuals, belonging to 18 species, and 8

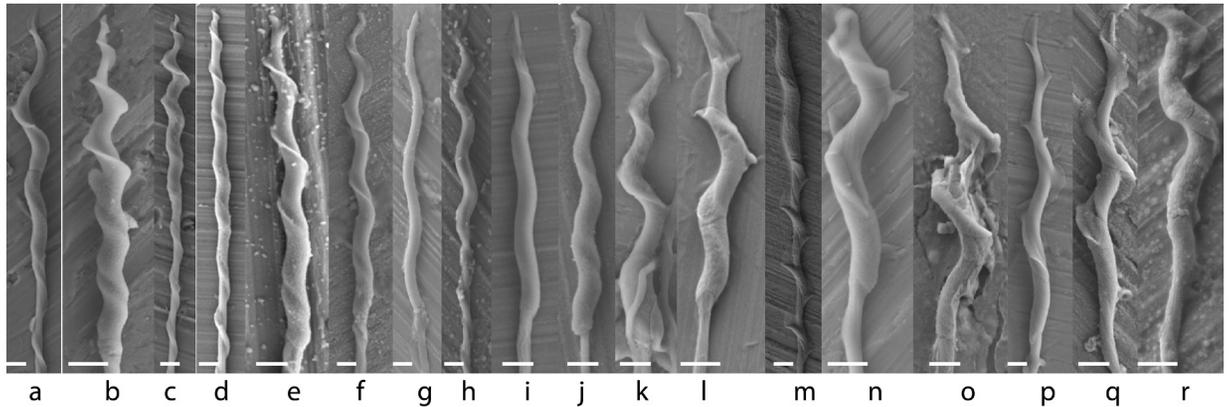


Figure 3. Scanning electron-micrographs of isolated sperm cell heads of various Passeriformes: a, *Hippolais polyglotta*; b, *Hippolais icitrina*; c, *Acrocephalus scirpaceus*; d, *Phylloscopus sibilatrix*; e, *Phylloscopus collybita*; f, *Phylloscopus trochilus*; g, *Sylvia melanocephala*; h, *Sylvia atricapilla*; i, *Sylvia curruca*; j, *Cistothorus palustris*; k, *Prunella modularis*; l, *Emberiza citrinella*; m, *Turdus merula*; n, *Fringilla coelebs*; o, *Acanthis cabaret*; p, *Chloris chloris*; q, *Carpodacus erythrinus*; r, *Haemorrhous purpureus*. All sperm cell heads have been shown with the tail down, and the acrosome up. Scale bars always represent 1  $\mu\text{m}$ .

families (SI3). Here, overall morphology was more uniform (Fig. 3), all sperm cells coiled sinistrally (SI4).

## Discussion

Our results suggest that sperm cell chirality is fixed in two large clades of animals: exclusively dextral in Stylommatophora, and exclusively sinistral in Passeriformes. Although we see interspecific differences in the number of gyres, the height of the crest, and the extent to which the helical structure dominates along the length of the acrosome, nucleus, midpiece, and tail, we found no indication of any intra-individual, intraspecific or interspecific variability in the *direction* of coiling. For Stylommatophora, this finding is consistent with the dextral sperm cells previously reported in the land snails *Amphidromus inversus* Müller, 1774, *Arion hortensis* (Férussac, 1819), *Deroceras reticulatum* (Müller, 1774), *Tandonia sowerbyi* (Férussac, 1823), and *Anguispira alternata* (Say, 1816) (Schilthuizen and van Heuven, 2011, and references therein).

For the Passeriformes, sinistrality has previously also been reported in, for example, *Hirundo rustica* Linnaeus, 1758, *Turdus merula* Linnaeus, 1758, *Fringilla coelebs* Linnaeus 1758, *Sturnus vulgaris* Linnaeus, 1758, and *Poephila acuticauda* (Gould, 1839) (Retzius, 1909; Vernon and Woolley, 1999; Hermosell et al., 2013; Rowe et al., 2015).

In contrast, a small number of papers depict stylommatophoran spermatozoa as sinistral helices (e.g.,

Maxwell, 1975, for *Cornu aspersum* (Müller, 1774), Selmi et al., 1989, for *Oxyloma elegans* (Risso, 1826)). Similarly, Birkhead and Immler (2007) depict a *Passer domesticus* (Linnaeus, 1758) sperm cell as a dextral helix. Because none of these papers explicitly state the chirality of their subjects, these inconsistencies could, in fact, be the result of inadvertent mirror-imaging of the scanning-electron micrographs. A more reliable deviation from our observations is Hickman (1931: 265), who states that in the amber snail *Novisuccinea ovalis* (Say, 1817), ‘there is no uniformity of direction of the spirals. [...] Out of many specimens examined, I find that the spirals may go in either a left or a right direction from head to tail, and that one condition is about as common as the other’. If confirmed by additional observations, this would suggest fixed coiling direction may be lost in some stylommatophoran lineages.

Despite the aforementioned deviations, and pending further investigations to augment the number of species and individuals sampled, we tentatively stress the uniformity in coiling direction of sperm cells in Stylommatophora and Passeriformes. It is tempting to speculate that such cellular homochirality is due to the same chiral cytoskeletal elements, such as actin filaments, that set in motion whole-body homochirality in the zygote (Levin and Palmer, 2007). Although, during spermiogenesis, the sperm cell nucleus retains little more than a near-crystalline DNA-histone-protamine complex, the acrosome, which, especially in birds, forms a considerable portion of the spiral sperm

head, is known to contain actin filaments (e.g., Breitbart *et al.*, 2005).

Another potentially relevant observation is that the sinistral and dextral sperm cells in birds (deuterostomes) and snails (protostomes) parallels the inversion of the antero-posterior and dorso-ventral axes in deuterostomes. The result in whole-body chirality is that the right of protostomes becomes the left in deuterostomes.

However, we doubt that such a one-to-one relation exists between spermatozoon homochirality and organismal homochirality. Two previous studies, by Selman and Waddington (1953) and Schilthuizen and van Heuven (2011), showed that snail species that are dimorphic for whole-body coiling direction (*Radix labiata* (Rossmässler, 1835) and *Amphidromus inversus*, respectively, which display both left-handed and right-handed shells), nonetheless always show dextral sperm. Similar studies have not yet been carried out in birds, where mirror-image reversal of the viscera is exceedingly rare (Palmer, 2004).

In conclusion, we suggest that a wider study of chirality in helical sperm cells in these and other taxa may prove a useful source for understanding symmetry breaking in development. We also suggest that studies of sperm morphology always explicitly record and identify the chirality of spiral structures and confirm that imaging methods did not yield inverted images.

## Acknowledgements

We thank Tim Birkhead for providing samples, and Bertie-Joan van Heuven and Ewan Richardson for technical assistance. Three reviewers, Rich Palmer, Marco Oliverio, and Yumi Nakadera provided comments that helped us improve the paper. The specimens of *Helix pomatia* were collected under permission FF/75A/2010/021a from the Netherlands Ministry of Agriculture, Nature, and Food Quality.

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### Online supplementary information

- SI1. A list of all Pulmonata samples used.
- SI2. Representative scanning electron micrographs of details of the sperm mass inside spermatophores for each of the Pulmonata samples in SI1.
- SI3. A list of all Passeriformes samples used.
- SI4. Scanning electron micrographs of representative sperm cells for each of the Passeriformes samples in SI3.

Received: 2 September 2017

Revised and accepted: 14 November 2017

Published online: 22 December 2017

Editor: A. Minelli