Flipping shells! Unwinding LR asymmetry in mirror-image molluscs

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In seeking to understand the establishment of left-right (LR) asymmetry, a limiting factor is that nearly all animals are ordinarily invariant in their asymmetry, except when manipulated or mutated. It is therefore surprising that the wider scientific field does not seem to appreciate the remarkable fact that normal development in molluscs, especially snails, can flip between two chiral types without pathology. Here, recent progress in understanding the evolution, development and genetics of chiral variation in snails is described, and put it in context with other animals. I argue that the natural variation of snails is a crucial resource towards understanding the invariance in other animal groups, and ultimately, will be key in revealing the common factors that define cellular and organismal LR asymmetry.

"In another moment Alice was through the glass, and had jumped lightly down into the Looking-glass room" [1]

The establishment of LR asymmetry

While most animal bodies are bilaterally symmetric on the outside, the internal organs usually show a consistent **left-right (LR) asymmetry**. Defining this LR asymmetry is a critical part of early development, such that left/right positional errors are an important class of human birth defect, and in later life numerous diseases affect apparently symmetric organs in an asymmetric fashion [2-4]. Yet, while a wealth of prior studies have been important in revealing the genes that promote the propagation of asymmetric signals [e.g. 5,6], the earliest LR symmetry-breaking events are not clear [2,3,7,8]. Is there a common pathway of LR symmetry-breaking across the breadth of animal life, or has it been independently derived [9]?

The fundamental problem in understanding LR asymmetry is that while it is relatively simple to conceive how the LR axis is established relative to the embryonic anterior-posterior (AP) and dorso-ventral (DV) axes, it is insufficient that left is *different* from right. Instead, it is a requirement that left and right are consistently orientated in the same manner. In the classic view, asymmetry is signalled by a **chiral** structure, the fabled "F-molecule" (**Figure 1a**), which is directionally orientated relative to the AP and DV axes [10].

There is now an emerging consensus that LR asymmetry likely originates from the cytoskeletal dynamics that underlie the asymmetric behaviour of individual cells (**Figure 1a**). The chirality that is fundamental to living cells is an ancient evolutionary feature that metazoans, including snails [11,12], exploit for macroscopic anatomical purposes [3,7,11,13].

However, in seeking to understand LR asymmetry [see recent reviews: 2,3,7,8,13-15], a continuing puzzle is that nearly all individuals in nearly all species have the same, fixed asymmetry. There is very limited natural variation, and so studies have necessarily been obliged to use rare mutants or manipulations to create wholly or partly **mirror-imaged** individuals. Unfortunately, while this approach has obviously been very valuable, it is also one-sided, and limiting, because most changes are either pathological, pleiotropic or

epistatic – if mutational changes are not pathological, at least in a laboratory setting, then why do they not sometimes rise to high frequency? A further complicating factor is that positional errors in LR asymmetry are often corrected, because of multiple redundancies in the pathway that produces asymmetric development [2].

In consequence, it is unfortunate that scientists across the world are using invariant animal models, largely vertebrate or fly/nematode, to understand the evolutionary and developmental origins of LR asymmetry, while largely ignoring snails, the only animal group in which individuals commonly show inherited variation in asymmetry [16-18]. The wider scientific field does not seem to fully appreciate the remarkable fact that inherited variation in snails ensures that ordinary development can flip between two **chiral** types, **dextral** or **sinistral (Figure 2)**. This originates in a stereotypic alternating **spiral cleavage** early in development that ultimately produces snails with clockwise or anticlockwise coiling shells, with mirror-image bodies to match (**Figure 1**). In all other animal taxa, including the other **Spiralia**, LR asymmetry does not vary, with very few exceptions [19], except when pathological [albeit sometimes at high rates e.g. in the frog 11,13].

Researchers are trying to see through the “Looking glass” to understand the mirror-image world on the other side [1], while not realising that they can use snails to just step through (**Figure 2a**). Moreover, the blunt tools that they are using to understand asymmetry – mutation and manipulation – often break the “Looking glass”, by causing pathology.

In comparison, the advantage of using snails is that the variation has already been sifted by natural selection – by and large, the mutations that reverse chirality are the ones that work. The repeated independent evolution of mirror-image snails therefore has the potential to be an unequalled natural genetic resource in understanding the early steps towards establishing LR asymmetry, before gastrulation and in the absence of cilia.

Fortunately – following a long aestivation – technological advances, alongside the discovery, isolation and distribution of sinistral pond snails *Lymnaea stagnalis* from a pond in Germany [20], are invigorating what has previously been a field of research that has advanced at a “snail’s pace”. The aim of this work, therefore, is consider recent advances in molluscan LR asymmetry, and reflect on the impact to the wider field. It is not the intention to consider the underlying mechanisms of symmetry breaking in detail, because several recent reviews have done this, including this journal [21], nor consider a possible deeper conservation of mechanism across kingdoms [9].

A twisted history of embryology and genetics in snails

It has long been known that the direction of the chiral twist that takes place during the third cleavage corresponds to the shell coiling direction and body asymmetry of the adult snail [22]. Then, in the early days of Mendelian genetics, Boycott and Diver [23] reported that shell coiling in the pond snail *Lymnaea* (*Radix*) *peregra* was a hereditary character, although the pattern of inheritance was difficult to understand. Sturtevant [24] responded and suggested that chirality is an exceptionally clear illustration of “maternal inheritance”, whereby the direction of coiling in the offspring is dictated by the genotype of the mother (**Figure 2c**). Since then, maternal inheritance of chirality has been demonstrated in all studied species of snail. In some but not all species, maternal *D* alleles dominantly determine a clockwise or dextral twist in embryos, so that during the third cleavage, four micromeres simultaneously emerge from four macromeres and twist clockwise [refs in 17].

Unfortunately, a neglected issue is that the species that have received the most study – because they are the ones that are easy to keep in culture – are those in which sinistrality is rare and usually associated with pathology. For example, in the pond snail *L. stagnalis*, only about 50% of offspring hatch from sinistral mothers. There is also occasional dextral cleavage and reversion to dextrality [11,25,26]. Similarly, development is pathological in *L.*

peregra, and in two newly characterised land snail species in which sinistrality is rare, the mutant allele apparently interferes with the chiral cleavage, so that wholly mixed broods are regularly produced [27-29].

In comparison, in species in which sinistrality is common, there is no evidence that chiral reversal is associated with pathology. Unfortunately, these species have rarely been studied from a genetic perspective, with just a few exceptions [30,31]. Fortunately, the increasing power and reduced costs of genome sequencing mean that it is now feasible to identify the chirality-determining genes in naturally variable species, even those that are not amenable to laboratory culture (**Box 1**).

Unwinding snail chirality

In an initially surprising finding (**Box 2**), it was discovered that at least two genes, including the TGF β gene *nodal*, are involved in LR signalling in both vertebrate (Deuterostomia) and molluscan (Lophotrochozoa) asymmetry, yet are absent in derived animals such as nematodes and fruitflies (Ecdysozoa) [5]. Then, it was shown that asymmetry in snails has much in common with other taxa that lack *nodal*. Thus, while the unequivocally first symmetry-breaking event in nematodes and snails is unknown, when the chiral arrangement of blastomeres in both is inverted by micromanipulation, embryos develop in a mirror-image form [32,33]. Further findings suggested strong similarities at the molecular level across diverse bilaterian animals. For example, observations on nematodes imply that during cytokinesis a chiral structure within the actin and microtubule skeleton translates intrinsic chirality to morphological asymmetry [34-36].

In the most recent breakthrough, it was established [11] and then proven [37-39] that a frameshift mutation in one copy of a duplicated cytoskeletal protein, a diaphanous-related **formin** is causative of variation in the chirality of *L. stagnalis*. A formin gene duplication is also associated with variation in LR asymmetry in a land snail, *Bradybaena similaris* [29]; similarities in the phenotype also suggest the involvement of a formin in *L. peregra*. The morphological LR asymmetry of single-cell snail embryos also varies within chirally dimorphic species [37], mirroring much earlier work comparing species of different chirality [38,40,41]. In comparison, the chirality of sperm does not vary with body asymmetry [42], perhaps indicating that any changes in the actin cytoskeleton are relatively subtle.

Together with studies in other animals [43,44] and individual cells, which can align their LR axis *in vitro* in the absence of organismal cues [45-48], these works show that LR asymmetry (possibly also including human handedness [49]) originates in the cellular architecture and is both defined and evident in the single cell embryo. Nonetheless, despite recent progress, there is a still only an embryonic knowledge of the molecular interactions that take place to induce symmetry-breaking in molluscs.

At the cellular level, how does a symmetric cell emerge from chiral cytoskeleton? Given a chiral cytoskeleton, what are the regulatory mechanisms and mechanics that create a first chiral cleavage, and then a second cleavage in the reverse direction?

At the organismal level, what is it about snails that makes them able to vary in their chirality when no other animals are able to do so? There has been a recent appreciation of the external factors that may impact upon chirality, such as natural and sexual selection [50-52], but less considered recently is how endogenous factors interact with the external environment to enable the evolution of sinistrality [25,26,53]. The following is therefore a view of this process of development, from the molecular and cellular to the organismal level.

Defining LR asymmetry in the cytoskeleton

Until recently, the means by which interacting sets of actin assembly factors work together in cells had remained obscure. This is unfortunate because a basic understanding of actin dynamics is a prerequisite towards understanding how the molecular chirality defines the cellular chirality in snails.

One point of progress has been in understanding how cellular chirality arises from the self-organization of the actin **cytoskeleton** [reviewed in 14,46], and biomechanical forces in general [15]. By studying actin cytoskeleton self-organization in circular fibroblasts, it was shown that a radially symmetrical system of actin bundles evolves spontaneously into a chiral system [47]. The handedness of the chiral pattern is regulated by alpha-actinin-1, with formin giving a directional rotation to actin filaments, resulting in a rightward tilting of radial fibres when triggered by a slight imbalance of transverse fibres. To interpret these findings, Tee at al. [47] used a computer model to demonstrate that the dynamics of the actinin-enriched radial fibres (RFs) and myosin-IIA-enriched transverse fibres (TFs) can explain the pattern transition from radial to chiral. A subsequent study showed that formin-driven actin polymerization is indispensable for the development of chiral actin patterns [48]. Thus, these studies and others demonstrate that the self-organization of the actin cytoskeleton provides built-in machinery that potentially allows cells to develop left-right asymmetry.

The main other avenue of advancement has been in using *in vitro* experimental set-ups to test the mechanics of individual components in actin assembly. The theory suggests that formin activity is important for maintaining tension at cell-cell junctions, because of local actin assembly and positioning of the actomyosin contractile apparatus. This is in part due to a twist that formin dimers impart to the actin cytoskeleton as it is extruded, likened to the swirl of a soft scoop ice cream [54]. Most recently, it has been shown that the rotation of mDia1 twists the helical structure of F-actin in the opposite direction to the cofilin-induced twist [55]. Specifically, the helical rotation of mDia1 untwists the helical structure of F-actin when mDia1 and the pointed end are immobilized, increasing the length of actin cross-overs by up to 10%. In comparison, in cofilin-assembled F-actin, the cross-overs are shortened by about 75% [56]. The precise twisting structure of F-actin is therefore determined by the opposite actions of formins and cofilin, and so the interplay between many different factors likely plays a pivotal role in the formation of high-order actin structure, such as actin stress fibres and actin cables [55].

Recent studies are beginning to elucidate how the antagonistic actions of cofilin and formin/myosin impart both twist and stability to the formation of high-order actin structure, such as actin stress fibres and actin cables F-actin [55,57,58]. Future studies that combine modelling with quantitative imaging analysis, especially of single-molecules, will give further clues towards understanding now chirality is transmitted from molecules to cells.

One suggestion arising from these findings is that in snails, both formin mutations [11] and misexpression [29] may impair the twist in the actin itself, which then impacts upon the tension and torque generation at cell-cell junctions and/or cortical flow [14]. Another consideration is that if formin inhibits cofilin depolymerisation, then perhaps there is a greater turnover of actin in mutant snails, which then causes the delayed twist in the third cleavage [12].

A future task must be to put these individual findings in context, incorporating the roles of cadherins, myosin and inversin in regulating the actin cytoskeleton [43,44,59-61]. For example, there is a suggestion that the involvement of myosin and inversin with cell-cell junctions might be another key part in defining asymmetry across diverse animals [61]. Moreover, as myosin has been shown to have a conserved LR asymmetry function in chordates and arthropods [43,59,62], then it is likely also involved in molluscs.

From chiral cells to chiral shells

How is chirality transmitted from molecules to cells? Studies in the nematode have shown that the clockwise tilt of two blastomeres at the four to six cell-stage transition, which marks the initiation of LR asymmetry, is matched by counter-rotating chiral cortical flows along the divisional axes. Changing the flow speed changes the degree of blastomere rotation, indicating that intracellular chiral flow is directing symmetry-breaking [63]. More generally, there is an increasing focus on LR asymmetry in cell-cell interactions – for example, in *Drosophila* an initial chirality of the shell shape is converted via LR asymmetric cell interactions into a directional hindgut rotation; myosin mutants twist in the opposite direction [64].

In the absence of similar data for molluscs, one approach has been to model spiral cleavage, where a combination of cellular development rules leads to biologically realistic outcomes. Brun-Usan et al. [65] found that a combination of cell polarization by an animal-vegetal gradient, a bias to orthogonality between consecutive cell divisions (Sachs' rule - arises from the stereotypic duplication of the centrioles, with their 90° angle, between cell divisions), cortical rotation and cell adhesion, reproduced the spiral cleavage, while other combinations of processes did not.

These theoretical findings are consistent with a unifying model that Levin and colleagues have put forward, whereby LR asymmetry is initiated during the early stages of development via the inherent chirality of the cytoskeleton [3]. The centrosome is the central actor, because it contains two centrioles that are asymmetric in size and at a right angle to each other, joining to form the microtubule organising centre (MTOC). The sperm entry point at fertilization produces the first “punctuation” mark, so setting this point as distinct from any other. The chirality of the actin cytoskeleton then establishes a LR axis, because the orientations of the AP and DV axes means that the MTOC is able to uniquely signal left and right [13].

The limited empirical data in snails are also consistent with Levin’s model. There is historic evidence that the DV axis is defined via the sperm entry point influencing the position of the spindles at the first two cleavages, and evidence for asymmetric trafficking of RNA involving centrosomes [66,67], and in the first cell division [11,68], albeit partly disputed [37-39,68]. In the future, it would be desirable to track asymmetric microfilament-dependent cortical contraction, the orientation of the MTOC and RNA trafficking. Although this evidence would be entirely correlative (without manipulations), inferential gains could be maximised by the identification of genes that determine the natural genetic variation in LR asymmetry of snails, with respect to transport of maternal determinants whose physiological activity amplifies cellular directionality into organism-wide positional information.

Overall, there are therefore several testable models of symmetry-breaking and spiral cleavage in molluscs, but there are few empirical data. As the molecular basis and *in vitro* mechanics of actin polymerisation becomes better understood, *in vivo* animal models such as snails will be essential in both providing empirical support for cell-based models of asymmetry and suggesting further avenues of research. Of course, many of the more general questions in terms of establishing LR asymmetry will always be best answered using *in vitro* systems, or other animal models for which there are far more sophisticated tracking methods [69]. Nonetheless, inferences gained from snails should feed into these other model systems.

Unfortunately, manipulative experiments that might be used to fluorescently-label proteins (such as actin) and enable fine-resolution real-time movies of cell cleavage and manipulative experiments have had a slow take-up in molluscs, with only a few studies

using RNAi [70,71] or CRISPR/Cas9 [37,72,73]. Germline transgenic methods will be essential in making progress, because of the maternal inheritance, but they currently require high skill in both delivering the vector and culturing the embryo to adulthood [37]. For more rapid progress, a modified virus and CRISPR/Cas9 construct combination would be desirable [74].

How do snails commonly vary in their LR asymmetry?

I began by highlighting the irony that molluscs – mainly snails – are the only animal group to vary in their LR asymmetry, yet many scientists are welded to studying invariant model species, mostly vertebrates, flies or nematodes. More specifically, the invariant LR asymmetry of other spiralian phyla is rarely considered against the varying LR asymmetry of snails, despite fundamental similarities in their development [75]. There is therefore an apparent paradox. Sinistral species of snails have evolved many times over, despite a mating disadvantage, yet sinistrals in other spiralian groups are absent, even though they develop in much the same way as snails and do not have conspicuous LR asymmetries that could cause mating problems.

From the perspective of the whole animal, snails vary in their chirality precisely because they are the only animal group that has a conspicuous outward asymmetry upon which natural and sexual selection can act (**Figure 3**) [25,75]. The fact that sinistrals have difficulty mating with dextrals, potentially selecting against new sinistrals, is a distraction — this is because the limited evidence suggests that the negative frequency-dependent selection only applies over a small range of morph frequencies, and only to some snails that are not able to mate, or mate with difficulty [25,76]. It also does not apply to any species that is broadcast spawning, such as many marine molluscs and annelids [25,53,77].

The most direct evidence for the role of selection in promoting the evolution of new chiral types comes from SE Asia. In that region, snakes predominantly predate dextral-coiling snails [50], which perhaps explains the high incidence of sinistrals [18]. Separately, in *Amphidromus* snails, inter-chiral mating is favoured because it is more likely to lead to fertilisation [51,78]. Finally, reproductive character displacement potentially provides a more general explanation for the evolution of chiral variation, although direct evidence is limited: if a new sinistral morph arises within a dextral species, then any mating disadvantage is counterbalanced if the sinistrals do not waste time (and gametes) mating with other species [25,79,80].

From the perspective of the embryo, it is more difficult to understand how chiral evolution is possible, given that the stereotypic alternating oblique cleavages of spiralians have mainly remained conserved over long evolutionary periods [75,81,82]. If the default character is that chirality does not evolve, then what are the special circumstances in molluscan development that make it possible? In other animal groups, it is hypothesised that there are multiple redundancies and/or constraints in the pathway that produces chiral development, such that positional errors tend to be corrected [2]. This is presumably a direct outcome of natural selection, because organ positioning defects are invariably pathological, and usually lethal. The fact that chiral rearrangements mostly occur at specific time points during embryonic development suggests that chiral properties are tightly regulated [14]. Pleiotropy is also implicated: it may be difficult to change the cytoskeletal genes involved in determining asymmetry without also incurring pathology. Another interesting perspective is that chirality emerges from multiple molecular interactions and is a summed effect of the interactions of many proteins [83]. Thus, perturbation of individual proteins may result in subtle deviations in twist, but they are insufficient on their own to reverse chirality [84].

A flip switch

A hypothesis is that snails vary in their chirality, because of the combined effect of natural selection on the outward phenotype and the dichotomous switch of spiral cleavage during early development (**Figure 3**). Thus, while a body of research shows that the LR asymmetry is coded in the single-cell embryo, the twist of the third cleavage is the key moment that defines the morphological body asymmetry. It is a dichotomous chiral switch which can not be supressed by other pathways or redundancies [32,85]. The strict maternal inheritance may also be a factor – as chirality is wholly determined by maternal inputs, then there is no possibility of a conflicting chiral signal coming from the zygote [86].

To date, formin is the only known gene that is able to accomplish this switch, but as mentioned changes in formin are associated with pathology in both *L. stagnalis* and *Bradybaena* [11,25,26,29]. In comparison, sinistrality is not associated with pathology in species that are frequently sinistral, but we do not know the identity of the genes involved. Therefore, despite recent progress it is still wholly unknown how genes determine natural chiral variation in snails, without pathological effect.

Fortunately, genome sequencing and genome mapping technologies are now sufficiently mature that it should be relatively straightforward to identify these genes. Only then can we determine if there is a universal pathway that sets up an asymmetric cellular architecture in all animals and plants [e.g. via microtubules, 9], and whether this fits into a unifying model of LR asymmetry. In snails, chiral mutations are likely due to regulatory changes in gene expression, or mutations in accessory factors are more likely to subtly alter the topology of the cytoskeleton, without pathology.

Concluding Remarks and Future Perspectives

Unlike any other animal group, snails vary in their LR asymmetry. Although recent progress has implicated the cytoskeleton as key, the set of genes that determine chiral change are not known, nor how this is possible without pathology (see Outstanding Questions). I argue that constraints to chiral change are likely the default in all animals, except in snails where new chiral variants can sometimes be favoured. This may be enabled by the dichotomous nature of spiralian cleavage and the action of selection on the outward shell phenotype.

LR asymmetry does not vary in most major taxonomic groups, yet the core features may be conserved across all groups. Therefore, the natural variation of snails will be crucial to understanding this invariance and revealing key parts of the pathway that defines asymmetry.

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Glossary

Chiral: an object that is asymmetric in such a way such that it is non-superimposable on its mirror image. A cell may have radial symmetry, by gross morphology, but contain a chiral cytoskeleton. A snail shell has LR asymmetry and is also chiral.

Cytoskeleton: the scaffolding of a cell, involving a network of protein filaments and microtubules in the cytoplasm that control cell shape, maintain intracellular organization, and are involved in cell movement.

Dextral or sinistral: in this context, whether the third cleavage is clockwise or anticlockwise with respect to the animal pole, or, whether the shell coiling direction is clockwise or anticlockwise when viewed from above.

Formin: a group of proteins that are involved in the polymerization of actin, via the fast-growing end (barbed end) of actin filaments. Formins regulate the actin and microtubule cytoskeleton and are involved in various cellular functions such as cell polarity, cytokinesis, and cell migration.

LR asymmetry: morphological asymmetries in otherwise bilaterally symmetric organisms. The left-right axis is defined in relation to the anterior-posterior and dorso-ventral axes, and is perpendicular to both.

Mirror-image: in this context, any individual for which some or all body organs are reflected as in a mirror. Normal individuals are *situs solitus*, with mirror-imaged individuals described as having *situs inversus*. *Heterotaxia* refers to the abnormal arrangement of visceral organs.

Spiralia: a morphologically diverse group of animals, united by their spiral cleavage, a means of early development found in many members of the Lophotrochozoa. The group includes molluscs, annelids, nemertean flatworms and some other groups.

Spiral cleavage: division of cells in the early embryo whereby the orientation of divisions alternates between clockwise and anticlockwise with respect to the animal pole. The third cleavage is dextral in most spiralian taxa, except some molluscs.

Box 1: Stepping through the ‘Looking-glass’

One peculiarity of chiral variation in snails is that individuals of different chiralities have difficulty mating, or are not able to mate at all. A mutation that flips chirality is in theory able to create a new species by “single-gene speciation”. This idea that a chiral change can cause speciation is remarkably persistent [e.g. 37,87], yet the combined weight of both theory and empirical data suggest that chiral change is *on its own* not likely to cause speciation.

Historically, the best-studied system for understanding chiral evolution and reproductive isolation have been *Partula* snails; they are now showing potential for future genomic studies [88]. The most well understood modern-day example are *Euhadra* snails from Japan. In this genus, it was originally suggested that as these snails mate in a face-to-face position, interchiral mating was not possible and so new species evolved because of changes in chirality [89]. Both a model and a RAD-seq based phylogenomic analysis have subsequently shown that that there is extensive gene-flow between the two chiral types [30,79]. This is because of rare matings (see Figure below), and also due to the maternal inheritance, which induces a generational delay in the expression of the chirality locus, and a discrepancy between individual phenotype and genotype.

Genomic studies of chiral reversal are not just of niche interest to evolutionary biologists. This is because one future avenue for research would be to use a genome wide association study (GWAS) to identify loci that are associated with non-pathological, natural chiral variation. There are already several emerging systems that could form the basis for a GWAS-based approach to identify chirality loci [30,31,50,51,90,91]. In doing so, any analysis would have to account for the uncertainty of chiral genotype inferences [79], due to the maternal inheritance, population structure and the number of times that a new chirality has evolved. For example, as the *de novo* mutations that produce mirror image snails must arise only rarely, the most straightforward explanation for multiple paraphyletic sinistral species or populations within a dextral group (or *vice versa*) is gene-flow or ancestral polymorphism (e.g. in *Euhadra* [79,89]). This rarity may also be used to benefit a GWAS study, because the expectation is that most loci will be divergent, except those that are linked to chirality.

Figure (in box): Dextral and sinistral snails have difficulty mating, especially species that mate in a face-to-face position. This image shows a rare mating between sinistral *Euhadra quaesita* and dextral *E. peliomphala*. Photo: Kentaro Nakao and Seiichi Takase, reproduced with permission.

Box 2: *Nodal* studies illuminate a diversity of model animals

Although LR asymmetry was likely a feature of the common ancestor of the three main bilaterian animal groups, the study of the molecular basis of LR asymmetry has traditionally concentrated on just a few model animals, such as vertebrates and nematodes, in the Deuterostomia and Ecdysozoa, respectively. The third main animal group, the Lophotrochozoa, which includes molluscs and the other spiralian lineages, has been neglected, partly because there is no single model taxon around which a research community has formed. As a result, progress in genome sequencing and developing transgenic methods for molluscs has lagged behind other groups (but is beginning to catch up e.g. [72,92]).

The danger of neglecting the Lophotrochozoa was highlighted with the surprising discovery of a *nodal* orthologue (and another gene family member, *pitx*) in snails and annelids [5]. Previously, *nodal* had been identified only in the deuterostomes, as a key signalling molecule that is asymmetrically expressed, and critical for establishing morphological LR asymmetry. As *nodal* was not found in the Ecdysozoa, and data were lacking for most Lophotrochozoa, it was assumed that the nodal pathway was a deuterostome innovation.

An early study, using undefined cross-phyla probes which may react to other TGF-beta genes, hinted that nodal might be present in the pond snail [93]. Then, Grande and Patel [5] discovered *nodal* in two different sinistral and dextral snails, showed that it is initially expressed on one side (unlike most vertebrates) and that the side of the embryo that expresses nodal is related to body chirality. Pharmacological knockdown of the nodal pathway produced some individuals with a loss of shell chirality [5].

The discovery of *nodal* – since validated by reports of nodal in other lophotrochozoans [75,94], including lineages without obvious adult left-right asymmetries, as well as the finding of a putative orthologue in the Cnidaria [95] – showed that the pathway must have evolved before the Bilateria split into the extant groups. This involvement of the same gene pathway therefore gives a tantalising hint of a conserved signalling system that may date back to the earliest origins of the Bilateria [96,97].

The key question for the future is to understand whether the use of the nodal pathway is indicative of a deeply conserved, ancient LR asymmetry, or else repeated use of the same pathway for the same function. Comparative phylogenetic studies are one approach e.g. [75,94]. Another approach would be to identify the gene regulation network [98] that leads to asymmetric nodal expression in spiralians. Present knowledge is very limited – as nodal expression in snails is asymmetric from the outset, and does not seem to be autoregulated or involve cilia, then at minimum, this indicates significant innovation or divergence in use. A key question is to understand how this asymmetric expression comes about, including the involvement of upstream gene networks and mechanical forces, such as the directional rotation of the micromeres [15].

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Figure 1. Development and genetics of LR asymmetry in snails. (A) LR asymmetry is hypothesised to originate in a chiral “F-molecule”, and transmitted via a chiral cytoskeleton to the embryo. In snails, LR asymmetry in morphology is visible at the third cleavage; viewed from the animal pole, four macromeres each bud off a small micromere. The direction of cleavage, whether clockwise or anti-clockwise, determines the chirality of the adult snail, including the shell chirality (dextral shown) and body asymmetry (right-sided genital pore shown). A anterior; P posterior; D dorsal; V ventral; R right; L left. (B) In wild-type *L. stagnalis* (top), a dextral third cleavage produces dextral-coiling offspring; when a formin inhibitor (SMIFH2) is added, the initial cleavage is neutral and then later dextral [11]. In sinistral mutant *L. stagnalis* (middle), the initial cleavage is neutral and then later sinistral [12]. In wholly sinistral taxa, such as *Physa* (bottom), the cleavage is immediately sinistral. (C) Chirality is a maternally inherited trait. If dextral is dominant, then individuals that are genetically dextral (*DD* or *Dd* – left) will bear dextral offspring, irrespective of their own chiral phenotype. Genetically sinistral individuals (*dd*; right) will bear sinistral offspring. Photo credits: Angus Davison, Lauren Holden, N. Yotarou Ester de Roij.

Figure 2. Mirror-images in literature, popular culture and in snails (A). Alice stepping through the mirror, image by John Tenniel from “Through the Looking Glass” by Lewis Carroll. (B) “Jeremy the snail” (top), a very rare sinistral garden snail that achieved world-wide fame and became an internet “shellebrity” [99] (C) Sinistral *Euhadra quaesita murayamai* from Japan. Species in this genus vary in their chirality depending upon location; maternal inheritance and rare matings means that there is gene flow between dextral and sinistral morphs [30] (D) New sinistral morphs have evolved at least three times on the Hawaiian islands, including in *Auricullela* (dextral and sinistral shown) and in a sinistral lymnaeaid (inset photo). Photo credits: Angus Davison.

Figure 3 (Key Figure). Graphic highlighting the similarities and differences in the establishment of LR asymmetry in snails (A) and a generic vertebrate (B). (A) In snails, LR asymmetry is a maternally inherited trait. Chirality originates in the cellular architecture, and is evident morphologically during early embryonic cleavages. The asymmetric expression of nodal is a key effector of organ LR asymmetry. Adult body asymmetry is both inwardly and outwardly chiral, including the shell and positioning of the reproductive organs. Inset box: external factors that impact upon the outward chirality. In the majority of snail species, the chiral position of the genitals and chiral behaviour during mating favours same-chiral mating. In most cases, this means that new chiral morphs are selected against, but reproductive character displacement could sometimes favour the evolution of a new chiral morph. In SE Asia, a snake predates on snails in a chiral manner, so

favouring the evolution of sinistral morphs; birds may also predate in a chiral manner, perhaps contributing to the evolution of sinistrals (e.g. on Hawaii). (B) As in snails, the architecture of animal cells is chiral. Early morphological development in vertebrates may appear symmetric but there are sometimes subtle or larger differences, such as the tilting of the node [100,101]. Gross morphological LR asymmetry is evident later, and follows the initially symmetric expression of nodal, usually involving cilia (no cilia, initial asymmetric expression in some e.g. chick). The pathway to asymmetry contains multiple redundancies, so that errors tend to be corrected. Adults are internally asymmetric (to enable a long gut [100]), but outwardly bilaterally symmetric. Inset box: Bilateral symmetry is superimposed upon an internal asymmetry, presumably to enable efficient movement through space, although some asymmetries, especially those that originate in brain sub-functionalisation e.g. handedness, are externally evident.