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Startle Response and Turning Bias in *Microhyla* Tadpoles

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ABSTRACT—*Rana* tadpoles are known to have a left-handed turning bias whereas *Xenopus* larvae lack such a preference in turning direction. Since *Rana* tadpoles have a single, external, left-handed spiracle, we previously suggested that a turning bias in tadpoles may be obligatorily linked to that external asymmetry. We have tested this idea by examining turning bias in the startle response of *Microhyla ornata* tadpoles. *Microhyla* tadpoles are, like *Xenopus*, externally symmetrical, but phylogenetically they are more closely related to *Rana*.

Individual *Microhyla* tadpoles were startled by a solenoid-driven plunger that sent a shock wave up through the bottom of a container holding each tadpole. High speed videography (250 frames per second) was used to witness the tadpole's response.

Microhyla tadpoles show no turning bias during the first few days post-hatching, when they are very small. However, they develop a left-handed turning bias while still in the earliest free-swimming stage (Gosner stage 25) and that bias persists through stage 41. At stage 42, after forelimbs emerge, the laterality in startle responses fades away.

Since *Microhyla* larvae are externally symmetrical, yet preferentially turn to the left, we can reject the hypothesis that a turning bias in tadpoles is obligated by external morphological asymmetry. An alternative working hypothesis, given the limited taxa that have been examined to date, is that handedness in tadpoles is phylogenetically conserved and independent of spiracle position in tadpoles.

INTRODUCTION

Recently we demonstrated that tadpoles of the genus *Rana* have a natural turning bias, and will—all else being equal—turn preferentially to the left about 60% of the time. In contrast, we found that *Xenopus* tadpoles lack such a turning bias, turning equally often to the right and left (Wassersug *et al.*, 1999).

Rana tadpoles are externally asymmetric, with a single sinistral spiracle from which water that has passed through their gill slits exits the body. *Xenopus* tadpoles, in contrast, are externally symmetrical with dual spiracles, one on each side of their body. This morphological distinction raises the possibility, as hypothesized by Wassersug *et al.* (1999), that the turning bias in *Rana* tadpoles is functionally linked to their external morphological asymmetry.

We test this hypothesis by examining the turning bias of *Microhyla ornata* tadpoles. Most modern authorities consider the Microhylidae to be ranoid-derived frogs and thus much more closely related to *Rana* than *Xenopus* (e.g., Ford and

Cannatella, 1993; Pough *et al.*, 1998; Wassersug, 1984, 1989). However *Microhyla*, especially *Microhyla ornata*, tadpoles are morphologically and ecologically convergent with *Xenopus* larvae. Like *Xenopus*, *Microhyla* tadpoles are externally symmetrical, but with a single midline spiracle rather than paired lateral ones. These features of *M. ornata* made it an ideal species for assessing whether external asymmetry in spiracle position is inseparably linked to biased turning in tadpoles.

METHODS

The tadpoles used in this study came from two populations. A small sample (N=12) of older tadpoles were collected in March, 1998, in an artificial pond on Amami Oshima, Kagoshima Prefecture, Japan. The majority of tadpoles tested (N=221) were collected in July, 1998, from a small mountain-side pool on Iriomote Island, Okinawa Prefecture, Japan. The number of clutches that produced these tadpoles was not ascertained, but the size range of the individuals in both groups and the large number of tadpoles at the two sites, suggests that the samples from both populations were derived from multiple clutches. Any malformed tadpoles were excluded from testing.

The tadpoles were tested individually, in a shallow watch glass. Each tadpole was tested in water from the tadpoles' stock tank and that water was replaced, after thoroughly rinsing the watch glass, with new water for each tadpole. The volume of water ranged from approximately 18–30 ml, with larger volumes used for larger tadpoles.

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This volume of water in the watch glass produced a small pool 100–130 mm in diameter. The depth of the water in the center of the watch glass ranged from 3–5 mm, which was adequate to allow the tadpoles to swim freely without necessarily touching the bottom and their backs exposed to the air.

The watch glass sat in a cradle made of plaster of Paris. The top of the cradle conformed to the shape of the watch glass. The bottom of the cradle was flat and sat on a flat, firm plastic sheet. This, in turn, was centered directly over a solenoid-driven plunger that, when fired, tapped the plate directly beneath the tadpole. The magnitude of mechanical shock was controlled by the voltage to the solenoid. The *Microhyla* tadpoles responded consistently and briskly to this startling stimulus.

For most of the data, turning was observed with illumination provided by two fiber optic microscope lamps directly opposite each other and tipped downward at *circa* 45°. The body of the camera and the arm to the tripod supporting it were perpendicular to the axis connecting the lights. The test container and camera were directly below the overhead fluorescent room light, with the body of the camera parallel to this ceiling fixture. The operator of the camera positioned himself directly opposite the camera.

For imaging in the dark, infrared light emitting diodes were employed for illumination. The central wavelength of the light from the diodes was 950 nm. A video camera with a Charge Coupled Device chip sensitive to infrared light (up to 1000 nm) took the image of the turning tadpoles. The operator of the camera remained outside the darkened room when these pictures were taken.

The tadpoles' movements were recorded at 250 frames per second with a Kodak Ektapro motion analyzer, Model 1000 HRC. The trigger for the solenoid was set to fire at the 40th frame into each Ektapro sequence. In other words, the camera always recorded 160 ms (=40 frames) of data before the solenoid fired.

Strict rules were adhered to in scoring a turn. The tadpole had to be completely still before the solenoid fired. Its tail had to extend directly backward. The individual had to be far enough away from the edge of the water to have full freedom to turn 180° either right or left. Tadpoles most often initiated movement in the 8th frame (=32 ms) or later, after the trigger had been fired. [A piezoelectric sensor on the platform established that of the approximately 32 ms from trigger firing to the reaction of the tadpole, 10 ms was the minimum lag time from solenoid impact to tadpole reaction.] Thus, the few tadpoles that started to move before the 8th frame were assumed to have coincidentally moved independent of the solenoid impact, and they were not scored.

For the group of 12 tadpoles collected in Amami Oshima, observations were made both in the dark and in the light, but on different days. Observation of turns for each condition was more than 10, and total number of turns scored for each tadpole ranged from 22 to 33. For the larger group, from Iriomote Island, each tadpole was scored at least three times in a row (total observations = 663), with a few minutes of rest in between. Each tadpole was ultimately designated either left or right "handed" based on the predominant direction that it turned to in the multiple trials. The vast majority of the tadpoles responded well to the stimulus; however some tadpoles were non-responsive and were recorded as such.

All tadpoles were staged according to Gosner (1960) and their total length measured to the nearest 0.1 mm.

RESULTS

The *Microhyla* tadpoles reacted to the solenoid once the magnitude of the mechanical shock exceeded a certain (but not quantified) threshold intensity. Their startle responses were stereotypic, involving a sharp bend in the base of the tail: i.e., a C-start as shown in Fig. 1. A QuickTime® video version of this event can be viewed at: <http://bio-q.surc.isas.ac.jp/frog/Microhyla.html>. This single lateral flexion of the tail was followed immediately by a locomotor stroke of the tail in which the tail momentarily took on an S-shape, with two points of flexion. The trajectory for forward movement was highly variable, but never in the direction that the tadpole was facing before it was startled. Thus, the startle reaction always involved a distinct turn on behalf of the tadpole. Latency for the startle reaction was distributed within a narrow range, between 10–20 ms after the mechanical shock.

Before being startled, tadpoles approaching metamorphosis (Gosner stage >38) rested with their hind limbs partially abducted and the knees flexed. When these tadpoles were startled, their hind limbs were promptly adducted and the knees extended into a position where they would not impede flow, however they could not assist in the generation of thrust (Liu *et al.*, 1996).

As the tadpoles entered metamorphic climax (stage ≥ 42)

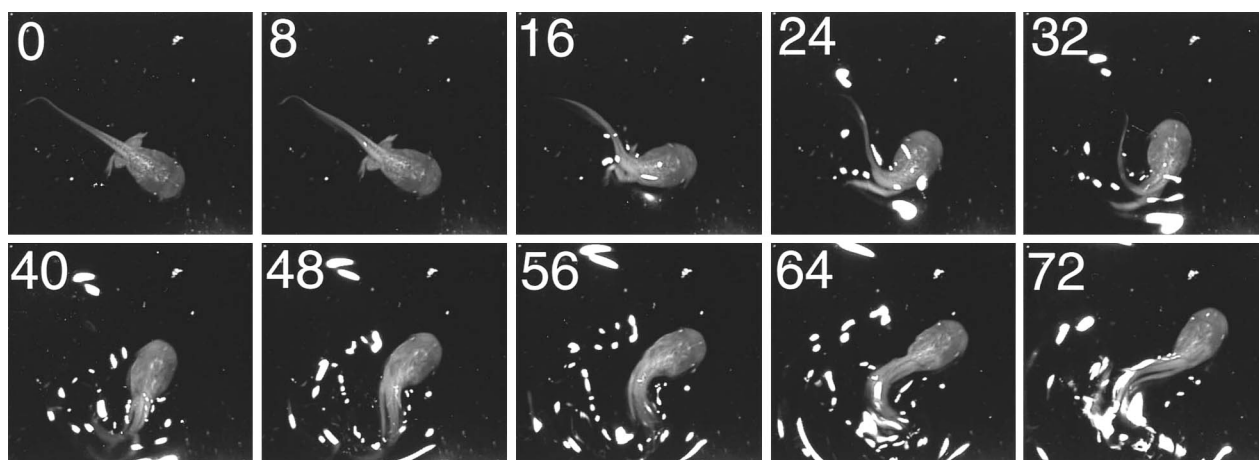


Fig. 1. The startle response of a *Microhyla ornata* larva induced by a mechanical stimulus pulse delivered to the bottom of its container, filmed from above. The number in each sequence frame indicates elapsed time in ms. This tadpole responded to the stimulus by an initial sharp turn to the left, such that it formed a "C" with its body at 32 ms. This was followed by a counter turn, leading to an "S" configuration by 48 ms.

startled individuals would cease moving their tails after several beats and then immediately abduct their hind limbs and flex their knees. This was followed by a synchronous kick of the hind limbs. At the same time, the tail made strong propulsive waves to initiate swimming. This "tail and legs" sequence was repeated several times before the tail and limbs ceased moving. As metamorphosis progressed, the synchronous limb movements began to predominate at the initiation of the startle response, with a less dramatic tail beat superimposed on the now dominant hind limb action.

Quiescent tadpoles, before being startled, were not completely immobile. The periodic pumping of water into their mouths and out the spiracle caused a slight back and forth oscillation of the resting tadpole. Furthermore, in the light, tadpoles oscillated their tail tips to keep themselves stable in the water column. We were concerned whether this oscillation in the tail tip, at the instance a tadpole was startled, could bias the direction that the base of the tail bent to at the beginning of a C-start. Preliminary observations showed however that resting tadpoles observed in the dark did not oscillate their tail tips when quiescent and their only movements were the slight rostro-caudal displacement due to pulsatile buccal pumping. We confirmed that the turning bias of the individual tadpoles was the same under both light and dark conditions and that oscillations of the tail tip in the light did not bias the direction of a tadpole's startle response. Thus, based on this finding, the remaining observations were performed solely in the light. Since scoring turns was recorded over a two day period, we were similarly able to establish that individual turning bias, in response to mechanical shock, was stable over that period.

Frequency of left turns, right turns and "no response" for the tadpoles from Amami Oshima is shown in Fig. 2. The bar graph there indicates that at the population level these tadpoles were predominantly left-handed (L:R ratio was 3:1 for N=12). However, there was much individual variation. At one extreme, one tadpole turned 10 times more often to the left than the right. At the other extreme, one tadpole turned 60% of the time to the right.

A sub-group of these tadpoles (N=5) were not responsive for a substantial proportion of the time (i.e., >20%). Such non-responsiveness may indicate habituation to the startle stimulus or a defensive reaction by the tadpoles (see Discussion).

How responsiveness and laterality change with development was further examined in the larger sample of 221 tadpoles from Iriomote Island (Tables 1 and 2 respectively). As

seen in Table 1, the amount of non-responsiveness increased as development progressed. At stage 25 the tadpoles were non-responsive to the mechanical stimulus less than 2% of the time. This rose to a full 50% once metamorphic climax was reached (Gosner stage 42) and the forelimbs erupted. In a contingency table χ^2 test (comparing the ratios of "response" to "no response" across stages) the decrease in responsiveness with developmental stage was highly significant ($p < 0.00001$).

In the youngest and smallest tadpoles (i.e., Gosner stage 25 and less than 11 mm in length) the L:R ratio for turning was only slightly greater than 1.0, with no significant laterality (χ^2 test, $p=0.50$). However once stage 25 tadpoles were greater than 11 mm in total length, their L:R ratio for turning increased to approximately 2:1 (χ^2 test, $p=0.022$). This left-handed turning bias was maintained through stage 41. When all premetamorphic tadpoles over 11 mm, from stage 25 through stage 41, were combined (N=134) the L:R ratio stayed close to 2:1 (L:R= 87:47; i.e., 65% left). This left-handed turning bias was highly significant (χ^2 test, $p=0.0006$), but disappeared

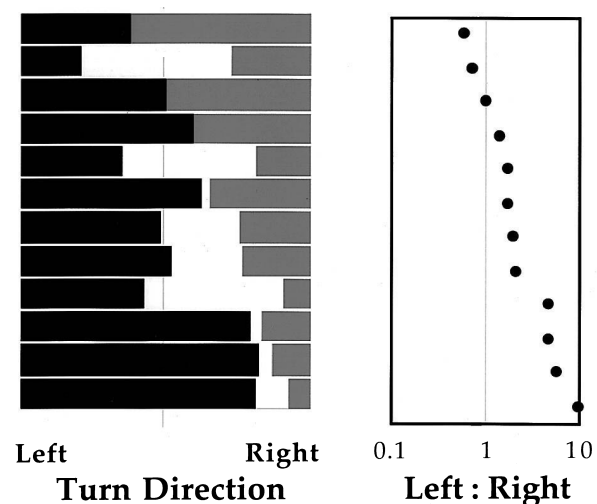


Fig. 2. Individual variation in the frequencies of left and right turns, plus "no response" for 12 *Microhyla ornata* tadpoles between Gosner (1960) stages 38-41, collected on Amami Oshima, Japan. Each tadpole was tested 22 or more times. The proportion of the total reactions that were either right or left turns for each tadpole are shown in the bar graph on the left. The white portion in between represents the portion of times that the tadpoles were non-responsive. The ratios of left to right turns are also plotted for each tadpole on the right. Tadpoles were observed both in the light and in the dark, where similar response patterns were recorded.

Table 1. Responsiveness of *Microhyla ornata* tadpoles to a mechanical startle stimulus.

Number of Tadpoles	Gosner (1960) Developmental Stage	Number of Non-responsive Tadpoles	Percent of Tadpoles that were Non-responsive
119	25	2	<2
40	27-36	1	2.5
44	37-41	11	25
18	42-45	9	50

Table 2. Turning bias in *Microhyla ornata* tadpoles.

Number that Responded to the Startle Stimulus	Gosner (1960) Development Stage	Total Body Length (mm) X $\pm$ SE	Turning Bias		χ^2	p
	X $\pm$ SE (Range)	X $\pm$ SE	Left:Right	% Left		
55	25 $\pm$ 0.0 (25)	9.1 $\pm$ 0.1	30:25	55	0.45	0.500
62	25 $\pm$ 0.0 (25)	12.6 $\pm$ 0.2	40:22	65	5.23	0.022
39	34.2 $\pm$ 0.3 (27–36)	27.9 $\pm$ 0.3	23:16	59	1.26	0.262
33	38.4 $\pm$ 0.2 (37–41)	29.6 $\pm$ 0.3	24:9	73	6.82	0.009
9	42.8 $\pm$ 0.2 (42–45)	24.9 $\pm$ 0.8	4:5	44	0.11	0.738

abruptly once the forelimbs erupted and the tadpoles entered the final stages of metamorphosis (i.e., stages ≥ 42 ; χ^2 test, $p=0.74$).

DISCUSSION

The startle response of the premetamorphic *Microhyla* tadpole is kinematically similar to that observed for *Rana* spp. by Hoff (1987; see also Hoff and Wassersug, 2000) and Will (1991). The increased incidence of non-responsiveness to the startling stimulus as the tadpoles approached metamorphosis has not been noted before for microhylid tadpoles, but may be adaptive. Such immobility responses have been previously documented for many other tadpoles in the presence of predators (literature reviewed in Wassersug, 1997). Wassersug and Sperry (1977) showed that *Pseudacris triseriata* tadpoles in metamorphic climax were more likely to attract predators if they moved, than if they remained stationary. Because of their mid-metamorphic morphology, the transforming froglets were not as efficient at escaping attacks and consequently their movements were more often fatal than effective, when near a predator.

The similarities in behavior and appearance between *Microhyla ornata* and *Xenopus laevis* larvae are striking. Tadpoles of both species are poorly pigmented, lack keratinized mouth parts, have very large branchial baskets with dense gill filters, dorso-ventrally flattened heads, eyes placed far laterally, and a filamentous tail (for illustrations see Liu, 1950, for *Microhyla* and Wager, 1965, for *Xenopus*). Tadpoles of both taxa are specialized midwater suspension-feeders that can hang in the water column by rapidly oscillating the tip of their tails. Tadpoles of both taxa form biosocial groups; i.e., schools (Wassersug, 1973; Maeda and Matsui, 1989). Yet, despite these morphological and behavioral similarities between *M. ornata* and *X. laevis* larvae, in its tadpole's turning bias *Microhyla* is clearly like *Rana*. In fact, when tadpoles of comparable stages are examined—e.g., 72 *M. ornata* tadpoles between stages 27–41 (in the present study) versus 55 *Rana catesbeiana* between stages 29–41 (see Table 2 in

Wassersug *et al.*, 1999)—the turning biases are identical, namely 65% to the left.

It is noteworthy that lateralized behavior in *Microhyla* tadpoles does not appear upon hatching, but only emerges later in development when the tadpoles have been free-swimming and feeding for some time; i.e., late stage 25 and over 11 mm in length. This developmental pattern is, once again, consistent with data on the development of the turning bias in *R. catesbeiana* tadpoles. Specifically before stage 29, the *Rana* tadpoles showed less bias in their turning than at later stages (Wassersug *et al.*, 1999).

We believe that this similarity in turning bias and its development in *Rana* and *Microhyla* tadpoles is more than coincidental. Microhylidae are considered ranoid frogs by most taxonomists (Cannatella and Hillis, 1993; Ford and Cannatella, 1993; Wassersug, 1984). Our results show that the turning bias in anurans is most parsimoniously interpreted as reflecting the phylogeny rather than external form of their tadpoles. The fact that the *Microhyla* tadpole is externally symmetrical yet has a strong behavioral laterality confirms that handedness in tadpoles is not obligatorily linked to gross external asymmetry, such as in spiracle position.

Many questions about behavioral handedness in tadpoles remain. We do not know, for instance, where in the central nervous system lies the laterality that accounts for the handedness in the fast-start. Asymmetries have been noted in the Mauthner neurons of some tadpoles (Moulton *et al.*, 1968) but have not been examined specifically in *Microhyla*. Neither have asymmetries in the Mauthner neurons been specifically linked to behavioral handedness in any anuran taxa.

Recently much attention has been given to handedness in postmetamorphic anurans (Bisazza *et al.*, 1996, 1997, 1998; Naitoh and Wassersug, 1996; Robins *et al.*, 1998). The behaviors that have been examined, to date, have been mainly righting reflexes and face wiping with the forelimb by postmetamorphic frogs. It is not known whether an individual anuran's particular handedness, exhibited as turning in a tadpole, survives metamorphosis to reappear in the froglet. Clearly, the peripheral motor systems for the pre- and

postmetamorphic displays of laterality have to differ, for the tail, that actuates turning in the tadpole, does not persist past metamorphosis. Conversely, the asymmetric limb motions used in both righting and face wiping in anurans are undeveloped in the tadpoles.

Of particular interest to us is the evolutionary origin of behavioral laterality in anurans. We do not know if it occurs solely in neobatrachian frogs, or has an earlier appearance in the phylogenetic tree of these organisms. In this regard, the few extant archeobatrachian genera—e.g., *Alytes*, *Bombina*, *Ascaphus* and *Discoglossus*—stand out as particularly interesting. Tadpoles of these genera, like those of *Microhyla*, have a single symmetrical median spiracle. Should they show a left-handed turning bias, then we would be led to conclude that handedness is a common feature in anuran larvae, perhaps secondarily lost in pipid/pipoid taxa, such as *Xenopus*. If, on the other hand, the archeobatrachian larvae lack a turning bias, then many more taxa will need to be examined to determine where and how often larval laterality has independently evolved in the more “advanced” Anura.

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