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RESEARCH

Antipredator response of Korean clawed salamander (*Onychodactylus koreanus*) larvae to odors of potential predators (Chinese minnow, *Rhynchocypris oxycephalus* and Korean freshwater crayfish, *Cambaroides similis*)

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Background: To identify and avoid predators, amphibians rely on chemical cues. *Onychodactylus koreanus* undergo two to three years of the larval stage in mountainous streams, where they encounter various predators. We aimed to identify the potential predators of *O. koreanus* larvae based on their antipredator responses to predator odors. Additionally, we examined whether the response was innate or can be strengthened by predator olfactory learning.

Results: In Experiment 1, *O. koreanus* larvae exhibited a substantial antipredator response to Chinese minnow (*Rhynchocypris oxycephalus)* odor but not to Korean freshwater crayfish (*Cambaroides similis*) odor. In Experiment 2, *O. koreanus* larvae, who did not previously expose to *R. oxycephalus* odor, demonstrated a substantial antipredator response to it. Experiment 3 indicated that predator olfactory learning of *R. oxycephalus* did not enhance the antipredator response of the larvae.

Conclusions: *Rhynchocypris oxycephalus* could prey on *O. koreanus* larvae, whose antipredator response to *R. oxycephalus* odor is innate and not enhanced by olfactory learning. Further investigation into the olfactory system of this species may provide insights into the life cycle of *O. koreanus*, uncovering hidden underground breeding sites and unknown breeding periods.

Keywords: conspecific alarm, imprinting, predation risk, predator olfactory learning, predator-prey interaction

Introduction

Animals must appropriately and accurately detect and identify the risk of predation for survival using various sensory systems, such as visual, auditory, olfactory, and mechanosensory systems (Crane et al. 2022; Dill 1987). Amphibians encounter various predators in terrestrial and aquatic environments (Quaranta et al. 2009) and employ various antipredator responses, including aposematic coloration, aposematism, hiding, and fleeing (Ferreira et al. 2019; Garcia and Sih 2003; Kang et al. 2017). Aquatic amphibians predominantly use olfactory cues for predator odor discrimination because the visual and tactile cues are limited by various obstacles and slow-moving water currents in aquatic environments (Hettyey et al. 2015; Mathis and Vincent 2000). Typical antipredator response in amphibians compromises an increased amount of time spent in shelters and reduced movement (Kats and Dill 1998). The reduced prey movement decreases the likelihood of predators detecting their locations (Hemnani et al. 2023). Antipredator responses entail energy and time costs, impacting essential life activities such as reduced foraging, mating, and territory defense. This trade-off between antipredator responses and essential life activities reflects an adaptive nature (Lima and Dill 1990). Studying amphibian antipredator response contributes to our understanding of their life histories and the selection of behaviors with optimal fitness (Dawkins and Krebs 1979).

Antipredator responses in amphibians can be either innate (Laurila et al. 1997) or acquired through learning (Hahn et al. 2023). For example, certain salamanders inherit antipredator responses, exhibiting an appropriate antipredator response to the odor of a predator even without prior exposure (DeSantis et al. 2013). Conversely, some salamanders engage in olfactory learning to develop appropriate olfactory antipredator response. This learning occurs when exposed to an injured conspecific cue (alarm cue) paired with the odor of a new predator (Ferrari and Chivers 2011). In some cases, prolonging the duration of predator olfactory learning often enhances the antipredator response (Crane et al. 2017). Even within the same species, the extent of antipredator responses can vary based on the predation pressure in their native habitat (Manenti et al. 2016).

Korean clawed salamander (*Onychodactylus koreanus*), inhabiting mountain streams (Jeon et al. 2023) with stable temperature and humidity, spend two to three years in the larval stage (Park 2005; Won 1971) within the streams. Their movement from the upper to lower mountain streams during the spring-to-summer transition could increase the likelihood of encountering novel predators in the lower parts of the stream (Lee et al. 2008). In Russia, the main predators of *Onychodactylus fischeri*, which is phylogenetically close to *O. koreanus*, are fish species, sharing the habitats, such as *Salvelinus malma* and *Cottus pollux* (Solkin 1993). Crayfish (Astacidea), who lives in mountain streams, exhibit diverse feeding habits, including feeding on various animal carcasses and hunting earthworms, snails, small invertebrates, and tadpoles (Kawai et al. 2015; Momot 1995). In the United States, *Procambarus clarkia* crayfish prey on aquatic salamanders such as *Eurycea sosorum* (Davis et al. 2017). Therefore, Chinese minnow (*Rhynchocypris oxycephalus*) and Korean freshwater crayfish (*Cambaroides similis*), which lives in mountain streams, is possibly a potential predator of *O. koreanus* larvae. The behavior of *O. fischeri* larvae to hide in gravel in response to predators suggests a potentially similar defensive response in *O. koreanus* larvae to that of *O. fischeri* (Regel and Epshtein 1975). To the best of our knowledge, studies on the antipredator responses of *O. koreanus* larvae are absent.

The habitats of organisms, including *O. koreanus*, are gradually decreasing due to climate change (Collins and Storfer 2003; Shin et al. 2021). Consequently, habitat changes can increase individual densities by crowding, raising the likelihood of predator–prey encounters. Investigating antipredator responses is crucial for understanding how a species balances the trade-off between minimizing predation risks and optimizing its reproductive fitness. This allows us to determine which factors affect their decision-making in life history. Consequently, it enhances our comprehension of the species, its life histories, and its interactions with other species.

In Experiment 1, we identified the potential predators of *O. koreanus* larvae through antipredator response experiments involving exposure to predator odors (*R. oxycephalus* and *C. similis*). In Experiment 2, we investigated whether antipredator response of *O. koreanus* larvae to predator odor is innate or not. Finally, in Experiment 3, we explored the potential enhancement of predator olfactory learning through antipredator responses of *O. koreanus* larvae.

Materials and Methods

Sampling and maintenance

For Experiment 1, we collected 39 *O. koreanus* larvae (mean snout-vent length [SVL] 25.0 ± 4.2 mm) from October 31 to December 8, 2022, at Wolgok-ri (37.91°N, 127.81°E) and Goeu-ri (37.84°N, 127.80°E), Chuncheon-si, Gangwon-do. Five *R. oxycephalus* and five *C. similis*, the potential predators, were collected on October 31, 2022, at Wolgok-ri and on November 25, 2022, at Geumak-ri, Yanggu-gun, and Gangwon-do (38.18°N, 127.95°E). *Onychodactylus koreanus* larvae and *C. similis* were confirmed to coexist at all sampling sites, and *R. oxycephalus* was found in the lower mountain streams where *O. koreanus* larvae existed.

We conducted Experiments 2 and 3 using less than one-year-old *O. koreanus* larvae, which exhibited less than 25 mm SVL and were previously unexposed to *R. oxycephalus* (Lee et al. 2008). We collected 58 *O. koreanus* larvae (mean SVL 22.6 ± 1.9 mm) at Goun-ri from February 7 to March 8, 2023. The sex of the larvae was not determined because it was morphologically impossible (Poyarkov et al. 2012). After sampling, we transported the individuals to the laboratory and measured the SVL to the nearest 0.1 mm using ImageJ (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih. gov/ij) and weighed the body weight (BW) to the nearest 0.1 g using a RE-700 digital pocket scale (CAS Korea, Seongnam, Korea).

Onychodactylus koreanus larvae were individually housed in 9.5 × 5 cm circular column containers with 70 mL of water. To ensure environmental diversity, unbleached paper towels were also used. *Rhynchocypris oxycephalus* and *C. similis* were kept in 26 × 17 × 18 cm containers with 2 L of water. Unbleached paper towels were also used. For all experiments, carbon-filtered water was used. We maintained all animals in a temperature-controlled set at 15°C–17°C with a photoperiod of 9 light:15 dark and fed them frozen bloodworm (Hikari Sales USA Inc., Hayward, CA, USA) and goldfish feedstuff (PSP, Yongin, Korea) once every 2 to 3 days. One-third of the water was replaced within 36 hours of feeding. All animals were deprived of food for at least 24 hours before the experiment to minimize the effects of emitted feces. Sampling of the animals was permitted by the local government (Chuncheon-si license # 4180 00085202 200004), and the experimental procedures were approved by the Institutions Animal Care and Committee (IACUC) of Kangwon National University (KW-221006-2).

Experimental setup and stimulus odor preparation

Antipredator responses were studied using the open-field test method, which is often used in personality experiments on amphibian larvae (Kelleher et al. 2018). The experiment included a petri dish of 90 mm diameter and 15 mm height filled with 30 mL of water. To control visibility, the sides of all the petri dishes were covered with opaque black tape (Kelly et al. 2023). A grid paper with a 5 × 5 mm grid was placed at the bottom of the petri dish to measure the distance the larvae travelled.

We extracted the potential predator odor by separately placing four *R. oxycephalus* and four *C. similis* (total 7 g; g/10 mL) in 70 mL of water for 24 hours (Lucon-Xiccato et al. 2018). The collected 70 mL odor was filtered once using a KIMTECH Science Wiper (Yuhan Kimberly, Seoul, Korea) to remove solid particles. The filtrate odor (100%) was diluted to 50% and 25% with the addition of water. We prepared four different predator odor concentrations: 100%, 50%, 25%, and 0% (blank water: control) (Souza-Bastos et al. 2014).

For Experiment 2, we collected *R. oxycephalus* odor by maintaining five *R. oxycephalus* individuals (total 8 g; g/10 mL) in 80 mL of water for 24 hours (Lucon-Xiccato et al. 2018). The 80 mL odor was filtered in the same way as previously explained and used as a predator odor stimulus. In Experiment 3, we extracted conspecific alarm cues from the *O. koreanus* larvae. Larvae were rapidly chilled to 2–4°C in 500 mL of ice and water (5:1) for 15–30 minutes (Galex et al. 2020). They were subsequently euthanized using the guillotine method (Gonzalo et al. 2012). The carcass was crushed using a mortar and pestle (Ferrari et al. 2012) with 60 mL of water per capita *O. koreanus* larvae, and the alarm cue was filtered in the same manner as previously explained to remove the remaining tissues. Each 0.5 mL of the prepared *R. oxycephalus* and *O. koreanus* larvae odor was mixed at a 1:1 ratio to create a 1 mL mixture odor stimulus (Ferrari et al. 2012). Prepared odor stimulus was stored in 1 mL aliquots in 1.5 mL tubes at −20°C and used within 7 days in all the experiments (Park and Sung 2006).

Experiment 1: antipredator response to potential predator odor

One of the 39 *O. koreanus* larvae was randomly selected, placed in an experimental petri dish, and acclimated for 2 minutes to determine the antipredator response to the odor of potential predators (Crowder and Ward 2022). Subsequently, we recorded its activity for 3 minutes using an iPhone XS (Apple, Cupertino, CA, USA) (Hahn et al. 2023). *Onychodactylus koreanus* larvae were then exposed to a potential predator (*R. oxycephalus* or *C. similis*) odor (100%, 50%, 25%, or 0% odor), which was randomly selected using a 1 mL pipette (P3960-1000A BioPette; Labnet International, Medley, FL, USA). The odor was slowly and manually injected into the center of the petri dish for 5 seconds. After injection, activity was recorded for an additional 3 minutes.

We analyzed the time spent moving and the distance travelled to evaluate the antipredator response of *O. koreanus* larvae to the odor of predators. The time spent moving was measured using a stopwatch (Morningglory, Hwaseong, Korea) as the sum of the time during which the tip of the snout moved for more than 0.1 seconds in the recorded video. The distance travelled was determined by counting the number of times the snouts of *O. koreanus* larvae crossed a 5 mm × 5 mm grid paper line (Mirza and Chivers 2000). Larval responses to odors were assessed by comparing the time spent moving and the distance travelled between pre- and post-exposure to potential predator odors. All individuals were tested at 100%, 50%, 25%, and 0% concentrations at one-day intervals in random order. We used 20 larvae in both odor tests, eight larvae only in *R. oxycephalus*, and five larvae in the *C. similis* test.

Experiment 2: innate antipredator response to predator odor

We used 58 less than one-year-old *O. koreanus* larvae that had not been previously exposed to *R. oxycephalus* to investigate whether the antipredator response to a specific predator odor was innate. We randomly selected 28 *O. koreanus* larvae between April 22 and 24, 2023, and measured their antipredator response to 1 mL of 0% odor (water) in the same manner as in Experiment 1. We also measured the antipredator response of the remaining 30 *O. koreanus* larvae to 1 mL of 100% *R. oxycephalus* odor in the same manner (Fig. 1).

Experiment 3: no enforcement of antipredator response by predator olfactory learning

After Experiment 2, we tested the effect of predator olfactory learning on the antipredator response to predator odor stimuli. All larvae were exposed twice to either predator olfactory learning or blank water learning between April 22 and 24 and May 17 and 18, 2023. Olfactory learning was executed in an opaque container of 105 mm diameter and 60 mm height filled with 30 mL of water for 1 hour. The control group (n = 28), which was exposed to 0% odor (water) in Experiment 2, received 1 mL of water and was maintained in the condition for 1 hour. The experimental group (n = 30), which was exposed to 100% *R. oxycephalus* odor in Experiment 2, received 1 mL of a mixed odor of *R. oxycephalus* and *O. koreanus* larvae and was maintained for 1 hour (Lucon-Xiccato et al. 2018). After each learning session, all the individuals were transferred to their individual containers. Between June 15 and June 16, we tested the antipredator response of the control and experimental groups to a 100% *R. oxycephalus* odor prepared as previously described (Fig. 1). We selected the 1

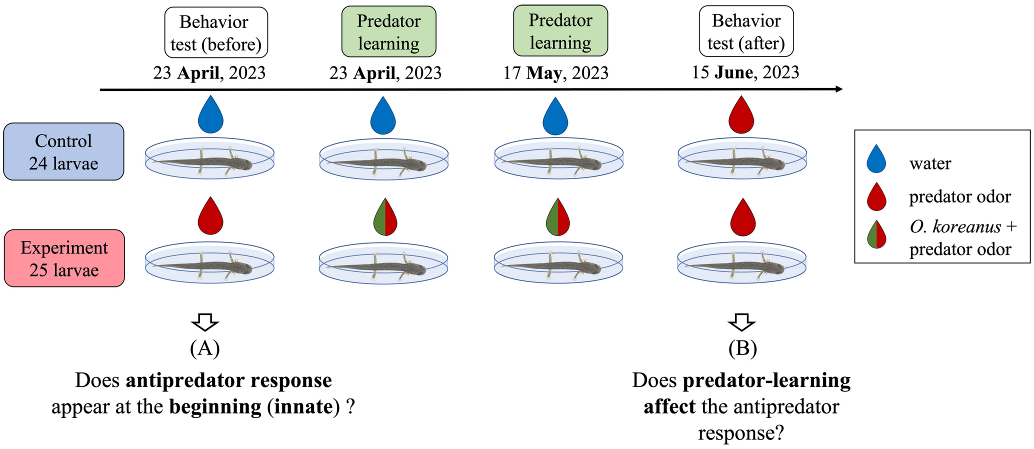

Fig. 1 A schematic diagram of experimental procedure. Antipredator response test before (A) and after (B) olfactory predator learning.

hour learning period based on previous predator learning studies in amphibians and fish (Chivers and Smith 1994; Ferrari et al. 2011). All experimental procedures and data analyses were the same as in Experiment 1. All experiments were conducted between 09:00 and 18:00.

Statistical analysis

We normalized all the data related to the time spent moving as log (time spent moving pre-exposure odor (seconds) + 10) / log (time spent moving post-exposure odor (seconds) + 10) × 100. The distance travelled was normalized in the same manner. We conducted a normality test (Shapiro–Wilk test, $p > 0.05$) and found the data to be normally distributed. We identified the outliers using Tukey fences (values ≤ Q1–1.5 × interquartile range (IQR) or values ≥ to Q3 + 1.5 × IQR) and removed two outliers from the *R. oxycephalus* odor group and four outliers from the *C. similis* odor group in Experiment I. Ultimately, we analyzed data from 28 larvae in the *R. oxycephalus* odor group and 25 larvae in the *C. similis* odor group. One-way analysis of variance was used to analyze the responses of *O. koreanus* larvae to *R. oxycephalus* and *C. similis* odors, followed by post-hoc comparisons (Tukey's honestly significant difference, $p < 0.05$).

For Experiments 2 and 3, we normalized the data in the same manner as previously described. After the normality test, we identified outliers and removed four and five larvae from the control and experimental groups, respectively. We analyzed data from 24 and 25 *O. koreanus* larvae in the control and experimental groups, respectively. We used a general linear model with BW and SVL as covariates to compare antipredator responses between the groups exposed to either 0% odor (water) or 100% *R. oxycephalus* odor in Experiment 2 and between the control and experimental (olfactory predator learning) groups in Experiment 3. All statistical analyses were performed using IBM SPSS version 26.0 (IBM Co., Armonk, NY, USA).

Results

Experiment 1: antipredator response to potential predator odor

Onychodactylus koreanus larvae exhibited significantly reduced time spent moving ($F_{3,\ 108} = 8.70$, $p < 0.001$; Fig. 2) and distance travelled ($F_{3,\ 108} = 10.86$, $p < 0.001$; Fig. 2) when exposed to different *R. oxycephalus* odor concentrations. The differences in the time spent moving and distance travelled between the three different *R. oxycephalus* odor concentrations (100%, 50%, and 25%) were not significantly different ($p > 0.05$; Fig. 2). The time spent moving ($F_{3,96} = 0.61$, $p = 0.609$; Fig. 2) and distance travelled ($F_{3,96} = 0.06$, $p = 0.981$; Fig. 2) by *O. koreanus* larvae exposed to *C. similis* odor did not significantly change.

Experiment 2: innate antipredator response to predator odor

Less than one-year-old *O. koreanus* larvae exhibited significantly reduced time spent moving ($F_{1,\ 47} = 9.38$, $p = 0.004$; Fig. 3) and distance travelled ($F_{1,\ 47} = 12.54$, $p < 0.001$; Fig. 3) when exposed to *R. oxycephalus* odor but not when exposed to water ($p > 0.05$; Fig. 3).

Experiment 3: no enforcement of antipredator response by predator olfactory learning

In June, after predator olfactory learning, the control and experimental groups did not exhibit different antipredator responses in the time spent moving ($F_{1,\ 47} = 1.93$, $p = 0.171$; Fig. 3) and distance travelled ($F_{1,\ 47} = 2.83$, $p = 0.100$; Fig. 3) when exposed to 100% *R. oxycephalus* odor.

Discussion

In this study, we identified the potential predators of *O. koreanus* larvae through antipredator response experiments involving exposure to predator odors (*R. oxycephalus* and *C. similis*). *Onychodactyuls koreanus* larvae sub-

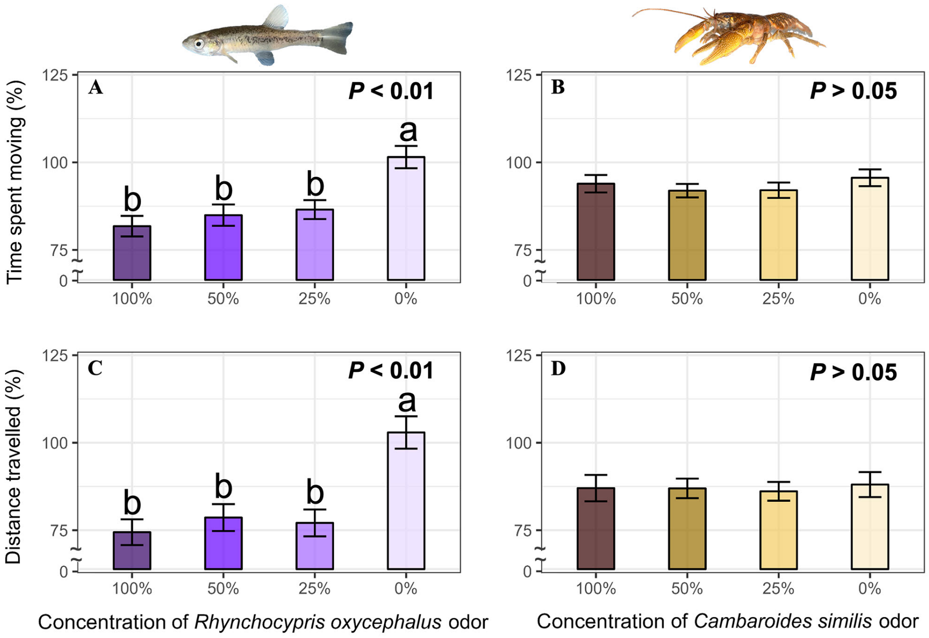

Fig. 2 The proportion of time spent moving (A, B) and distance travelled (C, D) by *Onychodactylus koreanus* larvae when exposed to the odor of *Rhynchocypris oxycephalus* (A, C) and *Cambaroides similis* (B, D). Different alphabets on the bars indicate statistically different values ($p < 0.05$).

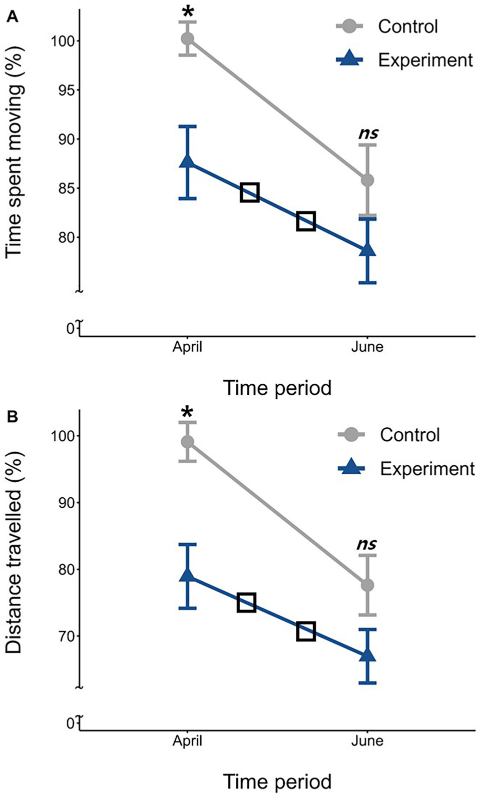

Fig. 3 Antipredator response (A, time spent moving and B, distance travelled) of *Onychodactylus koreanus* larvae before (in April) and after (in June) two instances of olfactory predator (*Rhynchocypris oxycephalus*) learning. The symbol of square indicates olfactory predator learning. *Indicates a significant difference at $p < 0.01$ and ns indicates no significant difference.

stantially decreased their time spent moving and the distance travelled when exposed to *R. oxycephalus* odor. This reduction is a typical antipredator response observed in amphibians (Mathis and Vincent 2000). These results suggest that *O. koreanus* larvae recognize *R. oxycephalus* as a predator. In Experiment 1, all tested *R. oxycephalus* odor concentrations above 25% induced significant antipredator responses. Although *R. oxycephalus* odor concentration in mountainous streams is unknown, these results suggest that all *R. oxycephalus* odor concentrations above 25% are stimuli above the threshold for *O. koreanus* larvae. *Amatitlania nigrofasciata* fish and *Cryptobranchus alleganiensis alleganiensis* salamander were previously exposed to different predator odor concentrations, and both species demonstrated the same magnitude of response above a certain concentration (Brown et al. 2014; Kenison and Williams 2018). In various species, the presence of predators limits the distribution range of prey or causes the prey to shift habitats (Kats and Sih 1992; Petranka 1983). Our results indicate that *R. oxycephalus* could be a predator of *O. koreanus* larvae and that the existence of *R. oxycephalus* in mountain streams potentially forces *O. koreanus* larvae to move to the upper mountain streams where *R. oxycephalus* individuals are absent.

The *O. koreanus* larvae did not respond to any *C. similis* odor concentrations. Therefore, *C. similis* may not be a predator of the *O. koreanus* larvae. *Procambarus clarkia* crayfish was previously found to prey on *E. sosorum* salamanders (Davis et al. 2017), indicating that crayfish can be a predator of salamanders. Several explanations exist for this difference in the results. First, the individuals of *C. similis* and *O. koreanus* larvae used in the experiment were collected from Yanggu and Chuncheon, respectively; therefore, *O. koreanus* larvae may not respond to *C. similis* odor. However, considering that the antipredator response of *O. koreanus* larvae is innate (see below), this possibility is unlikely. Second, in a study on *C. japonicus* crayfish, this species was found to rarely feed on salamander eggs, which were attached to rocks, or slow-moving snails (Sato 1990). Considering the fast movement of *O. koreanus* larvae, *C. similis* individuals could not prey the *O. koreanus* larvae. Therefore, although they share similar habitats, *C. similis* may not be a predator. Third, an open-field test of the antipredator response of *C. similis* may not be an appropriate method. Measuring the time of stay within the shelter is sometimes more appropriate for testing antipredator responses in some amphibians (Zabierek and Epp 2016).

Less than one-year-old *O. koreanus* larvae showed a considerable antipredator response to 100% of *R. oxycephalus* odor even without prior experience with *R. oxycephalus*. This suggests that the antipredator response to *R. oxycephalus* odor may be innate. In the previous study, three *Rana temporaria* tadpole populations were previously found to exhibit an innate antipredator response to *Perca fluviatilis* perch and *Aeshna juncea* dragonfly larvae odors, possibly because of their historical co-evolution of predator–prey interaction (Laurila 2000). *Onychodactylus koreanus* larvae innately recognizing *R. oxycephalus* might be another example of co-evolution, although this needs to be further tested. Innate antipredator responses can reduce potential threats from predators. Contrastingly, innate predator recognition may limit the number of detectable predator species (Wisenden 2003). In amphibians, novel predator recognition can occur through learning, although this requires a high initial predation rate (Mathis et al. 2008). Potential climate change or new artificially introduced predators could affect these aspects of antipredator recognition in mountain salamanders.

Predator olfactory learning did not considerably enhance the antipredator response of *O. koreanus* larvae to *R. oxycephalus* odor. This result suggests that predator olfactory learning did not reinforce the innate antipredator response of *O. koreanus* larvae to *R. oxycephalus* odor. Two possible explanations exist for this observation. First, additional predator olfactory learning will not enhance the antipredator response if the innate recognition of predator odors co-evolves. Co-evolution is known to underlie innate olfactory recognition in several amphibians, such as salamanders (*Plethodon cinereus*, *Eurycea nana*, and *E. sosorum*) (Anthony et al. 2007; DeSantis et al. 2013; Epp and Gabor 2008) and frogs (*Rana nigromaculata*, *R. limnocharis*, and *R. dalmatina*) (Hettyey et al. 2016; Zhang et al. 2015). Although reinforcing antipredator responses after predator olfactory learning has been reported in *Desmognathus quadramaculatus* and *Cryptobranchus alleganiensis*, this case was found only when novel predator odor was used (Crane and Mathis 2011; Dempsey et al. 2021). Another possibility is the long interval between learning and testing and/or a few learning sessions. Typically, the antipredator response diminishes over time after olfactory learning by predators. For example, *Rana sylvatica* tadpoles were taught the odor of *Ambystoma tigrinum* salamanders one, two, or four times. After 11–12 days, the effect of the antipredator response substantially decreased only when the tadpoles were taught the odor once or twice (weaker and earlier, respectively) (Ferrari et al. 2012).

Conclusions

Our results suggest that *R. oxycephalus* is a predator of *O. koreanus* larvae, whereas *C. similis* is not. Additionally, the antipredator response of *O. koreanus* to *R. oxycephalus* odor is innate. This innate antipredator response was not reinforced by additional olfactory learning in *R. oxycephalus*. Considering their secret life history, including underground breeding, further studies on *O. koreanus*'s olfactory system could enhance our understanding of how they locate potential mates, find breeding places, and limit their distribution by recognizing predators in mountain streams. In addition, our results could be useful to study prey-predator co-evolution in mountain streams as well as why *O. koreanus* have such a long larval period as two or three years unlike other Hynobiid salamanders.

Abbreviations

SVL: Snout-vent length
BW: Body weight

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Authors' contributions

JC did sample collection, data curation, investigation, formal analysis and writing-original draft. JK did supervision, sample collection, formal analysis, and writing-review and editing. HK sample collection, data curation, and writing-review and editing. JP sample collection, data curation, and writing-review and editing. DP did conceptualization, supervision, writing-original draft, and writing-review and editing. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was reviewed and approved by the Institutional Animal Care and Use Committee of Kangwon National University (KW-221006-2).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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