



# Lateralized Turning Biases in Two Neotropical Tadpoles

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Received: February 14, 2016

Initial acceptance: March 22, 2016

Final acceptance: April 21, 2016

(S. Foster)

doi: 10.1111/eth.12503

**Keywords:** laterality, tadpole, turning bias, behavioral lateralization, anuran, ontogeny

## Abstract

Lateralized turning behavior in startle responses and upon descent after surfacing for a breath of air has been documented for tadpoles in several anuran species. A left-handed preference is most common and was previously thought to be linked to the asymmetry in spiracle location. Here, we investigate the presence of behavioral asymmetries in tadpoles of *Agalychnis callidryas* and *Leptodactylus melanonotus* in explosive turns after air-breathing. Data were also collected on lateralized posture in the orientation of tails of embryonic *A. callidryas* within the egg case, as well as the startle response of free-swimming tadpoles exposed to mechanical stimulation. A left-curved tail bias was found among several clutches from Costa Rica, but this was not the case among clutches examined from Panama. Free-living tadpoles of *L. melanonotus* displayed a distinct right-handed preference during explosive turns. While some tadpoles of *A. callidryas* were at stages too early to detect any explosive turns when breathing, later-staged individuals did display a left-handed bias in startle response to mechanical stimulation. Additionally, it appears that the expression of behavioral lateralization of larvae (but not embryos) may predict whether or not the adults exhibit similar lateralization. Findings herein provide insight into the ontogeny and evolutionary origins of lateralized behavior in anurans.

## Introduction

Left–right behavioral asymmetries in bilateral mammalian organisms, particularly humans, have long been established (LeMay 1976). Within the past 30 yr, evidence has accumulated to support hypotheses on the presence of ‘handedness’ as well in fish, amphibians, reptiles, and birds (Rogers 1980; Bradshaw & Rogers 1993; Bisazza et al. 1998; Vallortigara et al. 1999; Dadda 2005; Robins 2005; Malashichev 2006). The ontogeny of lateralized behavior in larval forms has received some attention in amphibians (Bisazza et al. 1998; Malashichev & Wassersug 2004) and is particularly interesting given the metamorphic period that involves gross changes in both the morphology and ecology of individuals. Studies of anuran laterality during tadpole development present an interesting opportunity to investigate the dominance shift in the use of axial to appendicular musculature throughout metamorphosis (Wassersug & Yamashita 2002). In some tadpoles, lateralized behavior patterns

are apparent in the direction of whole body turns that individuals exhibit upon descent after taking a breath of air, and during startle response behavior (Wassersug et al. 1999; Yamashita et al. 2000; Goree & Wassersug 2001; Rogers 2002; Wassersug & Yamashita 2002). These explosive turns, referred to as ‘C-turns’ or ‘C-start’ responses, can occur within <100 ms and are likely to be Mauthner cell-mediated and ‘hard-wired’ in tadpoles (Bisazza et al. 1998; Rogers 2002; Wassersug & Yamashita 2002). Such fast reflex loops are adaptively significant as fast turns are necessary for predator avoidance, and lateralized perception centers (as opposed to redundant and competing bilateral centers) may reduce stimulus processing and response times (Vallortigara et al. 1999; Rogers 2000; Wassersug & Yamashita 2002; Robins 2005).

Turning biases do not occur in all anuran species, but there is typically a left-sided bias during explosive turns that was originally thought to be obligatorily linked to the common asymmetry of a left-sided spiracle (Wassersug et al. 1999). However, subsequent

data show that some species that are morphologically symmetrical nonetheless exhibit behavioral asymmetries during turning (reviewed in Malashichev & Wassersug 2004). These turning biases are apparent shortly after hatching, decrease throughout tadpole development, and disappear before metamorphosis, coinciding with the shift from axial musculature dependent movement in the larvae to post-metamorphic appendicular locomotion (Yamashita et al. 2000; Dadda 2005; Malashichev 2006).

Usually this laterality exists on the order of 60–90% of turns per individual (Wassersug & Yamashita 2002). Other forms of lateralized behavior may be apparent in anurans prior to the free-living larval stage. These include the side to which an embryo coils its tail within the egg case (Thibaudeau & Altig 1999) and the side to which a new hatchling leans when lying on a substrate (Wassersug & Yamashita 2002).

Published studies on tadpole laterality are restricted to the last two decades and include a small number of species, many of which occur in temperate zones, and none of which record handedness of tadpoles in the field. Here, we test the hypothesis that lateralized behavior, such as those used in descending after a breath of air and in startle responses to mechanical stimulation, is found in two distantly related species of New World tropical frogs. We provide data on embryonic tail orientation and turning bias as indicators of laterality in tadpoles of *Agalychnis callidryas* (Hylidae) and *Leptodactylus melanonotus* (Leptodactylidae) by implementing two previously developed protocols (Wassersug et al. 1999; Yamashita et al. 2000).

Whether behavioral lateralization in anurans is an evolutionarily conserved trait that is reflective of phylogeny (with more basal archaeobatrachian clades lacking behavioral lateralization) has been questioned by Malashichev (2006). As all chordates exhibit neural lateralization, *Xenopus* (Archaeobatrachia) larvae have been shown to exhibit visual perception laterality (Gouchie et al. 2008), and not all neobatrachians exhibit similar behavioral lateralization (Bisazza et al. 1997; Malashichev 2002; Malashichev 2006); we are inclined to agree with the idea that variation in type and degree of lateralization is more related to functional traits than phylogenetic relatedness. We also bring our results on previously unstudied species into this context to provide more resolution to the matter.

## Methods

We collected 10 egg masses for a total of 523 eggs of *A. callidryas* during June 27, 2002, to June 29, 2002, from the Research Swamp at the La Selva Biological

Station, Heredia Province, Costa Rica. In the laboratory, we staged egg development (Gosner 1960), counted number of eggs in each clutch, and assessed the tail orientation of the developing embryo in a subset of seven of the clutches. To supplement our data on embryonic tail orientation, we also examined photographs of 23 egg clutches of *A. callidryas* collected from a population at a man-made experimental pond at Smithsonian Tropical Research Institute facilities, adjacent to el Parque Nacional Soberania, Gamboa, Panama in 2005. Egg masses, attached to host leaves, were suspended above a cup of pond water (250 ml) until hatching, after which tadpoles were counted and relocated as a sibship to a larger container with 2000 ml of pond water. Tadpoles were kept in a laboratory that was open to ambient light and temperature fluctuations and were fed powdered fish food daily and water was changed regularly.

We placed two individuals in a translucent cup with 250 ml pond water providing sufficient room for swimming without contacting each other, nor the confines of the container nor be exposed to air. In this way, we could observe two tadpoles simultaneously in the laboratory. Following the protocol of Wassersug et al. (1999), we recorded whether turns made by each tadpole upon descent after taking a breath of air were toward the right or left. In addition to these trials, we also observed the combined turns of individuals found as an aggregate in the field to determine cohort-level handedness. We observed the group housed in the laboratory and recorded turns. Data on direction of explosive turns were recorded for each tadpole housed in pairs and tadpoles housed in a group as a representative of population handedness.

To investigate potential handedness in a wild population, we observed tadpole behavior of *L. melanonotus* in a small temporary pond (approximate area  $1 \times 0.5 \times 0.2$  m) at the Selva Tica Research Station, 10 km from La Selva in the corridor connecting La Selva to Parque Nacional Braulio Carrillo. We recorded turning direction of each individual that surfaced for air in the group (Wassersug et al. 1999). Individual turns were obvious because of water clarity and the time in between turns, both ensured that several different tadpoles were surfacing to breathe. Tadpoles were temporarily collected for staging and were found to be between Gosner stages 26–27 (Gosner 1960).

To investigate individual lateralized behavior in startle response among *A. callidryas* to mechanical stimulation (Wassersug et al. 1999), tadpoles were grouped in pairs for individual observation and a fine-point probe attached to an extendable handle was

directed toward the top of the tadpole's body on the midline. Tadpoles were stimulated only when in a resting position in the water column. Tadpoles that were actively swimming were not mechanically stimulated. The directional response (left or right) of each tadpole was recorded. To assess a response to mechanical stimulation when in a group setting, we examined natal cohorts in separate tanks by directing a probe toward the center of the container creating a small ripple in the water column that elicited a startle response.

We used a nonparametric chi-square test (IBM SPSS ver. 20) to test for significant differences in the direction of tail orientation of embryos and to investigate the direction of explosive turns (1) after tadpoles of *A. callidryas* and *L. melanonotus* surfaced for a breath of air and (2) after mechanically stimulating *A. callidryas* tadpoles in pairs and groups. All tadpoles at La Selva were returned to the Research Swamp, and no tadpoles of *L. melanonotus* were removed from Selva Tica. Eggs and embryos from the Panamanian population were used for other experiments, after which tadpoles and metamorphs of various stages were released at the collection site.

## Results

Costa Rican egg clutch size ranged from 14 to 94 eggs ( $\bar{x} = 51.7 \pm 8.83$  SD), and each of the ten clutches represented a cohort to determine handedness at the group level in the larval experiments. Seven egg masses were suitable for surveying embryonic orientation and contained 306 embryos of *A. callidryas* of which 162 embryos had curled tails toward the left representing 53% of the population and 144 had right-handed curled tails at 47%, but these were not significantly different ( $\chi^2 = 1.059$ ,  $df = 1$ ,  $p = 0.303$ ). However, when clutches were surveyed separately, five of the seven clutches showed a left-handed tendency in tail orientation of the developing embryos, and the two that did not reflect this tendency comprised larger egg masses ( $n = 94$ , and  $n = 55$ ). This may infer a genetic component, or the possibility of multiple clutches from different females, but could easily be an effect of a lower sample size at the clutch level.

Clutch size of 23 Panamanian egg clutches ranged from 31 to 71 eggs ( $\bar{x} = 44.4 \pm 10.64$  SD), totaling 1022 eggs. We similarly used each clutch to represent group handedness. Just over half of the eggs were visible in photographs to determine whether embryos had tails curled in either direction ( $n = 625$ ). Of the observable embryos, 300 had tails curled to the left

representing 48% of the population, while 325, 52%, had tails curled to the right. These were not significantly different ( $\chi^2 = 0.50$ ,  $df = 1$ ,  $p = 0.48$ ).

Upon hatching, we attempted to observe the turning bias of Gosner stage 23 tadpoles (Gosner 1960) of *A. callidryas* ( $n = 134$ ); however, tadpoles did not exhibit explosive turns after breathing and instead drifted toward the bottom of the container after surfacing. More developed tadpoles (Gosner stages 26–27) of a small population of *L. melanonotus* ( $n = 72$ ) in the wild showed a preference toward the right ( $n_{\text{right}} = 58$  vs.  $n_{\text{left}} = 14$ ) when descending after a breath of air ( $\chi^2 = 26.889$ ,  $df = 1$ ,  $p < 0.001$ ).

To test startle responses to mechanical stimulation in *A. callidryas*, we recorded the turn of each tadpole (Gosner stage 26) that were housed (1) in pairs ( $n = 54$  tadpoles) and (2) in natal cohorts ( $n = 134$  tadpoles). Tadpoles in pairs originated from three separate clutches, and more tadpoles displayed a left-handed tendency than a right-handed tendency (30 left vs. 24 right). However, this small difference was not significant ( $\chi^2 = 1.852$ ,  $df = 1$ ,  $p = 0.174$ ). When tadpoles of five separate clutches were observed in their natal cohort as an aggregated group, tadpoles displayed a significant left-handed turning preference at 59% vs. 41% right-handedness ( $n_{\text{left}} = 79$  vs.  $n_{\text{right}} = 55$ ) in their explosive turns ( $\chi^2 = 4.299$ ,  $df = 1$ ,  $p = 0.038$ ).

## Discussion

Embryos at Gosner stage 21 have well-developed tails and a coiled orientation within the egg that may be a precursor to lateralized movements post-hatching (Wassersug & Yamashita 2002). A previous study with *Eleutherodactylus coqui* suggested a left-sided coiling preference but no supporting data were presented (Thibaudeau & Altig 1999). In this study, we provide data on embryonic tail orientation as a precursor to tadpole lateralized behavior in *A. callidryas*. Results from the Costa Rican population may hint toward a left-handed clutch level bias, if a 'population' (in this case 'clutch') is said to be lateralized when more than 50% of the individuals are lateralized in the same direction (Bisazza et al. 1998). However, this observation was no longer apparent at the population level (all clutches), nor did the Panamanian population show any biases within clutches or when all embryos were grouped together.

Recently hatched pond tadpoles remain mostly stationary before they take up active swimming and are thought not to be capable of making explosive turns prior to Gosner stage 25 (Wassersug & Yamashita

2002; Wassersug & Yamashita 2002), and we confirm this with all 134 *A. callidryas* tadpoles that were at Gosner stage 23. Results of the startle response of *A. callidryas* tadpoles at Gosner stage 26 to mechanical stimulation illustrate that the observed left-handed bias was more apparent in larger groups. The amplification of apparent lateralization in behavioral responses has been linked to intraspecific aggregations (Bisazza et al. 2002; Dadda 2005; Gouchie et al. 2008; Karenina et al. 2013). Evidence also exists for lateralization of visual perception among certain larval anurans and fish, and this is believed to assist in both schooling with conspecifics as well as predator detection (Rogers 2000; Dadda 2005; Gouchie et al. 2008). *Agalychnis callidryas* tadpoles exhibit multiple predator-mediated phenotypically plastic traits (including timing of hatching and metamorphosis) and experience associated trade-offs with shifts in growth, behavior, and ontogeny (Warkentin 1995; Vonesh & Warkentin 2006; Gonzalez et al. 2010). Perhaps the direction of the C-turning bias in populations of larval anurans may be a consequence of exposure to certain predators.

Field observations of wild tadpoles of *L. melanonotus* in a small ephemeral pond revealed that these tadpoles exhibit immediate explosive turns upon descent after surfacing for a breath. We found an 81% right-handed bias. Leptodactylid tadpoles are externally asymmetrical with a single, lateral, and sinistral spiracle (Savage 2002). These findings taken alone support the early hypothesis that morphological asymmetry may be causal to behavioral asymmetries (Wassersug et al. 1999). However, in addition to successive findings in the literature (Malashichev 2002; Malashichev & Nikitina 2002; Malashichev & Wassersug 2004), we note inconsistencies with this hypothesis. Previously studied populations of *Rana catesbiana*, similarly externally asymmetric, exhibit a left-turning bias in the direction of spiracle location (Wassersug et al. 1999), whereas the turning behavior in this population of *L. melanonotus* was biased away from the spiracle. This difference between *L. melanonotus* and *R. catesbiana* may reflect phylogenetic distance and may be linked to possible asymmetry in the number of Mauthner neurons located in the brain of these two externally similar species (Wassersug & Yamashita 2002). However, our own results on *A. callidryas* also refute the hypothesis that larval symmetry is causal to behavioral laterality altogether.

Turning biases of tadpoles of *A. callidryas* are of particular interest because they are a neobatrachian (Hylidae) frog. They also exhibit near external symmetry in their body plan; larvae have a ventral spiracle that

opens on or near the midline of the body (Duellman 2001). Previous work has shown that *Xenopus laevis* and *Bombina orientalis* (both archaeobatrachians) that maintain a similarly symmetrical morphological arrangement show no turning bias (Wassersug et al. 1999; Goree & Wassersug 2001) while a left-handed bias has been observed in the neobatrachian microhylid, *Microhyla ornata*, which also has a symmetrical external body plan (Yamashita et al. 2000). Work on *R. catesbiana* (Wassersug et al. 1999) and *Rana sylvatica* (Oseen et al. 2001) also show consistency in the patterns of lateralized behavior among neobatrachians despite larval asymmetry. A rightward turning response has also been documented in the Australian neobatrachian, *Litoria latopalmata* (Rogers 2002). Considering that *L. melanonotus* is also a neobatrachian, the findings presented here, thus, support the hypothesis that lateralized behavior among tadpoles reflects phylogenetic relationships and is not coupled with morphological asymmetries (Malashichev 2002; Malashichev & Wassersug 2004). Lateralized behavior may be a highly conserved feature and may have been acquired at the base of the neobatrachian group. Thereafter, distinct evolutionary radiations may have resulted in some genera exhibiting a left-handed bias (e.g., *Rana*) and others a right-handed bias (e.g., *Leptodactylus*).

Furthermore, these findings also support the hypothesis of Malashichev (2006) that behavioral asymmetry may be an artifact of alternating limb locomotion. Thus, we propose that the potential for behavioral lateralization is an evolutionarily conserved trait that is mediated by ecological adaptations of locomotion. Indeed, even archaeobatrachians have been shown to possess strong lateralization of physiological traits, such as vision – which affects behavior, if not lateralization of a behavior itself (Bisazza et al. 2002; Gouchie et al. 2008). Because studies exploring behavioral lateralization in tadpoles are limited to movement, it seems logical that particular lateralized behaviors examined in the larval stage may also be a precursor to lateralization in locomotive strategy of the adult stage.

Assuming that tadpole behavioral laterality may be linked to laterality in the adults, our results fit the model outlined by Malashichev (2006). An arboreal Hylid (*A. callidryas*) that moves primarily by alternating limb movements should be expected to exhibit locomotive laterality, despite a symmetrical body plan. Tadpoles of *L. melanonotus* also exhibited behavioral laterality which has similar locomotive strategies as *Ceratophrys ornata*, which was shown to possess behavioral lateralization in Malashichev

(2006). Our results support the idea that the expression of behavioral lateralization (within the scope of these types of experiments) is inextricably linked to movement and thus locomotive strategy. It appears that the expression of behavioral lateralization of larvae (but not embryos) can be predicted by whether or not the adults exhibit similar lateralization. Our results have strong implications, not only for supporting the link between lateralization and functional traits as opposed to viewing lateralization as an independently inheritable trait, but also in demonstrating new ways for how functional traits of one life history stage can influence behavior earlier in an organism's ontogeny.

### Acknowledgements

Research was supported by the Organization of Tropical Studies and the University of Miami. We thank R. J. Wassersug for intellectual stimulation and assistance with manuscript production and J. Robertson, J. Hunt, and R. Rundell for field assistance. We are grateful to Sam C. for the opportunity to work on this project.

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