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A Latitudinal Variation in Growth Rate Caused by the Differences in the Length of the Period Suitable for Larval Growth in the Japanese Black Salamander, *Hynobius nigrescens*

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In ectotherms, body size differences between latitudes resulting from shorter activity periods at higher latitudes may disappear due to higher growth rates. Although such latitudinal variations have been examined for various taxa, only a few studies have examined such variations in Japanese vertebrates. *Hynobius nigrescens* is widely distributed in the northern part of Honshu, Japan, and although their larval period in the wild is shorter at high latitudes, there is no latitudinal variation in the body size of juveniles in the wild. Therefore, larvae may grow faster at high latitudes, and they can grow into juveniles with body sizes similar to those at low latitudes. To examine whether the growth rate of larvae is faster at high latitudes, we reared groups at 15°C and 20°C. We found that the larval period was shorter and the growth rate was greater at high latitudes. Next, we examined whether the body sizes of reared individuals differed between latitudes. We found that the body sizes of metamorphosed juveniles were smaller at high latitudes. Some ectotherms are known to feed more at high latitudes, leading to the disappearance of latitudinal differences in body size between latitudes. In this study, we provided the same amount of food regardless of latitude, which might have caused the differences in body size between latitudes. Our study suggested that the high growth rate at relatively high latitudes can compensate for the short activity period, leading to similar body sizes of juveniles among latitudes.

Key words: body size, larval stage, latitudinal compensation, salamander, water temperature

INTRODUCTION

Many organisms with broad distributions exhibit latitudinal variations in phenotypes such as body size (Bergmann, 1847; Ashton, 2002; Ashton and Feldman, 2003; Kojima et al., 2020; Campbell et al., 2021; Romano et al., 2021). For instance, the body sizes of endothermic animals such as mammals are known to be larger in cooler regions at high latitudes because individuals with lower surface area-to-volume ratios have greater heat retention (Bergmann, 1847). Such a variation in body size among latitudes has been viewed as a result of responses to the environment, such as differences in temperature. In some ectotherms, such as fish and amphibians, latitudinal variation in which body sizes are smaller at high latitudes, known as the converse Bergmann's rule, has also been observed (Belk and Houston, 2002; Ashton and Feldman, 2003; Cvetković et al., 2009). This pattern is explained by the shorter activity period at high latitudes than at low latitudes, which limits the time available for feeding, growth, and development, resulting in

a smaller body size at high latitudes (Blanckenhorn and Demont, 2004). However, because small body size has negative effects on fitness, such as increasing predation risk and decreasing the number of eggs produced, some types of adaptations, such as high growth rates and resistance to cold temperature, may occur to compensate for the short activity period; i.e., latitudinal variation in body size may disappear. In addition, some ectotherm species are known to grow faster at high latitudes than at low latitudes, leading to the disappearance of body size differences between latitudes in a phenomenon known as latitudinal compensation (Yamahira et al., 2007). Although the disappearance of body size differences between latitudes due to faster growth rates at high latitudes has been investigated for various taxa (Dehnel, 1955; Conover et al., 1990; Yamahira et al., 2007; Campos et al., 2009), only a few studies have examined such variation in the Japanese archipelago, which covers a wide latitudinal range in the middle latitudinal region and is considered appropriate for such studies. The Japanese black salamander (*Hynobius nigrescens*) is suitable for studying latitudinal compensation of body size. *Hynobius nigrescens* is widely distributed in the northern part of Honshu, Japan (approximately 35.6°N to 41.1°N latitude),

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and the breeding season varies among regions from the beginning of February to the end of July (Sato, 1943; R. Morii, personal observation). The larvae hatch out of the egg sacs in 4–5 weeks, and their metamorphosis is completed in September (Sato, 1943). We found that the eggs of *H. nigrescens* hatch in mid-May and metamorphose in late August in Aomori Prefecture at high latitude, while they hatch in early April and metamorphose in late August in Ishikawa Prefecture at low latitude (R. Morii, personal observation). Thus, hatch timing is later at high latitudes than at low latitudes, whereas the timing of metamorphosis does not differ among latitudes, leading to a shorter growing period at high latitudes. Nevertheless, we did not find a difference in body size (snout-vent length, hereinafter referred to as "SVL"; Fig. 1) of metamorphosed juveniles in the wild between latitudes (GLM: $t = -0.48$, $P = 0.636$; high latitude (site 7): 31.30 ± 2.93 mm (SD), $n = 10$; low latitude (sites 1 and 3): 32.11 ± 5.03 mm, $n = 24$; Fig. 2). These results suggest that a greater growth rate compensates for the shorter period of larval growth at high latitudes, leading to the disappearance of latitudinal variation in the body size of juveniles. The purpose of this study was to determine whether populations of Japanese black salamander at high latitudes have a greater growth rate during the larval stage than those at low latitudes, leading to the similar body sizes of juveniles among latitudes. First, we compared the water temperature during the larval stage among latitudes. Next, to examine whether the growth rate was greater at high latitudes than at low latitudes and to determine whether there was a difference in body size among juveniles at different latitudes, we compared the growth rates and body sizes of juveniles via a common garden experiment under laboratory conditions.

MATERIALS AND METHODS

Measurement of water temperature during the larval stage

To examine the water temperature during the larval stage at

each latitude, the water temperature was measured at four sites at high and low latitudes every 12 hours using data loggers (HOBO MX2201; Onset Computer Corporation, Bourne, MA, USA). Sites were selected from ponds with similar altitudes ranging from 36 m to 412 m to examine the differences in water temperature during the larval stage between latitudes without altitudinal differences. At high latitude, the water temperature was measured at site 1 (Aomori, Aomori Prefecture) and site 3 (Nishimeya, Aomori Prefecture; Fig. 2). At low latitude, the water temperature was measured at site 6

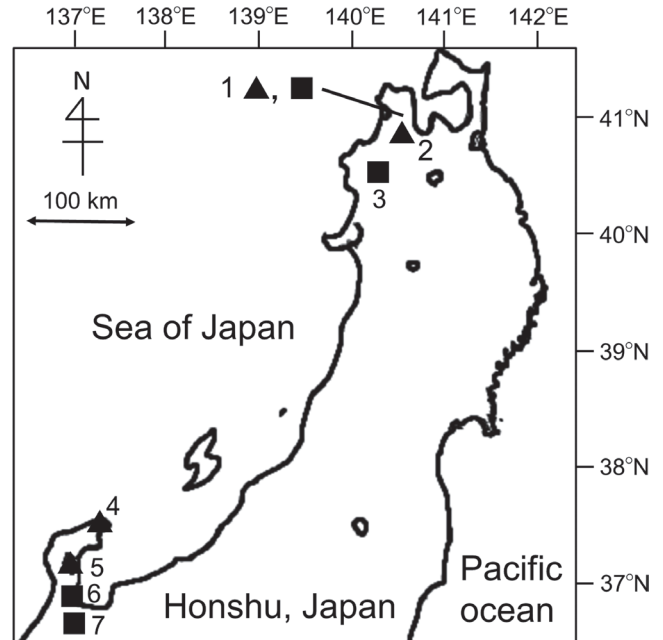


Fig. 2. Sites used in this study. Triangles indicate the sites where the samples for the common garden experiment were collected, and squares indicate the sites where water temperature was measured using data loggers.

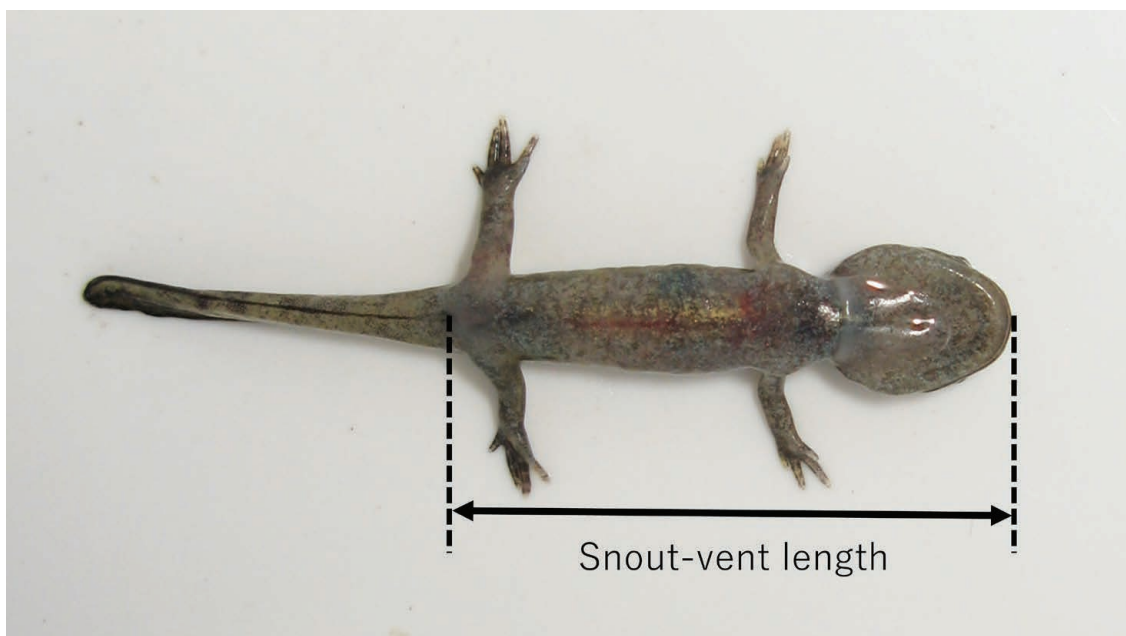


Fig. 1. Body parts measured in this study. The length from the snout to the vent is called the snout-vent length (SVL).

(Takaoka, Toyama Prefecture) and site 7 (Oyabe, Toyama Prefecture; Fig. 2). Each logger was attached to a fishing line 10 cm above a sinker (53 g), which was attached to the tip of the fishing line and sunk into each pond. The water temperature was measured at site 1 from 5 May to 25 October, at site 3 from 4 May to 16 October, at site 6 from 6 March to 30 September, and at site 7 from 5 March to 30 September in 2021. We compared water temperature during the period from larval hatching to the completion of metamorphosis in the wild between latitudes (low latitude: 1 April to 31 August; high latitude: 15 May to 31 August).

To examine whether there was a difference in water temperature during the larval stage between latitudes, we fit generalized linear models (GLMs) using R 4.2.0 (R core Team, 2022). We used mean water temperature per day during the larval stage as the response variable, and latitude (i.e., high or low latitude) and the number of days from hatched day as the explanatory variables. The normality was checked using the Shapiro–Wilk test. According to the results of the Shapiro–Wilk test, the normality was significantly rejected ($P < 0.001$); we assumed that the response variable was gamma distributed.

Common garden experiment

We conducted a common garden experiment using individuals from both high and low latitudes to examine whether the growth rate was faster at high latitudes. The study sites were selected from ponds with similar altitudes ranging from 36 m to 313 m to examine the differences in body size and growth rate between latitudes without altitudinal differences. We randomly collected 10 pairs of egg sacs of *H. nigrescens* at site 4 (Suzu, Ishikawa Prefecture) and site 5 (Hakui, Ishikawa Prefecture) as low-latitude sites in March 2019 and at site 1 (Aomori, Aomori Prefecture) and site 2 (Gosyogawara, Aomori Prefecture) as high-latitude sites in April 2019 (10 pairs of egg sacs \times four sites = 40 pairs of egg sacs in total; Fig. 2). We collected egg sacs in which the embryo was in the tail bud stage (i.e., with stage numbers ranging from 21 to 32: Iwasawa and Yamashita, 1991). We kept the egg sacs in plastic cases (150 mm \times 100 mm \times 50 mm high) filled with aged tap water in the experimental room at 20°C until they hatched. Just before hatching, we extracted two eggs from each pair of egg sacs (80 eggs from 40 pairs of egg sacs collected at four sites in total) and reared one of two eggs in a growth chamber at 15°C and the other egg in a growth chamber at 20°C (15°C: 20 individuals from two low-latitude sites and 20 individuals from two high-latitude sites; 20°C: 20 individuals from two low-latitude sites and 20 individuals from two high-latitude sites). We reared each individual separately in a circular plastic case (diameter: 100 mm \times height: 45 mm, water depth 25 mm) from just before hatching to metamorphosis. As food for the experimental groups, we provided tadpoles of montane brown frogs (*Rana ornativentris*) and frozen brine shrimp (Clean Brine Shrimp, 32 cubes, Kyorin Co.). The food content, feeding frequency, and amount of food are shown in Table 1. The water in the plastic case was changed every 3 days using aged tap water. We

measured the SVLs of hatched larvae (within 24 hours after hatching; developmental stage no. 40 to 41 in Iwasawa and Yamashita, 1991) and metamorphosed juveniles (within 3 days after metamorphosis; developmental stage no. 68 in Iwasawa and Yamashita, 1991) and recorded the number of days from hatching to metamorphosis. The growth rate per day was calculated by dividing the difference between the SVL of metamorphosed juveniles and that of hatched larvae by the number of days from hatching to metamorphosis ([SVL of metamorphosed juvenile – SVL of hatched larva]/the number of days from hatching to metamorphosis). Individuals that did not feed on the prescribed amount of food (100% of tadpoles and more than 80% of brine shrimp; Table 1) were removed from the rearing experiment.

We used R 4.2.0 (R core Team, 2022) for the statistical analysis. The normality was checked using the Shapiro–Wilk test. First, we fit a GLM with the SVL of hatched larvae as the response variable and latitude (high or low latitude) as the explanatory variable to examine whether there were differences in the body sizes of hatched larvae between latitudes used in the experiment. According to the results of the Shapiro–Wilk test, the normality was significantly rejected ($P = 0.013$); we assumed that the response variable was gamma distributed.

Next, we fit a GLM with the number of days from hatching to metamorphosis as the response variable and latitude (high or low latitude), rearing temperature (15°C or 20°C), and the interaction between latitude and rearing temperature as the explanatory variables to examine whether the number of days from hatching to metamorphosis was shorter at high latitudes than at low latitudes. According to the results of the Shapiro–Wilk test, the normality was significantly rejected ($P < 0.001$); we assumed that the response variable was gamma distributed. All candidate models were compared by model selection using the library “MuMIn”, and the best models with the lowest AIC scores were selected.

We fit a GLM with growth rate per day as the response variable and latitude (high or low latitude), rearing temperature (15°C or 20°C), and the interaction between latitude and rearing temperature as the explanatory variables to examine whether the growth rate was greater at high latitudes than at low latitudes. According to the results of the Shapiro–Wilk test, the normality was not significantly rejected ($P = 0.094$); we assumed that the response variable was Gaussian distributed. All candidate models were compared by model selection using the library “MuMIn”, and the best models with the lowest AIC scores were selected.

We fit a GLM with the SVL of metamorphosed juveniles in common garden experiments as the response variable and latitude (high or low latitude), rearing temperature (15°C or 20°C), and the interaction between latitude and rearing temperature as the explanatory variables to clarify whether there was no latitudinal variation in the SVL of metamorphosed juveniles in common garden experiments. According to the results of the Shapiro–Wilk test, the normality was not significantly rejected ($P = 0.077$); we assumed that the response variable was Gaussian distributed. All candidate models were compared by model selection using the library “MuMIn”, and the best models with the lowest AIC scores were selected.

If the growth rate is faster at high latitudes, the mortality risk may also be greater at high latitudes, and the mortality rates of reared individuals may be compared between latitudes. We fit Firth’s method with the library “logistf” with mortality (death, 0; survival, 1) as the response variable and latitude (high or low latitude), rearing temperature (15°C or 20°C), and the interaction between latitude and rearing temperature as the explanatory variables. Individuals that were removed from the rearing experiment because they did not feed on the prescribed amount of food were excluded from the analysis. We assumed that the response variable had a binomial distribution. All candidate models were compared by model selection using the library “MuMIn”, and the best models with the lowest AIC scores were selected.

Table 1. Rearing period, food, feeding frequency, and amount of food in the common garden experiment.

Rearing period	Food	Feeding frequency	Amount of food
Day 0–Day 15	<i>Rana ornativentris</i> (tadpole)	Once in 5 days	0.05 (g)
Day 15–Day 30	<i>Rana ornativentris</i> (tadpole)	Once in 3 days	0.05 (g)
Day 30–Day 51	<i>Rana ornativentris</i> (tadpole)	Once in 3 days	0.1 (g)
Day 51–metamorphosis	Brine shrimps	Once in 3 days	1/6 cube

RESULTS

Water temperature during the larval stage in the wild

Water temperature data were obtained at the four sites where data loggers had been placed (Fig. 3). The mean water temperatures during the larval stage were 19.14°C at site 1, 17.08°C at site 3, 14.24°C at site 6, and 14.70°C at site 7 (Table 2). In the GLM with mean water temperature per day during the larval stage as the response variable, a significant difference was detected between high and low latitudes ($t = -28.17, P < 0.001$). The number of days from hatched day was also significant ($t = 35.18, P < 0.001$). Therefore, the water temperature during the larval growth period in the wild was greater at high latitudes than at low latitudes.

Common garden experiment

Sixty-eight of the 80 individuals metamorphosed into juveniles, and a total of 12 individuals were removed from the common garden experiment; 11 individuals died and one individual did not feed on the prescribed amount of food. In the GLM with the SVL of hatched larvae as the response variable, a significant difference was not detected between high and low latitudes ($t = 1.281, P = 0.205$). Therefore, the body size of hatched larvae did not differ among latitudes (Fig. 4; high latitude: 9.16 ± 0.52 mm, low latitude: 9.31 ± 0.62 mm).

In the GLM with the number of days from hatching to metamorphosis as the response variable, latitude and rearing temperature were selected as explanatory variables by model selection, whereas the interaction between latitude and rearing temperature was excluded. A significant difference was detected between latitudes ($t = 8.13, P < 0.001$), and the number of days from hatching to metamorphosis was shorter at high latitudes than at low latitudes (Fig. 5; high latitude at

15°C: 95.25 ± 6.53 days, low latitude at 15°C: 109.35 ± 5.62 days, high latitude at 20°C: 66.30 ± 3.51 days, low latitude at 20°C: 79.07 ± 10.19 days). The rearing temperature was also significant ($t = -17.81, P < 0.001$), indicating that the number of days from hatching to metamorphosis was shorter when the larvae were reared at 20°C than at 15°C (Fig. 5).

In the GLM with growth rate as the response variable, latitude and rearing temperature and the interaction between latitude and rearing temperature were selected as explanatory variables by model selection. The interaction between latitude and temperature was significant ($t = -2.47, P = 0.016$), indicating that the growth rate of larvae was faster at high latitudes than at low latitudes, and this tendency was more prominent when the larvae were reared at 20°C than at 15°C (high latitude at 15°C: 0.18 ± 0.02 mm/day, low latitude at 15°C: 0.18 ± 0.02 mm/day, high latitude at 20°C: 0.22 ± 0.02 mm/day, low latitude at 20°C: 0.20 ± 0.03 mm/day; Fig. 6). Latitude and

Table 2. Estimations of the mean water temperature of the larval stage in the wild at each site and the number of days from hatching to metamorphosis based on the mean water temperature. The water temperature during the larval growth period in the wild was higher at high latitudes than at low latitudes. Additionally, the estimated number of days from hatching to metamorphosis was shorter at high latitudes than at low latitudes.

Site No.	Site name	Mean water temperature	Estimated number of days from hatching to metamorphosis
1	Aomori	19.14	70.9
3	Nishimeya	17.08	81.7
6	Takaoka	14.24	116.3
7	Oyabe	14.70	112.7

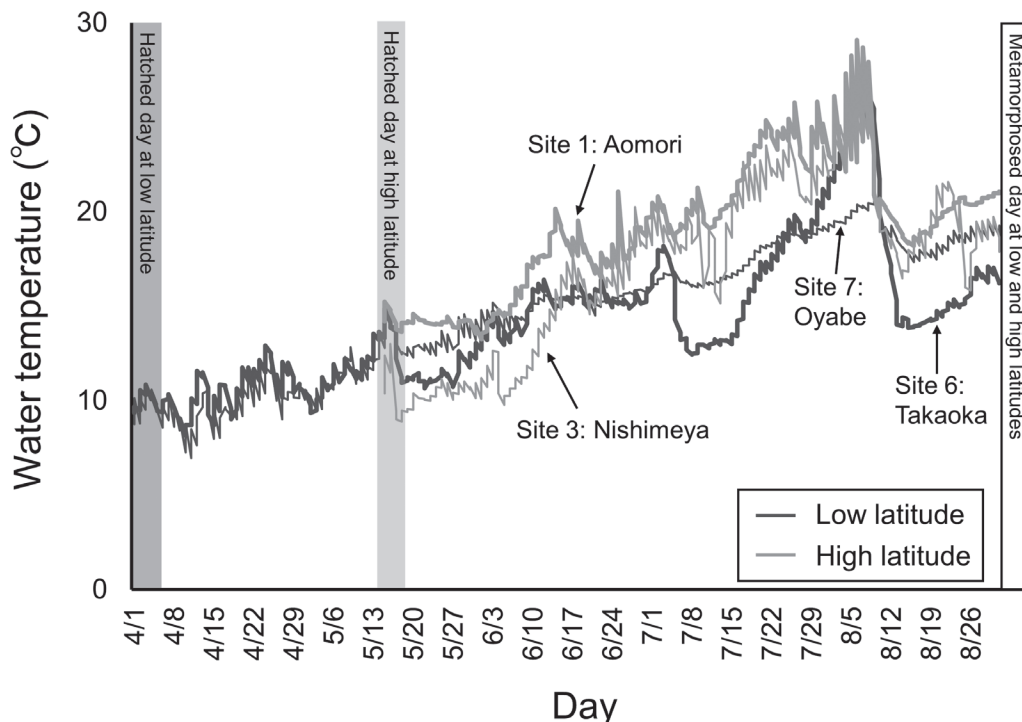


Fig. 3. Seasonal changes in water temperature during the larval stage at each site. Latitude had a significant effect on water temperature ($P < 0.001$), and the water temperature during the larval stage in the wild was higher at high latitudes than at low latitudes.

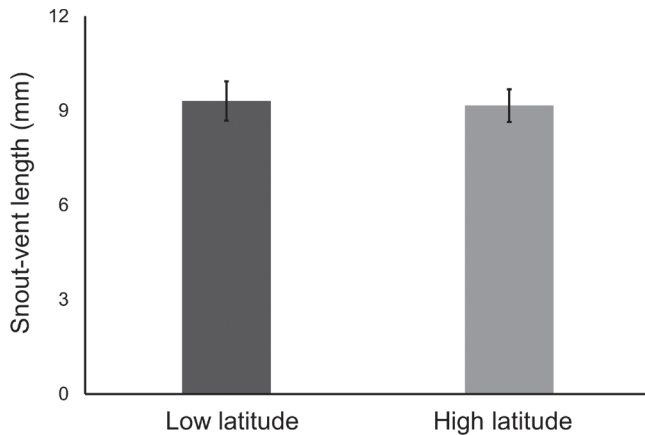


Fig. 4. Relationship between the SVL of hatched larvae and latitude in the common garden experiment. Error bars indicate standard deviations. Latitude did not have a significant effect on the SVL of hatched larvae ($P = 0.205$), and the body size of hatched larvae did not differ between latitudes.

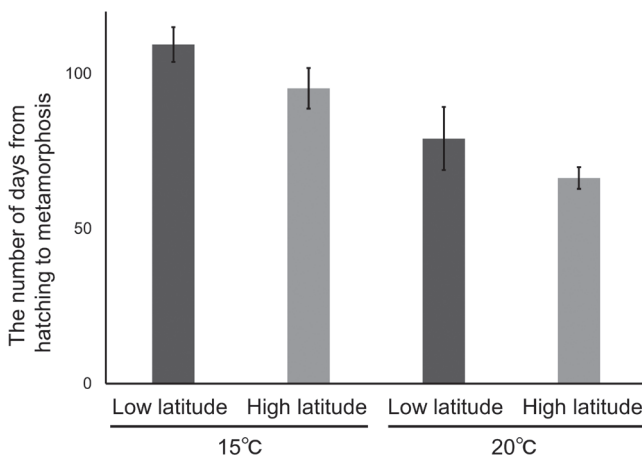


Fig. 5. Relationship between the number of days from hatching to metamorphosis and latitude in the common garden experiment. Error bars indicate standard deviations. Latitude and rearing temperature were selected as explanatory variables by model selection, whereas the interaction between latitude and rearing temperature was excluded. Latitude had a significant effect on the number of days from hatching to metamorphosis ($P < 0.001$), and the number of days from hatching to metamorphosis was shorter at high latitudes than at low latitudes.

rearing temperature were also significant (latitude: $t = 2.05$, $P = 0.045$; rearing temperature: $t = 6.45$, $P < 0.001$).

In the GLM with the SVL of reared juveniles as the response variable, latitude and rearing temperature and the interaction between latitude and rearing temperature were selected as explanatory variables by model selection. The interaction between latitude and rearing temperature was significant ($t = -2.43$, $P = 0.018$), indicating that the body size of juveniles after metamorphosis was smaller at high latitudes than at low latitudes, and this tendency was more prominent when individuals were reared at 15°C than at 20°C (high latitude at 15°C: 26.41 ± 1.13 mm, low latitude at 15°C: 28.80 ± 1.25 mm, high latitude at 20°C: 23.89 ± 1.10 mm, low latitude at 20°C: 24.95 ± 0.96 mm; Fig. 7). Latitude

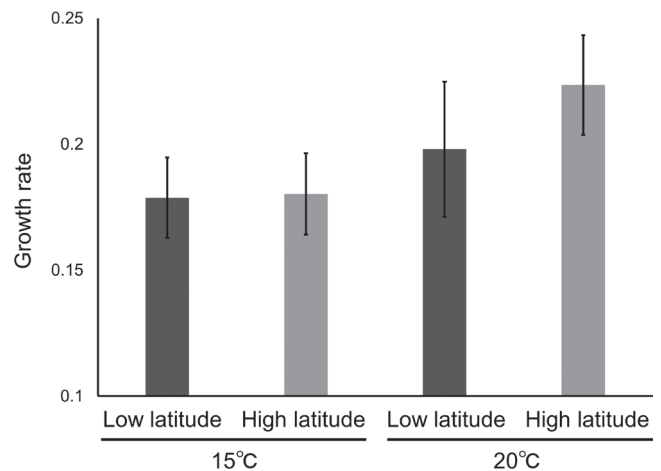


Fig. 6. Relationship between growth rates at the larval stage ($[\text{SVL of metamorphosed juveniles} - \text{SVL of hatched larvae}] / \text{the number of days from hatching to metamorphosis}$) and latitude in a common garden experiment. Error bars indicate standard deviations. Latitude, rearing temperature, and the interaction between latitude and rearing temperature were selected as explanatory variables for model selection. The interaction between latitude and rearing temperature had a significant effect on the growth rate ($P = 0.016$), indicating that the growth rate of larvae was faster at high latitudes than at low latitudes, and this tendency was more prominent when the larvae were reared at 20°C than at 15°C.

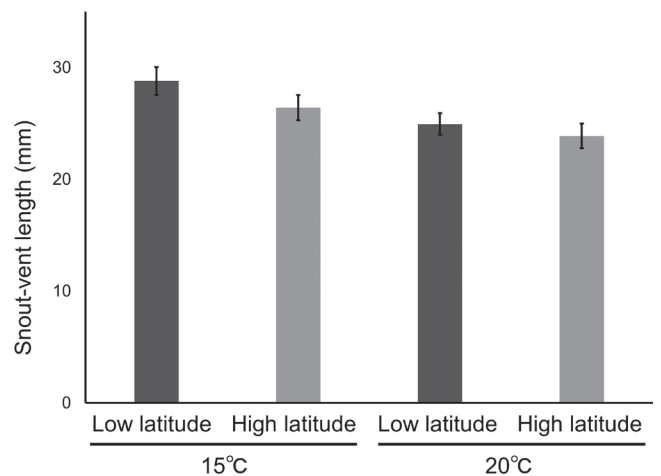


Fig. 7. Relationship between the SVL of juveniles and latitude in a common garden experiment. Error bars indicate standard deviations. Latitude, rearing temperature, and the interaction between latitude and rearing temperature were selected as explanatory variables. The interaction between latitude and rearing temperature had a significant effect on the SVL of juveniles ($P = 0.018$), indicating that the body size of metamorphosed juveniles was smaller at high latitudes than at low latitudes, and this tendency was more prominent when the larvae were reared at 15°C than at 20°C.

and rearing temperature were also significant (latitude: $t = 3.30$, $P = 0.002$; rearing temperature: $t = -6.73$, $P < 0.001$).

In the GLM using Firth's method with mortality as the response variable, only rearing temperature was selected as the explanatory variable by model selection, although no significant difference was detected ($P = 0.515$), whereas latitude and the interaction between latitude and rearing

temperature were excluded, indicating that the mortality of reared individuals did not differ between latitudes.

DISCUSSION

Our study revealed that the number of days from hatching to metamorphosis was shorter and the growth rate was greater at high latitudes than at low latitudes in *H. nigrescens* (Figs. 5, 6). These differences between latitudes were observed (Figs. 5, 6) under the same environmental (food and temperature) conditions, suggesting that the differences are genetically determined. At high latitudes, because environmental factors such as snow coverage limit the suitable period for larval growth to a short period of time, local adaptations such as a shorter number of days from hatching to metamorphosis and higher growth rates may occur. Although the disappearance of body size differences among latitudes due to higher growth rates at high latitudes has been studied for various organisms (Dehnel, 1955; Conover et al., 1990; Yamahira et al., 2007; Campos et al., 2009), only a few studies have examined such variations among Japanese vertebrates. Our study suggested that differences in growth rates between latitudes caused the disappearance of body size differences in *H. nigrescens*. Such latitudinal compensation of body size may be generally observed in Japanese vertebrates.

Although water temperature is normally considered to be lower at high latitudes than at low latitudes, the mean water temperatures during the larval stage in the wild were 19.14°C and 17.08°C at high latitudes but were 14.24°C and 14.70°C at low latitudes, indicating that water temperatures were higher at the sites at high latitudes than at the sites at low latitudes (Fig. 3). In addition, the number of days from hatching to metamorphosis was lower and the growth rate was higher when the animals were reared at 20°C compared with 15°C (Figs. 5, 6). The populations at high latitudes may only be able to survive in warmer habitats compared with those at low latitudes to compensate for the shorter period for larval growth at high latitudes due to the high growth rate at warm temperatures. To compare the number of days from hatching to metamorphosis at the mean water temperature at each site between latitudes, the number of days from hatching to metamorphosis at each water temperature was estimated by substituting the mean water temperature at each site into the equations for the relationship between the number of days from hatching to metamorphosis and the water temperature at low and high latitudes estimated in the GLM analysis. The results showed that the number of days differed between latitudes (from 31.0 to 45.4 days), with 70.9 days (site 1) and 81.7 days (site 3) at high latitudes and 116.3 days (site 6) and 112.7 days (site 7) at low latitudes (Tables 2, 3). The difference in the number of days from hatching to metamorphosis in the wild would be approximately 40 days between latitudes because hatching begins in mid-May at high latitudes and in early April at low latitudes. Thus, the difference in the number of days from hatching to metamorphosis between latitudes obtained in our common garden experiment should be the same as that in wild conditions. The metamorphosis occurs at the same seasonal time of the year (late August) regardless of latitude. This may be because the individuals that metamorphose after summer are likely to fail to overwinter due to the

Table 3. Differences in the number of days from hatching to metamorphosis estimated based on water temperature in the wild between latitudes. We calculated the number of days from hatching to metamorphosis by subtracting the number of days at the high-latitude site from that at the low-latitude site.

Low-latitude site – High-latitude site	Difference in the number of days from hatching to metamorphosis between latitudes
Site 6 – Site 1	45.4
Site 6 – Site 3	34.6
Site 7 – Site 1	41.8
Site 7 – Site 3	31.0

insufficient food intake on land after metamorphosis. These findings suggest that the results of our common garden experiment reflect the number of days from hatching to metamorphosis in the wild.

However, metamorphosed juveniles were smaller at high latitudes than at low latitudes (Fig. 7), although there was no difference in the body sizes of hatched larvae between latitudes (Fig. 4). When latitudinal compensation occurs, body size differences disappear due to higher growth rates at the cost of other factors at high latitudes. For example, some animal groups, such as fish, feed more regardless of predation risk at high latitudes, leading to no body size differences between latitudes (Suzuki et al., 2010). If the animals move around frequently to search for food, natural enemies can easily find them, and if they eat too much, they cannot escape quickly when attacked by natural enemies (Gotthard, 2000; Stoks et al., 2005; Suzuki et al., 2010). In the common garden experiment in this study, the same amount of food was provided regardless of latitude. In fact, the body size difference between wild individuals at high latitudes and individuals reared at 20°C collected at high latitudes (32.11 mm – 23.89 mm = 8.22 mm) was larger than that between wild individuals at low latitudes and individuals reared at 15°C collected at low latitudes (31.30 mm – 28.80 mm = 2.50 mm). Thus, individuals at high latitudes might not have had enough food to grow to the same body size as individuals at low latitudes in our common garden experiment.

Although we thought that the higher growth rate was related to higher mortality, mortality did not differ between latitudes in the common garden experiment, suggesting that the higher growth rate was not related to higher mortality at high latitudes. However, other effects, such as increased susceptibility to disease (De Block and Stoks, 2008) and lowered starvation endurance (Scharf et al., 2009), may also exist as trade-offs between the growth rate and these effects. Because the overwintering of larvae of *H. nigrescens* has been observed (Sato, 1943), more individuals may overwinter at high latitudes, where the suitable period for larval growth is shorter. However, we did not observe overwintering larvae at our surveyed sites, suggesting that most individuals do not overwinter regardless of latitude but metamorphose during their birth year. Therefore, it is unlikely that there were more overwintering larvae at the sites at high latitudes.

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COMPETING INTERESTS

We declare that we have no competing interests.

AUTHOR CONTRIBUTIONS

RM and HI conceived the project. RM, SY, and KH collected and analyzed the data. RM wrote the first draft of the manuscript. All authors contributed substantially to the revisions.

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