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Inversion of left–right asymmetry alters performance of *Xenopus* tadpoles in nonlateralized cognitive tasks

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Abstract

Left–right behavioural biases are well documented across the animal kingdom, and handedness has long been associated with cognitive performance. However, the relationship between body laterality and cognitive ability is poorly understood. The embryonic pathways dictating normal left–right patterning have been molecularly dissected in model vertebrates, and numerous genetic and pharmacological treatments now facilitate experimental randomization or reversal of the left–right axis in these animals. Several recent studies showed a link between brain asymmetry and strongly lateralized behaviours such as eye use preference. However, links between laterality of the body and performance on cognitive tasks utilizing nonlateralized cues remain unknown. *Xenopus* tadpoles are an established model for the study of early left–right patterning, and protocols were recently developed to quantitatively evaluate learning and memory in these animals. Using an automated testing and training platform, we tested wild-type, left–right-randomized and left–right-reversed tadpoles for their ability to learn colour cues in an automated assay. Our results indicate that animals with either randomization or reversal of somatic left–right patterning learned more slowly than wild-type siblings, although all groups were able to reach the same performance optimum given enough training sessions. These results are the first analysis of the link between body laterality and learning of nonlateralized cues, and they position the *Xenopus* tadpole as an attractive and tractable model for future studies of the links between asymmetry of the body, lateralization of the brain and behaviour.

Keywords

laterality; learning; left–right asymmetry; memory; *Xenopus*

The outward appearance of the vertebrate body plan is bilaterally symmetric. However, consistently biased asymmetries of the heart, visceral organs and central nervous system result from the activity of developmental pathways that underlie left–right (LR) patterning of the embryo (Speder et al. 2007; Basu & Brueckner 2008; Vandenberg & Levin 2009). Because large-scale body structure has inevitable implications for physiology and cognitive capabilities, a number of behaviours are also lateralized, as has been documented within many invertebrate (Frasnelli et al. 2012) and vertebrate phyla (Bisazza et al. 1998; MacNeilage et al. 2009; Rogers 2013). Examples include handedness in brushing material

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off an everted stomach in toads (Bisazza et al. 1996; Naitoh & Wassersug 1996), and even responses to exotic stimuli such as magnetic cues (Rogers et al. 2008), since chicks (as well as other avians) can respond to a magnetic compass when using only their right eye, but not when using only their left eye in location training experiments with natural and experimentally modified geomagnetic fields.

Zebrafish have become increasingly popular for such studies, as they show biases in the eye they use to inspect novel stimuli, the eye used when approaching prey and the direction turned when startled (Miklosi & Andrew 1999; Sovrano et al. 1999; Miklosi et al. 2001; Watkins et al. 2004). Zebrafish possess the same asymmetric anatomical regions as other vertebrates, and a mutant line now exists (*frequent-situs-inversus* [*fsi*]) in which offspring occur with either normal visceral and central nervous system (CNS) laterality or completely reversed visceral and central nervous system (CNS) structures. Interestingly, *fsi* zebrafish demonstrate a reversal of some lateralized behaviours compared to wild-type siblings (Barth et al. 2005). It is not known why some behaviours appear to correlate with body and brain laterality while others do not, although it has been suggested that there may be two separate pathways governing lateralized behaviours in these animals (McManus 2005).

Especially interesting but poorly understood are the causal links between evolution, brain development and behavioural programmes that deal with nonlateralized cues or responses, such as higher-level cognitive functions. Human left-handers show increased performance in associative memory tasks compared to the right-handed population (Christman & Propper 2001; Christman et al. 2004; Propper et al. 2005). In addition, left-handers tend to occupy both of the opposite ends of the performance spectrum, being more frequently observed in gifted individuals (Benbow 1986, 1987; Aggleton et al. 1994) as well as more than twice as common in children with Down's syndrome or autism (Batheja & McManus 1985; McManus et al. 1992).

We sought to test the hypothesis that body laterality affects cognitive functions in tasks that do not contain left–right decision making. We used *Xenopus laevis*, a model species that is highly amenable to the methods of modern molecular genetics (Beck & Slack 2001; Koide et al. 2005; Abu-Daya et al. 2012), chemical biology (Wheeler & Brandli 2009; Wheeler & Liu 2012) and developmental biophysics (Levin & Mercola 1998; Levin et al. 2002; Danilchik et al. 2006; Blackiston et al. 2011). Moreover, *Xenopus* is also the species in which most of the details about left–right patterning mechanisms are available (Lohr et al. 1997; Levin et al. 2002; Chen et al. 2004; Fukumoto & Kema 2005; Blum et al. 2009), and unlike zebrafish, has a fate map that allows the targeting of specific reagents to the prospective left or right halves of the embryo (Klein 1987; Kline & Moody 1987; Moody 1987, 2000; Masho 1990). More recently, standardized behaviour protocols have been established (Blackiston et al. 2010; Blackiston & Levin 2012) that allow the quantitative characterization not only of behaviour but also of complex learning and memory in this model system. In the current study, we used an automated training assay to determine the consequences of visceral laterality randomization and reversals on *Xenopus* cognition. We looked for evidence of new lateralized behaviours, previously undocumented in this model system, and specifically tested the hypothesis that randomization of laterality would negatively impact performance in learning and memory assays that did not involve asymmetry of cues or responses. Furthermore, we hypothesized that animals with complete laterality inversions would behave like wild-type siblings, as mirroring of asymmetry should result in no physiological or neurological mismatching among body components. Our results revealed the first link between fundamental left–right patterning of the body and nonlateralized learning in *Xenopus*, and position this model as an ideal system in which to unravel the mechanistic links spanning developmental genetics and cognitive performance.

METHODS

Animal Husbandry

Embryos were cultured according to standard protocols (Sive et al. 2000) in 0.1X MMR +0.1% gentamycin, with pH 7.8, and animals were staged according to Nieuwkoop & Faber (1967). All animals were reared under a 12:12 h light:dark cycle with the light phase spanning 0800–2000 hours. After reaching swimming stages, tadpoles were fed twice per day on standard Sera micron powdered food and were cleaned daily. All animals were reared at a temperature of 16 °C with a maximum concentration of 30 animals per 100×25 mm petri dish. Following the conclusion of the study, animals were euthanized in 0.02% triciane mesylate solution in 0.1% MMR media, filter-sterilized and pH buffered to 7.0. All experimental procedures involving the use of animals for experimental purposes were approved by the Tufts University Institutional Animal Care and Use Committee (IACUC) and Department of Lab Animal Medicine (DLAM) under the protocol number M2011-70.

Vibration of *Xenopus* Embryos

Embryos were vibrated between stages 1–18. Up to 200 embryos were transferred to 100×15 mm petri dishes and placed on 4-inch (10-cm) Sony model 1-544-670-11 speakers oriented in a vertical direction (the speaker was laid flat with the dishes resting on top). Speakers were connected to a Gwinstek GFG-812A function generator, which delivered a 7 or 15 Hz sinusoidal wave current that was found to induce laterality defects in a previous study (Vandenberg et al. 2011). Ambient temperature during vibration studies were 23 °C. Animals used as controls for behaviour studies were untreated siblings to those that were vibrated.

Laterality Assay

Laterality of the body organs was scored at tadpole stage 45. Individuals were anaesthetized in a 0.02% tricaine solution and sorted into groups based on positional alteration of the normally left coiling stomach, right looping heart and right positioned gall bladder. Heterotaxic individuals were those in which one or two of the organ positions were reversed with respect to wild-type animals, while situs inversus individuals were those in which all three organs were reversed. Any animals with developmental defects beyond laterality alterations were removed from the study.

Behaviour Apparatus

All behavioural and learning assays were completed using a custom machine vision system capable of both recording basic behaviours as well as training animals to avoid red or blue light. The design of the device has been previously documented (Blackiston et al. 2010). Briefly, a petri dish containing a single animal is placed above a motion-tracking camera capable of recording both the position and speed of the tadpoles, and 12 such chambers are run in parallel during an experiment. A series of light-emitting diodes provide narrow spectrum blue and red illumination from above the experimental environment, allowing the lighting of each dish quadrant to be controlled independently. In addition, a set of six iridium-oxide-coated titanium electrodes mounted flush along the walls of the experimental environment allow the delivery of mild to strong electric shocks in a uniform manner across the dish.

Computer software controlling the unit can be programmed to change the light conditions and/or deliver an electric shock based on specific timed events or animal behaviour in real-time. In addition, for each of the 12 chambers, animal position, speed, rotation direction and quadrant location are written to a log file that can be used to bin portions of behaviour trials in discrete blocks for statistical analysis. For all behavioural assays involving animals with

laterality alterations, animals from each group were run concurrently. Wild-type, heterotaxic and situs inversus ($N = 36$ for each treatment) animals were placed in the machine concurrently, with relative position of each treatment within the device randomized between trials to avoid any chamber-specific effects that could influence the outcome of an assay.

Learning Assay

Visual-training assays with *Xenopus* have been described previously (Blackiston & Levin 2012) and were used in the present study to generate red light aversion behaviour in tadpoles. After being placed individually in the automated training device, innate colour preference was assayed by illuminating half of the arena with red light and half with blue light. Tadpoles could swim freely between each half of the chamber during the 20 min assay, with the light regime rotating 180° after 10 min of evaluation. Following the innate colour preference assay, animals were presented with the same lighting regime, but individuals received an electric shock punishment when occupying the red half of the arena. Shocks were 1.2 mA in intensity and 96 ms in duration followed by a 300 ms period in which further shocks were inhibited, creating distinct pulses of current that were determined to be more effective in training during optimization of the protocol. This level of shock was found to be the minimum necessary current during previous amplitude–response assays: below this value, animals ignore the current, swim regularly and show no observable behavioural responses (Blackiston & Levin 2012). As with innate colour preference, the lighting regime was inverted after 10 min of training, and shock was inhibited for 4 s following light rotation to allow tadpoles to move to the unpunished blue half of the dish before shock was again delivered. Following training, animals were given a rest period of 90 min with blue light in all quadrants of the arena, where they could move freely without punishment. After resting, tadpoles were probed for learning by illuminating opposite halves of the arena with red and blue light. Individuals could choose between the two halves in the absence of punishment, and were given 5 min to choose to minimize extinction during the trial (pilot studies determined longer testing periods reduced performance as animals tended to revert to their innate preferences over time). The training, rest and testing regime of the trial was repeated a total of six times, allowing rates of learning to be calculated across the experiment and compared between treatments. Data were analysed by repeated measures ANOVA followed by Tukey post hoc comparisons between the innate colour preference of teach treatment with each of the proceeding testing values. All tests were two tailed and the sample size for each treatment was 29 wild type, 28 heterotaxia and 27 situs inversus individuals. To avoid the possibility of senstitization or habituation to the training apparatus, separate cohorts of animals were used for basic behaviours and learning assays.

RESULTS

Vibration of *Xenopus* Embryos Results in Laterality Alterations

To examine behaviour in animals with altered left–right asymmetry, we used a previously developed technique (Vandenberg et al. 2011, 2012) that facilitated the randomization of organ situs in animals that were otherwise anatomically normal. Beginning at tadpole stage 46, wild-type animals showed normal organ situs, as the stomach, heart and gall bladder assumed their asymmetric position with respect to the left–right axis. In these individuals, the stomach showed a leftward coiling, while the heart had a rightward looping and the gall bladder migrated to a rightward position (Fig. 1a). In contrast, animals exposed to low-frequency vibrations during stages 1–18 showed laterality alterations. These animals often showed independent randomization of one or two organs (see stomach and gall bladder reversal, Fig. 1b) or complete inversion of wild-type laterality (Fig. 1c). All animals chosen for all proceeding experiments were morphologically identical to untreated siblings, with no apparent disruptions to morphology other than reversals of the left–right axis. In addition,

vibrated animals developed at the same rate as control animals and began feeding at the same stage, suggesting no gross developmental defects resulted from the treatment.

Laterality Alterations Do Not Alter Basic Swimming and Exploration Behaviour

To determine whether laterality alterations affected basic tadpole swimming behaviours, we placed feeding-stage tadpoles containing wild-type organ placement, reversed hearts, reversed stomach and gall bladder, or situs inversus in a custom automated behaviour testing apparatus (Fig. 1d, e) for 40 min. Opposite halves of the dish were illuminated with red and blue light, respectively, and the positions of the lights were reversed every 10 min. While evaluating basic behaviours, no punishments were delivered during the course of the trial, allowing animals to explore all areas of the experimental environment.

Four behaviours were compared across treatments: the average speed of animals, the preference for the edge or the centre of the dish, the total area of the dish explored, and the preference for red or blue light, with no difference being observed between the four groups of animals. All animals showed an overwhelming preference for the edge of the dish, generally circling the perimeter of the experimental environment throughout the trial (Fig. 2a). On average, individuals moved at a rate of 8.9 mm/s although large individual variation was observed (Fig. 2b). Individuals showed no significant preference for either colour light, spending equal time in the red and blue halves of the dish (Fig. 2c). Tadpoles explored roughly half of the dish surface, and when looking at individual exploration behaviour, this was correlated with the outer portions of dish and was related to the strong edge preference in stage 46 tadpoles (Fig. 2d, e). Taken together, these results indicate that laterality inversions do not alter normal swimming behaviour in tadpoles: animals with randomized visceral asymmetry as well as those with complete reversal of asymmetries showed no significant differences in movement rates, location preference or colour preference.

Laterality Alterations Affect Biases for Clockwise versus Anticlockwise Swimming Direction

Xenopus tadpoles spent much of the time circling their rearing containers to facilitate filter feeding. We used the automated behaviour analysis device to calculate and summarize the amount of time that individuals spent swimming in both clockwise (CW) and anticlockwise (ACW) directions during the 40 min trials described above. The software was programmed to identify rotational swimming if an animal (1) covered an angular speed of 25°/s, (2) was at least 65% of the distance from the centre of the dish (i.e. within 35% of the edge) and (3) was moving at a rate of at least 3.3 mm/s (Fig. 3a). Using these criteria, the time that each tadpole spent swimming in both CW and ACW directions could be compared and pooled across each treatment.

The data revealed a surprising and consistent asymmetry in normal tadpoles. Wild-type animals tended to spend more of their time swimming in clockwise circles around the dish than in anticlockwise circles (Fig. 3b). Moreover, in contrast to the basic behaviours described previously, significant differences were noted in this novel marker of behavioural laterality among groups with different classes of laterality phenotype. Compared to -ype tadpoles, animals with situs inversus showed the opposite preference, spending significantly more time swimming in a ACW direction compared to wild-type individuals (ANOVA: $F_{4,175} = 2.44$; Tukey pairwise comparison: $P = 0.048$). Interestingly, tadpoles with randomized visceral asymmetry showed intermediate levels of rotation preference, spending approximately half of their time swimming CW and half ACW. We conclude that tadpoles have a chiral preference in the way their movements respond to a barrier, and that this preference is randomized or inverted by heterotaxia and situs inversus, respectively.

Animals with Randomized and Inverted Laterality Demonstrate Impaired Learning

To examine the effect of laterality alterations upon *Xenopus* tadpole cognitive ability, we created an associative learning task using a combination of coloured light and electric shock (Fig. 4a). Animals from each treatment showed no initial preference for red or blue light prior to training (Fig. 4b). In wild-type animals, the preference for red light decreased following each training session, with a significant aversion behaviour noted after three training periods (repeated measures ANOVA: $F_{6,28} = 10.29$, $P<0.001$). Following six training sessions at the completion of the trial, animals spend 17% of their time in the red half of the arena, compared to 50% during innate preference evaluate. Thus, *Xenopus* tadpoles were able to efficiently learn in this light discrimination task.

Heterotaxic animals were also able to learn in the visual discrimination task (Fig. 4b). However, in comparison to wild-type animals, it took an additional training session for heterotaxic individuals to achieve a significant decrease in red light preference, with significant differences only detected in the fourth and sixth testing periods (repeated measures ANOVA: $F_{6,27} = 3.101$, $P = 0.006$). These results support our original hypothesis that randomization of the left–right axis would negatively impact learning rates in the assay. The results were similar in the situs inversus tadpoles, which took longer to demonstrate learning than wild-type animals (Fig. 4b), requiring five training sessions before showing a significant decrease in red preference (repeated measures ANOVA: $F_{6,26} = 3.692$, $P = 0.001$). Interestingly, comparisons across treatments revealed no significant difference at the final testing period (ANOVA: $F_{2,81} = 0.494$, $P = 0.611$), demonstrating that while animals with laterality alterations took longer to achieve significant changes in light preference, they were able to achieve the same proficiency as wild-type animals at the completion of the trial. We conclude that perturbation of the normal left-right patterning mechanisms resulted in changes in the efficiency of learning and recall even when the task involved nonlateralized cues and responses.

DISCUSSION

We report here the first study of nonlateralized task learning in left-right altered *Xenopus laevis* tadpoles. Using a parallelized, fully automated training and testing system (Blackiston et al. 2010), we compared basic behaviours and learning ability in embryos with altered laterality and their wild-type siblings. Individual speed, preference for the centre or edge of the dish, area of the dish visited and red/blue colour preference did not vary between phenotypes. All animals spent the majority of the trial swimming around the edges of the dish in a circular motion, with no preference for either red or blue halves of the arena. Tadpoles were not fed during the course of the experiment, and, therefore, this circular swimming pattern is an indication of attempted filter feeding behaviour. In addition, we compared the behaviours of nonvibrated tadpoles with vibrated siblings containing wild-type organ *situs* and found no significant differences in any of the above metrics. Taken together, these results suggest that laterality defects resulting from early embryonic vibration do not inhibit basic swimming ability in any way that would confound movement-based learning trials.

In addition to basic behaviours, we tested each treatment for its effects on learning ability using a previously developed *Xenopus* tadpole colour training assay (Blackiston & Levin 2012). During each trial, animals received a shock punishment when occupying red portions of the arena, and subsequently spent the majority of their time in blue areas of the arena even when red areas no longer illicited punishment. As in our previous studies, wild-type animals showed no initial preference for red or blue light, but demonstrated a significant red light aversion following three training sessions within the device. By comparison, heterotaxic populations required four training sessions to show a significant change in light

memory, and situs inversus populations did not achieve significant performance until the fifth training period. We were surprised to find that situs inversus animals demonstrated poorer performance on our assay compared to wild-type siblings, since complete mirror-image inversion might be predicted to maintain functional connectivity among all body components. A likely explanation is that while we scored the three most common organ positions for reversals, other structures may possess cryptic asymmetries (undetectable at the level of morphology; Aw & Levin 2008; Pai et al. 2012) that were randomized but escaped our detection. In this sense it is possible that inverted animals behaved more like heterotaxic individuals in the associative learning assay. Interestingly, by the end of six training period, wild-type, heterotaxic and situs inversus tadpoles all showed indistinguishable levels of learning, suggesting that tadpoles in all three treatments achieved an equal level of proficiency given enough training.

While wild-type, heterotaxic and situs inversus tadpoles demonstrated similar swimming behaviour, the direction of rotation while circling the dish varied with laterality. Wild-type tadpoles, on average, spent the majority of their time swimming in a clockwise direction. In contrast, situs inversus tadpoles swam in an anticlockwise direction, and heterotaxic tadpoles as a population spent half of their time swimming in each direction. These differences were not the result of population preference differences between cohorts of tadpoles. Wild-type, heterotaxic and situs inversus siblings were tested simultaneously in the behaviour apparatus specifically to minimize cohort variability. The positions of each treatment within the behaviour machine were also varied during each trial to account for any unknown cues to which individual tadpoles may respond in a particular channel of the training device. The chirality of swimming direction thus represents a novel lateralized behaviour in *Xenopus laevis* larvae, a convenient read-out that can easily be added to any future genetic or pharmacological study to provide a behavioural endpoint accompanying the more common organ situs and molecular marker data. Interestingly, the preference among a population is close to 60%, about the same level as handedness in parrots and early hominids (Toth 1985; Tommasi & Vallortigara 1999; Vallortigara et al. 2011). Future work will dissect in detail the circuits guiding the handedness of movement; it will be particularly interesting to determine whether this process is biased by the intrinsic handedness of individual cells (Heacock & Agranoff 1977; Wan et al. 2011), or by a systems-level dynamics of neural networks in the brain.

All animals with visceral laterality alterations were produced through low-frequency vibration during early embryonic stages. This recently developed technique (Vandenberg et al. 2011) has a number of benefits for cognitive studies over other molecular-genetic or pharmacological LR randomizing methods. Vibration offered precise `on' and `off' periods of exposure, allowing us to limit its influence to very early stages (which is often difficult when using genetic or other means of perturbing laterality pathways). Furthermore, molecular constructs disrupting early LR patterning target pathways that often have additional roles at later time points, including in the patterning of the CNS and other target organs, which can cause confounding effects on behavioural assays that can be minimized by the temporal control afforded by the vibration technique.

There are many hypotheses about the origins of asymmetry and possible evolutionary advantages inherent to laterality (Morgan 1977; Bock & Marsh 1991). Although difficult to test, a plausible idea is that increasing the surface-to-volume ratio (to optimize diffusion-limited functions) of tube organs such as the intestine within a limited visceral cavity requires bending the tube; uniform bending (always folding cardiac and intestinal tubes in the same direction) may increase robustness of embryonic development, and indeed mutants in which this direction is randomized often present lower health (and thus fitness) compared to normal individuals (Burn 1991; Peeters & Devriendt 2006). Specifically within frogs, it

has been postulated that handedness may have evolved in response to pre-established organ laterality, as sick animals evert their stomach and wipe the contents with their forearms (Bisazza et al. 1996; Naitoh & Wassersug 1996). As the natural looping of the stomach causes it to evert to a specific side depending on the species, selection for handedness may have made this behaviour more efficient within the lineage. From this anatomical bias, further lateralized behaviours may have emerged (Yamashita et al. 2000; Oseen et al. 2001; Wassersug & Yamashita 2002; Malashichev & Wassersug 2004). However, asymmetry runs deep in the evolutionary tree and is present in a diverse array of species (spanning vertebrate, invertebrate and plant lineages), demonstrating that asymmetries may be vital in multiple aspects of an organism's life; there are even examples of single cells showing biases in pseudopodia extension behaviour (Heacock & Agranoff 1977; Xu et al. 2007; Tamada et al. 2010), suggesting that the origin of laterality is likely an ancient phenomenon. For example, beyond physiology and anatomy, asymmetry may have evolved to facilitate efficiency within the central nervous system, directing sensory stimuli to appropriate brain hemispheres for faster processing and decision making. The most predominant examples lateralized brain function in a behavioural setting include eye dominance in pigeons, which directs visual inputs to one hemisphere of the brain for object recognition (Gunturkun et al. 2000), and the alternating eye use in fish species to inspect and then strike a prey object (Miklosi & Andrew 1999; Sovrano et al. 1999; Miklosi et al. 2001; Watkins et al. 2004). This phenomenon is thought to maximize the speed at which decisions can be made and executed as it (1) decreases the possibility of conflicting information arising from opposing hemispheres, and (2) does not require information to be passed between lobes of the brain.

The current study builds on these observations and suggests that laterality may be used to increase cognitive performance even on nonlateralized behaviours, including generalized learning and memory. Indeed, within the vertebrate lineage, the evolution of anatomical asymmetry is likely critical to brain function as perturbation of natural asymmetry has been linked to anxiety, stress, attention, sleep and memory (Murphy et al. 1996; Valjakka et al. 1998; Haun et al. 1992; Amat et al. 2001; Lecourtier et al. 2004; Lecourtier & Kelly 2005). What remains unclear is the degree of crosstalk between visceral and neural laterality, and the result of discordance between the two. As a first step, the current study demonstrates that randomization of body laterality by disruption of developmental left-right patterning steps affects performance on a task that is fundamentally not asymmetric. Thus, this simple assay in the highly tractable *Xenopus* model is likely to be a useful system for beginning to understand the relationships between body/brain asymmetries and traits such as intelligence, creativity and social behaviour in vertebrates. Future work must identify and validate molecular markers of brain asymmetry in *Xenopus* (which do not yet exist) and explore the pathways linking mature neurobehavioural dynamics to very early developmental patterning events. Dissociating the contributions of asymmetries of neural and non-neural structures represents some of the most fascinating issues that impinge on developmental neurobiology, evolution of the brain and body, and cognitive science.

Acknowledgments

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- We show that *Xenopus* organ laterality is randomized by physical embryonic vibration.

- Normal tadpoles tended to swim in clockwise circles.

- Tadpoles with reversed body laterality swam in anticlockwise circles.

- *Xenopus* tadpoles demonstrated associative colour learning in an automated assay.

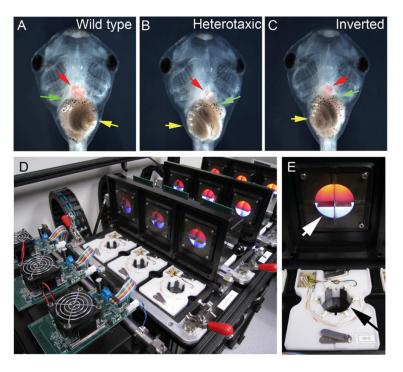- Tadpoles with alterations of body laterality learned more slowly than normal.

Figure 1.
Vibration-induced laterality alterations in *Xenopus laevis* tadpoles. *Xenopus* embryos were vibrated at 7 or 15 Hz from stage 1 to stage 18. At tadpole stage 46, individuals were scored for the position of the stomach, gall bladder and heart. (a) Wild-type animals had a leftward looping heart (red arrow), right-biased gall bladder (green arrow) and left coiling gut (yellow arrow). (b) Heterotaxic animals showed reversal of one or two organ positions. (c) Situs inversus tadpoles showed reversal of all three organ positions. Individuals were tested for basic behaviours, rotation direction and learning rates using an automated behaviour testing apparatus (d), which contained an arena with overhead illumination delivered through red and blue LEDs (e).
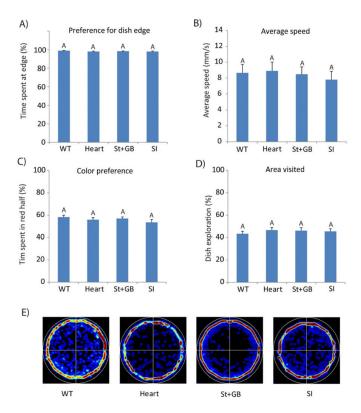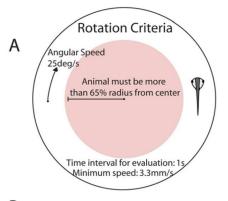
Figure 2.
Comparison of *Xenopus laevis* tadpoles' (a) average speed, (b) location at the centre or edge of the dish, (c) area of the experimental environment covered and (d) preference for red or blue halves of the dish, averaged across treatments. Values are means ± 1 SE (*N* = 36 per treatment). (e) Representative heat plots indicating tadpoles' positions in the dish as a function of colour (hotter colours indicate more time at a given position). WT: wild type; Heart: inverted heart; St+GB: inverted stomach and gall bladder; SI: situs inversus (complete reversal of asymmetry).
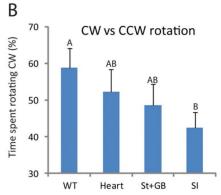
Figure 3.
(a) Swimming rotation criteria for wild-type *Xenopus laevis* tadpoles. (b) Time spent swimming in clockwise (CW) and anticlockwise (ACW) directions for wild-type tadpoles (WT), tadpoles with randomized organ asymmetry (inverted heart: Heart; inverted stomach/gall bladder: St+GB) and tadpoles with complete reversal of asymmetry (SI: situs inversus). Values are means $\pm$ 1 SE (N = 36 per treatment). Bars with different letters represent significant differences (ANOVA, followed by Tukey post hoc analysis).
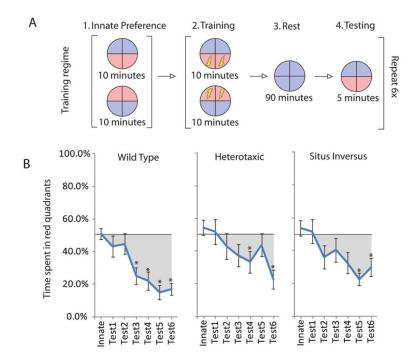
Figure 4.
(a) Training programme used to compare rates of learning in *Xenopus laevis* tadpoles. (b) Mean ± SE percentage of time spent by wild-type ($N = 29$), heterotaxic ($N = 28$) and situs inversus ($N = 27$) individuals under red light during testing periods. Asterisks indicate values that differed significantly from innate values (ANOVA, followed by Tukey post hoc analysis).