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盤古蟾蜍蝌蚪在不同水流狀態下的表型可塑性之探討

Phenotypic Plasticity of *Bufo bankorensis* Tadpoles

Under Different Flow Regimes

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謝誌




這篇研究能夠順利完成真的要感謝很多人、事、物！回想起當時推甄時，要不是有于綾學姐告訴我許老師他們那麼有趣的研究發現，接下來整個故事可能都要重寫了，這都要感謝當初師大杜銘章老師實驗室學長姐的建議跟幫忙！

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摘要



許多物種能因應棲地的環境條件來調整表型特徵，此能力稱為表型可塑性 (phenotypic plasticity)，可以提升個體在該棲地中的適存度。有些兩棲類的蝌蚪能夠生活在不同的水流環境之中，但針對水流這項環境因子的表型可塑性研究卻極度缺乏。本研究結合形態、生理、行為與生活史等面向，以實驗檢測盤古蟾蜍蝌蚪在靜止與流動水域環境下的表型可塑性。我們將野外帶回的 Gosner Stage 27 期 (G27) 蝌蚪隨機分成兩群，分別飼養於人工模擬的靜止與流動水域之中，待蝌蚪發育至 G35 時測量各項形態形值；隨後進行游泳耐力實驗和尾部肌肉的紅肌層數分析；最後測量變態 (G42) 時的體重。結果顯示：飼養在流動水域下的蝌蚪會擁有相對較小的體寬和體高，但具較長尾長和較寬尾肌，且尾肌的紅肌層數也較多，游泳耐力表現也比較好，但蝌蚪期較長且會以較輕的體重變態。此外，耐力游泳表現和相對體長 (+)、體寬 (-)、體高 (-)、尾長 (+) 和尾肌寬 (+) 組成之 PCA 軸 (PC1) 呈顯著正相關，顯示擁有相對流線型的身體、較長的尾長及較寬尾肌的個體，在耐力游泳上會有較好的表現。因此，生活在流動水域下的盤古蟾蜍蝌蚪可藉由表型可塑性來降低水中阻力，並提升尾部肌耐力以回應水流環境中持續游泳的需求。此一可塑性或許有提高覓食效率與避敵能力等優點，卻也必須因此付出代價：亦即較慢變態且變態時的體重較輕。另外，本研究也發現來自池塘與溪流的蝌蚪在尾長和肌肉層數上，對於水流的反應不同，這暗示著不同棲地類型之間可能有族群分化，但這需要從卵開始實驗研究才能給予有力的支持。

關鍵字：盤古蟾蜍、水流、尾長、紅肌、耐力游泳、棲地

Abstract



Phenotypic plasticity allows individuals respond to environmental challenges timely, thus generally promotes fitness. Tadpoles of some anuran species inhabit aquatic habitats with different flow regimes. However, the effects of flow regime on tadpoles are unclear due to the dearth of studies. In this study, I investigated the phenotypic plasticity of morphological, physiological, behavioral and life history traits of *Bufo bankorensis* tadpoles under different flow regimes. In the laboratory, I randomly assigned tadpoles at G27 stage to two treatments: static vs. flowing water. The results showed that tadpoles reared in flowing water had relative smaller body width and body height, but the values of relative tail length, tail muscle width and red muscle layers were larger than those reared in static water. Tadpoles living in flowing water also had better sustained swimming performance than those from static water groups. Furthermore, the regression analysis demonstrated a significant positive relationship between sustained swimming performance and PC1 composed by relative body length(+), body width (–), body height (–), tail length (+) and tail muscle width (+). It showed that individuals with a relatively narrow and shallow body, and had a long tail with thick tail muscle performed better in sustained swimming. Although tadpoles with such characteristics may gain benefits in foraging efficiency and predator

avoidance during larvae stage, they pay the costs of entering metamorphosis late and at a small size. Moreover, I also found that the responses of tail length and muscle layers to treatments were distinct between pond and stream populations, which suggested population divergence between habitats. Further research is needed to provide sound evidence of population differentiation.

Keywords: Bankoro toad, flow, tail length, red muscles, sustained swimming, habitat

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Introduction




Through various evolutionary processes, particularly natural selection, every creature has its own distinctive set of adaptive traits to match the challenges in their specific environments (Dobzhansky, 1956), in order to survive and reproduce successfully (Moran, 1992; Buskirk, 2002). For a given trait, the mean value of the trait in a population (the mean phenotype) is determined by genetic factors, environmental influences, and interactions between the two (genotype-environment interactions; Falconer & Mackay, 1996). A particular genotype often has the ability to produce alternative forms of morphology, physiology, behavior, and/or life history, called “phenotypic plasticity”, in response to environmental variations (West-Eberhard, 1989). Having phenotypic plasticity is frequently adaptive as it is beneficial for individuals facing variable environmental factors (Via & Lande, 1985; Schlichting & Pigliucci, 1998). Therefore, phenotypic plasticity is likely maintained by divergent natural selection forces across heterogeneous environments. The ability of producing alternative phenotypes is commonly seen in larval amphibians. For example, Benard (2006) showed that the pacific tree frog (*Pseudacris regilla*) tadpoles produced different body shapes when confronted different types of predation. When under the risks of diving beetle predation, tadpoles developed enlarged tails, which served to distract beetles

away from striking vital body parts. However, when the predator was bluegill sunfish, tadpoles developed a shape that improved swimming speed: shallow tails and large tail muscles.




So far, at least 13 environmental variables including temperature (Harkey & Semlitsch, 1988), predator (Touchon & Warkentin, 2008), altitude (Berven, 1987), food types (Michimae & Wakahara, 2002), salinity (Viertel, 1999), depth of water (Denver *et al.*, 1998), oxygen (Burggren & Mwalukoma, 1983), flow regime (Richards, 2002), canopy cover (Buskirk, 2011), UV (Smith *et al.*, 2000), population density (Loman, 2003), disease (Kilpatrick *et al.*, 2010) and parasite (Kupferberg *et al.*, 2009) have been shown to induce phenotypic plasticity in larval amphibians. Among the variables, the effects of “flow regime” have been rarely studied (but see Richards, 2002; Venesky & Parris, 2009; Kupferberg *et al.*, 2011), although many larval amphibians inhabit aquatic habitats with variable flow regimes. Nature aquatic habitats of most larval amphibians can be classified as one of two types: static and flowing water. It has been shown that species living in the former type usually have deep body, high fin, and low numbers of tooth rows. In contrast, species living in flowing water typically have flattened body, large mouthparts, high numbers of tooth rows, and heavy axial musculature (Orton, 1953; Altig & Johnston, 1989). In some species, tadpoles developed abdominal suckers



in order to suction-cup themselves to the bottom substrate for resisting strong stream flow (Inger, 1992). These presumable distinctive adaptive traits are identified between species inhabiting static vs. flowing aquatic environments. However, the effects of flow regime on phenotypic plasticity of tadpoles within species remain limited.

Under controlled laboratory conditions, Venesky and Parris (2009) tested if hydrological differences between lotic and lentic habitats contribute to within-species life history divergence in *Ambystoma barbouri*. They reared laboratory born larvae from pond- and stream-collected eggs in laboratory environments with hydrological patterns similar to their natal environments. The results showed that stream larvae metamorphosed faster and were smaller in body size compared to pond larvae. In a recent study, Kupferberg (2011) explored the effects of pulsed flows on tadpoles of the lotic-breeding Foothill Yellow-legged Frog, *Rana boylei*. They found that tadpoles reared in elevated flow velocities spent more time sheltering in the substrate, and their body mass, development rate, and survival rate were lower than those reared in low flow velocities. The results from limited studies imply that different flow regimes may induce variations in morphology, behavior, and life history within an amphibian species. A missing piece is flow-induced plasticity in “physiology” of amphibian larvae. However, in bony fish, there have been many well-documented experiments showing



the effects of flow regime on physiological performance. For example: after a endurance training program lasting 17 weeks, the red and intermediate muscle mass, fiber diameter and capillaries in two cyprinid species increased significantly, compared with control (Sänger, 1992).

In Taiwan, adults of the endemic *Bufo bankorensis* breed in both ponds and streams. A field study by Kuo *et al.* (2010) demonstrated that the body sizes of *Bufo bankorensis* tadpoles from the ponds were bigger than those from the streams; the relative tail lengths of stream tadpoles are longer than those of pond tadpoles. However, multiple field factors such as temperature, food availability, and flow regime may cause the different morphological traits between two habitat types. Without controlled experiments, the mechanism remains unknown. In this study, I investigated the effects of flow regime on *Bufo bankorensis* tadpoles further. I reared tadpoles collected from ponds and streams in laboratory environments with static vs. flowing water to examine the plastic responses in morphology, physiology, behavior, and life history by *Bufo bankorensis* tadpoles. I asked: (1) What are the patterns of phenotypic plasticity in morphology, physiology, behavior, and life history, respectively? (2) Does the phenotypic plasticity in morphology, behavior, physiology, and life history affect fitness? (3) Do populations from stream versus pond habitats have differential responses to the

flow regime? I aimed to integrate four aspects of flow-induced phenotypic plasticity.

Figure 1 shows the concept map for this study.



Materials and methods

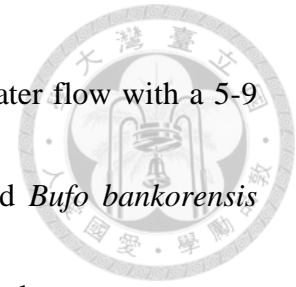


Tadpole collection and care

I collected 80 *Bufo bankorensis* tadpoles at Gosner stage 27 (Gosner, 1960) from 3 ponds and 3 streams each in Taipei City (Table 1), 6 batches in total. I spaced the collection of the 6 batches during October 2012 - February 2013 so I only worked on two batches at a time. Upon returning to the laboratory, I immediately randomly separated a batch into two groups of 40 tadpoles each. One group was reared in static water, and the other in flowing water. Each group was raised in a 55-cm-diameter plastic tub with a pebble substrate and 10-cm-depth tap water. At the center of each tub, I placed a 12-cm-diameter plastic flowerpot so the water body became doughnut-shaped (Fig. 2). I housed the tubs in a $20 \pm 0.5^\circ\text{C}$ incubator on a 12h:12h light/dark cycle. Tadpoles remained in the tub for the duration of the experiment, and were fed *ad libitum* with boiled spinach until they metamorphosed (except 2 batches. See below). Before they were released at collection sites, toadlets were temporarily housed in a tank provided with fruitflies.

I generated water flows by using water pumps in the flowing water treatment to simulate the stream environment (Fig. 2). The pump was housed inside the plastic flowerpot with a 4-pore PVC pipe extruding from the pot crossing the diameter of the

tub. Water pumped through the pores generated a unidirectional water flow with a 5-9 cm/s velocity. The flow velocity of natural streams where I found *Bufo bankorensis* tadpoles ranged between 0-16 cm/s. The static water treatment used the same apparatus except without the water pump.




Morphological traits

I took morphometric measurements of the G35 tadpoles when the majority (ranged between 16-27 individuals; Table 2) of tadpoles in a tub were at G35 (ranged between G33-G37). I recorded body weights using an electronic scale with an accuracy of 0.001 g. I then digitally photographed both dorsal and lateral views of each tadpole, and obtained the following 6 parameters (Fig. 3) by image analysis using ImageJ 1.45s: body length (BL), body width (BW), body height (BH), tail length (TL), tail height (TH) and tail muscle width (TMW).

Sustained swimming trials


After being photographed, tadpoles were held individually in 6-cm-diameter plastic containers before the swimming trials next day. I measured the sustained swimming performance of tadpoles in an apparatus (Fig. 4) following the design



described by Wassersug and Sperry (1977) with some modifications. The glass apparatus (LxWxH = 120x25x30 cm) uses the consistent difference in water levels between reservoir and sink to create a constant water flow entering the swimming pipe. The water levels are maintained by a submerged water pump that continuously recycles water from sink to reservoir. The straight plastic pipe (inner diameter = 2 cm; length = 60 cm) has a mesh at the far end and a vertical branch (chimney) located at 5 cm from the mesh end. The water speed channeling from the reservoir into the pipe is controlled by a valve, and water flow is rectified by a straw bundle. The 45-cm section of pipe between the straw bundle and mesh end is the swim way for tadpoles. I maintained the water flow at a constant velocity of 18 cm/s with the valve. The chimney served as the entrance for tadpoles entering the swimming trials.

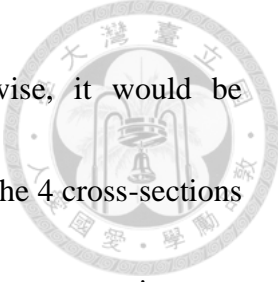
All swimming trials were performed at $20 \pm 1^\circ\text{C}$ water temperature, and recorded by a Sony HDR-SR12 digital HD video camera. Each tadpole was eased into the swimming pipe through the vertical chimney gently. The amount of time each tadpole actively swimming in the swim way was measured to the nearest 1 second. When a tadpole hit the mesh end and could no longer lift itself off the mesh for 10 consecutive seconds, the trial was terminated immediately. Each tadpole entered the trial only once, and was returned to the tub after the trial.

Muscle analyses



One pond (Shilin) and one stream (Tucheng) populations were sacrificed for muscle analyses (Table 2), upon the completion of sustained swimming trials. The tail of each tadpole was cut at base on ice, froze immediately in liquid nitrogen, and stored in a -80°C freezer. I obtained four consecutive 18- μ m cross-sections from the cut end of each tail on a cryostat (Leica CM1900) at -22°C temperature. Samples were affixed on slides without adhesive, dried at room temperature, stained with NBT (Nitro blue tetrazolium) in dark for 40 minutes, washed with 0.2M PBS three times, fixed in 4% PFA for 15 minutes, rinsed three times in distilled water, dehydrated in an alcohol series, counterstained in eosin, cleared with xylene, and finally mounted in Permount.

I used the succinate dehydrogenase (SDH) assay to identify muscle fiber types in the tails (Nachlas, *et al.*, 1957). The assay uses NBT (Nitro blue tetrazolium) to give mitochondria a purple color. The SDH enzyme located in the mitochondrion oxidizes succinate to fumarate in the citric acid cycle, thus can be used to distinguish between oxidative and “less” oxidative fibers. Red muscles have high mitochondrial content, thus stronger SDH reactions than “less” oxidative fibers, such as white and pink muscles. Because there is no sharp boundary between red and other muscle fiber types, I used an arbitrary criterion. If >50% cell circumference of a muscle cell was surrounded



by mitochondria, it would be classified as red muscle. Otherwise, it would be considered as non-red muscle cell. I chose the best stained one from the 4 cross-sections to quantify muscle fibers. Only one side (randomly chosen) of the cross-section was analyzed. I counted the numbers of all muscle cells and red muscle cells along three transect lines at the 1/4, 2/4 and 3/4 length of the muscle mass (Fig. 5). Values from the three transects were averaged.

Life history traits


For each tadpole that was not sacrificed for muscle analysis, I recorded the body weight at metamorphosis (G42) and the number of days required to reach metamorphosis.

Statistical analyses

To analyze the sustained swimming data set, I only included tadpoles at G35 to eliminate the influence of hindleg development on swimming performance, and also prevent the effects of allometry among different developmental stages.

Large individuals tend to have large values of morphological traits (Appendix 1).

To account for the effect of body size, I generated size-independent estimates of



morphological traits. I performed a principal component analysis that included 6 morphological variables (body length, body width, body height, tail length, tail height and tail muscle width) to produce the first principal component (PC1) to represent the overall body size. I regressed each morphological variable against PC1, and used the residuals as size-independent estimates of morphological traits. I used size-independent morphological traits in the subsequent analyses except that I did not adjust sustained swimming performance by body size because the former was independent of the latter ($r = -0.065$, $P = 0.401$).

Morphology, behavior and life history traits

The effects of flow regime (static vs. flowing water) and habitat type (pond vs. stream) on morphological, behavior and life history traits were analyzed by two-way ANOVAs with nested blocks. Based on the sampling scheme, habitat type and flow regime are the two fix factors. The 3 populations collected from each habitat were treated as random samples that nested within the habitat factor. Since tadpoles from each population were randomly assigned into two flow regime groups, the two groups were placed in a block. Tadpoles reared in the same tub were treated as random subsamples. The data set met the assumptions of parametric statistical analyses. When

an ANOVA showed significant results, I followed up with a *post hoc* analysis using Tukey's pair-wise comparisons. I performed all statistical analyses using JMP version 9.0.2 (SAS Institute Inc., Cary, North Carolina, USA).



Muscle layers

The effects of flow regime and habitat type on the number of muscle layers were analyzed by a generalized linear model. Since only one population from pond and stream habitat each was sacrificed for muscle analyses, tadpoles from the same habitat were treated as random samples. Flow regime was treated as a fix factor.

The relationship between body shape and swimming performance

To examine the relationship between body shape and swimming performance, I generated independent body shape parameters using PCA. I performed a PCA on 6 size-independent morphological variables (body length, body width, body height, tail length, tail height and tail muscle width), and produced 5 independent principle components. I used regression analyses to test whether swimming performance was associated with the principle components.

Results



Morphology and swimming performance

Habitat type had no significant effect on any size-independent morphological traits and weights at G35 (2-way ANOVA with nested blocks, habitat type effect, $P > 0.05$ in all cases; Table 3). In contrast, flow regime had significant effects on all morphological traits except tail height (Table 3). Tadpoles reared in flowing water were significantly lighter than those reared in static water (Tukey's *post hoc* pair-wise comparisons; Table 4; Fig. 6). Both body width and body height were significantly smaller in flowing than static water (Fig. 7b and c); whereas, body length and tail muscle width were significantly bigger in flowing than static water (Fig. 7a and d). Habitat type and flow regime interacted to affect tail length: tail lengths of stream tadpoles became longer in flowing water, while those of pond tadpoles had no response to flow regime (Fig. 7e). Tail height was not influenced by habitat type, flow regime, nor their interaction (Fig. 7f).

There was considerable variation among tadpoles in sustained swimming abilities. Tadpoles failed to swim or swam for less than 15 seconds were excluded from analysis. Habitat type had no significant effect on the performance of sustained swimming (2-way ANOVA with nested blocks, habitat type effect, $F_{1,6} = 0.001$, $P = 0.97$), but

there was a significant effect of flow condition on swimming performance ($F_{1,6} = 34.427, P < 0.001$): tadpoles reared in flowing water performed much better than those reared in static water (Fig. 8).



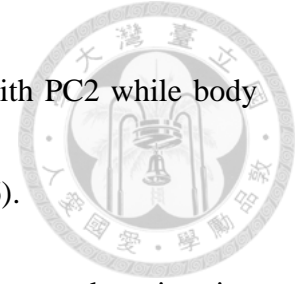
Muscle layers

Total muscle layers were significantly different between habitat types (Table 5). Stream tadpoles had more total muscle layers than pond tadpoles, but total muscle layers did not change with flow regime (Fig. 9a). Habitat type and flow regime interacted to affect red muscle layers. Although stream tadpoles generally had more red muscle layers than pond tadpoles, pond tadpoles reared in flowing water had more red muscle layers than those reared in static water (Fig. 9b).

The relationship between body shape and swimming performance

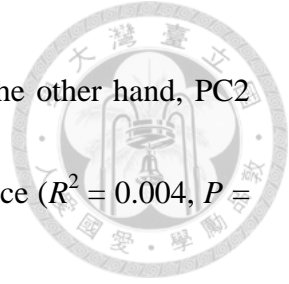
The PCA on 6 size-independent morphological variables produced 5 independent principle components. I only examine the effects of the first two components (PC1 and PC2) that had eigenvalues larger than one. Together, they accounted for 66% of variance in tadpole body shape. Body width and body height were negatively correlated with PC1 while body length, tail muscle width and tail length were positively correlated

with PC1. Tail length and tail height were negatively correlated with PC2 while body length and body height were positively correlated with PC2 (Table 6).



To find out the relationship between overall body shape and swimming performance, I used a quadratic regression analysis according to the fluid mechanics formula: $F_D = \frac{1}{2} \rho v^2 C_D A$. The formula states that the drag (F_D) encountered by a swimming tadpole is a function of four variables: liquid density (ρ), liquid velocity (v), drag coefficient (C_D), and cross-sectional area of the tadpole facing the current (A). In the swimming trials, liquid (water) density and liquid velocity are fixed values, thus the drag encountered by a swimming tadpole is positively correlated with drag coefficient (C_D) and cross-sectional area (A). However, drag coefficient (C_D) is itself a function of cross-sectional area (A), and the two variables change almost linearly when body size is controlled, as in my study (based on the formula “ $C_D = 0.013 \left[1 + \left(\frac{d}{l} \right)^{\frac{3}{2}} \right] + 0.15 \left(\frac{d}{l} \right)^2$ ”, where l could be consider as the value of body length and d as body width or body height; Hoerner, 1965). Thus, the drag of fluid encountered by a swimming tadpole (F_D) would be a quadratic function of cross-sectional area of body. Indeed, I found a quadratic regression yielded a significantly positive relationship between PC1 and the performance of sustained swimming ($R^2 = 0.371$, $P < 0.001$). Individuals with relatively wide and deep body (i.e., large cross-sectional area), thin tail muscle and short tail

performed relatively poorly in sustained swimming (Fig. 10). On the other hand, PC2 was not significantly correlated with sustained swimming performance ($R^2 = 0.004$, $P = 0.444$).



Life history traits


Habitat types had no significant effects on larvae periods and weights at G42 (2-way ANOVA with nested blocks, habitat type effect, $P > 0.05$ in both cases; Table 7). Both life history traits were significantly affected by flow regime. Tadpoles reared in flowing water took significantly longer to reach metamorphosis (G42) (larvae period: static vs. flowing = 34 vs. 37 days), and metamorphosed with lighter weights (weight at G42: static vs. flowing = 0.332g vs. 0.257g) compared to those reared in static water (Fig. 11).

Discussion




Although many larval amphibians inhabit aquatic habitats with variable flow regimes, studies investigating the phenotypic plasticity in response to different flow regimes are limited (but see Richards, 2002; Venesky & Parris, 2009; Kupferberg *et al.*, 2011). This study clearly demonstrated that *Bufo bankorensis* tadpoles respond to hydrologic regimes with phenotypic plasticity. Particularly, tadpoles reared in flowing water developed a body shape that had relatively narrow body width, shallow body height, long tail, and thick tail muscle. It seems such a shape is an adaptive response because it provides thrust and reduces drag in the flowing water.

First of all, the thick tail muscles contained not only high numbers of muscle layer, but also high numbers of red muscle layers that have abundant mitochondria which can promote aerobic capacity and power slow twitch during sustained swimming (Sasaki, 1974; Watanabe *et al.*, 1980; Meyer-Rochow & Ingram, 1993). In addition, the long tails can supply continuous propulsions as shown in *Pseudacris Triseriata* tadpoles (Wassersug & Sperry, 1977). Together, the three physiological and morphological features give *Bufo bankorensis* tadpoles the sustained thrust needed in the flowing water. Furthermore, the shape also reduces the drag of flowing water. According to the fundamental fluid mechanics model (as described in an early section), the drag of fluid



would be a quadratic function of the cross-sectional area of the swimming tadpole. This study found exactly that. There was a strong positive relationship between cross-sectional area and the performance of sustained swimming. Individuals with relatively narrow and shallow bodies, thus small cross-sectional areas, performed well in sustained swimming (Fig. 10). Hertel (1966) demonstrated that the ratio of body width to total length in most of streamlined swimmers and fast swimming aquatic creatures ranges from 0.1 to about 0.24. I measured the ratio in tadpoles, and found tadpoles reared in flowing water had a ratio of 0.24, which is closer to the ideal range than those reared in static water, which had a ratio of 0.26. Furthermore, long and thin body size is considered a shape beneficial for finding refuges such as interstices of rocks from running water (Richards, 2002; Altig & Johnston, 1989). Overall, a tadpole with a relatively narrow body width, shallow body height, long tail, and thick tail muscle has a shape similar to the streamlined body shapes of many aquatic vertebrates living in flowing water (Orton, 1953; Altig & Johnston, 1989; Fish, 1998; Pakkasmaa & Piironen, 2000; Langerhans, 2008). My study shows that *Bufo bankorensis* can potentially obtain the shape adaptively with phenotypic plasticity.

Peculiarly, I did not find tail height respond to flow regime and tail height was not significantly correlated with the body shape (PC1, Table 6) that supported sustained



swimming performance (Fig. 10). Past studies (McCollum & Leimberger, 1997; Dayton *et al.*, 2005; Johansson *et al.*, 2010) often found a positive relationship between tail height and burst swimming performance. Although the function of tail height on burst swimming was distinct among different species (Buskirk & McCollum, 2000; Buskirk *et al.*, 2003), tail height and burst swimming ability are often associated with escaping predators. Since I did not examine burst swimming performance, it's not clear how phenotypic plasticity in response to flow regime affect burst swimming performance.


Any plastic response to environmental demands has tradeoffs (Miner *et al.*, 2005). In this study, food was provided *ad libitum*, yet tadpoles reared in flowing water had a much lower growth rate which might be caused by the high metabolic demands of continuous swimming. These tadpoles developed slower and weighted much lighter at metamorphosis (G42) than tadpoles reared in static water. Both long larval period and small size at metamorphosis reduce fitness. It has been shown that prolonged larvae periods increase the risk of predation or habitat desiccation (Newman, 1992); whereas small individuals usually have poorer performance in locomotion, higher risk of predation, lower post-metamorphic growth rate, and longer time to reproductive maturity (Goater *et al.*, 1993; Kingsolver & Huey, 2008). Therefore, tadpoles reared in flowing water developed streamlined body shapes might gain benefits in energy

conservation and foraging efficiency in larvae stage, but they need to pay the costs of having extended larval period and small size at metamorphosis. However, the net consequence of phenotypic plasticity on fitness remains unclear.




It is notable that most *Bufo bankorensis* breed in stream habitat in the field; while tadpoles reared in flowing water indoor in my study have lower fitness (longer larvae periods and lighter in mass at G42). Such an inconsistency might be caused by the artifact in my study that tadpoles were forced to remain in a constant 5-9 cm/s water velocity rather than a variable 0-16 cm/s flow environment as seen in the fields. The enforced setting might enhance the consumption of energy since there would be no place to rest so that tadpoles would need more time to reach metamorphosis. However, besides flow variable, there are many distinct environmental factors between nature ponds and streams which are also concerned with alternation of performance of tadpoles, such as oxygen dissociation (Burggren & Mwalukoma, 1983), temperature (Harkey & Semlitsch, 1988), water level (Denver *et al.*, 1998), food source (Kupferberg, 1997; Alvarez & Nieceza, 2002), accumulation of nitrogenous wastes (Schmuck *et al.*, 1994) and taxa of predators (Benard, 2006; Touchon & Warkentin, 2008), etc. Therefore, it is hard to draw conclusions on which habitat type is better for *Bufo bankorensis* tadpoles.

Finally, but not the least, I found indications of differentiation between pond and



stream populations in this study. There were differential responses to flow regime by tadpoles came from pond and stream tadpoles in two traits: tail length and tail muscle layers. (The further analyses of oral disc also showed the differential responses between habitat types; Appendix 2 – 4) The tail length of stream, but not pond tadpoles became longer in flowing water. Tadpoles from stream habitats had more total muscle layers and red muscle layers than tadpoles from pond habitats. A potential explanation to such results is that differentiation may have occurred for populations inhabiting different habitats which impose different selection pressures (Slatkin, 1987; McRae *et al.*, 2005). Alternatively, the divergence of response in tail length and muscle layers between pond and stream populations may be due to historical effect. I collected tadpoles at G27 when they just came out of egg clutches (usually at G24) in the field. It is possible that habitat-specific development in these tadpoles have been induced in the fields before they were collected even though there was no significant difference among populations in morphological traits at G27. An experiment starting from eggs collected in the laboratory is needed to provide unequivocal evidence of differentiation between pond and stream populations.

In conclusion, my study demonstrated that tadpoles of *Bufo bankorensis* could show substantial phenotypic plasticity in morphological, physiological, behavior and

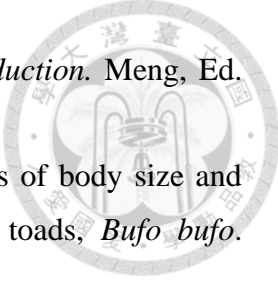


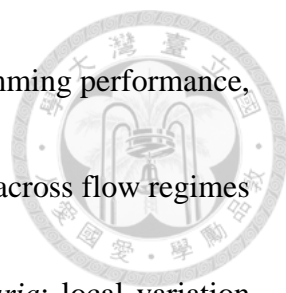
life history traits under different flow regimes. Tadpoles reared in flowing water had smaller body sizes to reduce water drag, longer tails and thicker tail muscles with more layers of red muscles to provide propelling force. Together, they maintain functional requirements necessary for sustained swimming in running water. Such phenotypic plasticity to flow regime might enable *Bufo bankorensis* to become a widely spread species, breeding in both pond and stream habitats in Taiwan. Finally, there is a possibility of differentiation between pond and stream populations. Further research is required to provide unequivocal evidence for differentiation between pond and stream populations.

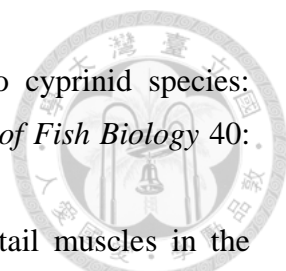


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Tables

Table 1. Collecting locations of the 6 populations in Taipei city.

	Ponds			Streams		
District	Shimen (石門區)	Da'an (大安區)	Shilin (士林區)	Xindian (新店區)	Shiding (石碇區)	Tucheng (土城區)
Altitude	185m	20m	382m	97m	131m	105m
Latitude and longitude	25.263, 121.582	25.017, 121.552	25.134, 121.544	24.949, 121.577	24.990, 121.659	24.959, 121.457





Table 2. Sample size (number of tadpoles) for each group.

Analyses	Groups	Ponds			Streams		
		Shimen (石門區)	Da'an (大安區)	Shilin (士林區)	Xindian (新店區)	Shiding (石碇區)	Tucheng (土城區)
Body shape	Static	24	23	20	25	24	21
	Flowing	24	18	21	20	27	16
Swimming	Static	13	11	11	14	15	11
	Flowing	12	13	14	12	17	12
Muscles	Static	—	—	15	—	—	17
	Flowing	—	—	20	—	—	15
Life history	Static	36	38	—	30	36	—
	Flowing	38	35	—	32	35	—

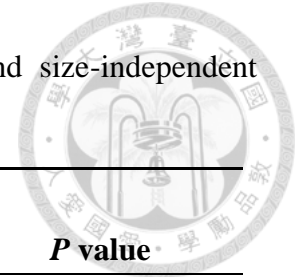
Note: Numbers in analyses of body shape and life history traits were considered as subsamples.

Table 3. Effects of habitat type (pond vs. stream) and flow regime (static vs. flowing water) on size-independent morphological traits at G35.

	Body length	Body width	Body height	Tail Muscle width	Tail length	Tail height
Habitat Type	3.578	0.008	1.344	0.715	0.445	0.269
Flow regime	6.56**	109.968***	83.789***	287.07***	36.96***	0.388
Interaction	1.012	0.048	2.728	0.633	20.143***	1.464

Note: Values are *F* ratios from univariate ANOVAs. Traits were adjusted for overall body size before analyses. Asterisks indicate levels of statistical significance between treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Table 4. Results of Tukey's *post hoc* pairwise comparisons for examining the effects of flow regime on weight and size-independent morphological traits at G35. The groups with significantly bigger values are highlighted in bold.



Trait	Mean \pm 1SE		<i>F</i> ratio	<i>df</i>	<i>P</i> value
	Static water	Flowing water			
Weight	0.301 \pm 0.003	0.252 \pm 0.003	170.349	1, 10	<0.001
Body length	-0.078 \pm 0.025	0.074 \pm 0.027	8.56	1, 10	0.007
Body width	0.127 \pm 0.018	- 0.147 \pm 0.019	109.968	1, 10	<0.001
Body height	0.099 \pm 0.016	- 0.115 \pm 0.017	83.789	1, 10	<0.001
Tail muscle width	- 0.079 \pm 0.007	0.088 \pm 0.007	287.07	1, 10	<0.001
Tail length (interaction)	- 0.252 \pm 0.059	0.291 \pm 0.063	36.96	1, 10	<0.001
Tail height	-0.013 \pm 0.021	0.015 \pm 0.023	0.388	1, 10	0.534

Table 5. Effects of habitat type (pond vs. stream) and flow regime (static vs. flowing water) on average number of muscle layers at G35.

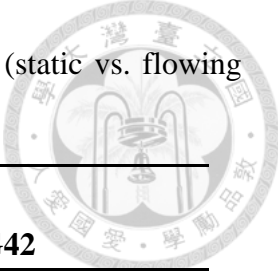
	Total muscle layers	Red muscle layers
Habitat Type	52.013***	47.248***
Flow regime	0.048	14.912***
Interaction	0.07	13.384***

Note: Values are *F* ratios from GLM. Only tadpoles from Shilin (pond) and Tucheng (stream) were sacrificed for muscle analysis. Asterisks indicate levels of statistical significance between treatments (**P* < 0.05, ** *P* < 0.01, *** *P* < 0.001).

Table 6. Summary of the results of 6 size-independent morphological traits from PCA based on correlation matrix; PC1 and PC2 refer to loadings of first and second eigenvectors. The predominant explanatory variables are highlighted in bold.

	PC1	PC2
Eigenvalue	2.491	1.488
% of variance	41.523	24.794
Eigenvectors		
Body length	0.201	0.651
Body width	- 0.521	0.024
Body height	- 0.481	0.192
Tail muscle width	0.446	- 0.125
Tail length	0.507	0.029
Tail height	- 0.021	- 0.722

Table 7. Effects of habitat type (pond vs. stream) and flow regime (static vs. flowing water) on larvae periods and weight at G42.



	Larvae periods (G27~G42)	Weights at G42
Habitat Type	0.0002	0.194
Flow regime	23.478***	194.217***
Interaction	1.086	3.87

Note: Values are *F* ratios from univariate ANOVAs. Excluding those sacrificing for muscle analyses, other groups of tadpoles were all continuously reared until metamorphosed at G42 for recording life history traits. Asterisks indicate levels of statistical significance between treatments (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figures

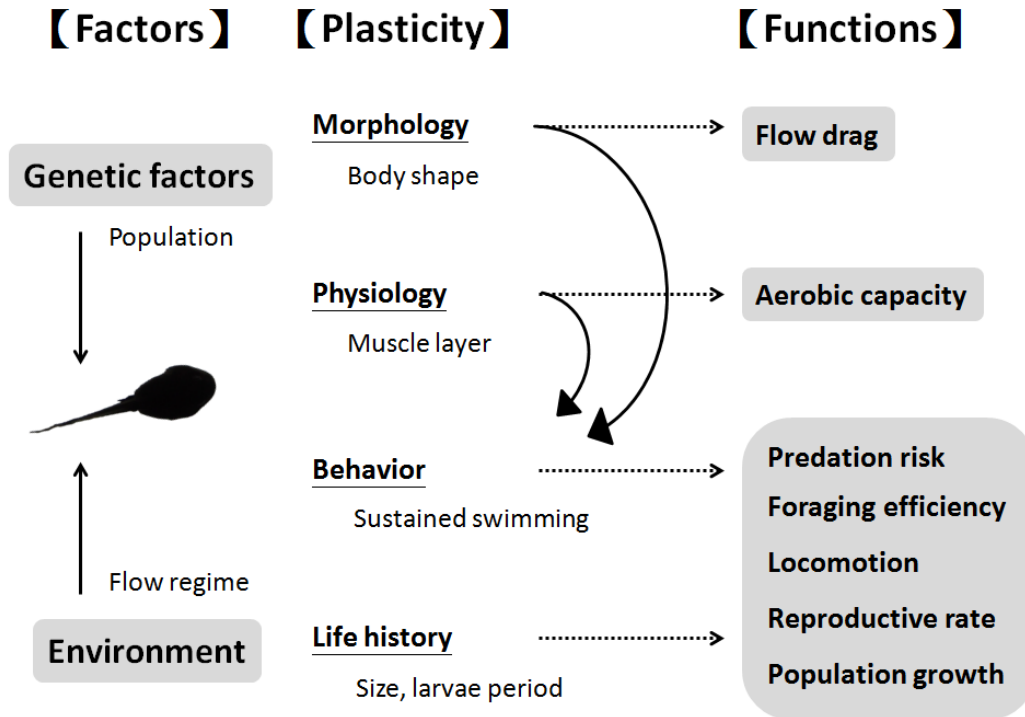


Figure 1. The concept map of this thesis.

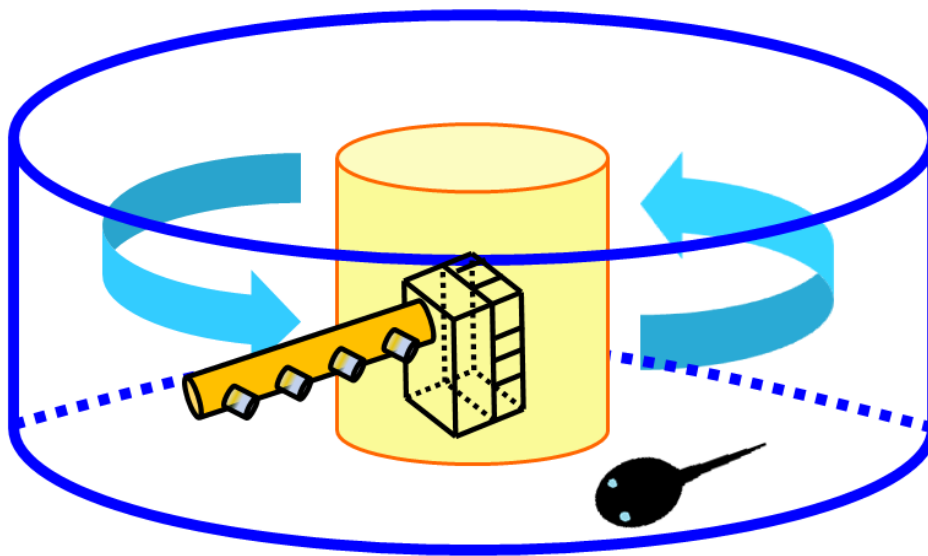


Figure 2. The flowing water tub. The 55-cm-diameter plastic tub has a pebble substrate and 10-cm-depth tap water. A pump was housed inside a 10-cm-diameter plastic flowerpot at the center with a 4-pore PVC pipe extruding from the pot crossing the diameter of the tub. Water pumped through the pores generated a unidirectional water flow with a 5-9 cm/s velocity. The static water treatment used the same apparatus except without the water pump.

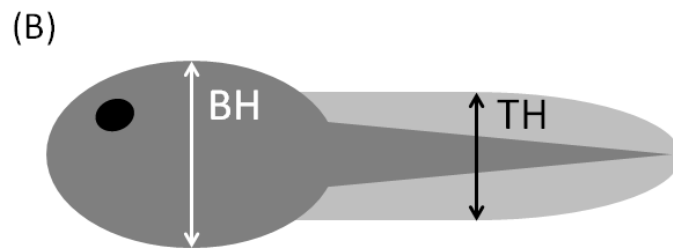
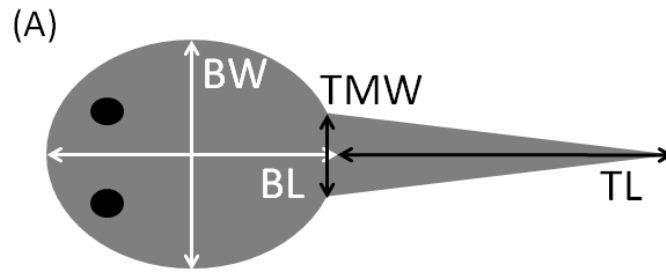


Figure 3. (A) Top and (B) lateral views of a tadpole showing the 6 morphological traits that were measured. BL = body length, BW = body width, BH = body height, TL = tail length, TH = tail height, TMW = tail muscle width.

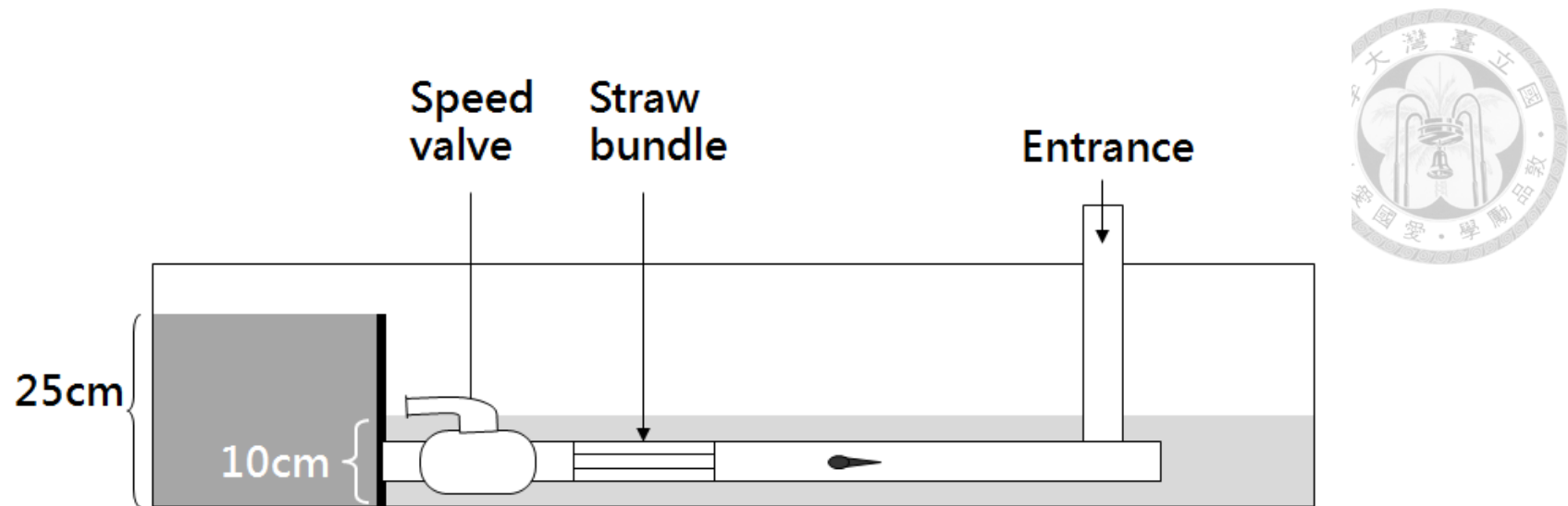


Figure 4. The sustained swimming apparatus. The glass tank ($L \times W \times H = 120 \times 25 \times 30$ cm) has a reservoir (dark area) and a sink (gray area) region to create differential water pressure. A submerged water pump maintains the difference in water levels between reservoir and sink to create a constant-pressure water flow entering the swimming pipe. The straight plastic pipe (inner diameter = 2 cm; length = 60 cm) has a mesh at the far end and a vertical branch (chimney) located at 5 cm from the mesh end. The water speed channeling from the reservoir into the pipe is controlled by a valve, and eddies are rectified by a straw bundle. The water flow velocity was maintained at 18 cm/s during all trials. The chimney served as the entrance for tadpoles entering the swimming trials.

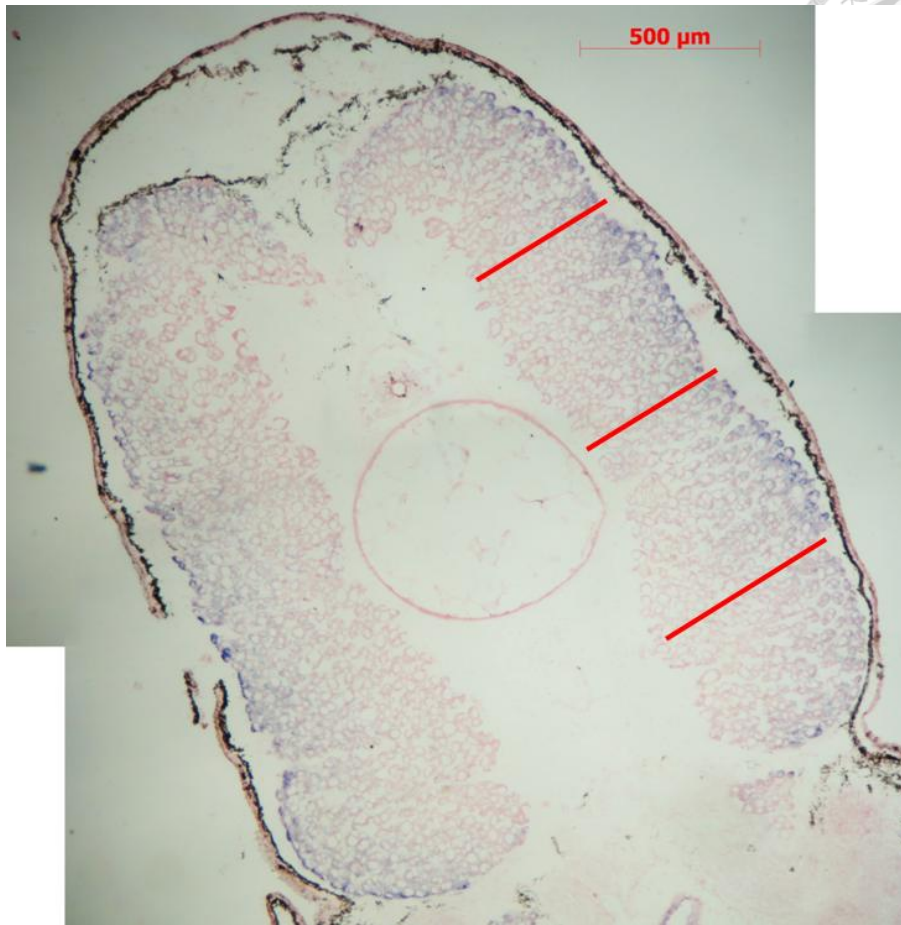


Figure 5. A cross-section of tail cut at tail base and stained with NBT (Nitro blue tetrazolium), which gives mitochondria purple color. Muscle cells with $>50\%$ cell circumference surrounded by mitochondria were classified as red muscles. Only one side (randomly chosen) of the cross-section was analyzed. I counted the numbers of all muscle cells and red muscle cells along three transect lines (red lines) at the $1/4$, $2/4$ and $3/4$ length of the muscle mass. Values from the three transects were averaged.

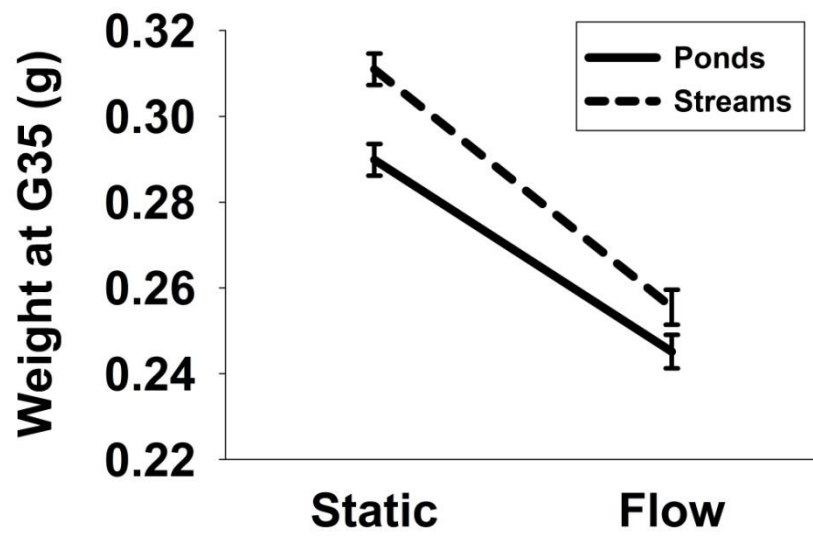


Figure 6. Weight at G35 under experimental treatments of habitat type (pond vs. stream) and flow regime (static vs. flowing water). The solid line represents pond tadpoles, and bars at the ends of lines depict mean \pm 1se.

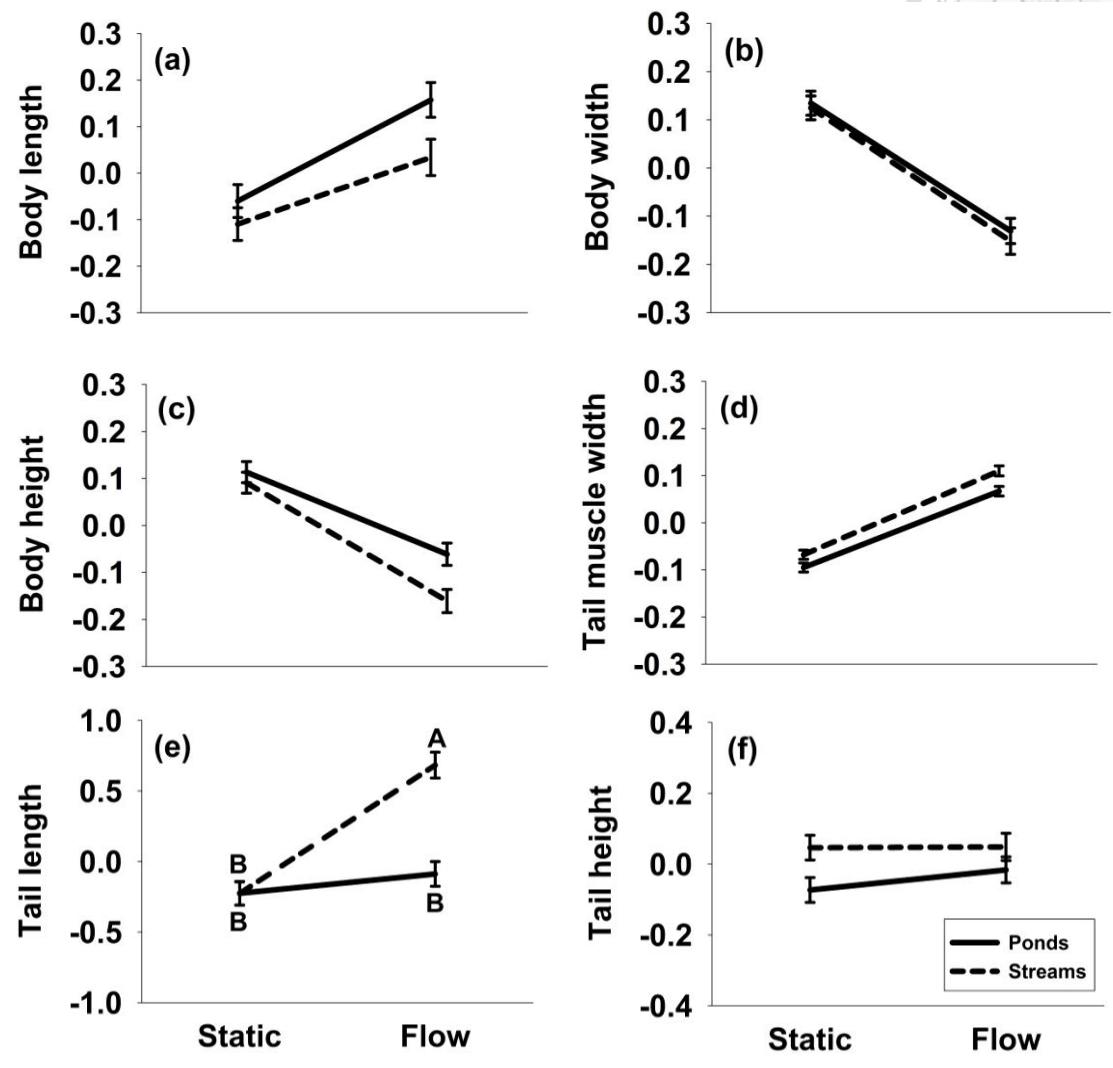


Figure 7. Size-independent body length (a), body width (b), body height (c), tail muscle width (d), tail length (e) and tail height (f) at G35 under experimental treatments of habitat type (pond vs. stream) and flow regime (static vs. flowing water). The solid lines represent pond tadpoles, and bars at the ends of lines depict mean \pm 1 se.

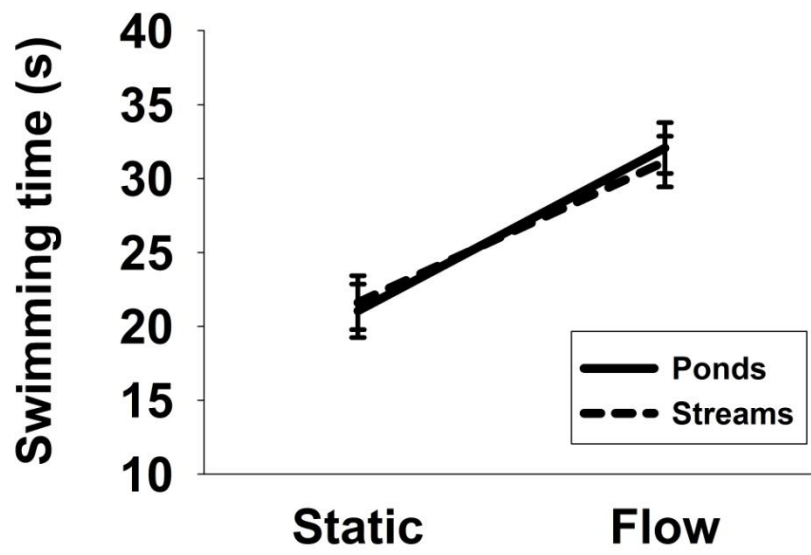


Figure 8. Sustained swimming performance at G35 under experimental treatments of habitat type (pond vs. stream) and flow regime (static vs. flowing water). The solid line represents pond tadpoles, and bars at the ends of lines depict mean \pm 1se.

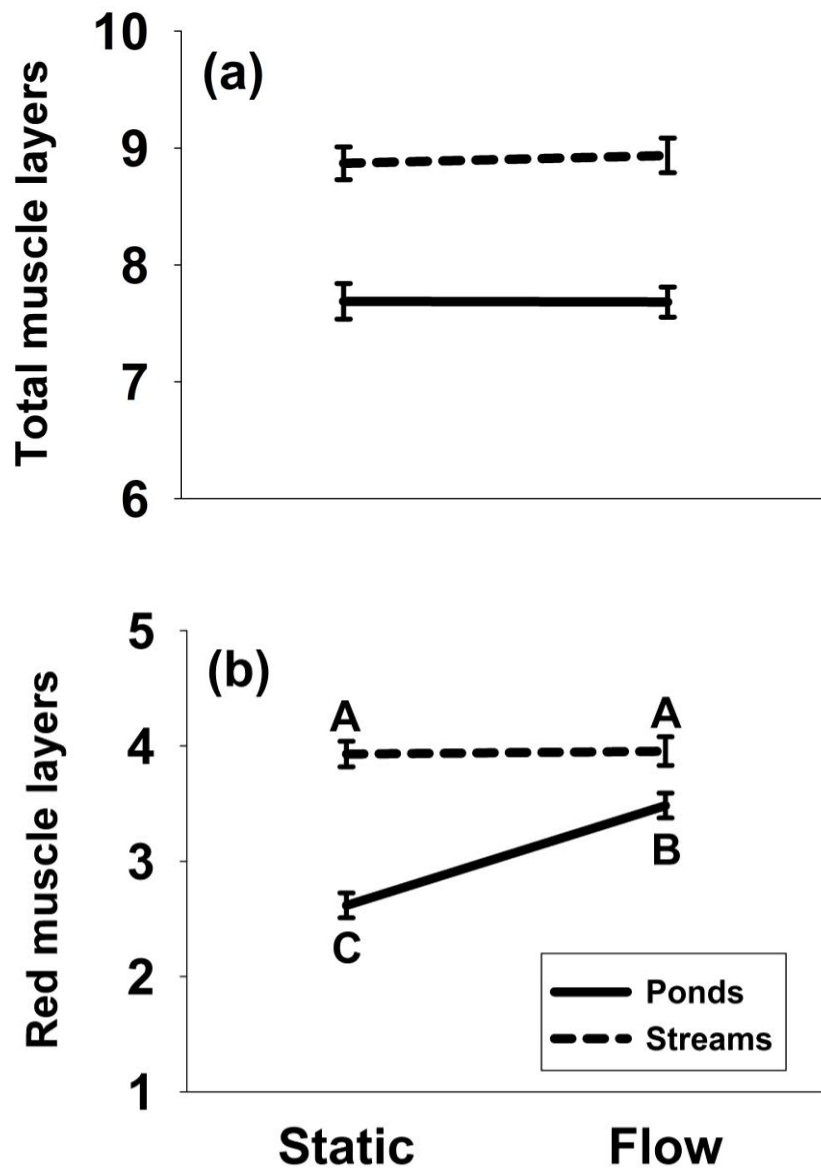


Figure 9. Average of total muscle layers (a) and red muscle layers (b) at G35 under experimental treatments of habitat type (pond vs. stream) and flow regime (static vs. flowing water). The solid lines represent pond tadpoles, and bars at the ends of lines depict mean \pm 1se.

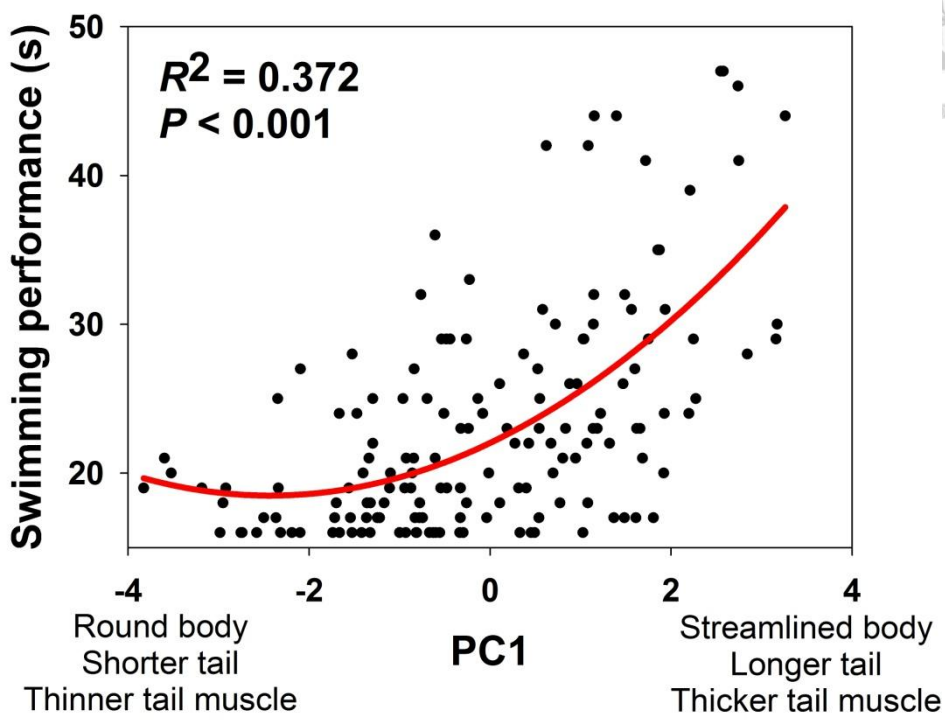


Figure 10. Relationship between morphology (PC1) and sustained swimming performance taken from the quadratic regression analysis. Tadpoles having streamlined body shape, thick tail muscle and long tail would perform better in terms of sustained swimming.

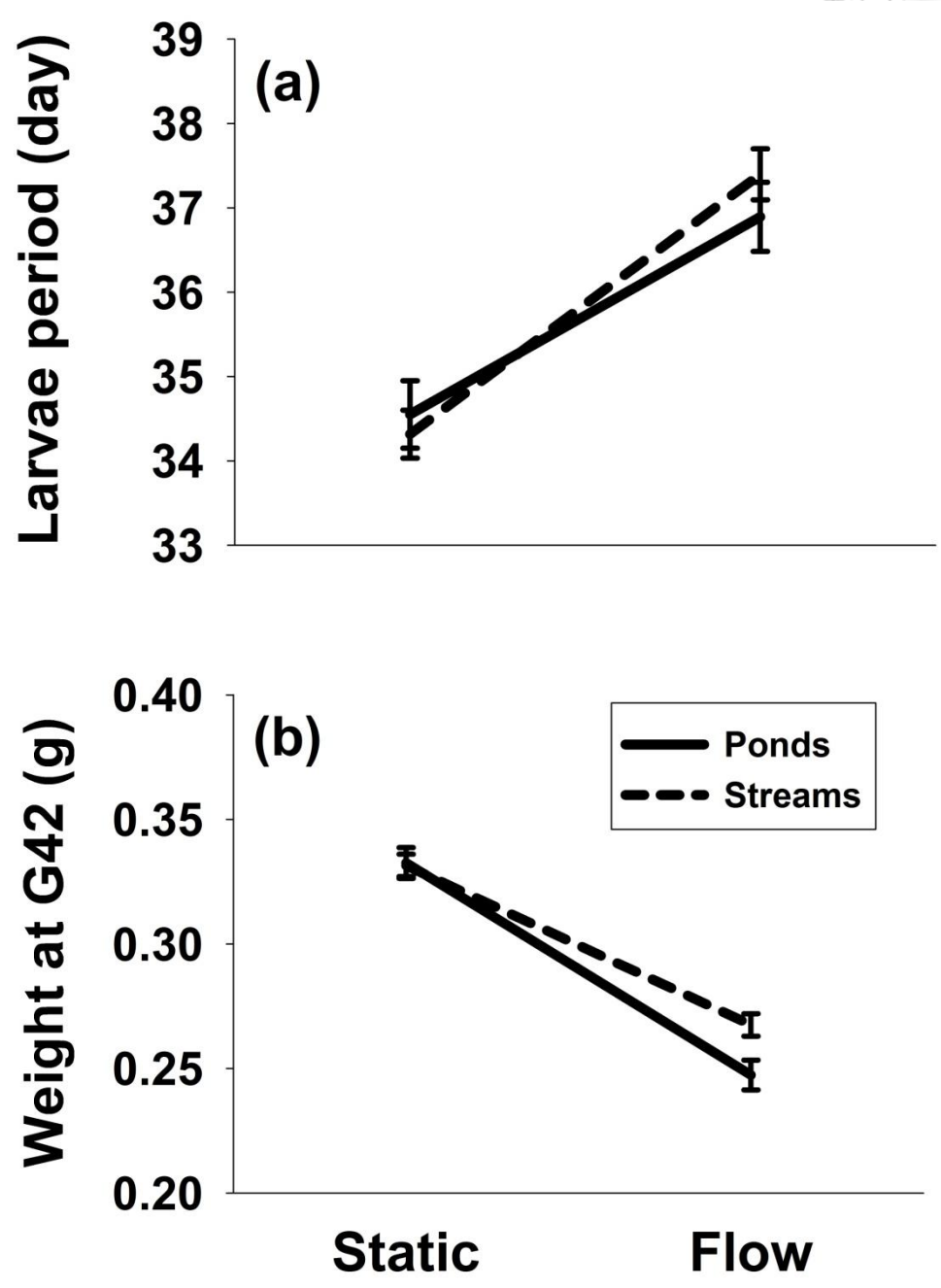


Figure 11. Life History traits of larvae periods (a) and weight at G42 (b) under experimental treatments of habitat type (pond vs. stream) and flow regime (static vs. flowing water). The solid line represents pond tadpoles, and bars at the ends of lines depict mean \pm 1se.

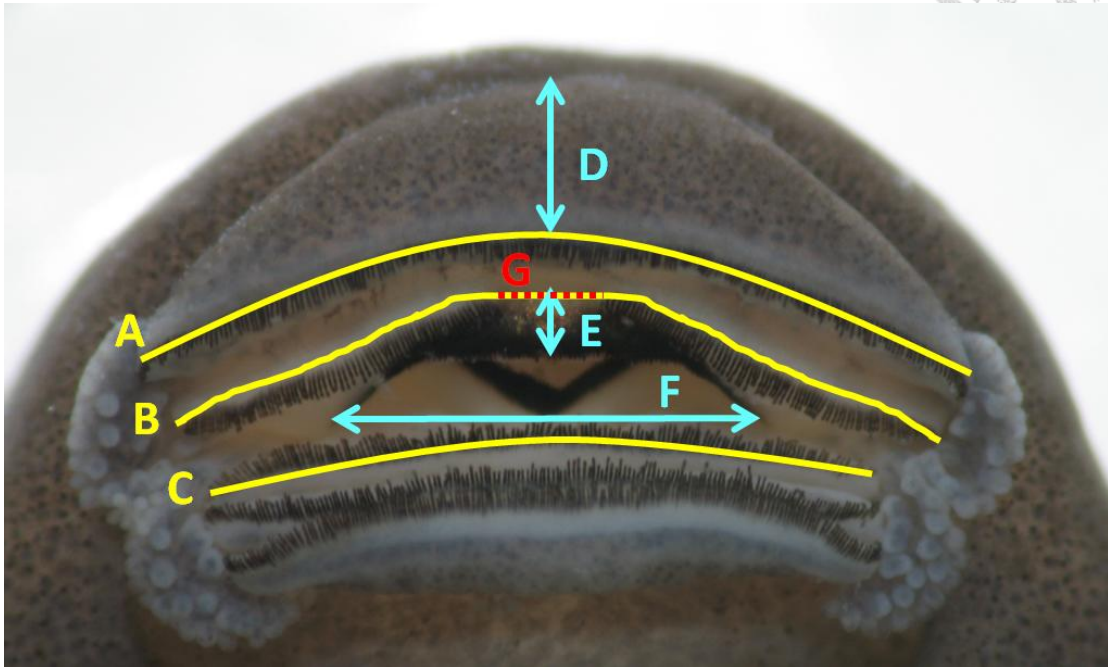
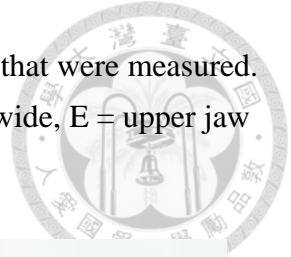
Appendix



Appendix 1. Raw data of 6 morphological traits before being adjusted by overall body size. Units: millimeter (mm.).

	Ponds		Streams	
	Static	Flowing	Static	Flowing
Body length	12.241	11.909	12.371	11.924
Body width	8.019	7.296	8.286	7.509
Body height	5.177	4.682	5.349	4.746
Tail muscle width	1.641	1.767	1.698	1.828
Tail length	18.672	18.241	19.013	19.302
Tail height	5.703	5.381	6.046	5.613

Appendix 2. Oral disc of a tadpole showing the morphological traits that were measured. A, B = 1st, 2nd upper tooth row, C = 1st lower tooth row, D = labium wide, E = upper jaw wide, F = upper jaw length, G = medial gap in 2nd upper tooth row.



Appendix 3. Effects of habitat type (pond vs. stream) and flow regime (static vs. flowing water) on oral disc morphological traits at G35.

	1st upper tooth row	2nd upper tooth row	1st lower tooth row	Labium wide	Upper jaw wide	Upper jaw length	Gap %	Tooth density	Tooth number
Habitat Type	14.694***	8.854**	11.337***	<0.001	10.099**	5.184*	66.745***	0.181	14.733***
Flow regime	2.654	1.9	2.308	1.755	2.361	1.268	0.31	20.36***	0.339
Interaction	8.345**	2.367	4.093*	0.295	1.748	1.301	0.99	13.2***	3.357

Note: All measurements were adjusted by body width except tooth density and tooth number. I measured the length of continuous 20 teeth on first upper tooth row to calculate the tooth density. Tooth number was calculated from the value of tooth density multiplied by the length of 1st upper tooth row. Gap % = Gap length/ 2nd upper tooth row. Values are *F* ratios from GLM. Only tadpoles from Shilin (pond) and Tucheng (stream) were sacrificed for oral disc analysis. Asterisks indicate levels of statistical significance between treatments (**P* < 0.05, ** *P* < 0.01, *** *P* < 0.001).

Appendix 4. Oral disc of 1st upper tooth row (a), 2nd upper tooth row (b), 1st lower tooth row (c), upper jaw wide (d), upper jaw length (e), Gap % (f), tooth density (g) and tooth number (h) at G35 under experimental treatments of habitat type (pond vs. stream) and flow regime (static vs. flowing water). The solid lines represent pond tadpoles, and bars at the ends of lines depict mean \pm 1se.

