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Author(s)
Michimae, Hirofumi

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Plasticity in the timing of a major life-history transition and resulting changes in the age structure of populations of the salamander *Hynobius retardatus*

Hirofumi Michimae

Division of Life System Sciences, Faculty of Advanced Life Science, Hokkaido University, Sapporo 060-0810, Japan

Running title: Plasticity and age structure changes

Correspondence and present address: H. Michimae, Division of Biostatistics, School of Pharmaceutical Sciences, Kitasato University, Tokyo 108-8641, Japan

Tel.: +81-3-5791-6322

Fax.: +81-3-3444-2546

email: michimaeh@pharm.kitasato-u.ac.jp
Variation in age and size at life-history transitions is a reflection of the diversifying influence of biotic or abiotic environmental change. Examples abound, but it is not well understood how such environmental change can influence a population's age structure. I experimentally investigated the effects of water temperature and food type on age and body size at metamorphosis in larvae of the salamander *Hynobius retardatus*. In individuals grown at a cold temperature (15 °C) or given Chironomidae as prey, the time to metamorphosis was significantly prolonged and body size at metamorphosis was significantly enlarged compared with individuals grown at a warmer temperature (20 °C) or fed larvae. I also examined whether larval density (a possible indicator of cannibalism in natural habitats) generated variation in the age structure of natural populations in Hokkaido, Japan, where the climate is subarctic. Natural ponds in Hokkaido may contain larvae that have overwintered for 1 or 2 years, as well as larvae of the current year, and I found that the number of age classes was related to larval density. Although cool water temperatures prolong the larval period and induce later metamorphosis, in natural ponds diet-based enhancement of development translated into a shorter larval duration and earlier metamorphosis. Geographic variation in the frequency of cannibalism resulted in population differences in metamorphic timing in *H. retardatus* larvae. It is important to understand how environmental effects are ultimately transduced through individual organisms into population-level phenomena, with the population response arising as the summation of individual responses. Without a thorough comprehension of the mechanisms through which population and individual responses to environmental conditions are mediated, we cannot interpret the relationship between population-level and individual-level phenomena.
ADDITIONAL KEYWORDS: amphibian - cannibalism - metamorphosis -

overwintered larvae - phenotypic plasticity
Almost all life-history traits are phenotypically plastic (West-Eberhard, 2003). Variation in age and size at life-history transitions is a reflection of the diversifying influence of biotic or abiotic environmental change, and, because it is tightly linked to fitness, it is a central topic in life-history evolution (Roff, 2002). The effects of variable environmental factors on life-history parameters, from the viewpoint of plasticity in the timing of life-history transitions, have been extensively studied in amphibians, which have complex life cycles. The larvae of many species of amphibians cannot escape their aquatic environment until metamorphosis, and thus plasticity in metamorphic timing may be important in these species, especially those that develop in ephemeral ponds (Travis, 1983; Denver et al., 1998; Laurila & Kujasalo, 1999). Changes in biotic or abiotic environmental factors such as larval density (Newman, 1998), presence of predators (Laurila & Kujasalo, 1999; Lardner, 2000), type and quantity of available food (Alford & Harris, 1988; Hensley, 1993), habitat desiccation (Travis, 1983; Denver et al., 1998; Laurila & Kujasalo, 1999), and water temperature (Stahlberg et al., 2001, Hickerson et al., 2005) can affect rates of growth and development, and thus the duration of the larval period and size at metamorphosis (Wilbur, 1980; Werner, 1986; Rose, 2005). Despite an abundance of examples of environmental changes affecting age and size at life-history transitions, how such environmental changes influence the age structure of larval populations has rarely been investigated, and it not well understood. The salamander Hynobius retardatus, which lives in Hokkaido, Japan, where the climate is subarctic, has long been noted for its variable life history (Sasaki, 1924; Iwasaki & Wakahara, 1999). This species spawns from early April to May in ponds, and
hatchlings appear from late May to June (Sato & Iwasawa, 1993). Most larvae in small, ephemeral ponds metamorphose into terrestrial juveniles by late autumn (October) of the same year. However, individuals in permanent ponds, ones that seldom dry up, may retain larval features such as external gills and tail fins and overwinter once or twice in their aquatic habitat, not metamorphosing until their second or third year (Iwasaki & Wakahara, 1999). Thus, such a pond habitat may contain several year classes.

In amphibians, high water temperature is often associated with a rapid larval development rate and therefore rapid timing of metamorphosis. In contrast, low water temperatures lead to a slower development rate and delayed metamorphosis (Voss, 1993; Walsh et al., 2008). Overwintering larvae obviously experience cooler temperatures (Petranka, 1998; but not necessarily, see Freeman & Bruce, 2001), but a previous field study of *H. retardatus* has shown that not only the water temperature but also the stability of the water level in a pond significantly affects the timing of metamorphosis (Iwasaki & Wakahara, 1999).

Theoretical and empirical studies have shown that cannibalism can affect the life history of various taxa (Elgar & Crespi, 1992; Wildy et al., 1998; De Block & Stoks, 2004; de Vries & Lakes-Harlan, 2007). In larval amphibian communities, cannibalism can directly affect population density, size, and structure, and therefore may play an important role in regulating populations (Crump, 1992; Maret & Collins, 1994). In general, cannibalistic individuals develop faster, are larger, and have higher survivorship and enhanced reproductive success than non-cannibalistic individuals, and these beneficial effects of cannibalism on life history may be more pronounced when food availability is low (Polis, 1981; Elgar & Crespi, 1992). In amphibians, however, fast-developing larvae metamorphose at a smaller body size than do slowly developing
larvae from the same cohort (Wilbur & Collins, 1973). In particular, *H. retardatus* larvae fed only conspecific larvae metamorphose much earlier and at a smaller size than those fed only their typical prey (freshwater oligochaetes) (Michimae & Wakahara, 2002). The fast development associated with cannibalism may result in the metamorphosis of larvae into terrestrial juveniles by late autumn, before ephemeral ponds dry up. In amphibians, therefore, cannibalism may be an important mechanism by which the larvae reach the necessary developmental stage and size before the pond in which they were spawned dries up, thus reducing mortality due to desiccation (Lannoo & Bachmann, 1984). Thus, there is a trade-off associated with cannibalism: even if it increases the likelihood of survival during the larval stage, the associated accelerated development can result in the larvae being smaller at metamorphosis, which can negatively affect fitness-related traits expressed later in life (Altwegg & Reyer, 2003).

I hypothesized that higher density in natural ponds is likely to trigger cannibalism and hasten metamorphosis, thus decreasing the number of age classes in a larval population and altering its age structure. Moreover, this may occur even in ponds with relatively cool water temperature, which tends to prolong the larval period and increase the number of age classes, with the benefit of a larger body size at metamorphosis. On the basis of this hypothesis I made the following predictions: (1) Relatively cool water temperature prolongs the larval period in *H. retardatus*, but cannibalism reduces the larval period. (2) In natural ponds characterized by cool temperatures, populations with high larval density should have fewer age classes than those with low larval density. I examined the first prediction by experimentally investigating the effect of water temperature and cannibalism on the duration of the larval period in *H. retardatus*. To my knowledge, this is the first study to jointly examine the relative influence of water
temperature and cannibalism. To test the second prediction, I conducted a field study of
larvae in seven natural ponds with either low or high larval density. In the field study I
addressed the following questions: (1) What is the age distribution of salamander larvae
in cool-water ponds in Hokkaido? (2) Is the number of age classes correlated with larval
density?

MATERIALS AND METHODS

REARING EXPERIMENT

I collected three fertilized egg clutches of *H. retardatus* in 2006 in the vicinity of
Sapporo, Japan, during the spawning season, transported them to the laboratory, and
placed all clutches in a large plastic tank (30 × 25 × 17.5 cm deep) filled with 5 L of
dechlorinated tap water. The tank was kept in the laboratory at 4 °C until use. The tank
was placed at room temperature (20–21 °C) to accelerate hatching before starting the
rearing experiment. All the clutches almost hatched on almost the same date (fewer than
3 days separated the earliest from the latest hatching), and then 60 of the newly hatched
larvae were separately reared for 1 week at room temperature (20–21 °C), each in a
small tank (8 × 8 × 8 cm) containing 0.3 L of dechlorinated tap water. The experimental
larvae were fed on days 3, 5, and 7 by being offered frozen Chironomidae from 20:00 to
22:00. They were always given enough food to eat within 2 h, and any food remaining
in their tanks was removed after the feeding period. The rearing water was also
exchanged on days 3, 5, and 7, after the feeding period.

Then, I randomly assigned a group of 15 1-week-old larvae to one of four
experimental conditions that were created by crossing two categories of water
temperature (15 °C or 20 °C) with two food type categories (Chironomidae or conspecific larvae). These water temperature choices in the experiment were based on the findings of our previous study (Sakata et al., 2005). That study found that *H. retardatus* larvae that were reared at 20 °C had the shortest larval period and those reared at 16 °C had the longest larval period (data not shown in Sakata *et al.* 2005), among larvae reared at four specific temperatures (16, 20, 23, and 28 ºC). Larvae continued to be reared separately in the small tanks in 0.3 L of dechlorinated tap water. Each larva was placed in an electric incubator set at 15 °C or 20 °C and fed with one of the two food type categories (one larva or frozen Chironomidae) from 20:00 to 22:00 every other day (no food remained at the end of the feeding period) until they completed metamorphosis. All food types had about the same wet weight (all fed larvae were about the same size, and frozen Chironomidae of about the same mass as one larva were fed). The fed larvae were smaller than the experimental larvae because they were reared after hatching in tanks (30 × 25 × 17.5 cm deep) maintained at 4 ºC, which retarded their growth. The wet weight of each food type was measured to the nearest 0.01 g with an electronic balance. The rearing water was also changed every other day after the feeding period. The time (days) from hatching to the completion of metamorphosis was recorded for each larva. To compare the effects of the two treatments (food type and water temperature) on body size at metamorphosis, I first anesthetized each new metamorph by immersion in 0.01% MS222 (Sandoz). I measured the total length and snout-vent length (SVL) of each metamorph to the nearest 0.05 mm with calipers and weighed each metamorph to the nearest 0.01 g with an electronic balance. Measurements of SVL were made from the tip of the snout to the anterior corner of the cloaca. The metamorphs were afterward released into the ponds from which they had
been collected from as eggs.

FIELD SURVEY

I chose seven discrete *H. retardatus* larval habitats (permanent ponds) in Hokkaido, Japan, each characterized by a different density of larvae, as described below (Fig. 1). The seven permanent ponds were visited every month from May or June through October in 2006 and 2007 to check whether spawning had begun and to monitor larval growth and measure water temperature. During the winter (December to next April) some sites were inaccessible because of the heavy snow fall. At each visit, water temperature in the ponds was measured at 5 cm depth, and captured larval salamanders were anesthetized by immersion in 0.01% MS222 (Sandoz). The SVL of each captured larval salamander was then measured, and the developmental stage was determined according to the normal table for *H. nigrescens* (Iwasawa & Yamashita, 1991). Iwasaki & Wakahara (1999) previously observed two or three size categories in some permanent ponds, but the categories may vary seasonally. Two studies used skeletochronology to determine the age of salamander larvae of different sizes from the same pond (Iwasaki unpublished data, Kanki & Wakahara, 2001). These studies identified larvae that had overwintered either 1 or 2 years, as well as a strong positive relationship between age and SVL (Iwasaki unpublished data, Kanki & Wakahara, 2001). I adopted the methodology of assigning age classes based on SVL, although the possibility cannot be excluded that the observed overlap in size of successive cohorts is due to differential feeding histories or other factors.

The number of *H. retardatus* egg clutches in each pond and the number of eggs in each
collected clutch were counted to estimate the annual recruitment in each pond. Ten egg clutches were collected from each pond in 2006 and ten in 2007. The annual recruitment in each pond was estimated by multiplying the mean size of the collected clutches by the estimated density of clutches in the pond. By visiting all seven ponds approximately monthly from May or June through October, I was able to ascertain both the end of the oviposition period of *H. retardatus* and also the total number of egg clutches of *H. retardatus* in each pond. The annual recruitment was calculated for each pond each year, and the mean annual recruitment (individuals/m² ± SD) in each pond was calculated by dividing the sum of the annual recruitment by the number of years that clutches were collected. Although total density in the ponds with two or three year classes cannot be estimated by this method, larval density can be approximated because the number of 2- or 3-year-old larvae was overwhelmingly smaller than the number of that year's recruitment (personal observation). I therefore categorized each of the seven ponds into two groups (low or high) according to the mean annual recruitment.

**STATISTICAL ANALYSES**

In the rearing experimental data, the effects of the two factors (food type and water temperature), and the interaction between them on body size (total length, SVL, and body weight) at metamorphosis were analyzed by multivariate analysis of variance (MANOVA). After MANOVA, I assessed which variables were responsible for the significant main effects by a univariate analysis of variance (two-way ANOVA) of each response variable. The Cox proportional hazards model was used to assess the effects of the two treatments and their interaction on time to metamorphosis, which was measured from
the date of hatching to the date of completion of metamorphosis. I conducted a stepwise model reduction and determined the final parsimonious model by comparing the deviance (the difference in the $-2\log$-likelihood values between two models) to evaluate the fit of the models, which consisted of the different combinations of, and the interaction between, the two independent variables. The effects of each independent variable on the time to metamorphosis in the final model were adjusted for the other independent variable by using the Cox proportional hazards model. Results were calculated as the hazard ratio and 95% confidence interval (CI). Also, the distributions of the time to metamorphosis between treatments were estimated by the Kaplan-Meier method, and compared by using the log-rank test.

In the field study, three ponds, Tomaru, Teine1, and Teine3, contained two age classes of larvae for 2006 and 2007, and the other four, Asari, Konuma, Jozankei, and Teine2, contained three age classes for 2006 and 2007 (Fig. 1). To compare the SVL of the oldest larvae just before metamorphosis (e.g., May) between the ponds with two age classes and those with three age classes, I used one-level nested ANOVA for the factor "age at metamorphosis" (two or three) and the subgroup "population" within age at metamorphosis.

I then categorized each of the seven populations in a two-by-two factorial of the factors larval density (low or high) and the number of age classes present (two or three). The mean annual recruitment (individuals/m² ± SD) was calculated for each pond: Asari (142.7 ± 10.1), Konuma (17.9 ± 1.16), Jozankei (431.6 ± 24.9), Teine2 (122.2 ± 21.7), Tomaru (2363.8 ± 170.1), Teine1 (896.9 ± 119.5), and Teine3 (928.7 ± 61.1). Larval density was low in Asari, Konuma, Jozankei, and Teine2, and high in Tomaru, Teine1, and Teine3. To find the relationship between the number of age classes present and
larval density, I analyzed a table of frequency data cross-classified according to these two categorical variables using Fisher’s exact test. I was not able to test for effects of water temperature differences between the natural permanent ponds on the number of age classes present, because no permanent ponds with a temperature exceeding 16 °C were found in my preliminary surveys. However, the temperature of some temporary ponds exceeds 16 °C (e.g., 20°C in summer, Iwasaki & Wakahara, 1999).

RESULTS

REARING EXPERIMENT

There were significant multivariate effects associated with both factors (food type and water temperature), but not with the interaction between food type and water temperature (Table 1). Subsequent ANOVAs detected that food type and water temperature significantly affected body size at metamorphosis by all three measures: total length, SVL, and body mass, but the interaction of the two factors had no effect on any of these three variables (Table 1). Salamander larvae consuming Chironomidae or reared at 15 °C had a larger body size at metamorphosis than those consuming larvae or reared at 20 °C (Fig. 2).

The final model included food type and water temperature as explanatory variables but no interactive effect was detected (Table 2). Therefore, only the additive effect of food type and water temperature explained the time to metamorphosis. The estimated hazard ratio of larvae to Chironomidae was 2.89 (95% CI, 2.03–4.27), and the estimated hazard ratio of a water temperature of 15 °C to one of 20 °C was 0.18 (95% CI, 0.11–0.29).
Kaplan–Meier curves for time to metamorphosis are displayed in Fig. 3 for each combination of water temperature (15 °C or 20 °C) and food type (Chironomidae or larvae). The time to metamorphosis between larvae fed Chironomidae (median time, 70 days; 95% CI, 69–74 days) and those fed conspecific larvae (median time, 55 days; 95% CI, 52–60 days) was significantly different (log-rank test, $P < 0.0001$) at the water temperature of 20 °C, and also at the water temperature of 15 °C: Chironomidae (median time, 85 days; 95% CI, 79–87 days); conspecific larvae (median time, 74 days; 95% CI, 71–80 days) (log-rank test, $P = 0.0003$). Similarly, the time to metamorphosis between water temperatures of 20 °C and 15 °C was significantly different in both larvae fed Chironomidae (log-rank test, $P < 0.0001$) and those fed conspecifics (log-rank test, $P < 0.0001$). Thus, the time to metamorphosis was prolonged by a decrease in the water temperature from 20 °C to 15 °C, and also by feeding the larvae Chironomidae instead of larvae (Fig. 3).

FIELD SURVEY

Figure 4 shows temporal changes in SVL (left axes: lines) of the collected *H. retardatus* larvae in relation to the water temperature (right axes, bars) in the seven ponds (Fig. 1). Larvae observed in May, the spawning season, at Tomaru, Teine1, and Teine3 were those that had developed from egg clutches spawned the year before and overwintered as larvae. These larvae were not observed in June (Teine1 and Teine3) or July (Tomaru), probably because they had metamorphosed. Newly hatched larvae were first observed in June at Tomaru and Teine3 and in July at Teine1. These larvae grew during the summer and some probably overwintered as larvae in the aquatic habitat while others metamorphosed by late autumn (October) of the year in which they were spawned. Only
full-grown larvae (stage 63) were observed at Tomaru, Teine1, and Teine3 in October of both 2006 and 2007. Some of these may have hibernated during the winter in muddy ground under the snow and not metamorphosed until the next year.

Larvae that were observed in the spawning season at Asari, Jozankei, Konuma, and Teine2 ponds belonged to two size categories, indicating the presence of larvae that had overwintered 1 year as well as ones that had overwintered 2 years. The larvae that had overwintered for 2 years (i.e., the larger larvae observed in the spawning season) probably completed metamorphosis during the summer of their third year, as by July they were no longer observed. The larvae that had overwintered for 1 year (i.e., the medium-sized larvae observed in the spawning season) continued to grow in these ponds during their second summer, and some may have completed metamorphosis by the late autumn of their second summer, whereas others presumably again overwintered as larvae in the aquatic habitat, to metamorphose during their third year. All 1 year-overwintered larvae observed at Asari, Jozankei, Konuma, and Teine2 ponds in October of both 2006 and 2007 were full grown (stage 63). Small, newly hatched larvae were first observed in June or July. These larvae grew during their first summer but probably did not metamorphose by late autumn (none of these larvae at Asari, Jozankei, Konuma, or Teine2 reached stage 63 in either 2006 and 2007). Instead, they probably hibernated during the winter to become 1 year-overwintered larvae the following year.

In 2007, SVL in the ponds with three age classes (Asari, Konuma, Jozankei, and Teine2) was significantly larger than that in those with two age classes (Tomaru, Teine1, and Teine3), but in 2006, SVL did not significantly differ between these two groups, indicating a very strong trend (Table 3). In natural environments as well as in the experimental laboratory environment, a prolonged larval period led to a slightly larger
SVL just before metamorphosis (Figs 4, 5).

The number of age classes was significantly affected by larval density in natural ponds (Fisher's exact test, \( P = 0.0286 \)). Ponds characterized by high larval density contained 1 year-overwintered larvae along with the current year's larvae (i.e., two age classes), but those characterized by low larval density had both 1-year- and 2-year-overwintered larvae along with the current year's larvae (i.e. three age classes).

**DISCUSSION**

The experimental results showed that in individuals grown at the relatively cold temperature of 15 °C the time to metamorphosis was significantly prolonged and their body size (total length, SVL, and body mass) at metamorphosis was significantly greater compared with individuals grown at the relatively warm temperature of 20 °C. This impact of water temperature on metamorphic timing and body size at metamorphosis is similar to the temperature effects seen in other amphibian larvae. For example, larval anurans grown at cold temperatures take longer to develop but the metamorphs are also larger than conspecifics grown at warmer temperatures (Smith-Gill & Berven, 1979; Voss, 1993; Walsh *et al*., 2008). After energy uptake, temperature can be considered the most important proximal cause of variation in size and age at metamorphosis in amphibians (Rose, 2005). In *H. retardatus* water temperature did not itself directly affect the body size at metamorphosis; rather, the prolongation of the larval period caused by the cooler water temperature caused the body size at metamorphosis to be larger, as described below. Food type, which is independent of water temperature, also affected the time to metamorphosis and body size at
metamorphosis. *Hynobius retardatus* larvae that consumed conspecifics had a shorter larval period (Fig. 3), indicating that cannibalism can cause a fast development rate (Michimae & Wakahara, 2002). This accelerated development led to smaller size at metamorphosis (Fig. 2, Michimae & Wakahara, 2002), implying that metamorphic timing may be accelerated by consumption of the thyroxine present in conspecific larvae (Pfennig, 1992).

Larvae of *H. retardatus* living in cool, permanent habitats may prolong the larval period into a second or third year by overwintering (Fig. 4), which ensures that they will have attained a larger size at metamorphosis (Figs 4, 5; Table 3). Iwasaki and Wakahara (1999) reported that the SVL of *H. retardatus* larvae just before completion of metamorphosis differs significantly among three age groups; their results showed that 2-year-overwintered larvae are significantly larger than both 1-year-overwintered larvae and those larvae that do not overwinter. Generally, low temperatures retard differentiation more than growth, thereby increasing stage-specific size (Berven et al., 1979; Voss, 1993; Walsh et al., 2008). The longer larval periods of overwintering larvae may benefit them by ensuring a larger body size at metamorphosis compared with their nonoverwintering conspecifics (Berven et al., 1979). In amphibians, a larger body size is directly related to increased fecundity, and, in many cases, reproductive success (Semlitsch et al., 1988; Goater, 1994; Scott, 1994; Altwegg & Reyer, 2003). In addition, larvae overwintering in cool permanent ponds may benefit by avoiding the additional costs of terrestrial migration incurred by smaller adults. Thus, in Hokkaido, growth conditions may be ideal for overwintering larvae.

The field survey results also suggest that time to metamorphosis in *H. retardatus* larvae is influenced by larval density, that is, by cannibalism (Fig. 4). Metamorphosis
proceeds as soon as larvae reach a certain stage of development (i.e., stage 63) (Rose, 2005). Cannibalistic salamander larvae in ponds with high larval density might grow faster and reach this stage earlier than non-cannibalistic larvae living in ponds with low larval density (Fig. 4). This diet-based enhancement of development might translate into a shorter larval duration and earlier metamorphosis, even though the cool water temperatures of ponds in Hokkaido tend to prolong the larval period, leading to later metamorphosis (Fig. 4). Thus, geographic variation in the frequency of cannibalism may result in population differences in the metamorphic timing of *H. retardatus* larvae (Fig. 4). Many *H. retardatus* larvae inhabiting permanent ponds in Hokkaido overwinter as larvae in the aquatic habitat in which they were spawned instead of metamorphosing during their first year, whereas most larvae inhabiting ephemeral ponds metamorphose by August or September of their first year, even though the water temperature is not different from that in the permanent ponds (Iwasaki & Wakahara, 1999). *Hynobius retardatus* larvae spawned in temporary ponds must metamorphose by August or September of the same year, like those of many other amphibians that breed in temporary ponds and metamorphose before the ponds dry up (Travis, 1983, Newman, 1988b, Denver *et al.*, 1998, Laurila & Kujasalo, 1999). Cannibalism is thus an adaptive behavior that, by accelerating larval development in drying ponds, reduces mortality due to desiccation, even though accelerated development is associated with smaller size at metamorphosis, which may negatively affect juvenile survival and the breeding success of adults (Altwegg & Reyer, 2003). Indeed, accelerated larval development in drying ponds is a classic example of adaptive plasticity (Travis, 1983; Lannoo & Bachmann, 1984; Newman, 1988b). However, this cannibalism-induced shortening of the larval period can be viewed as an unfavorable consequence for amphibian species in
permanent breeding habitats, where any extension of the larval period probably conveys increased fitness (Semlitsch et al., 1988; Goater, 1994; Scott, 1994; Altwegg & Reyer, 2003). Cannibalism is adaptive in that it reduces mortality due to desiccation by accelerating larval development in drying ponds, but in permanent habitats the effects of cannibalism on larval development might be maladaptive.

The aim of the laboratory experiment was to determine the association between key life-history characteristics of salamander larvae (body size and larval period) and environmental conditions (water temperature and diet). The extended temporal scope of the field observation allowed a description of the variation in population age structure under a range of environmental conditions (larval density) (Fig. 4). Populations of different density categories had very different population age structures and were composed of individuals with strikingly different life history characteristics (Table 3, Fig. 4). The population age structures of *H. retardatus* larvae may depend primarily on individual phenotypic plasticity in response to environmental variability. It is important to understand how environmental effects are ultimately transduced through individual organisms into population-level phenomena, with the population response arising as the summation of individual responses. Without a thorough comprehension of the mechanisms through which population and individual responses to environmental conditions are mediated, we cannot interpret the relationship between population-level and individual-level phenomena.

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Figure legends

Figure 1. Map of sampling sites and ponds used in the field study (open circles) in Hokkaido, Japan

Figure 2. Effects of water temperature and food type on body size at metamorphosis. Total length (a), SVL (b), and body mass (c) at metamorphosis of larvae under four experimental conditions created by crossing two categories of water temperature (15 °C or 20 °C) with two food types (Chironomidae or larvae). In each case the mean and SD are shown. Total length, SVL, and body mass at metamorphosis were significantly different between larvae reared at 15 °C or 20 °C and fed with Chironomidae or larvae. Total length (water temperature, \( P < 0.0001 \); food type, \( P < 0.0001 \)), SVL (water temperature, \( P < 0.0001 \); food type, \( P < 0.0001 \)) and body mass (water temperature, \( P < 0.0001 \); food type, \( P < 0.0001 \))

Figure 3. Kaplan–Meier estimates of time to metamorphosis for each of combination of water temperature (15 °C or 20 °C) and food type (Chironomidae or larvae).

Figure 4. Longitudinal growth data (snout-vent length, SVL) in larval Hynobius retardatus (left axes, symbols ± SD, lines) and water temperature (right axes, bars) in seven ponds surveyed in 2006 and 2007. Asari, Jozankei, Konuma, and Teine2, in which larval density was low, contained three age classes of larvae (larvae of the current year and 1-year- and 2-year-overwintered larvae), whereas Tomaru, Teine1, and Teine3, where larval density was high, contained two age classes of larvae (the current year's
larvae and 1-year-overwintered larvae). The numbers above each symbol show the sample size (n).

**Figure 5.** Numbers of larvae (larvae of the current year and 1-year- and 2-year-overwintered larvae) in each pond in relation to snout-vent length (SVL) during 2006 (left) and 2007 (right).
Table 1. Results of MANOVA for effects of food type and water temperature on body size (total length, SVL and body mass). ANOVA results for each response variable are also shown.

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<thead>
<tr>
<th>ANOVAs Variables</th>
<th>MS</th>
<th>d.f.</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food type</td>
<td>3.587</td>
<td>1, 56</td>
<td>51.763</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Water temperature</td>
<td>5.998</td>
<td>1, 56</td>
<td>86.555</td>
<td>&lt;0.0001</td>
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<tr>
<td>Food type x Water temperature</td>
<td>0.067</td>
<td>1, 56</td>
<td>0.972</td>
<td>0.3285</td>
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<tr>
<td>SVL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food type</td>
<td>0.905</td>
<td>1, 56</td>
<td>25.577</td>
<td>&lt;0.0001</td>
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<td>Water temperature</td>
<td>2.293</td>
<td>1, 56</td>
<td>64.790</td>
<td>&lt;0.0001</td>
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<td>Food type x Water temperature</td>
<td>0.076</td>
<td>1, 56</td>
<td>2.136</td>
<td>0.1494</td>
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<td>Body mass</td>
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<tr>
<td>Food type</td>
<td>1.162</td>
<td>1, 56</td>
<td>67.029</td>
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<td>Water temperature</td>
<td>2.485</td>
<td>1, 56</td>
<td>143.325</td>
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</tr>
<tr>
<td>Food type x Water temperature</td>
<td>0.051</td>
<td>1, 56</td>
<td>2.944</td>
<td>0.0917</td>
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</tbody>
</table>
Table 2. Models used in the Cox proportional hazards analysis, consisting of various combinations of two independent variables (T, water temperature; F, food type) and their interaction. Constant + T + F was selected as the final model.

<table>
<thead>
<tr>
<th>Variables in model</th>
<th>-2Log-Likelihood</th>
<th>df</th>
<th>Variable evaluated</th>
<th>Deviance (df)</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>Constant + T + F + T*F</td>
<td>279.0396</td>
<td>3</td>
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<tr>
<td>Constant + T + F</td>
<td>281.2178</td>
<td>2</td>
<td>T*F</td>
<td>2.1782 (2)</td>
<td>0.14</td>
</tr>
<tr>
<td>Constant + T</td>
<td>340.0858</td>
<td>1</td>
<td>F</td>
<td>58.8680 (1)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Constant + F</td>
<td>362.5096</td>
<td>1</td>
<td>T</td>
<td>81.2918 (1)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 3. Nested ANOVA results for the effect of age at metamorphosis (two or three) and population (Asari, Konuma, Tomaru, Jozankei, Teine1, Teine2, Teine3) within age at metamorphosis on SVL.

<table>
<thead>
<tr>
<th></th>
<th>2006</th>
<th></th>
<th>2007</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS</td>
<td>df</td>
<td>F</td>
<td>P</td>
</tr>
<tr>
<td>Age at metamorphosis</td>
<td>24.666</td>
<td>1</td>
<td>5.123</td>
<td>0.07</td>
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<tr>
<td>Population within Age</td>
<td>4.815</td>
<td>5</td>
<td>1.191</td>
<td>0.34</td>
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<tr>
<td>Error</td>
<td>4.043</td>
<td>23</td>
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<tr>
<td></td>
<td>44.947</td>
<td>1</td>
<td>10.264</td>
<td>0.02</td>
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<tr>
<td>Population within Age</td>
<td>4.379</td>
<td>5</td>
<td>1.151</td>
<td>0.36</td>
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<tr>
<td>Error</td>
<td>3.803</td>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1
Figure 2

(a) Total length (mm)

(b) SVL (mm)

(c) Body mass (g)

Water temperature (C)
Figure 3
Figure 4

(a) Asari

(b) Jozankei

(c) Konuma

(d) Tomaru
Figure 5

(a) Asari

Larvae of current year in 2006

Larvae of current year in 2007

One year-overwintered larvae in 2006

One year-overwintered larvae in 2007

Two year-overwintered larvae in 2006

Two year-overwintered larvae in 2007

Number of larvae

SVL (cm)

Number of larvae

SVL (cm)
(b) Jouzankei

Larvae of current year in 2006

Larvae of current year in 2007

One year-overwintered larvae in 2006

One year-overwintered larvae in 2007

Two year-overwintered larvae in 2006

Two year-overwintered larvae in 2007
(c) Konuma

- Larvae of current year in 2006
- One year-overwintered larvae in 2006
- Two year-overwintered larvae in 2006
- Larvae of current year in 2007
- One year-overwintered larvae in 2007
- Two year-overwintered larvae in 2007
(d) Tomaru

Larvae of current year in 2006

One year-overwintered larvae in 2006

Larvae of current year in 2007

One year-overwintered larvae in 2007
(e) Teine1

Larvae of current year in 2006

One year-overwintered larvae in 2006

Larvae of current year in 2007

One year-overwintered larvae in 2007
(f) Teine2

Larvae of current year in 2006

Larvae of current year in 2007

One year-overwintered larvae in 2006

One year-overwintered larvae in 2007

Two year-overwintered larvae in 2006

Two year-overwintered larvae in 2007
(g) Teine3

Larvae of current year in 2006

Number of larvae

Larvae of current year in 2007

Number of larvae

One year-overwintered larvae in 2006

Number of larvae

One year-overwintered larvae in 2007

Number of larvae

SVL (cm)