Chapter 20

Reversals of Bodies, Brains, and Behavior: Quantitative Analysis of Laterality and Its Disturbance in Model Species

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Abstract

Humans display fascinating left–right asymmetries at both the anatomical and behavioral level. These lateralized phenotypes are highly prevalent across metazoan phyla, with bilaterally symmetrical organisms exhibiting well-conserved, consistently sided positioning and anatomy of visceral organs and central nervous system structures. Deviations from normal laterality constitute an important class of birth defects, and much study has been devoted to the early mechanisms orienting the left–right axis during embryogenesis as well as lateralization of the brain. Far less understood are the potential links between laterality of the body and cognition, though recent work has begun to uncover a range of behaviors which are modified in organisms with altered left–right asymmetry. Here, we review regulatory events critical for the establishment of asymmetry and subsequent left–right patterning, using data from Xenopus, zebrafish, chick, invertebrates, Arabidopsis, and single cells, and discuss molecular and pharmacological reagents that disrupt these processes. We especially focus on behavioral assays which are sensitive to body laterality, presenting existing data for several model systems. Beyond classical conditioning and behavior screens, new automated machine vision platforms are powerful emerging tools to quantitatively examine the relationship between body asymmetry and lateralized and nonlateralized behaviors. This chapter serves as a primer for methods that allow the examination of cognitive and behavioral endpoints subsequent to molecular interventions in embryonic left–right asymmetry.

Key words Left–right asymmetry, Laterality, Behavior, Automated analysis, Quantification

1 Introduction

In many types of embryos, spanning diverse taxa across the evolutionary tree, left and right can be distinguished from the earliest stages of development [1]. The positioning and morphology of numerous structures within the bilaterian body plan are consistently asymmetric, deviating from perfect left–right symmetry in the same directions in all normal individuals [2, 3]. The consistent laterality of the heart, visceral organs, and even brain is well-conserved throughout the animal kingdom and suggests many fascinating puzzles regarding the evolution and ontogenetic

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mechanisms of this curious developmental and neurobehavioral feature [4, 5]. In contrast to fluctuating asymmetry (where bilateral traits are imperfectly synchronized but without consistent unilateral bias) [6] and externally/behaviorally imposed asymmetry [7], we focus on fixed asymmetry, in which the structure and function of specific organs are consistently oriented relative to the anterior–posterior and dorso-ventral axes.

Laterality is not only of basic interest, but has considerable biomedical implications. Errors in left–right patterning include loss of asymmetry (isomerism), reversal (*situs inversus*), or randomization (heterotaxia). These are an important class of human birth defects as a primary teratology [8, 9], but many other syndromes have a laterality component to their manifestation, both in fetal development [10, 11] and in adulthood, such as the relationship between asymmetry and cancer [12–16], immune response [17, 18], and schizophrenia [19]. Interestingly, recent work has begun to highlight the bioelectric aspects of asymmetry in cancer [20–22], connecting to the role of ion channels in establishing developmental organ asymmetry (see below). Beyond body anatomy and physiology, asymmetry of the brain and nervous system gives rise to lateralized behaviors, most frequently observed as handedness but influencing many aspects of an animal's function in their ecological niche [23–26].

In the last two decades, remarkable progress has been made in understanding the molecular mechanisms of left–right patterning throughout phyla [27]. The major steps (Fig. 1) involve symmetry breaking at the subcellular level [28–33], physiological signaling via small molecules [34–38], cascades of asymmetric gene expression [39–41], and asymmetric organogenesis driven by the resulting differential cell behaviors [42, 43]. Asymmetry is thus a fascinating example of multiscale integration [44–46], in which order is generated, amplified, and imposed on biological processes at many levels of organization, from molecular structures to tissues to the behavior of entire communities of individual organisms. Despite recent progress, many puzzles and open questions remain with respect to the mechanisms and consequences of asymmetry [47–50], making it essential to address the questions of asymmetry at multiple levels and in a wide variety of model systems. Here, we provide an overview of several model systems and the techniques of characterization of functional asymmetry and assays of lateralized behavior—an endpoint that enables study of the entire causal chain from genetics to CNS function.

2 Zebrafish

Among animal models used in laterality research, *Danio* spp., and related fish species, have contributed much to the field [25, 51, 52],

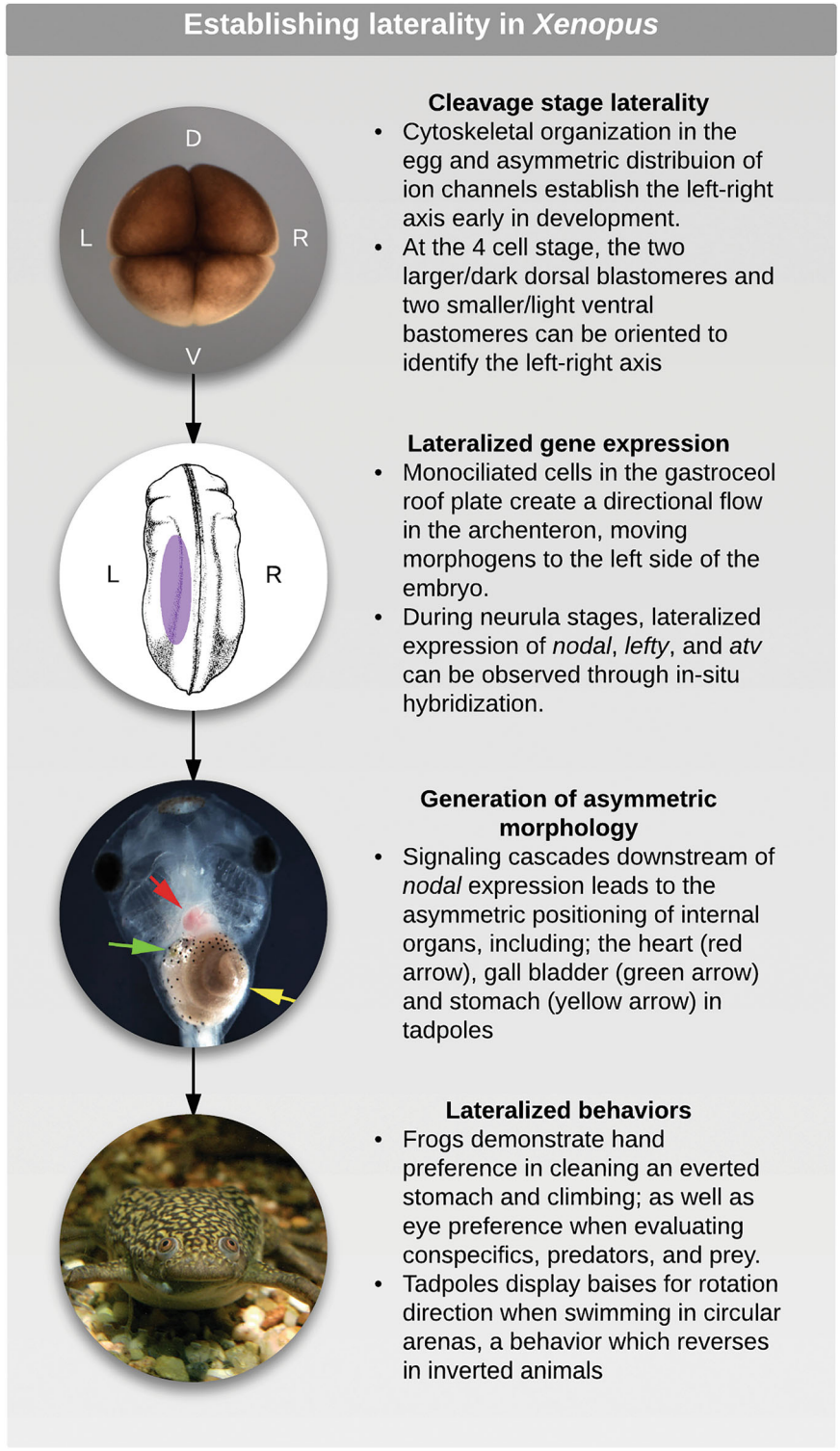

Fig. 1 Molecular, morphological, and behavioral laterality in *Xenopus laevis*. Shortly after fertilization, cytoskeletal organization initiates left–right axis specification. The resulting ion channel localization, intracellular morphogen redistribution, and chromatin acetylation serve as early markers of laterality. Following gastrulation, these early signaling events, and the later action of ciliated cells in the roof-plate, lead to

greatly facilitating the linking of genetics with behavioral tests. As a vertebrate model, they are an attractive entry point into translational research with the goals of improving health, as well as being the focus of both ecological and evolutionary work. Large numbers of fish can be raised in aquaculture, and fertilized eggs can be microinjected with synthetic RNAs to alter gene expression in specific tissues across developmental stages. Zebrafish also explore their environment; paying attention to stimuli in their habitats as well as the schedule of the individual who feeds them. Given their attention to visual stimuli, adult fish have been a favorite model for cognitive studies; examining visual processing, memory, sociality, addiction, and predator avoidance [53–62]. Less well studied but also capable learners nevertheless are zebrafish fry, which upon hatching becoming active hunters, chasing down paramecium and brine shrimp when raised in the lab and a variety of microorganisms in the wild. The real power of this model system emerges when the behavioral capabilities are combined with the powerful genetic tools available to zebrafish researchers, enabling laboratories to cross-breed and engineer offspring with precise control of the genotypic outcome.

A tremendous number of zebrafish mutant lines are currently available, and new lines can be generated through either targeted knockout methods or alternatively through random mutagenesis followed by screens for the phenotype of interest (known colloquially as fishing; pardon the pun). As such, these genetic tools position Zebrafish as a powerful model for all aspects of molecular development: from the role of transcription factors in mesodermal patterning to secreted morphogens that regulate neural induction. Zebrafish researchers have recently received an additional tool for their arsenal in the form of the CRISPR-Cas system, which has been successfully applied to *Danio* embryos as a less labor-intensive method of knocking in-or-out genes of interest [63, 64]. This system utilizes a bacterially derived CRISPR/Cas9 protein complex in conjunction with a synthetic RNA targeting the gene (or regulatory site) in question. On binding to the complementary sequence, the protein complex initiates a double stranded break by the Cas9 endonuclease in the genomic DNA; initiating an error-prone repair mechanism which ultimately leads to a frame-shift and silencing of the target gene. Further, by adding bridging RNAs, which span the region of the break, it is also possible to introduce novel sequence at the target locations, allowing researchers to add/substitute amino acid residues in a protein or regulatory

Fig. 1 (continued) asymmetric expression of *nodal* and several other laterality markers. These asymmetric cascades ensure the lateralized position of the internal organs. After hatching, tadpoles and frogs also demonstrate a number of lateralized behaviors, the relationship of which to internal left–right asymmetries is not yet known. Image of *Xenopus* frog provided under Wikipedia creative commons distribution license

sequence promotor regions of a genome. This technique has made an already established species for genetic work even more attractive, lowering the barriers of both time and cost and allowing laboratories of all sizes to edit the genome as required for their line of scientific inquiry. Another very powerful set of approaches, ideally suited to the transparency of zebrafish, is optogenetics [65, 66]—the use of light to modulate the function of specific components of the CNS with incredible spatio-temporal specificity. Optogenetics is now widely used in the zebrafish model to interrogate behavioral circuits [67–71].

Given these tools, it is perhaps not surprising that a number of laterality research groups have turned to zebrafish as their model of choice; working out many of the early pathways which correctly orient the left–right axis during early development. The majority of this work has focused on a structure present in all teleost fishes during embryogenesis known as Kupffer's vesicle, first described by its namesake in 1868 [72]. During gastrulation, cells occupying the animal pole of the embryo undergo epiboly to surround the yolk and move in a posterior direction; while also undergoing a second cell movement at one position along the equator, involuting and forming the embryonic shield. Along the leading edge of this structure approximately 25 cells maintain their position without involuting; a population referred to as the dorsal forerunner cells. At the conclusion of gastrulation, these cells occupy a position with the posterior of the tailbud and migrate to the interior of the embryo to form Kupffer's vesicle, a hollow region lined by monociliated cells. The rotation of these cilia coordinate in a counterclockwise motion and their combined action results in a directional fluid flow, which is thought to move morphogens to lateralized positions within the embryo [73–75]. These morphogens in turn signal downstream pathways which activate genes required for lateralization of visceral and central nervous system components of the developing zebrafish.

A number of techniques that disrupt aspects of Kupffer's vesicle result in animals with heterotaxia (randomized laterality) and *situs inversus* (reversed laterality). Genetic knockouts of genes expressed by the dorsal forerunner cells are sufficient to induce the phenotype [76], as is both laser ablation and surgical damage [75]. In addition, *left–right dynein-related 1* (*lrd1*) is expressed in the cells of Kupffer's vesicle and knockdown of this gene by morpholino disrupts fluid flow by the cilia and again induces both heterotaxia and *situs inversus* [75]. Further, the use of a morpholino allows specific targeting of the RNA through microinjection and knockdown of the transcript in regions unrelated to Kupffer's vesicle fail to induce any changes in laterality, restricting its action in left–right patterning to the monociliated cells only. Indeed, a number of research programs have examined the effects of disrupting genes in the formation of Kupffer's vesicle itself, or the directional action of

the monociliated cells, and all have found randomization of lateralized structures as a result [73, 77–81].

What pathways lie downstream of Kupffer's vesicle-mediated directional morphogen flow? Many transcripts show lateralized enrichment following gastrulation and three of the most well studied include transforming growth factor-β nodal-related family members (specifically *cyclops* and *southpaw*), *lefty 2*, as well as the homeobox gene *pitx2* [82–88]. The tgf-β family members *cyclops*, *southpaw*, and *lefty 2* are first observed in the lateral plate mesoderm of the developing embryo and demonstrate a left-only expression pattern. In situ hybridization reveals overlapping staining for the transcripts and misexpression on the contralateral side induces a randomization of asymmetric organ and CNS structures with the body [83]. *Pitx2* is expressed in a similar pattern to the above transcripts and is also capable of inducing heterotaxia when misexpressed on the right side of body following gastrulation. Further, *pitx2* lies downstream of both *nodal* signaling and *lefty* as the expression of either is sufficient to drive the activation of *pitx2* in a cell-autonomous manner. Together, these proteins constitute the canonical laterality specification pathway and are both necessary and sufficient to properly orient the left–right axis in developing zebrafish.

Both the heart and gut demonstrate asymmetries in shape and position, and expression of these genes can be found in both: *cyclops*, *lefty 2*, and *pitx2* in the left heart field; *lefty 2* and *pitx2* in the left gut precursors [89]. In addition to visceral structures, two distinct asymmetries have been noted within the brain of zebrafish, both within the epithalamus. The parapineal organ begins medially oriented before migrating to a final left-biased position in the fish diencephalon, where it is thought to signal the asymmetric growth of the habenula, a central component of the forebrain-midbrain limbic system [90, 91]. The habenula itself is present in both the left and right sides of the zebrafish brain, but shows distinct differences in nuclei organization and projections between the two. In addition, the left and the right habenula send projections posteriorly, which meet at the midline at the interpeduncular nucleus (IPN) within the midbrain. This structure does not show any left–right asymmetries but is highly patterned along the dorsal ventral axis. Projections from the left-habenula make up the dorsal portion of the IPN while those of the right-habenula those of the ventral IPN [92, 93]. While the functional roles of these asymmetries are not clear, the habenula has been implicated in many aspects of cognition including anxiety, memory, sleep, stress, and attention [94–99]. Further, disruption of left-sided gene flow, as well as left-sided migration of the parapineal, both result in habenular randomization, a finding which has helped establish fish as key species for studies linking brain/body laterality and behavior.

Zebrafish (as well as other fish species) exhibit lateralized behaviors in the wild and in the laboratory, and a large number have been described in the literature [25, 62, 100–104]. The most well-studied are the eye biases during inspection of objects in the fish's environment. When observing familiar objects including their reflection and conspecifics, zebrafish show a bias for their left eye (turning with the object to the left of their body). However, when the preparing to strike at food or prey, fish instead orient to place the object on their right, using their right-eye to examine the object before biting [105, 106]. The neurological and evolutionary basis of these behaviors is not clear; they are shared among many vertebrates [107–111], but in the case of zebrafish, there has been speculation the right eye possesses better depth perception and is thus more efficient at tasks requiring the animal to make precise movements in three-dimensional space. In addition, zebrafish exhibit turning biases when entering a novel environment or when startled in the laboratory setting. Using a set of rectangular chambers connected by central openings, zebrafish will demonstrate positive phototaxis and move between arenas if the proceeding areas are illuminated. When entering these novel arenas, fish may either move forward, turn left, or turn right, and in the absence of any other stimuli, demonstrate a bias for a left-sided turn upon exploring these new environments [100]. It is unclear why this bias occurs, though the authors of the study [100] speculate it may be due to the left eye creating a map of the recently exited area (as the left eye is used when viewing familiar objects [112, 113]) or it may indicate a more efficient spatial mapping function of the left eye in general. Inversely, when fish entering these environments are startled by the sudden dimming of lights, they show the opposite bias, turning to the right and swimming in the direction opposite to that seen under positive phototaxis [100].

How are these behaviors altered in fish with reversals of laterality? Recent work addressed this question using a genetic line of zebrafish known as *fsi*, or *frequent situs inversus*. Fish in this line display one of two phenotypes, either completely normal laterality or complete *situs inversus*. In addition, the laterality of both the visceral organs and central nervous system display the same laterality in these animals; if the visceral organs are reversed, then the asymmetry of neural structures are also reversed. This allows researchers to score animals quickly for visceral asymmetry (fish fry are transparent and can easily be assessed for laterality under a dissecting microscope) and simultaneously know the position of the parapineal organ and habenula (note: It is not known if visceral and neural asymmetries are patterned by the same mechanisms in other species; this may be a unique advantage, or disadvantage, to zebrafish depending on the study). Testing *fsi* zebrafish in the above lateralized assays yielded interesting results [100]. Two behaviors, the eye individuals use to inspect themselves in a mirror, and

the eye individuals use when striking food, were reversed in animals possessing laterality inversions. This finding was one of the first revealing a direct link between laterality and cognition, and has implications for a number of open questions in the field regarding brain laterality. However, more interestingly, two behaviors did not reverse in the zebrafish *fsi* line. Inverted fish still demonstrated a left-turn preference when entering an illuminated environment as well as a right turn bias when startled by rapid light dimming [100]. How these behaviors can maintain directionality irrespective of both neural and visceral asymmetry is not clear, but introduces a number of intriguing questions for to serve as the basis for future work.

Besides explicit lateralized behaviors, do fish with reversed neural asymmetry demonstrate any other cognitive differences compared to wild-type siblings? Here the answer is also affirmative. Zebrafish with reversed parapineal organ position spend more time inspecting a predator (a measure of boldness), spend more time in the center of an open field, and cover less distance when startled [114]. While none of these behaviors are lateralized per-se, they are still important metrics whose outcome are clearly directed by the positioning of central nervous system asymmetries and can be modulated through laterality inversions. Taken together, the large toolbox available to modulate and assay both molecular and cognitive laterality in zebrafish have made them a key species for investigations of laterality and cognition, and given modern advancements in genome editing, the species will no doubt continue to be a favorite for many research groups examining this interesting question.

3 Amphibians

Amphibians have long been a favorite model system in biological research, with early records of their use in physiology dating back to the 1700s [115]. Perhaps most famous are the studies of Luigi Galvani, who discovered that by passing an electric current through the dismembered legs of frogs he could provoke them to kick, "as in life." Galvani's studies, as well as those which followed, paved the way for advances in the fields of physiology, greatly enhanced our understanding of organ systems, and in turn significantly impacted human health and medicine. More recently (over the past century), frog research has diversified and many species' have been used as tools for the study of ecology, evolutionary biology, environmental health, neurodevelopmental disorders, and animal behavior [116–118], as well as regeneration [119–122], genetics [123], cancer biology [124, 125], and immunology [126–128]. A particularly profound impact in the fields of embryology and developmental

biology has been made by amphibians (particularly *Xenopus* and *Rana* species) [129].

In the laboratory, *Xenopus* species are capable of producing large clutches of eggs naturally, or through subcutaneous injection of human chorionic gonadotropin. An individual female is capable of laying thousands of eggs over the course of a day and can produce these clutches on a quarterly basis. Once fertilized, either naturally or through manual application of male sperm, the resulting embryos are extremely robust tolerating temperatures between 14 °C and 24 °C. Further, developing embryos are also amenable to cut-and-paste grafting experiments, allowing researchers to transplant ectoderm-, mesoderm-, or endoderm-derived tissues between individuals to elucidate cell fate and signaling events. Similar to zebrafish, with the advent of modern molecular tools during the past 30 years, frogs have become a leading model for developmental genetics, as microinjection of synthetic mRNA's into fate mapped blastomeres allows researchers to induce or inhibit the expression of specific genes across developmental stages, probing the specific pathways required for the transition from egg to organism.

Given this amenability to developmental manipulation, *Xenopus* species have become primary models for the study of left–right laterality and the early signaling events leading to asymmetric *nodal* expression [130–134]. Readouts for laterality in *Xenopus* are many: one can perform in situ hybridization for a number of asymmetrically expressed genes (such as *nodal*, *lefty*, and *pitx2*), or simply grow tadpoles to swimming stages where the asymmetric position of the heart, stomach, and gall bladder are visible through the transparent epidermis. Using the latter method, screens of molecules/pharmaceuticals which disrupt laterality can be readily accomplished [135–138]; developing embryos can simply be microinjected with constructs or raised in media containing chemicals of interest and scored at swimming stages for the position of the three lateralized visceral organs.

A number of techniques and tools exist to randomize or reverse visceral asymmetry in *Xenopus* tadpoles. *Xenopus nodal-related gene 1* is expressed asymmetrically in the left lateral plate mesoderm during neural stages of development. Knockdown of this gene on the left side, or misexpression of the wild-type construct on the right side induces both heterotaxia and *situs inversus* in experimental animals without altering other aspects of anatomy [139]. Further, alteration of upstream and downstream signaling molecules in the *nodal* pathway, such as *atv* and *lefty*, likewise result in randomization or reversal of lateralized organ placement [46, 140]. Targeted microinjection of wild-type or dominant-negative constructs of these genes can be done at early frog stages, as the left–right axis is established during the first cell cleavage (which divides the embryo into to right and left halves, though it is impossible to

know which side is which until the second cleavage). When visualizing four-cell embryos, two of the blastomeres are larger and more pigmented—these give rise to ventral tissues of the embryo—while the remaining two are smaller and lighter, giving rise to dorsal tissues. Orienting the embryo with the ventral cells pointing northward and the dorsal cells southward, the two left and right cells then correspond to the left and right hand sides of the developing animal. Following this fate-map allows researchers to easily target constructs of interest to the appropriate location, giving rise to laterality disruptions [141–143].

In addition to signaling factor cascades, a number of other mechanisms can be disrupted to alter left–right asymmetry in *Xenopus* prior to *nodal* expression. One is the action of monociliated cells lining the gastrocoel roof plate of early neurula stage embryos. These cilia beat with a bias, resulting in a strong leftward flow within the interior of the gastrocoel (mirroring the Kupffer's vesicle in zebrafish), which have been proposed to move secreted determinants to the left side of the archenteron. Molecular disruption of the cilia (either by removal or disruption of their movement) results in tadpoles with randomized laterality, although the majority of treatments used to target this ciliary motion in fact also disrupt earlier intracellular (cytoskeletal) components of asymmetry [48, 49, 130]. It has also been reported that inhibition of fluid flow within the gastrocoel (by microinjection of a viscous mixture of methylcellulose to impede movement of fluid by cilia) is sufficient to induce heterotaxia and *situs* inversus [144].

In addition to motile cilia, bioelectrical gradients resulting from ion channel and pump function are also important regulators of left–right laterality in frog embryos. Early in development (between the 1 cell and 32 cell stage), asymmetric localization of potassium channels and the two proton pumps (the H,K-ATPase and the V-ATPase) can be observed between the left and right hand sides of the embryos [37, 145–148]. This differential expression leads to changes in membrane voltage potentials on opposite halves of the developing animal, with cells on the right-hand side being more negatively polarized than those on the right. Given this voltage difference, charged morphogens (such as serotonin, which is weakly positive at physiological pH) progressively move through gap junction-mediated transcellular paths and subsequently signal downstream to epigenetically regulate the expression of asymmetric genes [149]. This physiological system amplifies the initial chirality of the cytoskeleton [33, 130, 150], enabling intracellular directional asymmetries to ultimately impose positional asymmetries (with respect to the midline) of gene expression in large cell fields [151, 152]. As such, it provides a number of convenient and tractable control points, at which specific pharmacological or genetic reagents can be introduced to disrupt this sequence of events and induce heterotaxia. The pharmacological toolkit

includes cytoskeletal or motor protein disruptors, potassium channel blockers, inhibitors of gap junctions, and serotonergic pathway modulators [33, 36, 37, 142, 145, 153–155]. Similarly, the heterotaxia or *situs inversus* can be induced by misexpression of mutated cytoskeletal components, mutant ion channels that regulate resting potential, dominant-negative connexin proteins (on the dorsal side), constitutively open connexin proteins (across the ventral side), or mutant serotonin transporters. Since these signaling events occur extremely early in development, animals can be treated within the first 24 h of fertilization (often within the first few hours only), and then raised under normal conditions; this does not require exposure at later stages that could potentially impact additional tissues or patterning pathways. Indeed, it has proven possible to dissociate the left–right patterning roles of all of the abovementioned components from other functions; the randomization obtained with these reagents can be very specific, giving rise to otherwise quite normal embryos with no generalized toxicity or defects that can perform well in behavioral assays.

Another convenient method for generating asymmetry defects in tadpoles is through the application of low frequency vibration, is sufficient to induce left–right randomization and reversals in frog embryos (the target is not known definitively but is likely to involve subtle cytoskeletal disruption). Using this method, fertilized eggs are placed on a simple audio speaker, driven by a sinusoidal function generator set to a frequency of 7 or 15 Hz [156]. Vibration at these frequencies across the first 24 h of development is sufficient to induce heterotaxia in 30% or more of treated embryos, in line with other methods of left–right disruption [156, 157]. The greatest advantage of this method is the experimenter can establish precise "on-off" times for treatment, as opposed to molecular or pharmaceutical treatments, where determining definitive drug washout can be very difficult.

Disruption of *nodal*-related signaling pathways, motile cilia in the gastrocoel roof plate, ion-channel-mediated bioelectricity, and early cytoskeletal organization are all established methods to randomize and reverse *Xenopus* visceral asymmetry. What about assays for cognitive and neural asymmetry? Here the research is far less established in *Xenopus*. Frog species (not *Xenopus* in particular) show hand preferences in a number of tasks including climbing and the limb used to clean an everted stomach [158]. Evolutionarily this was thought to be due to the asymmetry in gut position; an everted stomach will typically evert in the direction opposite its normal position within the body; thus, the hand closest to the everted stomach may be more effective at wiping and cleaning if the frog ingested hazardous material. However, counter examples exist in which hand preference is absent or varies between tasks [110, 159, 160], suggesting the everted stomach hypotheses as an unlikely driver of behavioral laterality in amphibians. Beyond

handedness, frogs also show a bias for specific eye use based on particular tasks. In Australian green tree frogs and Bufonids, individuals demonstrate a left-eye bias when engaging in antagonistic behavior with conspecifics or when evaluating potential predators, as well as a right eye bias when assessing prey items [158]. However, it is not currently known if any of these behaviors are reversed in animals possessing laterality alterations, as these species have not been reared in laboratories performing functional left–right asymmetry studies.

However, in *Xenopus,* one behavioral assay has recently been developed, which allows investigation of behavioral laterality. Tadpoles feed by filtering particulate matter from their environment, a process which is facilitated by natural swimming movements. When placed in circular containers, tadpoles (both individuals and groups) readily adopt a circular swimming pattern, following the edge of the arena as they feed in the water column. Observation of individuals revealed a clockwise rotation preference for wild-type animals [161]. Interestingly, populations of tadpoles with reversed visceral laterality (reversed heart, gall bladder, and stomach) revealed a counterclockwise bias for swimming direction, and tadpoles with randomized laterality showed no bias for either direction. To date, this was the first report of a lateralized behavior in *Xenopus* that tracks with organ placement. While only tested in larvae, it would be interesting to determine if adults had these same biases in swimming to any number of task—including those currently used in zebrafish (bias when navigating around an obstacle, direction turned when startled, or location preferences when entering a novel environment).

Given the amount of work dedicated to molecular left–right signaling in early *Xenopus* development, why are there so few cognitive assays of lateralized behavior in this species? The answer is twofold. First, *Xenopus* tapdoles and adults are notoriously difficult to use in studies of behavior, learning, and memory. Numerous attempts were made in through the 1970s, at which point one group frustratingly stated, "It appears that attempts to train frogs have been rather unproductive. . . .after several years of unsuccessful and unpublished studies with frogs we were inclined to agree" [162, 163]. Only recently has this line of research been reopened, and with the help of automated computer software, some preliminary success has been made in training *Xenopus* tadpoles using associative assays [164–166]. The recent development of several different quantitative behavioral paradigms [167–171] in this species is sure to facilitate the gathering of data that can be readily mined for directional asymmetries.

Second, and equally frustrating, is that there are currently no asymmetric brain markers in *Xenopus* or *Rana*, although asymmetries in cell behavior during neurulation have been described [172]. Thus, it is currently unknown whether alteration of visceral

laterality also impacts mature neural asymmetry. A primary step toward overcoming this barrier could be cloning frog transcripts of zebrafish markers for asymmetric structures within the epithalamus including the parapineal organ and habenula. Beyond this candidate gene approach, microarrays comparing the left and right portions of the developing brain for enriched transcripts would likely yield hitherto unknown markers for cerebral asymmetry and help establish the relationship between visceral and CNS laterality in amphibian species.

4 Chick

In addition to the aquatic model organisms described above, the domestic chick also represents a powerful model for studies of developmental and behavioral laterality [173]. As an amniote, this model system rests closer to humans on the evolutionary tree than both teleost and amphibians. Moreover, the flat blastodisc embryonic architecture of the chick is more similar to that of humans than the popular mouse model, which gastrulates as a cylinder-like morphology [174]. Indeed, the chick model system was the first in which molecular pathways regulating organ asymmetry were characterized [27] and also contributed to the understanding of laterality disturbances in conjoined twins [175].

The chick's development provides a contrast to the epiboly-based movements present in early fish and frog embryogenesis, and presents a flat, optically accessible system in which to study asymmetry. Amniotes begin development by forming a disc of epiblast tissue covering a region known as the area pellucida. Within this area, at a location that will later become the posterior of the animal, the epiblast undergoes a coordinated moment forming the primitive streak; a visible line from the posterior to anterior, dividing the left and right hand sides of the developing organism (the leading edge of this structure is known as Henson's node, named after its discoverer Victor Hensen). It is thought that the embryonic midline is first determined with the formation of the primitive streak; however, possible very early (pregastrulation) mechanisms have not yet been examined due to the difficulty of working with chick embryos prior to egg-laying (by which time tens of thousands of cells are already present in the embryo). Following the formation of the primitive streak, a number of transcripts are expressed in an asymmetric manner; *activin-inducible type II activating receptor* to the right of Henson's node, left-sided expression of *sonic hedgehog*, followed by *nodal* expression to the left of the primitive streak [27, 176, 177]. These gene cascades progress in a similar fashion to those in zebrafish and *Xenopus*, eventually establishing the asymmetric placement of visceral organs and CNS structures in an otherwise bilateral organism.

However, unlike fish and frog, the chick does not possess a ciliated fluid-filled organ for chiral flow [174, 178]. How do cells occupying positions to the left and right relative to the primitive streak receive positional information in the absence of ciliated cells? The most recent data pointed to planar cell polarity as a mechanism for orientation within this two-dimensional plane. Immunohistochemistry against *vangl2*, a core planar cell polarity protein, revealed a polarized expression pattern with all labeled cells pointing in the direction of the primitive streak [179]. Cells to the left of the streak localized *vangl2* to their rightmost position, while those to the right of the streak localized the protein to leftward positions. Further, early disruption of *vangl2* function through electroporation of a dominant-negative transcript was sufficient to randomize *sonic hedgehog* expression later in development. These novel data were significant in that such a mechanism may be possible in humans as well, as all amniotes share the formation of an embryonic disc prior to tissue specification. In addition to this, the familiar ion channels, gap junctions, and serotonergic signaling have all been implicated in chick [36, 37, 145, 154] as in frog [38, 180] and more distant species, such as sea urchins [181] and protochordates [182].

In addition to shared molecular markers of laterality between chick and aquatic species, lateralized behaviors are also similar between the models. As with zebrafish, chicks also demonstrate characteristic eye biases when examining their environment. Viewing familiar objects and conspecifics favors right-eye usage after hatching, although this appears to reverse to a left-eye preference as animals age [183]. In addition, when bypassing a centrally located barrier, chicks prefer to detour around the object to the left, using their right eye in circumnavigation [184]. Covering one eye or the other during the task revealed that animals with their right eyes exposed took less time to move around the barrier than those with exposed left eyes. Chicks are also more responsive to predators when observed with the left eye (similar to zebrafish) [185, see also Chapters 3 and 21].

However, while these behaviors demonstrate lateralized biases, it is not clear how they relate to lateralization of the viscera and central nervous system as few studies exist explicitly testing this correlation [186]. It would be interesting to raise randomized or reversed animals (produced through disruption of *vangl2*, *sonic hedgehog*, or *nodal*) and determine if the above behaviors randomize in a comparable manner. Given the differences between chick and fish/frog development, similarities in the outcomes of such experiments would strengthen the idea that findings in these species would also be applicable to humans, as the results would apply to both amniotes and anamniotes (and, thus, potentially all vertebrates). Future multispecies comparisons to similar lateralized tasks

would be greatly informative to our understanding of the evolutionary relationship between body laterality and cognition.

5 Invertebrates

Invertebrates represent an emerging and fascinating set of taxa for the study of laterality. The diversity of species within this umbrella is myriad, including multiple members of the nematode, mollusk, and arthropod phyla. Further, these species can be classified amongst a sliding scale of colony complexity, from solitary individuals to social swarms, including several eusocial species with caste differentiation [187, 188], and the degree to which lateralized behaviors may emerge at these higher scales remains a fascinating question. Indeed, due to the breadth of species represented by the invertebrate label, studies in these systems often fall under the umbrella of evolutionary development and may help shed light on conserved laterality pathways among the various animal branches of the phylogenetic tree [189].

While lateralized behaviors have been broadly described within ecological and behavioral disciplines, less is known about the molecular developmental regulation of laterality among invertebrates compared to their chordate counterparts. The most resolved pathways come from the model species *Drosophila melanogaster* due to the powerful genetic tools commonly employed with the system [189]. Through the work of numerous knockout studies, the motor protein Myosin 1D (Myo1D) has been identified as a key regulator of asymmetric development in fruit fly embryos [190–192]. Knockout of the Myo1D protein leads to complete situs inversus, and expression of the protein is regulated through tissue specific organizers, including the atypical cadherin Dashsous (Ds) in the hindgut [193], and puckered (puc) in the midgut [194]. Further, motor function of the protein is necessary for its role, as point mutations targeting either the actin- or (ATP)-binding sites lead to systemic laterality defects in the resultant larvae [195]. Perhaps most interesting, this protein can induce chirality, even in tissues that naturally have no asymmetric properties. When ectopically expressed in epidermal lineages, the entire larval body twists dextrally, creating a coiled morphology with the mouthparts facing upward. Interestingly, movement in the subsequent animals is altered from the typical crawling locomotion to a barrel-rolling-like behavior [195]. Similar results could also be produced in the trachea, where Myo1D expression likewise induced a dextral twisting. Thus, within the Drosophila genera, Myo1D has been demonstrated to be both necessary and sufficient for chirality in expressing tissues. Additional factors now known to be important in the chirality of the gut in Drosophila, via effects on cell shape, include

E and ID proteins [196], and the actin nucleator DAAM [197], which also interact with the MyoID signaling pathways.

Myosins, along with several other factors, have also been implicated in the establishment of laterality in model nematode *C. elegans*, which begins during early cleavage stages in the embryo [198, 199]. While intermediate steps in the left–right asymmetry generation have not been fully resolved, early steps such as the generation of cell size differences in asymmetric cell division [200, 201] and downstream anatomical asymmetries have been thoroughly documented due to the deterministic nature of *C. elegans* development [199]. Of the total 302 total neurons in the nematode, four which are located within the head ganglia are present unilaterally, and many within the ventral nerve cord are positioned asymmetrically [202], including those involved in sensing oxidative stress [203]. Further, within a subset of the 198 bilaterally symmetrical neurons, some of these members show differential receptor expression on the left or right side, as in the case of olfactory neurons which express the str-2 gene in only the right or left, determined stochastically during development [199]. In contrast, other expression patterns demonstrate a fixed asymmetric expression pattern, including classes of guanyl cyclase receptors in paired gustatory sensory neurons [204] and other aspects of the ventral nerve cord [205]. These results provide fascinating new insight into laterality, as they show that anatomical structures, or even individual cells, which are bilaterally symmetrical may differ at the protein and functional level. It is only through the tools available to these model systems that such resolution could be achieved, and forthcoming work will surely shed more light on these subtle differences.

While Drosophila and *C. elegans* have been the focus of much molecular developmental work on laterality in invertebrates, the species in which lateralized behaviors have been described are far broader [26, 206, see also Chapters 7 and 17]. At the individual level, the arachnid *Scytodes globula* demonstrates left leg bias when manipulating prey [207] and locusts present a right leg bias when grooming [208]. Giant water bugs show a left turning bias when placed in a T-maze [209], and two shrimp species initiate lateralized escape responses when surprised [210]. Outside of solitary behavior, laterality has been observed at the colony level among social insects [211]. Several ant species demonstrate a leftward turning bias, both when exploring and in the absence orienting cues [212, 213], and honeybees show lateralized antennae usage when determining responses to individuals from the same or different hives [214]. Among noninsect invertebrates, octopus species demonstrate leg preferences when walking as well as eye use when inspecting objects [215–217], though in the case of the latter, these asymmetric behaviors manifest at the individual and not population level. Likewise, a cuttlefish species demonstrated a

side-turning bias in a T-maze choice test and preferential eye usage for different tasks [218–220]. These examples are by no means exhaustive and serve only to illustrate a handful of representative behaviors across diverse taxa.

As the species used to examine the developmental control of laterality and those used in behavioral work are largely nonoverlapping, the degree to which visceral and CNS laterality dictate asymmetric behavior and cognition in invertebrates remains unclear [221]. Of note, however, are several examples where changes in external anatomical asymmetries can be altered independent of internal organ situs. These include lobsters whose crusher claw bias does not correlate with internal structures [222], flounders where reversal of eye position does not predict underlying optic chiasma anatomy [223], and *C. elegans* Roller (Rol) mutants where reversed organ laterality does not show concordant reversals in cuticle chirality [224]. Thus, visceral laterality is often referred to as primary asymmetry and gross morphology/behavior is referred to as secondary asymmetry [189] to differentiate between tissue and organism level phenotypes. How these primary and secondary asymmetries are regulated is not yet clear and will require the establishment of new invertebrate model systems. Thus, there remains much promise to understand the evolutionary basis of laterality through the continued study of these diverse taxa.

6 Single Cells

While studies of laterality tend to focus on whole organisms, or even vertebrates (given the link to human health), asymmetry runs deep in the evolutionary tree [189, 225]. Perhaps this idea is no better illustrated than by the finding that even single cells, grown in culture, are capable of consistently lateralized behavior, such as preferential bending to one side during neurite outgrowth or biased migration in patterned surfaces [226–231]. How can directional axes be determined in eukaryotic cells? In the case of epithelial sheets, there is often a well-defined apical-basal polarity, and other tissues are known to use planar cell polarity to orient with regard to one another. But how can individual cells, unattached to neighbors, demonstrate a directional bias in the absence of external coordinates? To answer this, one must look to two landmark structures within the cell, such as the nucleus and the centrosome. While the centrosome is duplicated during mitosis to aid in chromosome segregation, it exists as a single structure directing microtubule organization during other phases of the cell cycle. After identifying these two structures, one can draw a line through the cell which bisects both the nucleus and the centrosome. By doing so, the center of the nucleus can be considered 0 in Cartesian coordinates and the direction of the centriole the Y axis (or "north"

alternatively). With the cell oriented in such a way, it is possible to label half of the embryo as "left" and the other half as "right" with regards to their location relative to these two structures.

Having created such a direction system, a cellular behavior has been discovered that demonstrates a left–right bias, the direction of polarization and extension in cultured neutrophil-like dHL60 cells [226]. When these cells are cultured in uniform media (no flow or gradients of chemicals or attractants), 88% showed a leftward bias of extension and cytoplasm distribution. It is important to note here that the cells were in no way directing their growth toward one another; in fact, the directions of cell growth appeared random with respect to the position under the cover slip. However, by orienting their growth the to the left–right axis established by transecting the nucleus and centrosome, this asymmetric polarization becomes clear.

This behavior could be abolished through disruption of centrosome or microtubule organization. Molecular knockdown of *par6*, a component of the centrosome orientation pathway, as well as application of the microtubule polymerization inhibitor nocodazole, both randomized dHL60 polarity with regard to the left–right axis. Perhaps most interesting is the role of *GSK3β* in this process, a protein known to be involved in the polarity of dHL60 cells. Over-expression of wild-type *GSK3β* resulted in a reversal of laterality, with 83% of cells polarizing to the right. Similar to the original finding, this effect could be abolished through the application of nocodazole; producing cells with randomized polarity [226]. Interestingly, nocodazole randomizes asymmetry in *Xenopus* as well [155, 156].

It is unclear why individual cells would possess an internal laterality, although there are multiple theories. A number of single celled organisms exhibit radial asymmetry and as such an inherent left–right establishing mechanism could be a pathway upon which selection pressures could shape cellular morphology [232, 233]. Further, developing tissues, as well as communal unicellular organisms, must orient themselves in three-dimensional space and an intrinsic laterality could help coordinate these complicated movements. Finally, the centrosome is a key component of the primary cilia of eukaryotic cells, which act as sensors for the external environment in a wide variety of contexts. Disruption of cytoskeletal components affects chiral intracellular transport as well as primary cilia. The tantalizing connection between left–right asymmetry of individual cells and complex multicellular animals could thus be a sign that asymmetry is extremely ancient—a feature that predates multicellularity but was exploited during metazoan development by novel amplification and long-range coordination mechanisms.

7 Evolutionary Conserved Laterality Mechanisms Across Kingdoms of Life

While plants are less common models for studies of lateralization [152, 234–239], the kingdom has made a significant impact nonetheless. Studies of plant asymmetry typically focus on helical growth of climbing plants—honeysuckles form left-handed (clockwise) helices as they grow while flowering bindweeds form right-handed (counterclockwise) helices. As the genetic model for much of plant research, *Arabidopsis* has been the primary species to examine the molecular pathways underlying these phenotypes, and two primary mechanisms have been identified which contribute to helical growth. Wild-type *Arabidopsis* plants demonstrate no native helical asymmetries during growth, producing uncoiled vertical hypocotyls (the stem between the root and cotyledon of the germinating seedling) and radial cotyledons. However, dominant-negative mutations to two α-tubulins, *α-tubulin 4* and *α-tubulin 6*, results in plants with left-handed helical growth and clockwise twisting of the developing cotyledons [235, 239].

This finding extends far beyond the plant kingdom. Recently, researchers have examined how mutations directing Arabidopsis chirality affect laterality in animal models, introducing the α-tubulin and γ-tubulin mutants to *C. elegans* and *Xenopus laevis* during development. The result was a randomization of laterality in both species; *Xenopus* tadpoles demonstrated contralateral or bilateral expression of the marker *nodal* as well as randomized organ placement, while *C. elegans* demonstrated a mirroring of asymmetric olfactory neurons which would otherwise express different receptors on the right and left sides [33]. This study identified tubulin genes as a mechanism for orienting laterality conserved across kingdoms, further strengthening the idea of potential conservation between unicellular organisms, plants, and animals. Similarities between the auxin and serotonin pathways in plants and animals have been noted [45]; however, roles of serotonin-like signaling in plant asymmetry remain to be tested.

8 Automation

Beyond additional tools to specifically alter early left–right patterning events during embryogenesis, what other advances would significantly enhance study of the relationship between brain/body laterality and cognition? The primary roadblock to date has been the basic nature of many behavioral assays in model organisms: researchers have molecular tools to alter phenotype but few, if any, cognitive tools to examine the result on animal behavior. The barriers here are many; behavioral trials typically involve a researcher manually watching an animal perform a task in an

arena, or a video recording of the animal, which is potentially scored at a later time by a blind observer. This process is extremely time consuming; given a full workday without breaks, only 8–9 h of footage can be parsed by hand, limiting the ability to do rapid large-scale screens of behaviors. In addition, animals must be handled repeatedly as they are introduced and removed from arenas, potentially adding variability to the results. Primary data generated through manual scoring can also be difficult to standardize and share between laboratories, as the data is generally coded in shorthand to increase the speed of recording. Finally, it is challenging to reevaluate trials for additional behaviors at a later date when using recorded footage (and impossible to do with live scoring). For example, if researchers were initially interested in animal movement rate but later wanted to re-evaluate all of their data for clockwise or counterclockwise rotation within the arena, the process would likely require reanalysis of all primary data.

Automation has the potential to overcome these barriers. Using a combination of motion tracking cameras and computer software, a machine vision robot can track animals in real time; recording complex behavior (such as distance and location between conspecific animals, social behavior, grooming, courtship, aggression, movement in three-dimensional space, and predator responses, among others) as well as fine movements that would be impossible to evaluate by eye. Computer software is generally free of observer bias and can run continually for days, removing the need to constantly introduce and remove animals from experimental environments. Multiple animals can be tracked simultaneously, either through the use of multiple arenas under the field of view of a single camera, or by stitching together the fields of view of multiple cameras (potentially allowing for thousands of behavior trials to be run in parallel). Further, complicated controls are readily automated through software. For example, a yoked control involves delivering a stimulus (reward or punishment) to an animal not for its own behavior, but for the behavior of its paired neighbor. Using manual training by hand, even with only two individuals, these trials are challenging to say the least. However, computers can perform these controls with minimal computing power for many animals simultaneously, greatly increasing the throughput of cognitive data. Once obtained, data can rapidly be parsed for multiple behaviors at a later date. In addition, log files or spreadsheets can be stored on collaborative servers allowing researchers to mine the results for their behaviors of interest; one laboratory may be investigating animal speed and locomotion, another color preference in the arena, and a final group in collisions with barriers within the environment: All three of these could be analyzed using separate algorithms applied to the same original log file.

A number of automated systems have been developed over the past decade and are currently used with organisms ranging from

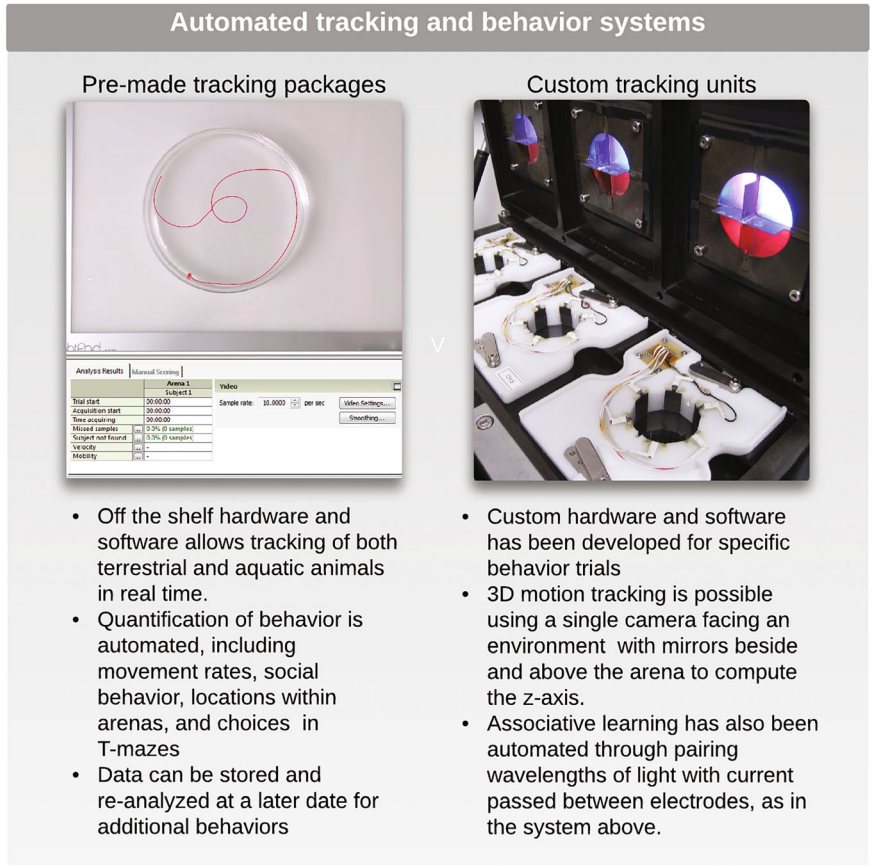

Fig. 2 Turnkey and custom analysis software for monitoring lateralized behaviors. A number of tracking and analysis hardware systems exist, allowing researchers to define custom "arenas" in which behaviors including movement rates, escape responses, sociality, aggression, and fine movements can be quantified. Analysis can happen in real-time or from prerecorded video. Custom hardware and software also exists for specialized analysis that allows not only tracking, but real-time feedback (rewards and punishments) for the animals. Both types of systems allow for rapid and parallel analysis of multiple individuals, resulting in high throughput behavioral testing of animals with altered genetics, physiology, or anatomy. The latter class of systems in addition facilitates training paradigms, which enables assays of learning and memory

invertebrates to vertebrates. These systems fall into two distinct categories (Fig. 2): off-the-shelf turn-key units that run on pre-made software and custom units typically designed for specific tasks (such as 3D motion, or to deliver current or vibration as a punishment). For the off-the-shelf units, two products see frequent use; Noldus Ethovision (Wageningen, Ne.) and Viewpoint Behavior Technology (Lyon, Fr.) [240, 241]. Both are similar in operation (although at the moment Noldus Ethovision delivers more flexibility) utilizing a single camera connected to a PC capture card and operated with through a visual interface. Cameras are generally placed above the animal of interest, and multiple individuals can be tracked simultaneously within the field of view of the camera.

Animals can be tracked as individuals (placed in separate wells of a 96 well plate) or as a group to examine social behaviors. The researcher can also outline a testing area through the live-feed, allowing any number of environments to be monitored; circular dishes, T-mazes, Y-tubes, or other shapes that can be seen by the camera. Data acquisition and analysis is automated, providing rapid summary of animal movement rates, color preferences, choices in T-mazes, or position within the arena. Indeed, these software packages have proved extremely powerful and have been used with vertebrates, especially zebrafish, to analyze a number of behaviors, including: response to predators [242–244], drug addiction [245], sleep [246, 247], associative learning [248], social learning [249], and alcohol exposure [59, 250]. It is also important to note that while these systems have historically been used with aquatic vertebrates, the motion tracking algorithms could work equally well with single cells, birds, mice, and even humans. Very flexible software platforms, such as the JAABA system [251, 252], are coming on-line with increasingly sophisticated capabilities powered by machine learning and statistical mechanics algorithms.

These approaches are seeing increased integration in laterality related research programs [253–255], and their potential is immense. The direction animals turn when entering environments, in response to light cues, or when bypassing barriers, would not be difficult to set up using the prepackaged software. In addition, it is possible to record direction vectors (indicating the angle at which the anterior of the animal is pointing in reference to the camera's position), which would allow researchers to automate trials examining the eye used to examine prey or familiar objects. Finally, fine movements can also be calculated, such as alternating left–right body arching while zebrafish fry and adults swim. Analysis of such fine-scale motor movement may reveal yet undocumented lateralized behavior and act as new metrics for body and brain laterality studies.

However, while these software packages are powerful, there also exist limitations to their use in certain types of trials. Real-time feedback between tracking and punishment/reward in the environment is difficult to implement. In addition, the background subtraction algorithms used to track animals requires constant lighting conditions within the environment during of the trail. Further, while training multiple animals, it is difficult to deliver a stimulus (changes in light intensity, refreshing or altering chemicals in the media, etc.) to one animal/arena without indirectly affecting the surrounding animals; adding variability to results. Faced with these challenges, a number of custom behavior monitoring systems have been developed which may be of use to those examining the link between anatomical and behavioral asymmetries.

One of these systems has been developed to specifically track aquatic animals in three-dimensional spaces [256–259]. Using a

single camera, mirrors beside and above the tank provide the software with XYZ coordinates for the animal, which are then combined to create a 3D movement track for individuals. This additional dimension within the water column has already revealed a number of interesting behaviors which would be missed in two-dimensional summaries of results. For example, fish exposed to ethanol show significant movement in the vertical direction of the water column, a direction which would not be obvious from the more standard overhead two-dimensional view [259]. In addition, when using many overhead 2D tracking systems the water level is kept intentionally low to maintain a fine focal plane during image acquisition. However, low water levels are fairly unnatural for aquatic animals in the wild and may in fact stress fish, frogs, and tadpoles; leading to variability or inconsistencies within the results. It is likely there is much more to be learned from three-dimensional tracking of animals and would be exciting to examine how laterality alterations may impact movement and behavior in 3D space.

An integrated motion tracking and feedback system has been developed (suitable, e.g., for planaria, zebrafish, axolotl, and *Xenopus* tadpoles), which enables automated associative training assays using separate wavelengths of light and electric current [164–166]. As opposed to top-mounted camera units, this device uses under-mounted cameras; one for each of the individual arenas in the device. Illumination is provided from above by LEDs of different wavelengths and current can be applied to the media by six electrodes mounted around the edges of the circular arena. The power of this hardware and software is that shock can be delivered in real-time to each of the experimental arenas independently in response to animal behavior, allowing for associative color learning assays to specific wavelengths of light. This particular device was used in the first study to demonstrate associative learning in *Xenopus* tadpoles [165] and has also been used to show that posteriorly located ectopic eyes confer vision to blinded animals [166]. In addition, this machine vision system was employed to explicitly test the effects of laterality inversions on tadpole cognitive ability; determining that randomization of visceral organs resulted in decreased learning compared to unaltered siblings [161].

9 Summary

Several vertebrate and invertebrate models are now routinely used to produce animals with altered laterality. A number of pharmacological, biophysical, and molecular-genetic tools exist to perturb normal asymmetry, and versatile assays are being developed to track not only the resulting neural structures but also the behavior. With continued work, even more subtle lateralized aspects of cognition will be discovered, and linked to molecular-genetic pathways for a

fuller, multiscale understanding of asymmetry and its importance. Moving forward, automated devices such as those outlined above, as well as forthcoming next-generation motion tracking and real-time training systems, will be key tools for investigating the link between laterality and cognition. Such closed-loop platforms will overcome many of the barriers in the field, allowing multiple animals to be tracked simultaneously, long recording sessions leading to decreased animal handling, examination of fine motor movement possibly in three-dimensional space, rapid reanalysis of previous trials for lateralized behavior, and easy implementation of complicated controls. It is likely that a marriage of modern molecular functional techniques with robotics technology will uncover a wide variety of subtle behavioral changes related to left–right patterning and in turn give us new insight into the link between laterality and cognition.

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