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Correspondence

Overwintering larvae of *Hynobius retardatus* salamanders can consume invertebrate prey at very low temperatures

Aya Yamaguchi[1] & Osamu Kishida[2]

[1] Graduate School of Environmental Science, Hokkaido University, Toikanbetsu, Horonobe, Hokkaido 098-2943, Japan
[2] Teshio Experimental Forest, Field Center for Northern Biosphere, Hokkaido University, Toikanbetsu, Horonobe, Hokkaido 098-2943, Japan

Correspondent author: Aya Yamaguchi, e-mail: cryamaguchi.514@gmail.com

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Thirty-three species of salamanders of the genus *Hynobius*, family *Hynobiidae*, are known to be distributed in East Asia (Sparreboom 2014). Life histories of *Hynobius* species are categorized into stream-type or pond-type, according to their preferred type of spawning site and larval habitat. The duration of the larval period varies greatly from several months to several years between and within the individual *Hynobius* species, regardless of their life-history types, and overwintered larvae of most *Hynobius* species are frequently observed in aquatic habitats. The larvae of many pond-type species facultatively overwinter, depending on environmental conditions (Kusano 1981, Goris & Maeda 2004, Michimae 2011). Because pond-type species are found either in boreal regions or at high altitudes in temperate regions, overwintering larvae must be able to withstand extremely low water temperatures in ponds covered with ice. The question arises, are those larvae actively carnivorous in winter pond ecosystems? Although the answer to this question is important to our understanding of the ecology of *Hynobius* salamanders, to our knowledge, the possible foraging activity of *Hynobius* larvae during very cold periods has never been documented. In this study, we experimentally tested the foraging activity of *H. retardatus* larvae at very low temperatures corresponding to winter pond conditions.

Hynobius retardatus Dunn, 1923 (Hokkaido salamander) is a typical pond-type species that is endemic to Hokkaido Island, Japan. The foraging ecology of *H. retardatus* larvae has been extensively investigated because they can have a strong predatory impact on their prey communities (Ohdachi 1994, Kohmatsu 2001, Kishida et al. 2014, Takatsu & Kishida 2015). During warm periods of the year (spring to fall), *H. retardatus* larvae generally feed on amphibian larvae, including conspecifics, as well as on aquatic invertebrates such as mayfly and chironomid larvae (Kohmatsu 2001, Kishida et al. 2009, Kishida et al. 2014). However, we have no knowledge of their foraging ecology in winter, even though *H. retardatus* larvae conditionally overwinter in their spawning ponds. Some larvae will have metamorphosed by the fall of their first year (Fig. 1), while others pass their first winter in the pond and metamorphose only in the following year, depending on biotic and abiotic conditions encountered in the pond (Iwasaki & Wakahara 1999, Michimae 2011). Hokkaido Island is located in a boreal climate region, and the average temperature in most areas of the island during January and February will lie below 0°C (but > -30°C). As a consequence, the natural ponds inhabited by the larvae are covered with ice during winter. Under the ice, water temperatures remain very low (near 0°C), And such low values will typically cause an poikilothermic species to be less active. In this study, we conducted an experiment in which we examined whether *H. retardatus* larvae would feed on offered prey when maintained at low temperatures (i.e., 2 or 4°C).

We used mayfly larvae (*Cloeon ryogokuense*) as a prospective wintertime prey species, because mayfly larvae are a common prey item of *H. retardatus* larvae from spring to fall (O. Kishida unpubl. data), and they are widely observed in both early spring and late fall, suggesting that they are present in the ponds during the winter. We selected temperatures of 2 and 4°C, respectively, for our experiments for the following reasons: Because water density is highest at a temperature of approximately 4°C, water of that temperature will descend to the bottom of the water column, whereas water of less than 4°C rises and freezes at the surface (Moore et al. 2009). Because *H. retardatus* larvae typically rest on the bottom of the pond (O. Kishida pers. obs.), it is likely that overwintering larvae will typically stay in that temperature zone in mid-winter. On the other hand, although overwintering *H. retardatus* larvae are not likely to spend very much time in the middle or upper water layers with temperatures below 4°C, we alternatively applied a water temperature of 2°C in order to examine the foraging capability of overwintering *H. retardatus* larvae at such an extremely low temperature.

We collected a sufficient number of mayfly larvae and 33 overwintering *H. retardatus* larvae from several ponds in the Teshio Experimental Forest of Hokkaido University, Hokkaido, Japan, on 14 and 15 November 2013. We took photographs of 46 randomly selected mayfly larvae and measured their lengths on the digitised images by using ImageJ 1.45s software (Schneider et al. 2012). The mean ± SD snout–vent length of these mayfly larvae was 4.9 ± 1.0 mm (N = 46). Similarly, we scanned all *H. retardatus* larvae in ventral aspect (Canon, CanoScan 9000F Mark II) and measured in the same manner their lengths on the digitised images. The mean ± SD snout–vent length of the *H. retardatus* larvae was 24.9 ± 2.7 mm (N = 33). Before the experiment, we kept the mayfly larvae and *H. retardatus* larvae in separate 4-l storage containers filled with 2 l of matured tap water and stored in a refrigerator at 4°C (i.e., five salamander larvae or about 100–200 mayfly larvae were kept in each storage container). The water in all containers was replaced on 4 December. On 18 December 2013, we placed the *H. retardatus* larvae individually into small plastic containers (length × width × height: 84 × 57 × 44 mm) filled with 100 ml of matured tap water, which were kept in the refrigerator at 4°C. After 4 days, we added two mayfly larvae to each container as potential prey items for the resident *H. retardatus* larvae. We kept the larvae in these containers until 18 January 2014, when they were assigned to experimental units.

Figure 1. Overwintering larva of *Hynobius retardatus* (right) and a hatchling from the current year (left) on 4 June 2007.

Each experimental unit was a 4.5-l (285 × 165 × 95 mm) aquaria filled with 2 l of matured tap water that was exposed to a natural photoperiod of 10:14 hours. We performed 15 replicates of each of four trials, by ticking off the presence or absence of a *H. retardatus* larva at a temperature of 2 or 4°C as follows: (1) no salamander at 2°C (2°C-NS), (2) one salamander at 2°C (2°C-S), (3) no salamander at 4°C (4°C-NS), and (4) one salamander at 4°C (4°C-S). Each *H. retardatus* larva was randomly assigned to either the 2 or 4°C temperature exposure. Following a 5-day acclimation period, we added two mayfly larvae that were randomly selected from the storage containers to all experimental tanks on 18 January (the starting day of the experiment). The realized density ($0.28/m^3$) of mayfly larvae was within the range of their observed density in natural ponds (K. Takatsu pers. comm.). The effective average temperatures of the 2 and 4°C experiments were 2.3 ± 0.4°C (N = 5) and 3.8 ± 0.3°C (N = 5), respectively. We counted the number of surviving mayfly larvae after 2, 5, 8, and 11 days (i.e., we checked how many mayfly larvae had disappeared by each census day). At these census times, we removed all surviving mayfly larvae from the experimental tanks and released two fresh mayfly larvae randomly selected from the storage containers into each tank. To assess whether the *H. retardatus* larvae predated on the mayfly larvae, we used a chi square test in which the null hypothesis was that the disappearance of mayfly larvae occurred independently of whether a *H. retardatus* larva was present during the 11-day experimental period. As a result, the null hypothesis was clearly rejected ($\chi^2 = 83.17$, df = 1, $p < 0.0001$). Indeed, in the absence of *H. retardatus* larva (i.e., in the 2°C-NS and 4°C-NS control group setups), all mayfly larvae survived, whereas in the presence of a predator, 1.22 ± 0.30 and 1.15 ± 0.41 (mean ± SD) individual mayfly larvae disappeared between census days in the 2 °C-S and 4°C-S setups, respectively. Analysis of variance revealed that the feeding activity of *H. retardatus* larvae did not differ between the two temperature regimes ($F_{1,28} = 0.261$, $p = 0.61$). These results indicate that overwintering *H. retardatus* larvae are capable of consuming small aquatic invertebrates at very low temperatures.

Although several studies have suggested that overwintering salamander larvae of the genus *Hynobius* (Kusano et al. 1985, Kishida & Tezuka 2013) and *Ambystoma* (Stenhouse 1985, Urban 2007, Anderson et al. 2013) play important predatory roles in aquatic ecosystems, these studies investigated the predatory impacts of the overwintering larvae only during the warmer seasons from spring to fall. For example, in spring, cannibalism by overwintering *H. tokyoensis* larvae can greatly reduce the abundance of conspecific hatchlings (Kusano et al. 1985), and predation by overwintering *Ambystoma opacum* larvae can be the most important factor in reducing the abundance of the hatchling cohort of *A. maculatum* (Stenhouse 1985, Urban 2007). Our results suggest that overwintering *H. retardatus* larvae may actively forage under the ice of a natural pond during winter. Thus, *H. retardatus* larvae may have a significant predatory impact on the prey community in a pond not only during the warm seasons of the year but also during winter. To better understand the role of salamander larvae in pond ecosystems all year round, future studies should examine whether predation by overwintering salamander larvae reduces the abundance of prey species in natural ponds during winter. In addition, an examination of the possible contribution of prey consumption in winter on the growth of overwintering larvae would be informative, because both prey-capturing ability and food intake may influence the growth of salamander larvae (Nosaka et al. 2015, Takatsu & Kishida 2015). Moreover, if overwintering salamander larvae grow substantially during winter, their predatory effects in the subsequent season would likely be intensified. Therefore, the next step should be to explore the trait- and community-level consequences of winter-foraging by overwintering larvae of *H. retardatus* and other salamander species.

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