AN ECOMORPHOLOGICAL ANALYSIS OF LOCOMOTION IN LARVAE AND

NEOTENES OF TWO SALAMANDER SPECIES: *DICAMPTODON TENEBROSUS*

(STREAM-TYPE) AND *AMBYSTOMA GRACILE* (POND-TYPE).

By

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ABSTRACT

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Morphology is the physical expression of a species' evolutionary history and

adaptation to its environment and as such is tied to ecology. Salamander larvae have

historically been separated into "pond-type" and "stream-type" groups based on their

morphology, however no studies have been performed quantifying the relationship

between morphology and ecology. In this study I utilized in-situ behavioral observations,

morphological measurements, and in-lab performance tests of *Dicamptodon tenebrosus*

(stream-type) and *Ambystoma gracile* (pond-type) to examine the relationship between

salamander larval morphology and ecology. In the field, behavior was videorecorded

during nighttime surveys; afterwards animals were captured and limb measurements were

taken. Flow resistance was measured in the lab using a flow chamber and water velocity

meter. Swim escapes were videorecorded in lab trials and analyzed using video analysis

software. In the field, aquatic walking was the predominant form of movement observed

in *D. tenebrosus*, constituting 98.1 percent of all movements; by contrast, aquatic walking

made up only 65.4 percent of all movements in *A. gracile*. Aquatic walking was

correlated with more robust forelimbs with a smaller length:width ratio. Swimming was

correlated with slender forelimbs with a larger length:width ratio. Forelimb ratio,

hindlimb ratio, and caudal fin area were all significantly different between species. Of the fourteen muscles measured, seven were significantly heavier in relative mass in *D. tenebrosus*. Specifically, muscles that are used in moving the animals forward or closer to the substrate. *Dicamptodon tenebrosus* were significantly more adept at resisting flows than *A. gracile*. There was no significant difference in mean swim escape velocities between *A. gracile* and *D. tenebrosus*. However, there was a significant difference in variability of swim escape, with *D. tenebrosus* more variable. These results support the hypothesis that the salamander larval morphotypes are connected to their ecology by behavior and performance. The larval types are not just morphotypes, they are ecomorphotypes.

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INTRODUCTION

While terrestrial salamanders can inhabit a wide range of habitats, their aquatic

larvae fall into three morphological types named for the typical habitat they are

associated with – pond-type, stream-type, and mountain brook-type (Noble, 1927;

Valentine & Dennis, 1964). Pond-type larvae have dorsal fins that extend onto the back

of the animal, a long gill rachis, a lack of functional limbs at hatching, and a laterally

compressed body. Stream-type larvae have dorsal fins that end near the hind limbs, a

reduced gill rachis with many branches, functional limbs upon hatching, and a more

dorso-ventrally compressed body (Noble 1927; Valentine & Dennis, 1964; Petranka,

1998). The mountain brook-type is a more extreme stream-type with even more reduced

gill rachis, and more reduced caudal fin (Valentine & Dennis, 1964).Yet while the

general features of the larval types are well known, most morphological and performance

studies involving larval salamanders focus on a single species (Frolich & Biewener,

1992; Fitzpatrick et al., 2003; Landberg & Azizi, 2010), with comparisons being solely

morphological (Valentine & Dennis, 1964; Ashley-Ross, 1992). No previous studies

combine morphology, ecology, and behavior/performance in comparing larval

salamander types. In this study, I examined the ecomorphology of aquatic locomotion in

larval and paedomorphic salamanders. Specifically, the Coastal Giant Salamander

Dicamptodon tenebrosus was chosen as the stream-type model, and the Northwestern

Salamander *Ambystoma gracile* was chosen as the pond-type model, due to the two

species occurring in the same region and their relatively close relation in sister families

(Larson & Dimmick, 1993; Pyron & Wiens, 2011). Due to the difficulty in locating,

videorecording, and similarity to the stream-type, the mountain brook-type was not

included in this study.

A species' physical adaptation to its environment is a fundamental theme of

evolutionary biology and, specifically, ecomorphology (Wainwright 1994). From an

ecomorphological perspective, morphology is the phenotypic expression of the

relationship between an organism and the environments that it inhabits (Ricklefs & Miles

1994). Ecomorphology also constitutes a core concept of paleontology, used when

reconstructing the life of extinct vertebrates (Van Valkenburgh, 1994). However,

morphology can only be related to ecology if morphological characteristics are deeply

connected to behavior and in particular to performance of the species at ecologically

relevant tasks, such as feeding and locomotion (where performance is defined as how

well an individual completes a task). By studying extant species, where the relation

between morphology and ecology can be tested, I hoped to get a glimpse into the

paleoecology, behavior, and performance of aquatic locomotion in extinct early fossil

tetrapods (Ashley-Ross, 2004).

Larval morphotypes seem to be based on analogous traits and more tied to

ecology than phylogeny. Pond-type larvae are found in many different salamander groups

that are not closely related, such as Salamandridae (e.g., *Notophthalmus*) and the

Ambystomatidae (e.g., *Ambystoma*). The stream-type condition shows this same

dispersion through the families of salamanders such as Proteidae (e.g., *Necturus*) and

Dicamptodontidae (e.g., *Dicamptodon*) (Valentine & Dennis, 1964). However, the larval

type does not change within a species based on rearing conditions, which suggests these larval types are genetically fixed (Valentine & Dennis, 1964). Whether the stream-type larva or pond-type larva is the basal condition in salamanders is not well researched, however the morphology of early tetrapods and their relatives may give a glimpse into the phylogenetic history of these traits.

The caudal fins of *Ichthyostega*, *Acanthostega*, and *Tiktaalik roseae* – early tetrapods and their fishlike relative – ended well posterior to the hind limbs, similar to the stream-type condition (Daeschler et al., 2006). Daeschler et al. (2006) described *Tiktaalik* as living in a shallow, meandering river-like environment. Other sarcopterygian fishes also show morphology similar to that of extant larval salamanders. Dipnoans (lungfish) have a derived diphycercal caudal fin which is similar to the condition found in extant pond type larval salamanders (Kemp, 1986). The morphology of early tetrapod ancestors and sarcopterygian fishes shows that these morphological caudal fin traits are found in groups other than Caudata even though they appear to have evolved independently and may be associated with a distinct style of locomotion expressed by these species. Evidence of aquatic walking behaviors similar to those of extant salamanders have been found in *Acanthostega* (Coates & Clack, 1991) and even extant lungfishes (King et al., 2011). The similarity of the caudal fin of lungfish to that of pond type larvae, and the similarity of the caudal fin of early tetrapods like *Ichthyostega*, *Acanthostega*, and *Tiktaalik*, hints at a functional similarity between derived lungfish, some early tetrapods, and extant salamanders.

Study Species – Natural History and Feeding Ecology

Salamanders have a wide diversity of life history patterns, however many species display a biphasic life cycle with an aquatic larval stage and a terrestrial or sometimes semiaquatic adult stage (Petranka, 1998). After hatching, the larvae may stay in the streams or ponds for weeks to several years (Petranka, 1998). Upon metamorphosing, the morphology of the salamander changes, with the most noticeable external changes being resorption of the gills and reduction of the fins. Some species are paedomorphic, retaining their larval form and reaching sexual maturity without ever leaving the aquatic habitat. *Ambystoma gracile* and *Dicamptodon tenebrosus* both exhibit facultative paedomorphosis, where metamorphosed terrestrial adults and non-metamorphosed aquatic adults occur in the same populations (Denoël et al., 2005).

The natural history of a species determines which tasks and behaviors are ecologically relevant. What an animal eats determines the behaviors it uses to feed and stalk prey. The presence of predators can also change behaviors and even life history patterns (Efford & Mathias, 1969; Sprules, 1974; Taylor, 1983a, 1983b; Rundio & Olson, 2003). Here I will give an overview of the life history, feeding ecology, and predators of *D. tenebrosus* and *A. gracile* in order to provide context for this ecomorphological comparison.

Dicamptodon tenebrosus, is one of four species within the monogeneric family Dicamptodontidae, the sister family to Ambystomatidae (Pyron & Wiens 2011). It is found in and around semi-permanent streams, permanent streams, and occasionally lakes

and ponds in mesic coastal forests ranging from southern Sonoma Co. in northern coastal California to southwestern British Columbia (Maughan et al., 1976; Nussbaum, 1976; Good, 1989; Petranka, 1998). Hatching occurs 6-7 months after oviposition and hatchlings average 18-19 mm SVL (Nussbaum & Clothier, 1973). The length of the larval stage varies between 18 months to > 3 years, depending on the permanence and size of the streams they are inhabiting, and they typically metamorphose between 53-93 mm SVL (Nussbaum 1976). Gilled, neotenic adults are found in higher percentages in larger, permanent streams (Petranka, 1998; Coriell, 2003). Larvae typically mature into gilled adults at about 115 mm SVL, however some populations have smaller gilled adults, ranging from 85-107 mm SVL (Nussbaum 1976). Larvae have been found in the hyporheic zones of streams (Feral et al., 2005) and a neotenic individual was found in a pitfall trap outside of the stream (Welsh, 1986) showing that they can use locomotor modes other than swimming.

Dicamptodon tenebrosus larvae are frequently the most abundant aquatic predator in their habitat (Petranka 1998) and can constitute > 90% of the predator biomass in the streams of Oregon and Northern California (Murphy & Hall, 1981). Larvae and neotenes are typically hidden under cover during daylight hours and are most active at night (Parker, 1994). The diet of larval *D. tenebrosus* is made up mostly of stream invertebrates, however large larvae and neotenic individuals have been known to eat vertebrate prey (Petranka, 1998). In particular, Antonelli et al. (1972) found *D. tenebrosus* larvae to be opportunistic benthic feeders. Their diet consists of amphipods, caddisflies, beetles, odonates, mayflies, isopods, other arthropods, and vertebrates such as

rainbow trout (*Oncorhynchus mykiss*), sculpin (*Cottus* spp.), northwestern salamanders, and other *D. tenebrosus* larvae (Schonberger 1944; Johnson & Schreck 1969; Antonelli et al 1972; Garland & Losos, 1994; Parker 1994). *Dicamptodon tenebrosus* are preyed upon by fish, garter snakes (*Thamnophis* spp.), river otters (*Lontra canadensis*), shrews (*Sorex*), and weasels (*Mustela* spp.) as well as other *D. tenebrosus* (Nussbaum & Maser 1969; Nussbaum et al. 1983).

      Ambystoma gracile is one of thirty-two species in the monogeneric family Ambystomatidae (Larson & Dimmick, 1993; Pyron & Wiens, 2011). *Ambystoma gracile* ranges from the Pacific coast of northern California through British Columbia up to southern Alaska (Petranka, 1998). It inhabits mesic environments, with breeding occurring in permanent and semipermanent ponds to large lakes. Occasionally, adult *A. gracile* are found in pools of slow flowing streams (Petranka, 1998). Northwestern Salamanders typically hatch 2-9 weeks after oviposition, depending on the temperature of the water (Slater, 1936; Watney, 1941; Licht, 1975; Brown, 1976). The average size of *A. gracile* after hatching is 8 mm SVL (Licht, 1975). Both lowland and montane populations become sexually mature at about 70-75 mm snout-vent length (SVL). Eagleson (1976) suggested that genetic differences between low elevation and high elevation populations have an influence on the proportion of gilled adults. Very little is known about the ecology of *A. gracile* specifically in northwestern California, other than its geographic range.

      Aquatic larvae of *Ambystoma gracile* feed primarily on zooplankton, with feeding beginning shortly after hatching (Petranka 1998). As the size of the larvae increase, so

does their prey size. Larger larvae and gilled adults will feed on benthic prey items such as annelids, ostracods, amphipods, isopods, and a host of other arthropods, as well as frog tadpoles in lowland populations (Henderson, 1973; Licht 1975; Peterson & Blaustein, 1991; Petranka 1998). Larvae and neotenes typically inhabit ponds that lack predatory fishes, but when predatory fish are present their behavior changes, as they adopt a nocturnal lifestyle (Efford & Mathias, 1969; Sprules, 1974; Taylor, 1983a, 1983b). *Ambystoma gracile* larvae can be prey items for the larger *D. tenebrosus* larvae when *D. tenebrosus* occasionally occurs in lentic habitats (Johnson & Schreck 1969).

Aquatic Locomotion in Salamanders

Locomotion is an ideal behavior for ecomorphological studies because most behaviors—such as foraging, escaping, and relocating—involve locomotion (Garland & Losos, 1994). Salamanders maneuver in aquatic environments via two basic methods: axial propulsion (swimming) and aquatic walking (Ashley-Ross, 2004). Yet in contrast to terrestrial locomotion, which has been quite well-studied (Edwards, 1977; Full et al., 1990; Frolich & Biewener, 1992; Ashley-Ross, 1994; Ashley-Ross, 1995; Ijspeert et al., 2005; Kawano & Blob, 2013), only a few studies have examined aquatic locomotion in salamanders.

Axial propulsion in swimming salamanders is a standing wave lateral undulation (Frolich & Biewener 1992) similar to that of anguilliform fishes, but with the limbs compressed along the body (D'Août & Aerts, 1997). However, salamanders swim much

less efficiently, defined by stride length and propeller efficiency, during steady speeds

than anguilliform fishes and anuran tadpoles (D'Août & Aerts, 1997), even more so

during metamorphosis (Landberg & Azizi, 2010). In the wild, *Ambystoma* species use

axial propulsion to move up the water column to feed on invertebrate larvae (Anderson &

Graham, 1967). Axial propulsion in *Ambystoma tigrinum* is also used as a mode of

escape from predators, and in this context speed and maneuverability are more important

than in steady swimming (Fitzpatrick et al., 2003).

Aquatic walking on a level surface has been described in the California newt

Taricha torosa—it is a trot-like gait, weight is supported on diagonal limbs during stride

(i.e. right hindlimb and left forelimb), with a lower duty factor than the terrestrial

diagonal-couplets lateral sequence walk (77% in terrestrial vs. 41% in aquatic) (Ashley-

Ross et al., 2009). The lower duty factor during aquatic walking indicates that the weight

bearing limbs are only touching the substrate for 41% of their stride instead of 77%

during terrestrial walking. While the stride in aquatic walking was more variable than that

of terrestrial walking, elements of the terrestrial stride were highly conserved in aquatic

walking (Ashley-Ross et al. 2009). The ecological use of aquatic walking is unclear.

Parker (1994) described *Dicamptodon* as "active along the streambed" but did not

describe locomotor mode. The streambed rocks are used as refuge from predators

(Rundio & Olson, 2003) and the small spaces between may be more accessible by

walking than swimming. Duellman and Trueb (1986) describe stream type larvae in

general as locomoting primarily by walking along the substrate. Water velocity tends to

slow down towards the bottom of streams which could facilitate easier movement along a

streambed (Westenbroek, 2006). Friction could also assist in resistance to being swept downstream. Pond-type larvae have also been noted to utilize aquatic walking along the substrate after feeding (Anderson & Graham, 1967). These scattered reports make it clear that much remains to be learned about the ecomorphology of aquatic locomotion in salamanders.

Study Design and Predictions

In this study, I performed an ecomorphological comparison of locomotion in *Dicamptodon tenebrosus* and *Ambystoma gracile* – comparing aquatic locomotor behaviors, kinematics, and escape and flow resistance performance. I tested the assumption that morphology is connected to ecology by behavior and performance by attempting to determine whether each morphological type performs best at ecologically relevant tasks associated with its environment. My predictions were that:

1) The stream-type larva of *Dicamptodon tenebrosus* would perform better at resisting increasing water flow velocities by using its limbs to hold onto the substrate,

2) The pond-type larva of *Ambystoma gracile* would display higher swimming escape velocities due to the increased caudal fin size, and finally,

3) The stream type *Dicamptodon tenebrosus* would primarily utilize aquatic walking as a means of locomotion in the field, while the pond type *Ambystoma gracile* would primarily utilize swimming.

In addition to performance tests and observations on locomotor mode, I also aimed to better characterize the divergent morphology of the pond type and stream type by measuring hindlimb, forelimb, and tail features. I predicted that the morphological characteristics measured would be correlated with water velocity (resisting flow and escape speed) and locomotor behaviors (walking vs. swimming).

METHODS

I coupled in situ behavioral surveys of locomotion in the wild with performance tests in the lab to examine how the following differ between the two species: 1) limb and tail fin proportions, 2) primary mode of locomotion, and 3) performance in ecologically relevant tasks, including escape velocity and ability to resist water flow. All procedures were approved by the HSU IACUC (Protocol # 16/17.B.37-A).

Field Observations

To test my hypothesis that different modes of locomotion are used by the two larval types, I conducted field observations of both salamander species between May and October, 2018. My field sites for *D. tenebrosus* included different reaches of four streams in Humboldt and Trinity counties: Madden Creek, located east of Willow Creek, CA; East Fork of Willow Creek, located west of Willow Creek, CA; Jolly Giant Creek, in Arcata, CA; and North Fork of the Trinity River (Figure 1). My field sites for *A. gracile* were numerous perennial ponds located on Green Diamond Resource Co. property north of Arcata, CA, and on private land in Maple Creek, CA. The field observations involved first year larvae to neotenic individuals (3+ years) of *D. tenebrosus* and *A. gracile*; with those animals that were 70 mm SVL or larger classified as neotenic (Nussbaum and Clothier 1963; Sagar et al., 2007).

Figure 1: Perennial streams and ponds where *Dicamptodon tenebrosus* and *Ambystoma gracile* behavior was videorecorded. Sites were located in Humboldt and Trinity counties.

Observations were conducted during the most active hours for *D. tenebrosus* and *A. gracile*. I videorecorded fifty *D. tenebrosus* (average SVL = 79.01mm, range = 33.45mm – 132.15mm) and thirty-eight *A. gracile* (average SVL = 48.39mm, range = 25.00mm – 91.90mm). In this study, *A. gracile* was only observed at night, in situations where fish were present. I observed the animals using an Eyoyo Underwater Fishing Camera (Eyoyo, Guangdong, China) attached to a recording system consisting of a Coomatec Mini DVR (Coomatec Intelligence, Shenzhen, China) powered by a Talentcell Li-ion battery (Talentcell Technology, Shenzhen, China) (Figure 2). The camera was attached to a telescoping pole by a microphone clip epoxied onto a paint roller frame.
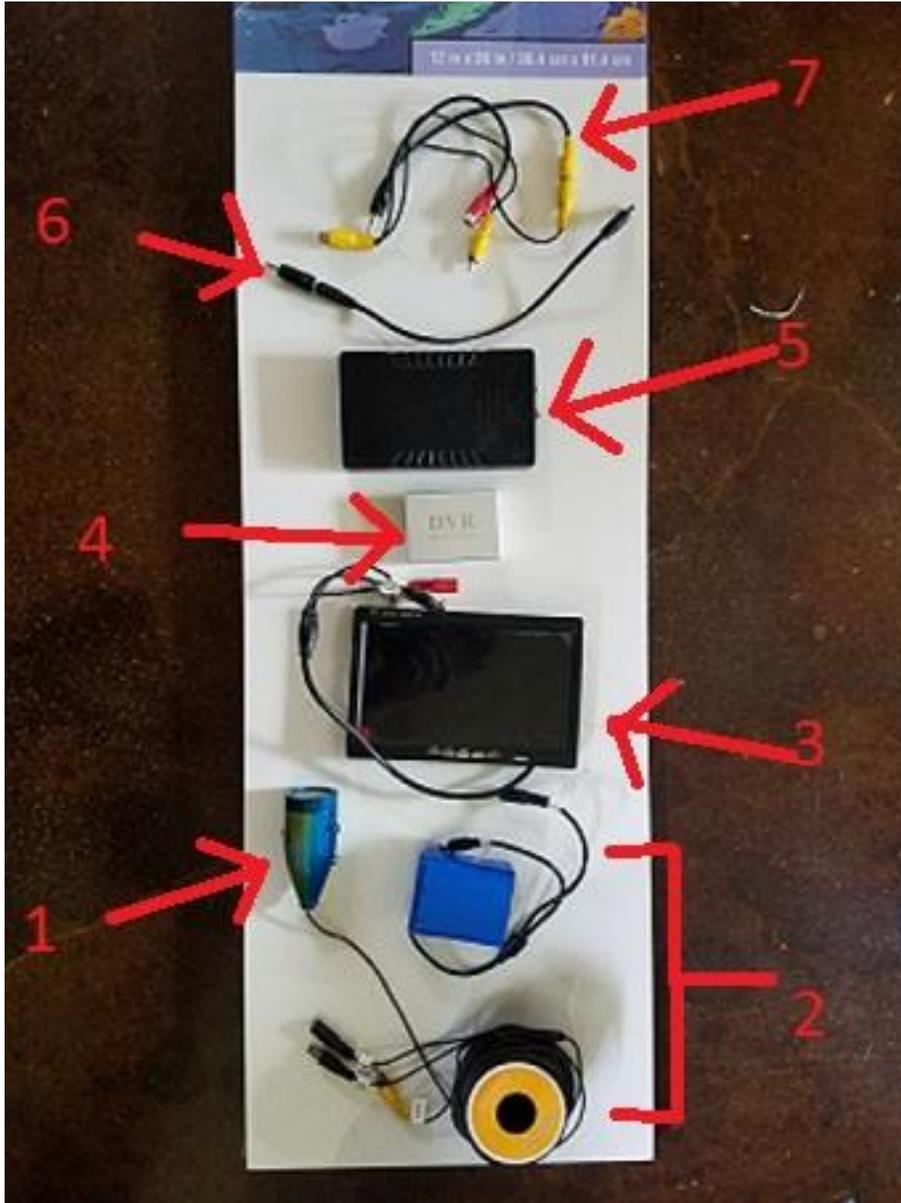
Figure 2: The Eyoyo Infrared Fish Camera, recording system, and battery pack. The

system is a configuration of 1) the infrared camera with built in infrared lights, 2) the

30m line and camera battery, 3) the display 4) the external DVR recording system, 5) the

DVR's external battery pack, 6) the battery connection cord and 7) the A/V cords

connecting the camera to the display and external DVR.

The camera was infrared sensitive, with an attached infrared light source; this prevented shorter wavelength light disturbing the behavior of the animals (Buchanan, 2006). While water absorbs infrared light more readily than other wavelengths, Chidami et al., (2007) showed that infrared light can be used in underwater behavioral surveys effectively. I found the animals by walking along the bank of the stream or pond with a flashlight. Once found the animal was allowed an acclimation period of 10 minutes before any locomotor behavior was recorded. Animals were then videorecorded (Figure 3) for 15 minutes or until they were lost from view (focal survey method). I later analyzed these recordings in the lab for more accurate determination of timing of locomotor behaviors.

Figure 3: An example of use of the recording apparatus. Typically, a second observer is holding the screen for better control of the camera by the main observer.

Videos were processed using Kinovea Video Software® (Version 0.9.3,

www.kinovea.org). A locomotor event was defined as beginning at the first frame where

movement was observed. The end of the locomotor event was defined as the last frame

where movement was observed and there was no other movement observed for at least

three seconds afterwards. Total movement times for locomotor events were recorded to

the nearest second. In order to facilitate assessment of the primary locomotor mode used,

an animal was categorized as either a walker, swimmer, or mixed locomotor based on the

percentage of time spent exhibiting each movement type. The animals were designated a

walker or swimmer if the time spend walking or swimming was greater than 75 percent.

Animals were categorized as mixed if there was no locomotion type greater than 75

percent. I chose this threshold based on similar locomotor categories in *Anolis* lizards

(Moermond, 1979) but was adjusted to account for only two locomotor types instead of

three.

After the digital recording was completed, I captured the animals by net, if

possible, to obtain morphological data. In order to facilitate measurement, I placed the

animals into individual plastic bags filled with water from the stream or pond where they

were caught. This facilitated measurements by restricting movements of the animals

during the process. The following data were collected: SVL, total length (TL), forelimb

and hindlimb lengths (forelimb forearm and upper arm, hindlimb shank and crus), tail

length, tail width, and caudal fin area (Figure 4). Measurements were taken at the end of

each survey night (Figure 4). Total length, SVL, and limb measurements were taken

using dial calipers (± 0.01mm). For tail measurements, I captured a digital image of the

caudal fin and tail in a lateral view, taken alongside a centimeter scale. I processed the

image in the lab using ImageJ (https://imagej.nih.gov/ij/) software. After measurements

were taken, I released the animal back into the pond or stream where it was captured.
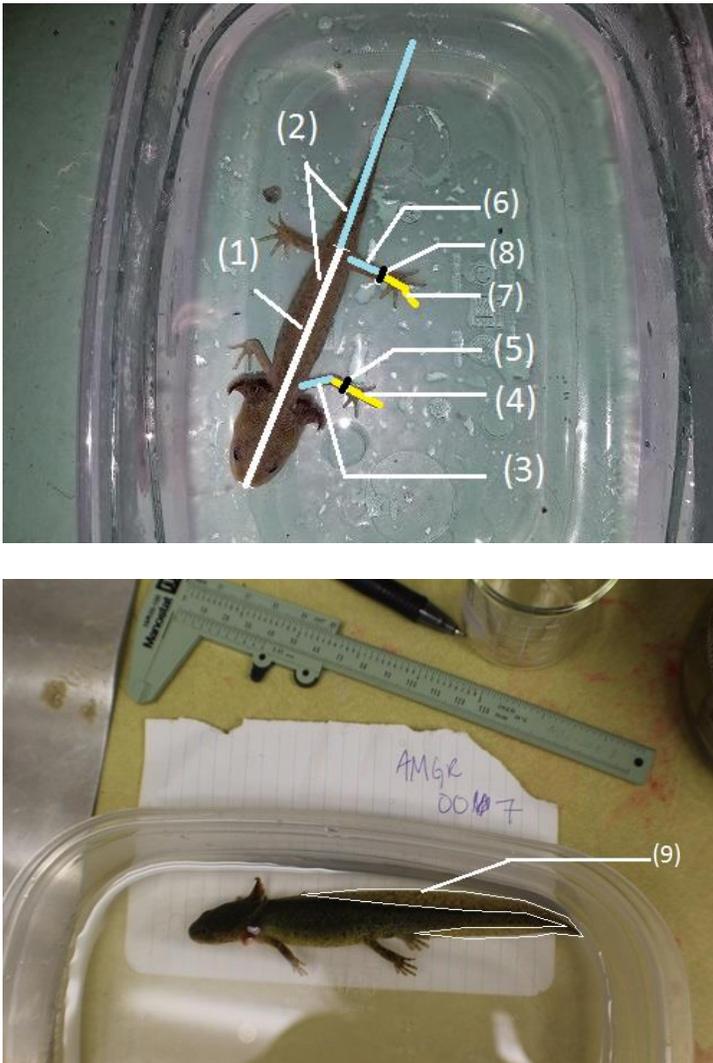

Figure 4: Measurements taken on field and lab animals. Measurements include: (Top) (1)

SVL, (2) TL, (3) forelimb upper arm length, (4) forelimb forearm length and (5) width,

(6) hindlimb upper limb (thigh) length, (7) hindlimb lower limb (shank) length and (8)

width, and (Bottom) (9) caudal fin area.

I recorded flow velocity at the specific area the animal's movements were recorded using a flow velocity meter (Swoffer Instruments Inc, 2100 series current velocity meter, Seattle, Washington, USA).

The first five captured individuals for each species (10 total) were kept and transported by cooler to holding tanks at HSU to be used in the performance observations. Five additional animals were collected after five individuals perished prior to performance observations.

Lab Studies

Housing

I housed the animals in environmental control chambers (Conviron, Winnipeg, Canada, BDR16-plant growth chamber). The temperature was set at 14° C, the average of both the stream and pond temperatures, with a light cycle of 12/12 hours. Seven *D. tenebrosus* were housed in the same tray system consisting of three plastic trays sectioned off into three sections each by screening to allow water to pass through (Figure 5). Each section was 0.15 m wide x 0.3 m long x 0.1 m depth to allow full submersion of the animal. Each animal was confined to a separate section. Filtered tap water was circulated by pumping water up into one end of the container from a 30-gallon reservoir and then drained through an overflow pipe, on the other end, back into the reservoir. The flow rate was a constant 0.1 m/s. I housed the *A. gracile* in a similar situation to the *D. tenebrosus* but in standing water (i.e. no circulated water). Animals were fed chopped earthworms

three times per week at 1g/salamander/feeding . I changed the water out three times a

week or more frequently if needed.

Figure 5: Photograph of the holding tray system for *Dicamptodon tenebrosus*. Each tray

was divided into three sections by flow-through partitions. The water was recirculated by

a pump kept in a 30-gallon reservoir situated under the trays.

Performance Tests

     I conducted swim escape velocity tests with cameras to record swimming

velocity, and flow regime tests to determine an animal's ability to withstand increased

water velocity. The swim escape velocity test was conducted in a 2 m long x 0.5 m tall x

0.5 m wide tank. I positioned a high-speed camera (Panasonic, HC-VX870K, Lake

Forest, California) lateral to the tank to record the velocity. The back of the tank was

lined with 1 cm grid markings. I induced the escape response by agitating the animal—by

touch with a gloved finger—starting at one end of the tank and recording its escape to the other end. I recorded at least 18 escape responses for each individual, for a total of 198 responses. Escape velocities were standardized by body length (SVL/s) to account for the effect of body length on swim velocity (Landberg & Azizi, 2010).

I conducted the flow resistance test in a flow tank (1 m length x 30 cm height x 30 cm width) at Humboldt State University's Fish Hatchery (Figure 6). The flow tank had a separate holding container for the animals to prevent them from escaping into the flow tank. A pebble sized (10 – 15 mm diameter) rocky substrate was present to allow for gripping by the animals, however the size of the rocks was such that the animals could not use them for cover. Each animal was placed in the tank with the water circulation pump on the lowest setting (0 m/s). Every 30 seconds the flow was increased by 0.15 m/s. I increased the flow rate until the animal was no longer able to resist and was pushed back into a safety net secured behind it. I measured water velocity using a flow meter throughout the entire trial. The first and last reading were recorded.

Figure 6: Above: Photograph of the flow chamber used for flow resistance tests. Below:

A neotenic *Dicamptodon tenebrosus* during acclimation prior to testing.

After all lab performance testing was complete, I euthanized the animals in 2.5 percent MS-222 (tricaine methane sulfonate, Western Chemical, Ferndale, Washington) buffered to a pH of 7.0 with sodium bicarbonate. Each animal was then fixed in a 5 percent formalin solution for two weeks, then preserved in 70 percent ethanol. I dissected all lab animals and measured weights for a series of muscles associated with the pectoral and pelvic girdles. Working under a dissecting scope, I removed muscles from both the left and right side of the animal at the insertions and origins (Table 1). I then placed each muscle into 70 percent ethanol. For measurements, I removed the muscle from the ethanol, blotted it dry on paper towels, weighed it on a digital scale to the nearest 0.01 g, then placed it back into 70 percent ethanol. I repeated this process three times for each muscle on each side.

Table 1: Muscles weighed after euthanasia, preservation in formalin, and dissection, the respective limbs from which they are derived, and hypothesized functions. CPIT = caudalipuboischiotibialis, PIT = puboischiotibialis, PTB = pubotibialis, CDF = caudofemoralis, ISF = ischioflexorius, PIFI = puboischiofemoralis internus, PIFE = puboischiofemoralis externus (Duellman and Trueb, 1986; Ashley-Ross, 1995; Peters & Goslow, Jr., 1983; Székely et al., 1969).

MUSCLE	LIMB	HYPOTHESIZED MAJOR ACTION
M. pectoralis	pectoral	Adducts and retracts forelimb
M. supracoracoideus	pectoral	Adducts forelimb
M. procoracohumeralis	pectoral	Protracts forelimb
M. humeroantebrachialis	pectoral	Flexes elbow
M. latissimus dorsi	pectoral	Elevates and retracts forelimb
forearm flexors	pectoral	Flexes wrist
M. CPIT	pelvic	Tenses the PIT and retracts hindlimb
M. PIT	pelvic	Flexes knee
M. PTB	pelvic	Adducts hindlimb
M. CDF	pelvic	Retracts femur/laterally flexes tail
M. ISF	pelvic	Flexes knee
M. PIFI	pelvic	Protracts femur
M. PIFE	pelvic	Adducts femur
trunk muscles	trunk	Laterally flex trunk

Analysis

I compared morphological measurements using a t-test (accounting for non-equal variance) of the length to width ratios of the forelimb and hindlimb, and the area of the caudal fin. All measurements were standardized to SVL to account for size differences between species and among specimens.

For the analysis of field locomotor behavior, I categorized each individual by behavior type (aquatic walking, swimming, or mixed) based on the primary locomotor behavior exhibited (see above). I used a step-wise two way discriminant analysis to determine if the locomotor behaviors differed between species. My response variable was the percent of time spent swimming and my predictors were the species, forelimb and hindlimb length to width ratios, and caudal fin area. Afterwards, I performed a principal components analysis to determine the relationship between the predictors and species.

For the performance analyses I used a simple paired t-test since the same individual was tested more than once. My predictors were the individual animal and the species. These were the same for both the swim escape velocity and the water flow resistance tests. The response variable for the swim escape velocity was the average velocity of escape and the response variable for the water flow resistance was the measured water velocity at the time the animal is swept away.

All statistical analyses were carried out in R (R Core Team, 2020). All measurements and ratios are reported as mean +/- SE of the mean.

RESULTS

Field Behavior

As a species, *D. tenebrosus* are primarily walkers, with 45 (90%) out of the 50 animals observed locomoting by aquatic walking >75% of the time. *Ambystoma gracile* are more variable, with 16 (42%) of the animals categorized as walkers, 10 (26%) animals categorized as swimmers, and 12 (32%) animals placed into the mixed category. During all observations, *D. tenebrosus* collectively walked 98.1% of the time with only 1.9% of the time spent swimming. Movement in *A. gracile* was more variable with aquatic walking being 65.4% of all movement and swimming being 34.6% of all movement. Swimming was significantly positively correlated with forelimb length:width ratio (adjusted $R^2 = 0.4552$, df $= 51$, p-value < 0.001) while aquatic walking was significantly negatively correlated with forelimb length:width ratio (adjusted $R^2 = 0.4346$, df $= 51$, p-value < 0.001). All other morphological predictors (hindlimb ratio and caudal fin area) were not significant. Species was highly correlated (adjusted $R^2 = 0.82$) with all other predictors so it was not included in the final model to account for duplicate predictors.

Morphology

The mean forelimb ratio, defined as length/width, of *D. tenebrosus* (3.91 ± 0.18) was significantly smaller than that of *A. gracile* (7.72 ± 0.37) which indicates a shorter,

more robust forelimb (T-stat = -9.103, df = 29, p-value = <0.001) in *D. tenebrosus*, with a

ratio closer to one, and a more elongate forelimb in *A. gracile*. The differences in

hindlimb ratios are similar to the forelimb ratios: *D. tenebrosus* (3.56 ± 0.07) had a ratio

closer to 1 than *A. gracile* (6.13 ± 0.31). This indicates significantly shorter, more robust

hindlimbs in *D. tenebrosus* and more elongate hindlimbs in *A. gracile* (T-stat = -8.153, df

= 29, p-value < 0.001). Caudal fin area, standardized by SVL, was significantly larger in

A. gracile (8.62 ± 0.31) than in *D. tenebrosus* (4.82 ± 0.65) (T-stat = -5.227, df = 29, p-

value < 0.001). Forelimb ratio, hindlimb ratio, and caudal fin area were all significantly

correlated with species (adjusted R^2 = 0.82, df = 49, p-value < 0.001) as evidenced by the

principal components analysis (Figure 7).
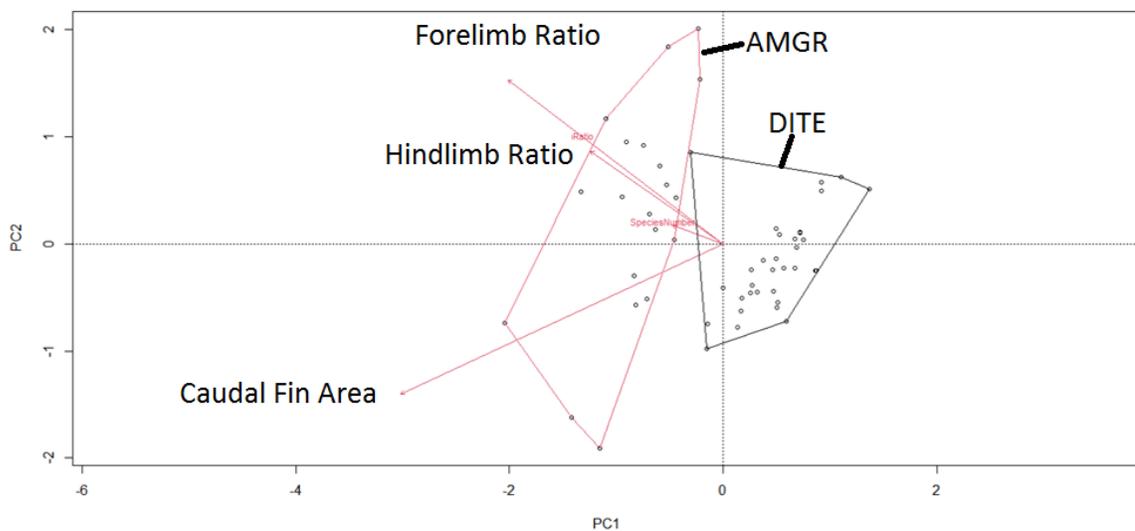
Figure 7: Principal component analysis of salamander morphology. The pink polygon

encompasses *A. gracile* and the black polygon encompasses *D. tenebrosus*.

<u>Muscle Weights</u>

Out of the fourteen muscles or muscle groups that were weighed, seven showed a significant difference between *D. tenebrosus* and *A. gracile* (Table 2). *Dicamptodon tenebrosus* has larger mean weights in all significantly different muscles or muscle groups.

Table 2: A table of the mean, variance (SE), p-value, and significance of muscle weights. Data are reported as a percentage of total body weight. CPIT = caudalipuboischiotibialis, PIT = puboischiotibialis, PTB = pubotibialis, CDF = caudofemoralis, ISF = ischioflexorius, PIFI = puboischiofemoralis internus, PIFE = puboischiofemoralis externus. * = Significant difference (p < 0.05) between *D. tenebrosus* and *A. gracile*. Df = 9 for all results. *DITE = Dicamptodon tenebrosus, AMRG = Ambystoma gracile.*

MUSCLE	*DITE* MEAN	*DITE* SE	*AMGR* MEAN	*AMGR* SE	P-VALUE
M. pectoralis*	0.246	0.019	0.123	0.031	0.006
M. supracoracoideus	0.079	0.012	0.059	0.006	0.094
M. procoracohumeralis*	0.068	0.007	0.046	0.005	0.042
M. humeroantebrachialis	0.029	0.004	0.023	0.003	0.329
M. latissimus dorsi*	0.072	0.008	0.038	0.004	0.007
Forearm flexors*	0.081	0.007	0.003	0.004	0.005
M. CPIT	0.077	0.008	0.059	0.009	0.196
M. PIT	0.160	0.032	0.099	0.026	0.189
M. PTB	0.031	0.004	0.027	0.006	0.579
M. CDF*	0.116	0.011	0.043	0.014	0.003
M. ISF*	0.052	0.004	0.030	0.006	0.012
M. PIFI	0.056	0.004	0.039	0.008	0.073
M. PIFE	0.033	0.009	0.033	0.003	0.914
Trunk muscles*	1.790	0.066	1.315	0.055	0.0004

Flow Resistance

Flow resistance between species was significantly different (T-stat = 2.741, df = 57, p-value = 0.025). *D. tenebrosus* (4.19 ± 0.25 svl/sec) showed on average a higher resistance to water flows than *A. gracile* (2.51 ± 0.29 svl/sec). During flow resistance trials I noted that *D. tenebrosus* pulled their bodies close to the substrate as the flow began. However, I noted that *A. gracile* made no efforts to move closer to the substrate during trials.

Swim Escape Velocity

Swim escape velocity between species was not significantly different (Table 6) between species (T-stat = -0.293, df = 103, p-value = 0.77). However, *D. tenebrosus* (2.52 ± 0.25) had highly variable swim velocities (range: 0.18 - 9.71 svl/sec) while *A. gracile* (2.60 ± 0.13) had significantly less variable swim velocities (range: 1.15 - 5.24 svl/sec) (Fstat = 4.505, df = 70, p-value < 0.001).

DISCUSSION

Salamander larval types are defined by morphological characteristics—difference in gill rachis length, degree of hind limb development, and whether the caudal fin extends past the pelvic girdle (Noble 1927)—and by their habitat. Yet until the present study, there had been no studies on how the morphotypes interact with their respective habitats.

The results of the field behavioral observations I made show a significant difference in locomotor modes between *D. tenebrosus* and *A. gracile* in their natural environment. Larviform (larval or neotenic) *D. tenebrosus* exhibited walking behavior (98.1%) at a higher frequency than *A. gracile* (65.4%) and had a higher percentage of individuals categorized as walkers. These results support the prediction that *D. tenebrosus* utilize aquatic walking more. Interestingly, *A. gracile* individuals observed varied as to whether they were predominantly walkers, swimmers, or had mixed locomotion; I had expected them to be predominantly swimmers.

One explanation of this observed difference is that stream-type larvae benefit more from continually being at or near the bottom of the water column. In a streambed, water velocity nears zero as it gets closer to the bottom of the column (Westenbroek, 2006). While the lab performance results of this study show that *D. tenebrosus* are more adept at resisting flows, constant resistance would be energetically costly. Also, because *D. tenebrosus* are opportunistic benthic feeders, movement along the bottom of the water column is also better suited for prey location, capture, and consumption (Antonelli et al, 1972).

Ambystoma gracile, on the other hand, is able to spend time moving throughout the water column of lentic water bodies without being subject to an increase in water velocity. In the field, I frequently observed *A. gracile* suspended in the water column. Furthermore, species in the genus *Ambystoma* are known to move throughout the water column to feed (Anderson & Graham, 1967). In a lentic habitat, swimming along the bottom is not less energetically costly than swimming in the water column. Comparing walking versus swimming, Tucker (1975) found walking to be more energetically costly than swimming in aquatic environments. However, Enders et al. (2005), found that as flow turbulence increases, the energetic cost of swimming in fish significantly increases. Arguably, these same conditions could increase the energetic cost of swimming in salamanders as well. Thus in lentic conditions swimming is the most energetically conservative locomotor type; as expected, I found a significantly higher percentage of swimming in *A. gracile* than I found in *D. tenebrosus*. However, that dynamic changes when flow velocity and turbulence increase, creating an environment where walking along the bottom – where flows are near zero – is the more energetically conservative locomotor type. In this environment, I saw an almost complete reliance on walking as a locomotor type in the stream-type *D. tenebrosus*.

Whether such observed differences in locomotor mode between the species are behavioral responses to the environment or intrinsic behavioral traits is not clear. This study did not observe stream-type larvae in lentic habitats or pond-type larvae in lotic habitats. Observing locomotor behavior in alternative habitats would help clarify whether the behavior is partly or wholly a response to ecology. Future studies should conduct a

reciprocal transplant experiment in the lab – by placing *D. tenebrosus* in a pond like

mesocosm and *A. gracile* in a stream-like mesocosm and recording locomotor behaviors.

This would serve to test whether behavioral differences are responses to habitat

conditions or if they are genetic (i.e. do *D. tenebrosus* utilize walking the same amount

even when in lentic conditions).

The results of my morphological comparisons show a significant difference

between species in forelimb and hindlimb length:width ratio, caudal fin area, and the

mass of seven out of fourteen muscles or muscle groups (Table 2). These results show

that there are morphological differences between types beyond those first described by

Noble in 1927. A likely explanation for the significant differences in forelimb and

hindlimb ratios is that limb robustness plays an important role in navigating moving

waters by walking along and gripping the substrate. *D. tenebrosus* showed more robust

hindlimbs and forelimbs than the *A. gracile*, with robust being defined as a length to

width ratio closer to one. The principal components analysis (Figure 7) showed that

hindlimb ratio, forelimb ratio, and caudal fin area were all correlated to species. *D.*

tenebrosus have limb proportions more in line with terrestrial lizards that are vertical

climbers than those that are walkers (Herrel, Meyers, & Vanhooydonck, 2001), meaning

more robust limbs with the forelimb and hindlimb being similar in size. While this seems

unintuitive, the forces acting on vertical climbers move from anterior to posterior, the

same direction that is being acted on in animals living in high flows in a stream. In the

flow experiments, *D. tenebrosus* had a higher mean resistance than *A. gracile*. During the

performance experiments, *D. tenebrosus* were anecdotally noted moving their bodies

closer to the substrate when the flows started, whereas *A. gracile* showed no inclination towards this motion. The shorter, more robust, limbs and significantly larger pectoralis, latissimus dorsi, forearm flexors, caudofemoralis and ischioflexorius support a "climbing" like lifestyle based on the hypothesized muscle actions (Peters and Goslow 1983; Duellman & Trueb, 1989) and electromyographic (EMG) analyses of locomotion (Szekely et al 1969; Ashley-Ross, 1995).

Contraction of the pectoralis adducts and retracts the forelimbs and the latissimus dorsi elevates and retracts the forelimb. The contraction of the pectoralis would push the animal off the substrate to lift the body during movement. If the animals are moving along a vertically sloped surface during high velocity flows, more power would be needed to push the animal off the substrate during locomotion. The latissimus dorsi brings the body closer to the substrate during contraction. Both muscles retract the forelimbs which would move the animal forward during locomotion. In high flows larger muscles would aid in this motion due to increased forces (water pressure) acting in the opposite direction. The procoracohumeralis protracts the forelimb. In especially high flows, such as during winter and spring, this could assist the animal with limb placement during locomotor activities. The forearm flexors depress the manus towards the substrate and flex the digits, allowing the animal to grip. While this hasn't been explored in salamanders, Sustaita et al. (2013) showed that gripping in anurans is due to the contraction of the flexor digitorum communis longus.

In the hindlimb region of the animal, the ischioflexorius flexes the knee joint, which would cause the body to move closer to the substrate, while the caudofemoralis

activates in femoral retraction which is used to push the animal forward. The hypaxial and epaxial myomeres, which flex the body laterally, were significantly larger in *D. tenebrosus*. The body musculature is typically activated during both aquatic swimming and walking (Frolich & Biewener, 1992; Deban & Schilling, 2009). More force would need to be generated to swim against a moving current than in still water. The enlarged caudofemoralis also serves to laterally flex the tail which would further assist in swimming against strong currents.

However, some muscles that would play similar roles were not significantly different between species. The humeroantebrachialis and pubotibialis would assist in movements that would bring the body closer to the substrate, while the supracoracoideus would elevate the body, similar to the pectoralis. While they do serve similar functional roles to muscles that were significantly different in weight, a likely explanation is that the majority of the force is generated by the larger muscles. Young et al. (2010) showed that functional redundancies do occur and can allow for ecological convergences. The mean size of the pectoralis is three times larger than that of the supracoracoideus in *D. tenebrosus* and twice as large in *A. gracile*. The humeroantebrachialis is half the mean size of both the forearm flexors and latissimus dorsi. The pubotibialis is half the size of the ischioflexorius in *D. tenebrosus*, however the sizes are equal in *A. gracile*.

When standardized by SVL, *A. gracile* had a significantly larger caudal fin area than *D. tenebrosus*. However caudal fin area was not a significant predictor of locomotor mode. Similarly, while caudal fin area has been shown to be positively correlated with swim escape acceleration in *Ambystoma tigrinum mavortium* (Fitzpatrick et al., 2003),

there was no significant difference in mean swim escape speeds between *D. tenebrosus* and *A. gracile*. *D. tenebrosus* had much more variable escape speeds while mean speeds of *A. gracile* were more uniform. Furthermore, the maximum escape speed was greater in *D. tenebrosus* as compared to *A. gracile*. The power needed to swim against flowing water would require the ability to generate a large amount of force. The larger caudofemoralis and trunk muscles of *D. tenebrosus* allow for generation of more force in lateral bending and tail flexion, movements necessary for axial propulsion.

Webb (1977) showed – by removing the caudal fin of trout (*Oncorhyncus mykiss*) – that in fish the caudal fin is used in fast-start propulsion. However, removing the caudal fin does not affect the steady swimming performance. Steady swimming was more reliant on the trunk musculature. My results showed *D. tenebrosus* to have both a higher maximum velocity and larger trunk musculature than *A. gracile*. Swimming against a current has similar energetic costs and physiomotor requirements as fast steady swimming, similar to behaviors exhibited by sub-carangiform fish such as trout. The form of *A. gracile* is more similar to that of anguilliform fish (Hoff et al., 1989) such as eels. Even though they have a relatively large caudal fin area, anguilliform fish are on the lowest end of swimming performance (Blake, 2004).

While this study aimed to complete a comprehensive examination of salamander morphotypes, some aspects were not covered. Valentine and Dennis (1964) briefly mentioned that phenotypic plasticity does not appear to play a role in larval morphotypes. However, this was not quantified by measurements. Further studies should quantify any variation due to plasticity. An easy way to do this would be to raise *Ambystoma gracile*

hatched from eggs in both lotic and lentic conditions; similar experiments on

Dicamptodon tenebrosus would be much harder, due to the difficulty of finding egg

masses.

This study was limited to two local species. Future studies should attempt to

examine larval morphology, behavior, and performance of a wide range of species,

spanning other salamander families. Young et al. (2010) showed that functional

morphological redundancies could allow for convergent evolution. Having two muscles

that serve the same function allows for selective forces to act on either muscle. For

example, if the pectoralis is enlarged in all stream-type species then it's possible that that

form is derived from a single ancestor. However, if in some species the supracoracoideus,

which serves the same function as the pectoralis, is enlarged then it is possible that the

stream-type condition arose in different lineages. Examination of the morphological

characteristics of multiple species spanning all salamander families for these functional

redundancies could elucidate whether these ecomorphotypes (stream-type and pond-type)

arose from a common ancestor or evolved in separate lineages.

I showed that easily measurable morphological characteristics, forelimb

length:width ratio, are correlated with locomotor behavior. While direct observation is

always the best method for determining behavior, paleoecological studies do not have the

ability to observe behavior. Extinct tetrapod ancestors with more robust limbs would

likely utilize aquatic walking more than tetrapods with slender limbs. While there are

caudal fin similarities in extant salamander and lungfishes and tetrapod ancestors (Kemp,

1986; Daeschler et al 2006), there was no significant behavior or performance correlates

with caudal fin size so no assumptions about behavior or performance can be elucidated from these measurements. However, based on the more robust limbs of tetrapod ancestors my results suggest that early tetrapod vertebrates were in a more lotic environment.

Behavior and performance have been used to link morphology and ecology of many vertebrates – *Anolis* lizards, bats, fish, and migratory birds (Losos, 1990; Winemiller, 1991; Aguirre et al., 2002; Wainwright et al., 2002; Winkler & Leisler, 2008; Ginot et al., 2016). Ricklefs and Miles (1994) describe this relationship as two distributions (morphology and ecology) linked by "intervening variables" (behavior and performance at an ecologically relevant task). Salamander larval morphotypes have been described since Noble (1927), however the ways in which these morphotypes interact with their environment has been largely neglected. The correlation of morphological measurements with behavior, and differing performance in ecologically relevant tasks shows how stream-type and pond-type salamander larvae are physically adapted to the environments they inhabit and their morphology and ecology are linked by these variables: they are not just morphotypes but ecomorphotypes.

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