Predator-Induced Tail Coloration Toward Diverse Dragonfly Nymphs in Tadpoles of the Japanese Tree Frog (*Dryophytes japonicus)*

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Abstract

Anuran larvae can change their coloration and morphology to avoid predation. Particularly, some tadpoles in the genus *Dryophytes* in the Americas develop deep tail fins and bright orange tail coloration in response to predators such as dragonfly nymphs. These conspicuous tails are hypothesized to attract predator attacks, protecting vital body parts from fatal injuries. However, it remains unclear whether the extent of the induced phenotypic changes differs between various predators with distinct feeding patterns and habitats. To explore this, we reared tadpoles of the Japanese tree frog, *Dryophytes japonicus*, with four dragonfly species, water bug, water scorpion, or newt, and measured phenotypic changes, especially focusing on tail coloration. We found that the aeshnid *Anax nigrofasciatus*, a sit-and-wait predator that climbs on aquatic vegetation, induced bright orange and broad tails of tadpoles. Other odonate species that forage in and on the substrate also elicited tail coloration or deeper larval tails, but it took more time to induce the tadpoles' response compared to *A. nigrofasciatus*. In contrast, non-odonate predators did not induce color and morphological changes. This study provides the first evidence of predator-induced tail coloration in an Asian species of *Dryophytes*, which appears to be a defensive response specifically to dragonfly nymphs, as visually adept predators. Moreover, the differences in the extent and timing of the phenotypic changes among odonate species suggest that anti-predator phenotypes are modulated based on relative predation risks, considering the predators' foraging patterns and visual capabilities, thereby allowing tadpoles to minimize physiological costs and unintended risks associated with the induced traits.

Introduction

The body color of organisms has diverse implications for predator–prey interactions. Many species exhibit cryptic body color, which suggests significant selective pressure from predators (Norris and Lowe, 1964). Cryptic coloration contributes to predation avoidance by representing background matching, countershading, and the disruption of body shape or outlines (Thibaudeau and Altig, 2012). In contrast, some organisms use bright colors and expendable body parts to divert predator attention away from more vulnerable areas (Hawlena et al., 2006). Consequently, body color contributes to predation avoidance by fulfilling either of two primary functions: enhancing prey crypticity or creating a defensive lure phenotype (Touchon and Warkentin, 2008).

Changes in body color are often induced by predators through phenotypic plasticity. The utilization of a prey's body coloration is highly dependent on its context, including the sensory capabilities of predators and the background color (e.g., Espanha et al., 2016). For example, although the poison dart frog (*Dendrobates tinctorius*) is well recognized for its typical aposematic signaling, its conspicuous coloration also functions as camouflage when viewed from a distance, blending with the background (Barnett et al., 2018). Especially in aquatic habitats, substantial variation exists in background conditions and the composition and abundance of predators across different localities, making it difficult for prey to achieve a uniform predation avoidance ability across their range (Van Buskirk, 2002; McIntyre et al., 2004). Therefore, such environmental uncertainty has generally been believed to favor plastic rather than constitutive anti-predator defenses (Agrawal and Karban, 1999; McIntyre et al., 2004).

Anuran larvae, or tadpoles, have served for decades as a model system for investigating induced defense mechanisms, as well as predator avoidance strategies and trade-offs mediated by plastic traits (Kasumovic, 2013; Kruger and Morin, 2020). Phenotypes such as body color and shape are generally subject to predator selection for survival and mate choice, but the latter can be ruled out in the case of tadpoles. They often rely on chemical cues

to evaluate predation risk through continually released predator-borne cues and digestion-released, prey-borne cues, and then exhibit predator-induced defense mechanisms (Brönmark and Hansson, 2000; Hettyey et al., 2010, 2015).

Predator-induced plastic responses in tadpoles can be categorized into four primary domains: behavioral, morphological, physiological, and chemical defenses. Behavioral responses encompass a reduction in overall activity (e.g., Lawler, 1989; Takahara et al., 2012a), increased burst swimming speed (Takahara et al., 2008), and spatial avoidance of predators (e.g., Laurila et al., 1998; Nunes et al., 2013). Morphological changes for predator avoidance have been well documented in tadpoles of various species (Relyea, 2001; Van Buskirk, 2002; Richter-Boix et al. 2007). For instance, tadpoles undergo changes in body size (i.e., correlated with growth rate and metamorphosis timing) and shape, such as the development of deeper tail fins (Lardner, 1998, 2000). Behavioral and morphological responses are sometimes co-expressed (Hossie et al., 2017). Physiological responses include changes in respiration rate and gut evacuation rate (Steiner, 2007; Steiner and Van Buskirk, 2009; Costello and Michel, 2013). These may arise due to trade-offs in predator avoidance (Relyea and Auld, 2004) or as responses to partially offset the costs of lower energy intake resulting from evasion (Barry and Syal, 2013). Additionally, tadpoles of certain groups, particularly toads, evade predation through toxin production (Bókony et al., 2016; Hettyey et al., 2019).

The types and intensities of induced responses in tadpoles often differ according to predator types (Relyea, 2001; Teplitsky et al., 2004, 2005; Hettyey et al., 2011). For instance, Kishida and Nishimura (2005) revealed that tadpoles of *Rana pirica* developed deeper tail fins in response to dragonfly nymphs, whereas in the presence of salamander larvae, they exhibited not only deeper tails but also bulging heads and bodies. Tadpoles expressing these predator-specific traits should have a survival advantage over those with mismatched or non-induced phenotypes when faced with predation. Conversely, expressing such defense traits incurs energetic costs and can increase vulnerability to non-focal predators (Touchon and

Warkentin, 2008; Innes-Gold et al., 2019). Due to these costs, defense traits are expected to remain plastic rather than fixed, and their types and intensities can vary depending on predator species, sometimes exhibiting predator-specific responses.

Dragonfly nymphs, as one of the major predators of tadpoles, are known to induce morphological responses characterized by deep tail fins across many anuran species (Relyea and Werner, 2000; Van Buskirk, 2002). This induced broad tail has been repeatedly hypothesized to improve the swimming performance of tadpoles and facilitate their escape from predators (Smith and Van Buskirk, 1995; McCollum and Leimberger, 1997; Van Buskirk and McCollum, 2000a). However, experimental evidence of this benefit is lacking (Van Buskirk et al., 2003; Johnson et al., 2008). Moreover, a few tree frog species in the genus *Dryophytes* respond to odonate predators by not only exhibiting deeper tails but also turning their entire tails bright orange (McCollum and Van Buskirk, 1996; Van Buskirk and McCollum, 1999; LaFiandra and Babbitt, 2004). Thus, as another function, the induced deep and brightly colored fin is thought to serve as a lure, diverting predator attacks away from the more vulnerable head and body regions (Van Buskirk et al., 2003, 2004; Johnson et al., 2008). Given that tail fins tear easily (Doherty et al., 1998) and that tadpoles can maintain their swimming performance even after partial tail loss (Hoff and Wassersug, 2000; Van Buskirk and McCollim, 2000b), the tail is considered expendable relative to other body parts. Because dragonfly nymphs are predominantly visual predators, exhibiting the ability to discern both the shape and color of immobile as well as moving prey (Bybee et al., 2012; Espanha et al., 2016), color and shape changes in tadpoles are likely to be effective in avoiding predation by odonates.

Among dragonfly nymphs, several ecological types have been identified based on microhabitat usage and foraging behavior (Pritchard, 1965; Corbet, 1999), including climbers (e.g., Green darner, *Anax junius*), moving among aquatic vegetation and ambushing prey on plants; sprawlers (e.g., Globe skimmer, *Pantala flavescens*; Sherratt and Harvey, 1989), inhabiting pond bottoms and swimming through the water column to attack; and burrowers

(e.g., Golden-ringed dragonfly, *Cordulegaster boltonii*), burrowing in mud or bottom debris and often lying in wait for prey with only the eyes protruding. The reliance on vision for foraging varies among these types. For example, climbers that often have large compound eyes rely extensively on vision for prey detection, while other types with smaller eyes may rely more on tactile stimuli (Pritchard, 1966; Corbet, 1999). For tadpoles, the frequency of encounters with and relative predation risk of these predators differ among habitats and seasons. These differential predation risks likely determine the cost−benefit balance of induced traits, thereby suggesting that tadpoles may exhibit different defense responses. However, prior experimental studies have primarily focused on a single dragonfly species and have not examined how tadpole responses may vary among odonate (and other) species.

The Japanese tree frog, *Dryophytes japonicus*, is a prevalent species with a wide distribution encompassing entire regions of Japan (Honshu, Hokkaido, Shikoku, and Kyushu islands), as well as regions outside Japan including Sakhalin Island, Jeju Island, and the Korean Peninsula (Matsui and Maeda, 2018). Its breeding typically occurs from May to July (in Honshu), with breeding pairs depositing small clutches of eggs at multiple sites. Tadpoles of this species inhabit various environments such as rice paddies, marshes, ponds, puddles, and other ephemeral water bodies (Matsui and Maeda, 2018). They are classified as "nektonic" raspers, moving around the mid-water layer, characterized by distinctive body shapes with tall tail fins that originate relatively anterior to the body (Orton, 1953). Tadpoles of Japanese tree frog serve as important prey for numerous aquatic predators, including dragonfly nymphs, water scorpions, and red-bellied newts (Ohba and Nakatsuji, 2006; Hirai et al., 2020; Kita et al. 2020; Ozaki and Nishikawa, 2023). Morphological responses to predators have also been documented in this species, with dragonfly nymphs inducing deep tail fins, while chemical cues from goldfish prompt the development of shallow fins (Takahara et al., 2012b). The occurrence of orange tail coloration has also been briefly reported, although it has been attributed to ingested algae rather than predator-induced plasticity (Doi et al., 2005; Yuasa, 2006, 2007). Despite being rarely documented, tadpoles

exhibiting brightly colored tails were frequently observed in our study site in Kyoto, central Japan (Noda, personal observation).

To clarify the cause and function of the orange tail coloration in tadpoles of *D. japonicus*, we conducted laboratory experiments with the tadpoles and several predator species. These experiments aimed to achieve two primary objectives. First, we investigated whether the tail coloration of tadpoles is induced by dragonfly nymphs, and if so, whether the intensity and speed of this response vary among different odonate species with different ecological characteristics. Simultaneously, we examined changes in body shape that are known to be plastically induced by predators. The second objective was to assess whether tadpoles exhibit color/morphological responses to predators characterized by a lower reliance on vision for foraging compared to odonates. We used water bugs (*Appasus major*), water scorpions (*Laccotrephes japonensis*), and newts (*Cynops pyrrhogaster*) as non-odonate predators. For such predators, predation avoidance strategies involving tail coloration are expected to be less effective than those against dragonfly nymphs. Thus, tadpoles may exhibit weakened or no color/morphological responses to minimize the costs associated with developing plastic traits. In both experiments, tadpoles were reared with a single predator species, and their morphological traits of interest were compared with those of tadpoles raised in predator-free tanks. By analyzing the extent of tail coloration in tadpoles exposed to diverse predators, we revealed that tadpoles modulate their induced responses according to the risks posed by predators with different foraging types and sensory capabilities.

Materials and Methods

Two series of laboratory experiments were carried out: Experiment I involved the comparison of induction of color/morphological changes in tadpoles by four different dragonfly species as predators, and Experiment II employed three non-odonate predators.

Experiment I

Adults of *Dryophytes japonicus* were captured in the paddy fields at Kyoto University (35°01'N, 135°47'E) on June 9 and 10, 2022. Following capture, mating pairs were housed overnight in plastic cages (19 × 19 × 14 cm; 6 cm water depth), and eggs were obtained the following day. After hatching, tadpoles were separately kept in plastic containers (43 × 31 × 16 cm; 6 cm water depth), as those from the same clutch were kept together in a single container. A room temperature of 20°C was maintained until the experiments began, and tadpoles were fed powdered food (Kyorin Co., Ltd.) every two days. In this experiment, four species of dragonfly nymphs were used as predators taking into account their foraging styles: *Anax nigrofasciatus* (Black-striped lesser emperor; climber), *Gynacantha japonica* (Blue-spotted dusk-hawker; climber), *Orthetrum albistylum speciosum* (White-tailed skimmer; burrower), and *Pantala flavescens* (Wandering glider; sprawler). Nymphs of *A. nigrofasciatus* collected in Sakura City, Chiba Prefecture, and those of *G. japonica* collected in Higashihiroshima City, Hiroshima Prefecture, were obtained from an online vendor, and *O. a. speciosum* and *P. flavescens* were collected from the same paddy field as the mating frog pairs. The dragonfly nymphs were maintained individually in plastic trays (23 × 17 × 8 cm) and fed tadpoles of *D. japonicus* every two days until the beginning of the induction experiment.

The induction experiment commenced on July 12, 2022. Each experimental tank housed 21 tadpoles, with three individuals randomly selected from each of the seven egg clutches to randomize genetic effects in each tank. Tadpoles in a tank were assigned to one of five treatments (Ax group with *A. nigrofasciatus*; Gy group with *G. japonica*; Or group with *O. a. speciosum*; Pf group with *P. flavescens*; and the control Ct1 group) with eight replicates per treatment. Each experimental tank (31 × 18 × 24 cm; 10 cm water depth) were equipped with a transparent isolation case (17 × 9 × 10 cm) with approximately 5 mm diameter holes spaced about 5 mm apart, where one dragonfly nymph was introduced. This setup allowed visual and chemical-mediated interaction between the predator and tadpoles without physical

contact. The tanks were aerated and maintained under a 14-h light/10-h dark photoperiod at a room temperature of 22°C, with half of the water in all tanks changed every three days. Air was continuously supplied to the isolation case within each tank through an aquarium air stone. Tanks designated for the Ct1 group lacked predators and utilized an empty isolation case. Tadpoles in the experimental tanks were fed every two days with commercial food for Axolotl, *Ambystoma mexicanum* (Kyorin Co., Ltd.), while dragonfly nymphs received two tadpoles as food every two days.

Experiment II

The design of Experiment II mirrored that of Experiment I, with adjustments made for sampling dates, the interval between hatching and the experiment's commencement, and the selection of predators. For this experiment, adults of *D. japonicus* were captured from the paddy fields at Kyoto University on May 18 and 20, 2022. Eggs were collected and tadpoles from clutches were reared in the same way as in Experiment I until the experiments began. Three predator species were chosen based on their foraging style and the primary sensory organs used for prey detection: Ferocious water bug, *Appasus major* (sit-and-wait predator using tactile and olfactory senses), water scorpion, *Laccotrephes japonensis* (sit-and-wait predator using tactile and olfactory senses), and Japanese fire belly newt, *Cynops pyrrhogaste*r (active tracker using olfactory senses). Aquatic insects, collected in Higashihiroshima City, were purchased from the online vendor, while red-bellied newts were collected from irrigation ditches in Kyoto City (35°05'N 135°47'E). These predators were individually housed in plastic trays and fed with tadpoles every two days.

The induction experiment began on June 18, 2022. Each experimental tank housed 21 tadpoles, with seven individuals randomly selected from each of the three egg clutches. Tadpoles in each tank were allocated to one of four treatments (Be group with *A. major*; Ws group with *L. japonensis*; Fn group with *C. pyrrhogaster*; and the control Ct2 group) with eight replicates per treatment. One predator was introduced into an isolation case in each tank.

Other experimental setups, i.e., tank design, room temperature, light condition, and feeding frequency, were kept the same as in Experiment I.

Measuring Coloration and Morphological Responses

All tadpoles were photographed two and three weeks after the start of the experiment using a Canon® EOS RP mirrorless camera. Individual tadpoles were placed in a narrow acrylic aquarium with a ruler and color chart in the background. The distance between the aquarium and the camera was kept constant for all individuals. All photos were taken with a F/16 aperture, a 1/20th second exposure time, and ISO 3200. Consistency in artificial lighting conditions was maintained for individuals photographed on the same day. Some individuals were excluded from the analysis due to poor nutritional condition or significant tail loss during rearing. The exclusion criteria included: (1) head and body length less than 6 mm, (2) severe tail damage or loss, and (3) inability to maintain posture in water.

Tail parts excluding the tail muscle were trimmed using the image measuring software, Natsumushi version 1.12.2 (Tanahashi and Fukatsu, 2018; https://sites.google.com/site/mtahashilucanid/program/natsumushi). The color of the tail was quantified using the average of CIELAB values (CIE 1976) calculated in Adobe Photoshop 25.5.1 (Adobe Inc.). In the CIELAB color space, color was represented using three components: the lightness component L* and the color components a* and b*. The L* component ranged from black to white between 0–100, while a* and b* components ranged from green to red and blue to yellow, respectively, on a scale of −128 to 127. As Photoshop displays images in 8-bit format, these values were rescaled to range from 0 to 255. Therefore, L* was multiplied by 100/255 and a* and b* were adjusted by subtracting 128, respectively, to facilitate analysis (Fig. 1). Due to an incorrect white balance, photographs taken at the second week of Experiment II were excluded from the analysis of tail coloration. During the third week of Experiment II, photographs of 11 individuals from the Ct2 group were also taken with an incorrect white balance, so the color tone was later corrected using Photoshop,

referencing one of the other photographs from the Ct2 group. Additionally, morphological measurements were conducted based on the photographs using Natsumushi. Morphological traits included tadpole body length (BL), tail length (TAL), and tail depth (TD) were measured using the scale within each image.

Statistical Analyses

All statistical analyses were conducted in R version 4.3.2 (R Core Team, 2023). Linear mixed models (LMMs) were employed based on the measured traits, using the lmerTest package (Kuznetsova et al., 2017) to estimate differences between the control group and the other treatments. The experimental tank was treated as a random factor in the LMMs. When color components (e.g., a* and b*) were utilized as the response variables, the treatment (predator) was used as the explanatory variable in the models to compare the treatment groups to the control group. In the same way, when body length (BL) was utilized as the response variables, the treatment was used as the explanatory variable in the models. On the other hand, in analyses for TAL and TD as the response variable, four models were constructed for each trait. These models included: the model with only BL as the explanatory variable, the model with only the treatment as the explanatory variable, the model with both BL and treatment as explanatory variables, and the model incorporating BL, treatment, and their interactions. The final model was determined via backward elimination, by applying likelihood ratio (LR) tests. Specifically, all explanatory variables (with their interactions) were entered into the full model, then removed one by one until a final model was selected. At each step, the reduced model was compared with the former model by LR test. The significance level was set at 5%. If a significant difference was found between the former and reduced models, the former model was adopted as the final model for further analysis.

After constructing final models, comparisons between each treatment group and the control were performed using Dunnett's test with the glht function in the multcomp package (Hothorn et al., 2008). The significance level was set at 5%, with treatment groups considered

significantly different from the control group if the *P* values were below this threshold. Orange tail coloration and increased tail depth were reported as predator-induced traits in previous studies, so we decided to focus primarily on b* values and tail depth.

We also conducted a principal component analysis (PCA) for color and morphological traits at the third week after the start of the experiment to concisely visualize the multivariate responses of the tadpoles to the predator treatments. The analysis included total length (TL; the sum of BL and TAL), standardized body length (sBL), standardized tail length (sTAL), standardized tail depth (sTD), and coloration values (a* and b*), The sBL, sTAL, and sTD were calculated by dividing the BL, TAL, and TD measurements by TL, then subtracting their respective means and dividing by their standard deviations, in order to use variables with different units together. Pearson's correlation matrices were used to compare the principal component scores with the original variables to interpret the principal components. We also used Pearson's correlation matrices to assess the relationships among tadpole traits.

Results

Coloration

In Experiment I, the values of a* were lower in the Ax group compared to the Ct1 group (Dunnett's test, $P < 0.001$; Fig. 2). Additionally, the Ax group had a higher value of b* ($P < 0.001$). The latter indicates that tadpoles in the Ax group showed more orange tails than in the control group. Also at the third week, the values of a* were lower ($P = 0.0283$) and b* were higher ($P < 0.001$) in the Ax group than in the control group. In addition, the Or group showed higher b* values than the Ct1 group ($P = 0.0443$), indicating that orange tails were induced not only by *Anax nigrofasciatus*, but also by *Orthetrum albistylum speciosum*. Some individuals of the Pf group also exhibited tail coloration, but this tendency was not

statistically significant ($P = 0.1426$ in b* value). However, in Experiment II, no group showed significant differences from the Ct2 group in the a* or b* values at the third week (Fig. 3).

Morphological Responses

In Experiment I, whether tail length or height was used as the response variable, models with BL and treatment as explanatory variables were selected as the final models at both the second and third weeks after the start of the experiment. At the second week, the Pf group had a larger body compared to the Ct1 group (Dunnett's test, $P = 0.0471$; Fig. 4), and tails were shorter in the Ax and Pf groups than in the Ct1 group ($P < 0.001$). Moreover, the Ax group had a higher tail than the Ct1 group ($P = 0.0092$). At the third week, only the Gy group had a shorter tail than the Ct1 group ($P = 0.047$), while the Pf group, as well as the Ax group, showed a higher tail than the Ct1 group ($P = 0.0215$ for Pf, $P < 0.001$ for Ax). The Or group also tended to have higher tails compared to the Ct1 group, but the difference was not statistically significant ($P = 0.1132$).

In Experiment II, the models with BL and treatment as explanatory variables were chosen in the model selection for tail length at the second week. On the other hand, at the third week, the models with only BL were selected. As for tail depth, the models with only BL were also selected. Although the models with only BL as explanatory variable indicated that the effect of the treatment on tail length or tail depth was not large, post-hoc tests were conducted to assess differences between treatments. At the second week, the Be group exhibited a shorter tail ($P < 0.001$), but no significant differences in tail depth were observed between any group and the Ct2 group ($P > 0.05$ for all Be, Ws, and Fn; Fig. 5). At the third week, there was no significant difference in tail depth compared to the Ct2 ($P > 0.05$ for all Be, Ws, and Fn). Consequently, tadpoles in all non-odonate predator treatments were not induced to develop significantly deeper tail fins compared to the control group.

Principal Component Analysis

In Experiment I, the first two principal components (PC1 and PC2) for larval coloration and morphology accounted for 67.3% of the variation, then combined with PC3, they explained 83% (Table 1). PC1 showed strong correlations with all morphological traits except sTD, indicating that it is likely influenced by the larval body proportion between body length and tail length. On the other hand, PC2 was strongly correlated with sTD (Pearson's correlation coefficients, -0.84) and b* (-0.65), suggesting that it represented the direction of predator-induced responses of *Dryophytes japonicus*. The 95% probability ellipse of the Ax group was conspicuously elongated in the negative direction along the PC2 axis, followed by the Pf, Or and Gy groups (Fig. 6). An increase in tail height and coloration change occurred together; the correlation coefficient between sTD and b* value was 0.34, ranking as the fourth highest after the coefficients between TL and other morphological traits (sBL, sTAL), and between TL and b* value (Table 2).

In Experiment II, the first two principal components explained 77.5% of the variation, and combined with PC3, they accounted for 90.4% (Table 1). PC1 largely explained color trait variation, as well as morphological traits except sTD. PC2 exhibited strong correlations with sBL, sTAL, and sTD, while PC3 showed relatively strong correlations with sTD and b*. Although PC3 may represent the direction of response in this species, its overall contribution to the total variation was limited (12.9%). Furthermore, the correlation between sTD and b* value was 0.16, which was not particularly large compared to other coefficients (Table 2), indicating that changes in tail color and shape did not necessarily occur together.

Discussion

Tail Coloration in Asian Species of *Dryophytes*

Our experiments revealed that tadpoles of *Dryophytes japonicus* developed induced changes in tail coloration as well as tail shape in response to dragonfly nymphs. Although tail coloration has rarely been documented in this species, its occurrence has been reported from

several locations in Japan, including Hokkaido, Shiga, Hyogo, Hiroshima, Shimane, Kouchi and Ehime prefectures (Doi et al., 2005), as well as from our collection site in Kyoto (Noda, personal observation). The phenotypes induced by *Anax nigrofasciatus*, i.e., deep tail fins and bright orange tails, closely resemble those previously observed in other species in *Dryophytes* in the New World, Cope's Gray Treefrog (*Dryophytes chrysoscelis*; McCollum and Van Buskirk, 1996), Pine Woods Treefrog (*Dryophytes femoralis*; LaFiandra and Babbitt, 2004), and Gray Treefrog (*Dryophytes versicolor*; Van Buskirk and McCollum, 1999). Thus, our findings represent the first documented case of a predator-induced tail coloration phenotype in Asian species of *Dryophytes*.

Our discoveries of the plasticity in the tail coloration in the Asian species of *Dryophytes* suggests that the trait is symplesiomorphically shared between the North American and ancestral Asian lineages, and has been inherited by present-day Asian species, including *D. japonicus*. An ancestral stock of *Dryophytes* is inferred to have migrated from North America to East Asia across the Bering Land Bridge during the Miocene (Duellman et al. 2016; Dufresnes et al., 2016). Present-day Asian species of *Dryophytes* are monophyletic, having diversified from this ancestral stock. It is plausible that tail coloration has persisted as a phenotypic plasticity in both North American and East Asian species of *Dryophytes* due to the consistent importance of dragonfly nymphs as predators.

It remains unknown whether tail coloration can be induced in the three other Asian species, *Dryophytes flaviventris*, *Dryophytes immaculates*, and *Dryophytes suweonensis* (see Borzée et al., 2020). In North America, Pine Barrens Treefrog, *Dryophytes andersonii* is induced to have a darker tail, not an orange tail, by *Anax junius*, which induces orange tails in other species of *Dryophytes* (Kruger and Morin, 2020). Furthermore, Squirrel Treefrog, *Dryophytes squirellus* exhibits no morphological changes in response to odonate species (McCoy and Bolker 2008; Baker, 2021). These cases indicate that the plastic responses in *Dryophytes* have diversified, and some may have lost the ability to develop orange tails.

Future studies are needed to elucidate the interspecific variation in plastic responses to predation risk and the evolutionary causes underlying this variation.

Differences in the Extent of Coloration by Predators

Our experiments demonstrated that the extent of induced responses varied depending on the dragonfly species reared with tadpoles. Two weeks after the start of the experiment, nymphs of *A. nigrofasciatus* induced both deep fin and tail coloration in tadpoles, whereas, at the third week, *Pantala flavescens* also induced deep tail and *Orthetrum albistylum speciosum* induced tail coloration. Furthermore, although weak, some tadpole individuals exposed to *P. flavescens* showed tail coloration, and those exposed to *O. albistylum speciosum* also tended to have higher tails. These results indicate that the defense traits (coloration and deep tail) were induced by the nymphs of *A. nigrofasciatus* more rapidly and strongly, but similar responses were also elicited by the other two predator species. The probability ellipses in PCA further suggest that the direction of larval responses to these benthic predators (*P. flavescens* and *O. albistylum speciosum*) were similar to that to *A. nigrofasciatus* (Fig. 6).

The differences in the degree and rate of responses induced by different dragonfly species may reflect varying levels of predation risk for tadpoles posed by each species. Plastic phenotypes with significant tail coloration should result in trade-offs because of energetic costs associated with synthesizing pigmentation (Grether et al., 2001) or costs due to the increased vulnerability to mismatched predators. Consequently, tadpoles are expected to minimize these costs by developing effective defense traits for each predator as much as possible based on the perceived predation risk (Touchon and Warkentin, 2008; Innes-Gold et al., 2019). The predation risk for tadpoles would be different among dragonfly species according to (1) the foraging style of dragonfly nymphs, (2) their color vision, and (3) the total amount of intake related to their body size and occurrence period.

Firstly, the four dragonfly species used in our experiment exhibit different foraging styles. According to the classification by Corbet (1999), *A. nigrofasciatus* and *G. japonica* are

climbers, sit-and-wait predators on aquatic vegetation, while *P. flavescens* is a sprawler, foraging while moving along the bottom, and *O. albistylum speciosum* is a burrower, residing within the substrate and ambushing prey. For sit-and-wait predators, there is often a time lag between identifying prey and launching an attack, during which they can choose which part of tadpole's body to target. Therefore, the lure effect of tadpole tails is likely to attract ambush predators more effectively than those that actively chase their prey. In addition, as *A. nigrofasciatus* prefers habitats abundant in floating-leaved plants (Ozono et al., 2021), it can move freely along the plant structures from roots anchored at the bottom to leaves extending above the water surface. As a result, the risk of predation on tadpoles could be present throughout the entire water column, from the bottom to the surface, making behavioral responses like moving to a specific depth insufficient as a means of avoiding predation. Conversely, *P. flavescens* and *O. albistylum speciosum* primarily forage at the bottom, so tadpoles may evade these predators by relocating to higher water layers. However, such avoidance behavior often leads to a decrease in growth rate due to reduced foraging efficiency (Teplitsky et al., 2003); i.e., for tadpoles that primarily feed on detritus, avoiding the bottom layer may result in malnutrition. The combination of moderate morphological responses with behavioral responses (the "double defense" strategy; Dewitt and Hucko, 1999) may help mitigate the impact of malnutrition from behavioral changes while minimizing the costs of defensive traits.

Secondly, differences in eyesight, particularly color vision among dragonfly species, may influence the extent of the tail coloration changes in the tadpoles. Pritchard (1966) noted a correlation between foraging habit and compound eye size in dragonfly nymphs, with climbers typically possessing larger compound eyes compared to sprawlers and burrowers. He also stated that the climbers, such as species in *Anax*, rely heavily on vision for prey detection, whereas other foraging types depend more on tactile stimuli. Hence, it is plausible that the lure effect of the brightly colored tails is more effective for climbers, which are more visually oriented. Furthermore, Futahashi et al. (2015) investigated the genetic basis of color

vision in dragonflies and redocumented the number of opsin genes and the characteristic spatiotemporal expression patterns in the nymphs of various species, including those of the burrower *O. albistylim* and the climber *A. parthenope*, a species closely related to *A. nigrofasciatus* used in our experiment. They found that dragonflies possess a greater number of opsin genes than other insects, and *A. parthenope* possesses and expresses a greater number of opsin genes, particularly those sensitive to long-wavelength light. Although the functions of these diverse genes are not fully understood, this suggest that species of *Anax* may have enhanced abilities to perceive reddish colors, potentially enabling tadpoles with bright orange tails to more effectively avoid predation by using their tails as lures. Thus, the larval antipredator defense of *D. japonicus*, which involves tail coloration, may have been driven by the sensory systems of predatory dragonfly nymphs, and subsequently evolved specifically against color-sensitive predators, represented by dragonfly nymphs of *Anax*.

Thirdly, the variation in the degree of induced responses may reflect the relative predation risks associated with body sizes or occurrence periods of respective odonate predators. In nature, it is generally difficult to determine which predators exert higher predation pressure on tadpoles, and few studies have investigated such differences. However, during the rearing process, we observed that the nymphs of *A. nigrofasciatus* exhibited higher food demands and displayed more frequent attacks on tadpoles compared to other species. Additionally, they are approximately twice the size of the nymphs of *P. flavescens* and *O. albistylum speciosum* (see Ishida et al., 1988), suggesting that size-related predation pressures could influence the necessity and effectiveness of tadpole morphological responses. However, tadpoles did not exhibit significant responses to *G. japonica*, which is similar in size to *A. nigrofasciatus* (see Ishida et al., 1988). This indicates that tadpoles likely respond not only to size-related predation pressure but also to other characteristics of dragonfly species, such as foraging style or habitat overlap, thereby modulating the extent of induced responses. Since the habitat of nymphal *G. japonica* overlaps with that of tadpoles of *D. japonicus* (Nakagawa et al., 2004), the lack of an apparent induced response may be due to the limited period in

which they co-occur. This species grows more rapidly compared to other species in Aeshnidae in Japan, occurring in aquatic environments only from May to August (Ozono et al., 2021). Considering that the nearly-metamorphosing nymphs cease foraging, the period during which this species poses a predation threat to *D. japonicus* is even shorter. This brief coexistence period suggests that this dragonfly species may not be an important predator for the tadpoles.

In addition to the tail coloration and depth we focused on, a tendency toward larger body lengths and shorter tail lengths compared to those in the control group was observed, although not statistically significant in most treatment groups. These changes may represent general responses to predator treatments, as noted in previous studies. The increase in body length could be attributed to enhanced availability of food resources resulting from leftovers and feces of caged predators (e.g., McIntyre et al., 2004; Richardson, 2006). Similarly, shorter tail lengths in the presence of predators have been reported in several frog species (Laurila et al., 2002; LaFiandra and Babbitt, 2004).

Responses Toward Non-Odonate Predators

Tadpoles of *D. japonicus* showed neither conspicuous tail coloration nor deep fin when exposed to newts or aquatic insects other than dragonfly nymphs in our experiment, likely because the costs of expressing these defenses outweighed their benefits for avoiding predation. Although Experiment II did not include a positive control (i.e., treatment with dragonfly nymphs, such as *A. nigrofasciatus*), we believe that the absence of color induction was unlikely caused by experimental failure, because preliminary experiments demonstrated the high reproducibility of the induced responses when using dragonfly nymphs. Therefore, our results suggest that changes in body color and tail depth are not strongly induced by these predators and may not serve as effective predation avoidance strategies against them. An important point in understanding why tadpoles did not show tail coloration is that defensive traits come with physiological costs and may increase predation pressure from non-focal predators. If changes in tail color and shape do not significantly contribute to non-odonate

predator avoidance, tadpole should minimize these disadvantages by refraining from developing these antipredator defenses.

Given that the non-odonate predators are presumably less dependent on vision than odonate species when foraging, the strategy of using colored deep tails as lures may be ineffective and provides only limited benefits against them. For instance, diving beetles, water bugs, and water scorpions capture prey by grasping the entire body with their arms, so the tail of tadpoles should not serve as a lure for them. Additionally, they use tactile, visual, and olfactory senses for locating prey (Babbitt and Jordan, 1996; Kehr and Gómez, 2009; Mogali et al., 2019; Nowińska and Brożek, 2019), and possess well-developed organs for detecting auditory and mechanical signals (Yager, 1999; Cocroft and Rodríguez, 2005), suggesting they are relatively less dependent on vision. Previous studies have reported predator-induced changes in larval morphology in response to these predators (Benard, 2006; McCoy, 2007; Michel, 2012), but few have reported effects on larval tail coloration (Touchon and Warkentin, 2011). Similarly, newts and salamanders, both as larvae and adults, are active foragers that swallow their prey whole like fish (Wilson et al., 2005; Kishida and Nishimura, 2007). Consequently, the tails of the tadpoles are unlikely to function as a lure for these predators. Newts tend to rely on olfaction cues as well as vision for food recognition (Copeland, 1913; Lindquist and Bachmann, 1982; Schwarz et al., 2020). Few studies have reported that tadpoles exhibit induced defensive traits in response to these predators, and none have documented induced changes in body coloration (Kishida and Nishimura, 2004; Hettyey et al., 2011; Urban et al., 2017). These facts support the hypothesis that the brightly colored tails of *D. japonicus* do not function as lures against the non-odonate predators, and that, as a result, tadpoles do not express costly defensive traits in response to them.

The lack of induction of defensive phenotypes may also be attributed to physiological costs associated with developing tail coloration, although the magnitude of such costs in tadpoles remains poorly understood. Since most animals are unable to biosynthesize carotenoids, the pigments thought to be causing the bright orange coloration, they metabolize

and accumulate these pigments by absorbing them from their diet (Bertrand et al., 2006; Thibaudeau and Altig, 2012). Thus, the allocation costs of expressing coloration by transporting these pigments may not be as high as commonly assumed. On the other hand, the potential for increased vulnerability to non-focal predators may also interfere with the expression of defensive traits (Innes-Gold et al., 2019). This disadvantage may sometimes outweigh the energetic costs of expressing a defensive phenotype. Carfagno et al. (2011) found that tadpoles of *Acris* exhibit darker a tail spot, which is considered a defensive trait against dragonfly nymphs, even in the absence of predators. However, the presence of fish predators induces the loss of this spot. This suggests that the cost of expressing defensive traits may be lower than typically expected, and the disadvantage of increased vulnerability to mismatched predators may prevent these phenotypes from being constitutively expressed. Similarly, in tadpoles of *D. japonicus*, the elevated risk of predation from non-odonate predators, considered non-focal predators, due to expressing bright tail coloration, rather than its energetic cost, could explain why they did not exhibit deep and brightly-colored tail in response to these predators. These defense traits against dragonfly nymphs may not only be ineffective in preventing predation by non-odonate predators but could also increase the potential vulnerability to them. For example, the deepened tail may increase the vibrations produced during swimming, thereby heightening predation pressure on tadpoles by tactile or olfactory predators (i.e., water bugs, water scorpions, and newts). Additionally, for other predators such as fish, tail coloration may make the tadpoles more vulnerable by increasing their visibility. However, further experiments are warranted to assess and compare the effectiveness of these defense traits toward each predator, including evaluating how tadpole coloration influence their survival rate in the presence of various aquatic predators.

Acknowledgements

We thank the Experimental Farm, Graduate School of Agriculture, Kyoto University, for providing the research environment, and Mr. Kagata, Mr. Yasui, and Mr. Sakakibara, technical staff, for their assistance with observations. This work was supported by JST SPRING, Grant Number JPMJSP2110.

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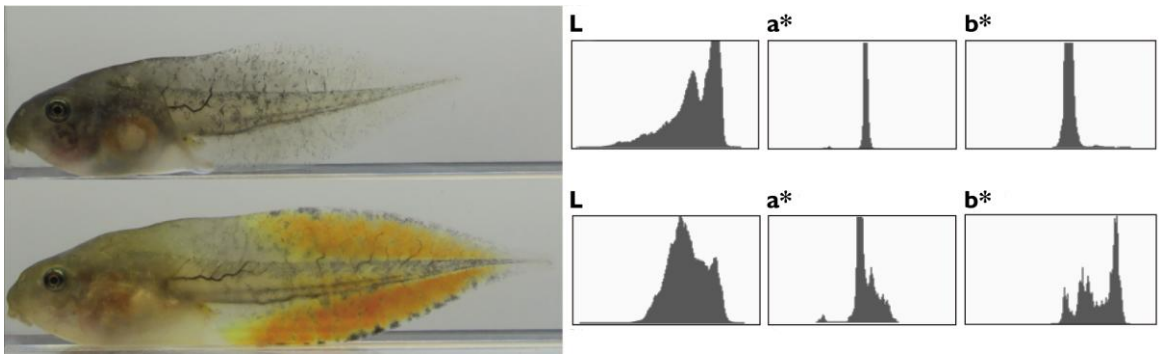

Fig. 1. Photographs of tadpoles of *Dryophytes japonicus* reared without predator (top) and with *Anax nigrofasciatus* (bottom) and their corresponding Photoshop Lab color histograms. The mean values of tail coloration are L = 61.2, a* = -2.5, and b* = 7.96 (without predator), and L = 57.96, a* = 1.75, and b* = 35.51 (with *A. nigrofasciatus*), respectively.

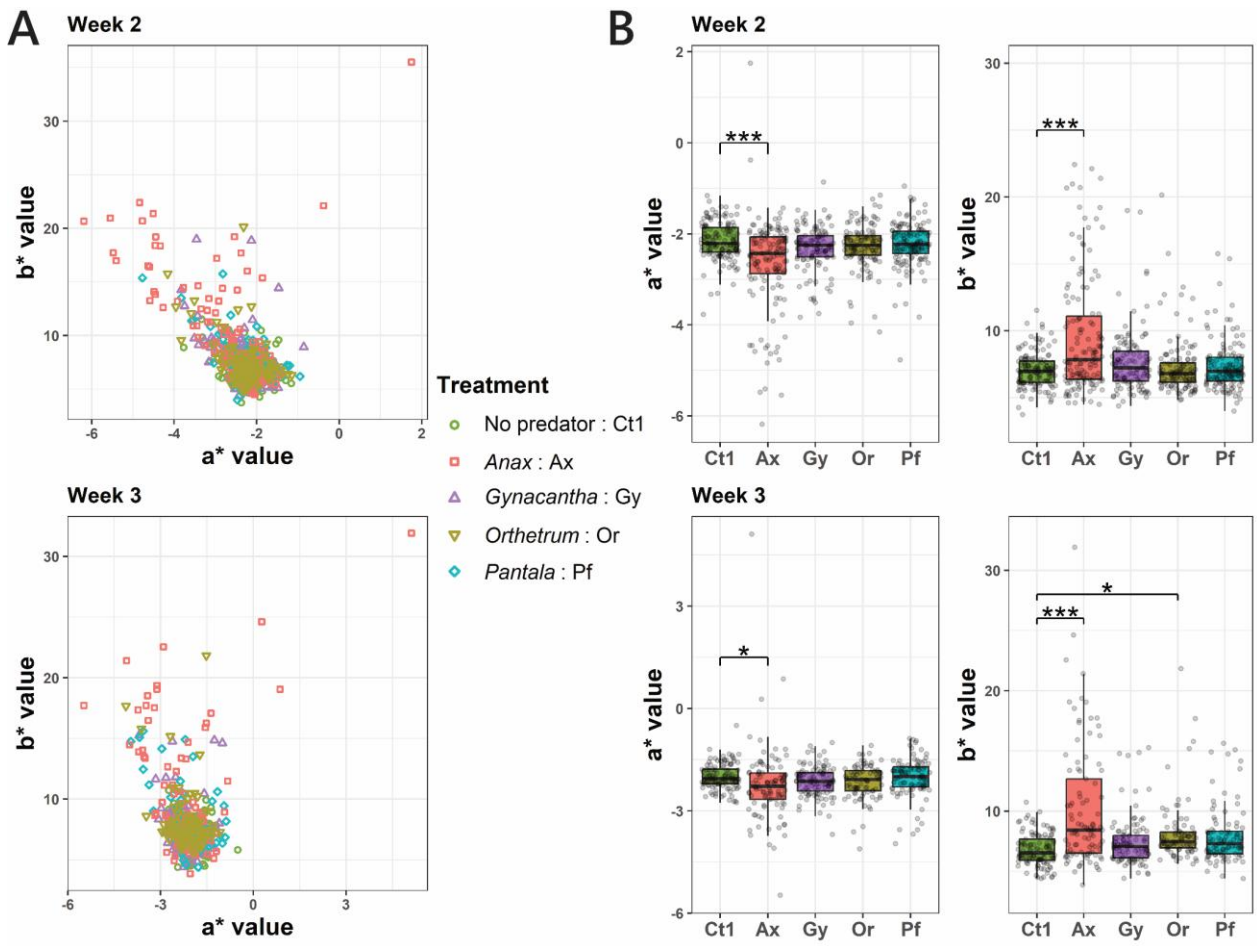

Fig. 2. (A) Scatterplots of tail coloration parameters (a* and b* values) of tadpoles two weeks (top) and three weeks (bottom) after the start of the Experiment I, using different dragonfly species. Tadpoles were reared in the presence of no predators (the control group; Ct1), *Anax nigrofasciatus* (Ax), *Gynacantha japonica* (Gy), *Orthetrum albistylum speciosum* (Or), or *Pantala flavescens* (Pf). (B) Box-and-whisker plots displaying the range of the tail color traits (a* and b* values) two weeks (top) and three weeks (bottom) after the start of the Experiment I. The asterisks in the figure indicate that there were significant differences compared to the control group, which were led by the LMM analysis (see Materials and Methods). *$P < 0.05$, ***$P < 0.001$.

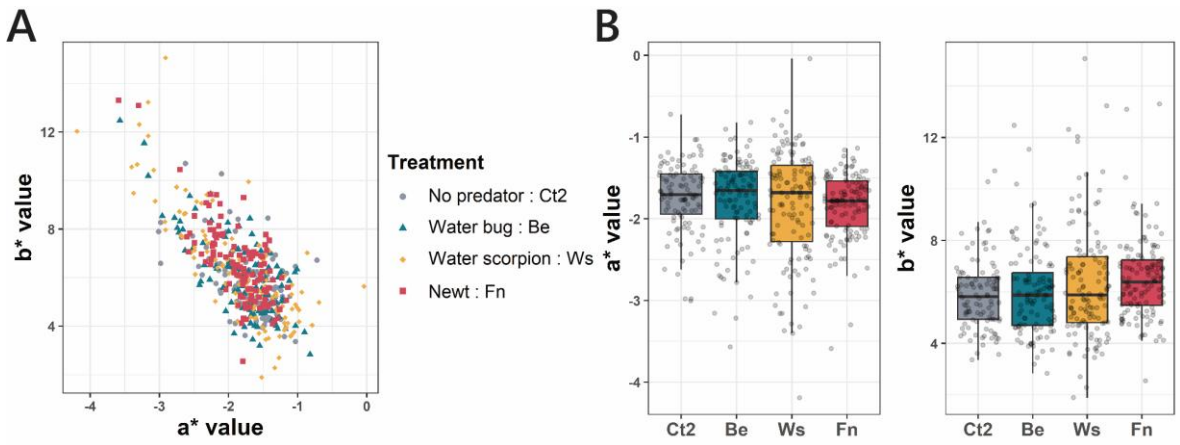

Fig. 3. (A) Scatterplots of tail coloration parameters (a* and b* values) of tadpoles two weeks (top) and three weeks (bottom) after the start of the Experiment II, using non-odonate species. Tadpoles were reared in the presence of no predators (the control group; Ct2), water bug (*Appasus major*; Be), water scorpion (*Laccotrephes japonensis*; Ws), and fire belly newt (*Cynops pyrrhogaster*; Fn). (B) Box-and-whisker plots displaying the range of the tail color traits (a* and b* values) three weeks after the start of the Experiment II.

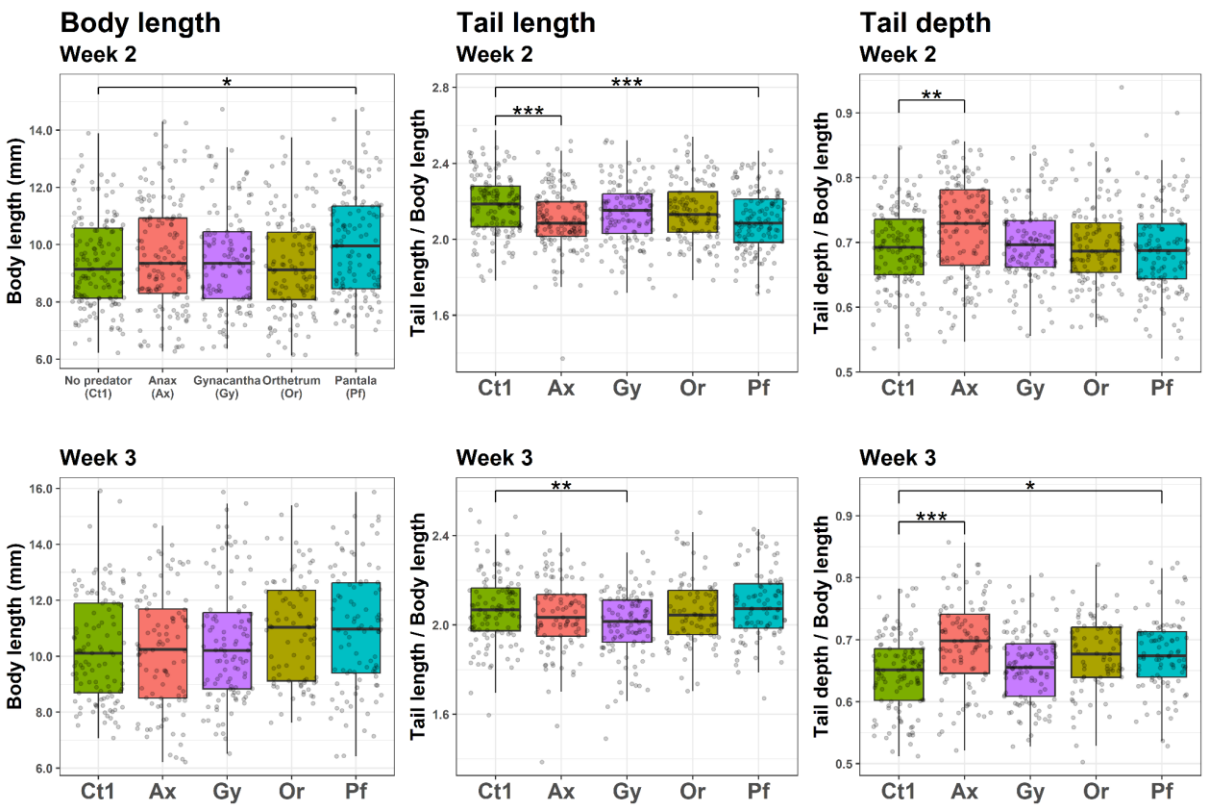

Fig. 4. Box-and-whisker plots showing the range of tadpole morphological traits, BL (body length), TAL (tail length), and TD (tail depth) in Experiment I, using different dragonfly species. Tadpoles were reared in the presence of no predators (the control group; Ct1), *Anax nigrofasciatus* (Ax), *Gynacantha japonica* (Gy), *Orthetrum albistylum speciosum* (Or), or *Pantala flavescens* (Pf). TAL and TD values were divided by body length to account for size differences among individual tadpoles. The asterisks in the figure indicate that there were significant differences compared to the control group, which were led by the LMM analysis (see Materials and Methods). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

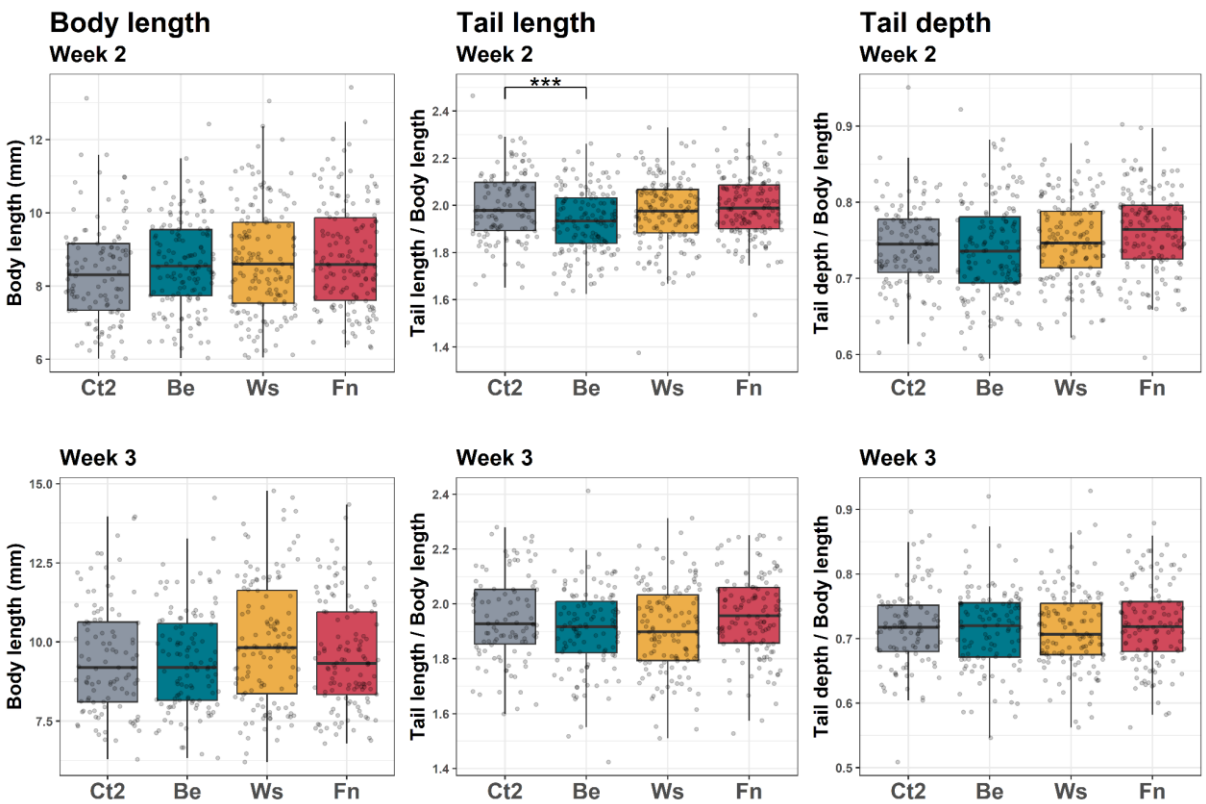

Fig. 5. Box-and-whisker plots showing the range of tadpole morphological traits, BL (body length), TAL (tail length), and TD (tail depth) in Experiment II, using non-odonate predators. Tadpoles were reared in the presence of no predators (the control group; Ct2), water bug (*Appasus major*; Be), water scorpion (*Laccotrephes japonensis*; Ws), and fire belly newt (*Cynops pyrrhogaster*; Fn). TAL and TD values were divided by body length to account for size differences among individual tadpoles. The asterisks in the figure indicate that there were significant differences compared to the control group, which were led by the LMM analysis (see Materials and Methods). ***P <0.001.

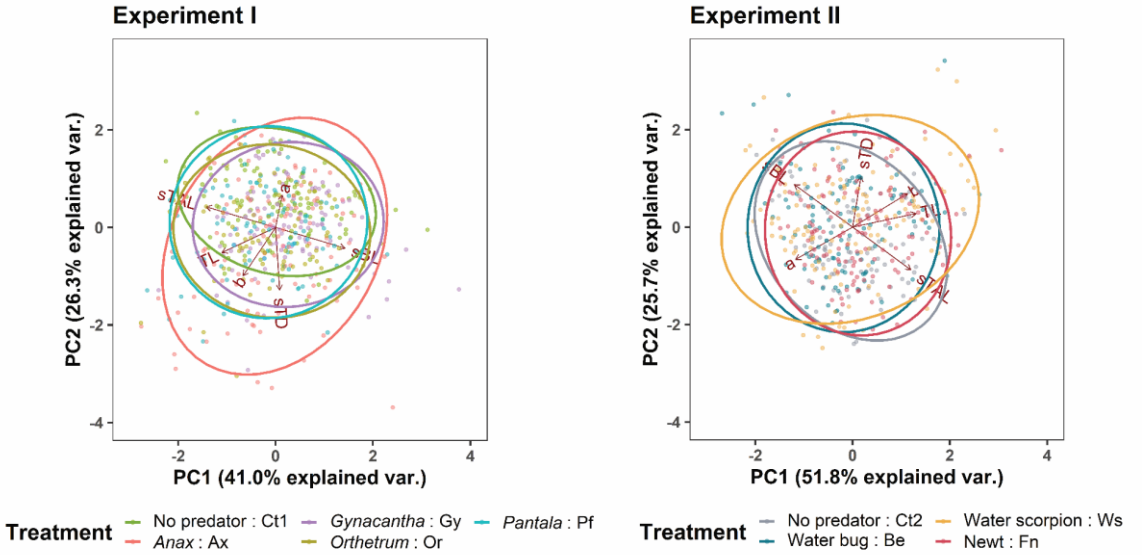

Fig. 6. Plots of principal components 1 and 2, with their contribution shown in parentheses, based on four morphological traits, total length [TL; the sum of body length (BL) and tail length (TAL)], standardized body length (sBL; BL/TL), standardized tail length (sTAL; TAL/TL), and standardized tail depth (sTD; TD/TL), and the coloration values (a* and b*), sampled three weeks after the start of Experiment I (left) and Experiment II (right). In Experiment I, tadpoles were reared in the presence of no predators (the control group; Ct1), *Anax nigrofasciatus* (Ax), *Gynacantha japonica* (Gy), *Orthetrum albistylum speciosum* (Or), or *Pantala flavescens* (Pf). In Experiment II, they were reared in the presence of no predators (the control group; Ct2), water bug (*Appasus major*; Be), water scorpion (*Laccotrephes japonensis*; Ws), and fire belly newt (*Cynops pyrrhogaster*; Fn). Ellipses represent the 95% probability ellipses of each treatment group. The red arrows indicate the direction in which the variables of each trait contribute to the principal components. Data for one individual in the Ax group, which appeared as an outlier in the bottom left-hand side, was omitted in this figure.

Table 1. Results of principal component analysis of coloration and morphological traits of tadpoles in Experiment I (top) and Experiment II (bottom). PC1, PC2, and PC3 indicate the first three principal components. The values in the table are the correlation coefficients of each morphological trait with the principal component scores.

Experiment I

Response variable	PC1	PC2	PC3
Total Length	–0.72	–0.34	0.19
Standardized body length	0.93	–0.28	0.10
Standardized tail length	–0.93	0.28	–0.10
Standardized tail depth	0.05	–0.84	0.07
a*	0.09	0.43	0.88
b*	–0.44	–0.65	0.31
% of variance explained	41.0%	26.3%	15.7%

Experiment II

Response variable	PC1	PC2	PC3
Total Length	0.85	0.18	0.06
Standardized body length	–0.78	0.57	0.22
Standardized tail length	0.78	–0.57	–0.22
Standardized tail depth	0.11	0.68	–0.72
a*	–0.77	–0.43	–0.15
b*	0.74	0.45	0.36
% of variance explained	51.8%	25.7%	12.9%

Table 2. The Pearson's correlation coefficients between morphological trait pairs, total length [TL; the sum of body length (BL) and tail length (TAL)], standardized body length (sBL; BL/TL), standardized tail length (sTAL; TAL/TL), and standardized tail depth (sTD; TD/TL), and the coloration values (a* and b*). Diagonal cells, representing self-correlations, are denoted by dashes. Results from Experiment I are shown above the diagonal, while results from Experiment II are presented below the diagonal.

Experiment I

	TL	sBL	sTAL	sTD	a*	b*
TL	–	–0.47	0.47	0.18	–0.07	0.43
sBL	–0.51	–	–1.00	0.20	0.03	–0.18
sTAL	0.51	–1.00	–	–0.20	–0.03	0.18
sTD	0.16	0.14	–0.14	–	–0.21	0.34
a*	–0.62	0.33	–0.33	–0.26	–	–0.10
b*	0.67	–0.26	0.26	0.16	–0.72	–

Experiment II