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Correlated evolution of phenotypic plasticity in metamorphic timing

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1   **Abstract**

2   Phenotypic plasticity has long been a focus of research, but the mechanisms of its

3   evolution remain controversial. Many amphibian species exhibit a similar plastic

4   response in metamorphic timing in response to multiple environmental factors;

5   therefore, more than one environmental factor has likely influenced the evolution of

6   plasticity. However, it is unclear whether the plastic responses to different factors have

7   evolved independently. In this study, we examined the relationship between the plastic

8   responses to two experimental factors (water level and food type) in larvae of the

9   salamander *Hynobius retardatus*, using a cause-specific Cox proportional hazards

10   model on the time to completion of metamorphosis. Larvae from ephemeral ponds

11   metamorphosed earlier than those from permanent ponds when kept at a low water level

12   or fed conspecific larvae instead of larval Chironomidae. This acceleration of

13   metamorphosis depended only on the permanency of the larvae's pond of origin, but not

14   on the conspecific larval density (an indicator of the frequency of cannibalism) in the

15   ponds. The two plastic responses were significantly correlated, indicating that they may

16   evolve correlatively. Once plasticity evolved as an adaptation to habitat desiccation, it

17   might have relatively easily become a response to other ecological factors, such as food

18   type via the pre-existing developmental pathway.

1

2   *Keywords:* amphibian; cannibalism; desiccation risk; metamorphosis; phenotypic

3   plasticity; water level

1 **Introduction**

2 Many organisms exhibit plasticity in their morphological and life-history traits (Pfennig

3 *et al.*, 2010). Although the evolution of phenotypic plasticity has long been a focus of

4 research, the mechanisms of this evolution remain controversial (Pfennig *et al.*, 2010).

5 As described in greater detail below, many organisms can exhibit a similar plastic

6 response to multiple environmental factors, with this response mediated by different

7 environmental cues. To advance the field of evolutionary biology, it is essential to

8 reveal both the original selective factors favoring the evolution of phenotypic plasticity

9 and the historical sequence in which plastic responses to different environmental cues

10 have evolved (Gomez-Mestre *et al.*, 2008).

11 Phenotypic plasticity has been extensively studied in amphibian larvae with

12 complex life cycles. The larvae of many amphibian species cannot escape their aquatic

13 environment until metamorphosis, which might be one factor indicating that these

14 larvae exhibit phenotypic plasticity in response to variable environmental conditions. In

15 fact, amphibian larvae exhibit phenotypic plasticity in their development and timing of

16 metamorphosis (which affects their size at metamorphosis) in response to variation in

17 biotic or abiotic environmental factors, such as larval density (Newman, 1998), the

18 presence of predators (Laurila & Kujasalo, 1999; Lardner, 2000), the type and quantity

1   of available food (Hensley, 1993), habitat desiccation (Denver *et al.*, 1998; Laurila &

2   Kujasalo, 1999), and water temperature (Walsh *et al.*, 2008).

3   Therefore, more than one environmental factor has likely influenced the evolution

4   of plasticity, and interactions among different factors acting simultaneously may also be

5   important. However, it is unclear whether the plastic responses to different factors have

6   evolved independently. Developmental events in amphibians are regulated primarily by

7   the hypothalamus–pituitary–thyroid (HPT) axis, through the production of thyroid

8   hormone, which is produced enzymatically and regulates metabolic and developmental

9   processes (Rose, 2005). In particular, the metamorphosis of amphibian larvae is a

10   thyroid hormone-dependent developmental process (Rose, 2005). Sharing of the same

11   developmental pathways by the plastic responses might influence the capacity of a

12   population to evolve in response to selective factors (Derrickson & Ricklefs, 1988),

13   implying that it may not allow for their independent evolution.

14   Developmental plasticity is often adaptive. For example, on one hand, amphibian

15   larvae dwelling in a permanent water body can delay their metamorphosis and use the

16   extra time for additional growth in order to attain a larger size before metamorphosis

17   (Berven *et al.,* 1979; Alvarez & Nicieza, 2002). Having a larger body size at

18   metamorphosis may directly increase an organism's survival to maturity (Scott, 1994)

1   and size at reproduction (Altwegg & Reyer, 2003), which would, in turn, increase the

2   individual's fecundity and reproductive success (Altwegg & Reyer, 2003). On the other

3   hand, developmental plasticity can allow larval amphibians inhabiting temporary or

4   ephemeral water bodies to complete their metamorphosis more quickly, before their

5   habitat dries up. In this case, acceleration of larval development may be adaptive in

6   drying ponds because it reduces mortality due to desiccation (Laurila & Kujasalo, 1999),

7   even though it is associated with a smaller size at metamorphosis, which can negatively

8   affect the survival of juveniles and breeding success of adults (Altwegg & Reyer, 2003).

9   The salamander *Hynobius retardatus*, which lives in Hokkaido, Japan, has long been

10   noted to have variable life history (Iwasaki & Wakahara, 1999). This species spawns in

11   ponds from early April to May, and has free-living aquatic larvae. Some individuals of

12   this species born in permanent ponds retain certain larval features, such as external gills

13   and tail fins, and overwinter once or twice in their aquatic habitat as larva, not

14   metamorphosing until their second or third year of life (Iwasaki & Wakahara, 1999;

15   Michimae, 2011). In contrast, most larvae spawned in small, ephemeral ponds created

16   by melting snow metamorphose into terrestrial juveniles by late autumn (October) of

17   the same year they are spawned (Iwasaki & Wakahara, 1999). Ponds of different

18   permanency exert different selective pressures on organisms, which, in response, locally

1 evolve to attain different plastic responses (Leips *et al.,* 2000; Lake, 2003). Hence, as *H.*

2 *retardatus* utilizes both permanent and ephemeral ponds for spawning, it is possible that

3 the species requires different reaction norms for adaptation to larval pond habitats of

4 different permanency.

5 The larvae of *H. retardatus* are both carnivorous and cannibalistic (Michimae,

6 2006). In general, cannibalistic individuals develop faster, are larger, and have higher

7 survivorship and enhanced reproductive success than non-cannibalistic individuals, and

8 these beneficial effects of cannibalism on an individual's life history may be more

9 pronounced when food availability is low (Elgar & Crespi, 1992). Thus, organisms may

10 adopt cannibalistic behavior mainly to enhance their mass and body condition. In

11 amphibians, however, fast-developing larvae metamorphose at a smaller body size than

12 do slowly developing larvae from the same cohort (Wilbur & Collins, 1973). In

13 particular, *H. retardatus* larvae fed only conspecific larvae metamorphose much earlier

14 and at a smaller size than those fed only their typical prey (freshwater oligochaetes and

15 Chironomidae) (Michimae & Wakahara, 2002; Michimae, 2011). Therefore,

16 cannibalism in *H. retardatus* leads to accelerated metamorphosis (Michimae &

17 Wakahara, 2002; Michimae, 2011). Specifically, interpopulational variation in

18 conspecific larval density (an indicator of the frequency of cannibalism in natural

1   habitats (Whiteman *et al.*, 2003)) in natural ponds can promote population differences

2   in cannibalism-mediated developmental plasticity.

3   Intraspecific studies of geographic variation in phenotypic plasticity have

4   provided convincing evidence of both organismal adaptation to varying environmental

5   conditions (Pigliucci, 2005) and the ecological consequences of environmentally

6   induced plasticity (e.g., phenotypic plasticity can alter interactions between organisms

7   and their abiotic and biotic environments; (Miner *et al.,* 2005)). Therefore, we are

8   interested in studying the relationship between habitat type and the reaction norm in *H.*

9   *retardatus*, especially the geographical variation in phenotypic plasticity in

10   metamorphic timing in response to a reduction in the water level, which can be used as

11   a reliable cue indicating habitat desiccation. We are also interested in examining the

12   interpopulational variation in phenotypic plasticity in the metamorphic timing of the

13   species in response to being fed different food types (i.e., conspecific larvae and larval

14   Chironomidae), corresponding to the diets available to *H. retardatus* larvae living in

15   ponds with different larval densities.

16   In the present study, to analyze the evolution of the phenotypically plastic

17   responses in the metamorphic timing of *H. retardatus*, we surveyed 9 ponds in

18   Hokkaido, Japan (see Table 1), whose specific populations have been described

1   previously (Iwasaki & Wakahara, 1999; Michimae, 2006; Michimae *et al.,* 2009;

2   Michimae, 2011). The preliminary surveys quantified the variability among the ponds in

3   terms of pond permanency and the density of conspecific larvae (Table 1). The primary

4   aims of our present study were as follows: (1) to identify phenotypically plastic

5   responses in the metamorphic timing of *H. retardatus* larvae that are induced by either

6   the experimental water level or food type, the latter of which was previously shown to

7   affect metamorphic timing of larvae (Michimae & Wakahara, 2002; Michimae, 2011);

8   (2) to examine whether each plastic response in metamorphic timing of *H. retardatus*

9   larvae due to experimental changes in water level or food type is dependent on

10   differences in the permanency of the natural pond from which the larvae were derived

11   (permanent or ephemeral), on differences in conspecific larval density in their source

12   pond, or both; and (3) to examine the spatial population heterogeneity and the

13   relationships between these environmental conditions (i.e., pond permanency and

14   conspecific larval density) and the reaction norm at the population level.

15   In addition, we are interested in determining whether the two plastic responses

16   evolve independently. Thus, the secondary aim of this study was to estimate and

17   interpret patterns of phenotypic correlation between the two plastic responses in

18   metamorphic timing and the two different experimental factors of interest—water level

9

1   and food type.

2

3   **Materials and methods**

4   **Study populations and sites**

5   We categorized Konuma, Tomaru, and Asari as permanent ponds because they never

6   dried up during the years they were monitored (Table 1). In contrast, Okusawa, Atsuta,

7   Kamitobetsu, Toyoha, Erimo, and Nopporo were categorized as ephemeral ponds

8   because they dried up nearly every year that they were monitored (Table 1).

9   Ten egg clutches were collected from each pond. The annual density of

10   conspecific larvae in each pond was estimated by multiplying the mean size of the

11   collected clutches by the estimated density of clutches in the pond (see Michimae,

12   2006). The mean density of conspecific larvae (individuals/m^2 ± SD) in each pond was

13   calculated each year, and the mean total annual density of conspecific larvae in each

14   pond was calculated by dividing the sum of the annual total larval densities by the

15   number of years that clutches were collected. Using data from 2 to 5 years (from 2002

16   to 2007, except for 2005) and a calculation method previously detailed by Michimae

17   (2006), we determined the mean density of conspecific larvae (Table 1). The conditions

18   of natural ponds, i.e., pond permanency and larval density, were the covariates in the

10

1   evaluation of the effects in the following experiment.

2

3   **Rearing experiment**

4   We collected 20 fertilized egg clutches of *H. retardatus* from each of the 9 ponds during

5   the spawning season (early April to late May) in 2006. Each clutch was placed in a

6   stock tank filled with 1.6 L of dechlorinated tap water and was kept with a natural

7   light/dark schedule in an incubator at 4 °C or in the laboratory at 20–21 °C until

8   hatching (because the hatching day of the embryos was controlled). After the egg

9   clutches hatched, 5 newly hatched larvae from each clutch were reared as a group for 2

10   weeks in the laboratory at 20–21 °C, with each group in a plastic tank (30-cm long ×

11   25-cm wide × 17.5-cm deep) containing 5 L of dechlorinated tap water. The larvae were

12   maintained in the plastic tank until the experiment described below was established. The

13   experimental larvae were fed on days 3, 5, 7, 9, 11, and 13 by being offered frozen

14   Chironomidae from 20:00 to 22:00. The larvae were always given enough food to eat

15   within 2 h, and any food remaining in their tanks after the feeding period was removed.

16   The rearing water in each tank was also exchanged after each feeding period.

17   We established four experimental treatments by crossing two water levels (low vs.

18   high) and two food types (Chironomidae vs. conspecific salamander larvae). For each

11

1 pond, we randomly assigned five sets of the five 2-week-old larvae to one of the

2 experimental treatments. Hence, each treatment group contained 225 larvae (45

3 clutches) in total, including 25 (5 clutches) larvae from each of the 9 ponds. The rearing

4 experiment was designed to have five cages (clutches) for each treatment. Five cages

5 were hung in each experimental tank. In each of the two water-level treatments, five

6 larvae were raised as a group in a cage (15-cm long × 15-cm wide) with plastic mesh

7 sides (mesh size, 3 mm) and a plastic plate bottom (1.6-mm thick) that hung from the

8 edge of the experimental tank (86-cm long × 66-cm wide × 27-cm deep) containing 145

9 L of dechlorinated tap water. For the high water-level treatment, the distance from the

10 bottom of each cage to the water's surface (i.e., the water depth) was approximately 25

11 cm, whereas it was approximately 2 cm for the low water-level treatment. Larvae in the

12 cages were individually fed with one of the two food types (one conspecific larva or

13 frozen Chironomidae) from 20:00 to 22:00 every other day, until they completed

14 metamorphosis. During feeding, larvae in the cages of each experimental treatment

15 were individually transferred from the experimental tank to another tank (8 cm × 8 cm ×

16 8 cm), allowed 2 h to eat the food, and then transferred back to their cages in the

17 experimental tanks. Both diets had constant and similar wet weights, as all larvae used

18 for feeding were about the same size, and enough frozen Chironomidae were fed to

1 match the mass of the larvae used for feeding. The wet weight of each food type was

2 measured to the nearest 0.01 g with an electronic balance. The water in the experimental

3 tanks was changed every other day during the experiment. Four experimental tanks and

4 five cages in experimental tanks were randomly redistributed after every water

5 exchange. The time to event (days from hatching to the completion of metamorphosis or

6 larval death) was recorded for each larva.

7   Each new metamorph was anesthetized by immersion in 0.01% MS222 (Sandoz),

8 and its snout-vent length (SVL) was measured to the nearest 0.05 mm with calipers, and

9 its body mass was measured to the nearest 0.01 g with an electronic balance. SVL

10 measurements were made from the tip of the snout to the anterior end of the cloaca.

11 Body mass was transformed to the same linear scale as SVL in all analyses by taking its

12 cube root before analysis.

13

14 **Analyses**

15 A cause-specific Cox proportional hazards model was used to assess the effects of the

16 two experimental factors (water level and food type), the two environmental factors

17 (pond permanency as a categorical covariate and pond larval density as a continuous

18 covariate), and their interactions on time to completion of metamorphosis. Identities for

1 the egg clutch were also incorporated into the Cox proportional hazards model as a

2 random effect, accounting for the within-clutch correlation. We used a general strategy

3 for model selection, as recommended by Collett (2003), rather than information

4 theoretic criteria such as Akaike's Information Criterion (AIC) or Bayesian Information

5 Criterion (BIC). The analysis results are reported as hazard ratios with the 95%

6 confidence interval (CI).

7 We also characterized the completion of metamorphosis over time using the

8 cumulative incidence function, i.e., the overall probability of metamorphosis in the

9 presence of death (Fine & Gray, 1999).

10 To examine the correlation between the plastic responses in metamorphic timing

11 at the population level, we conducted correlation analysis based on the difference

12 between the median times to metamorphosis for the level of each experimental factor

13 for each population.

14 To examine the correlation between phenotypic plasticity in body size (SVL and

15 body mass) at metamorphosis with the two experimental factors at the population level,

16 we conducted multivariate analysis of covariance (MANCOVA) and correlation

17 analysis as follows. The mean body size at metamorphosis with each treatment was

18 calculated for each population. The effects of the two experimental factors, the two

1   environmental factors (pond larval density as a covariate), and their interactions on the

2   mean body size at metamorphosis were calculated using MANCOVA. This test was

3   carried out by re-running the models excluding the interaction terms. If there was no

4   interaction between the two experimental factors, we conducted correlation analysis

5   based on the difference between the mean body sizes for the level of each experimental

6   factor for each population. If a larva of a treatment had died, we deleted the associated

7   measurements of body size before calculating the mean body size.

8   To examine the relationship between body size (SVL and body mass) at

9   metamorphosis and age at metamorphosis (days from hatching to the completion of

10   metamorphosis), we conducted correlation analysis. For this analysis, larval

11   measurements of body size were averaged to calculate the treatment means, and the

12   median time to metamorphosis was estimated by each treatment for each population. If

13   a larva of a treatment had died, we deleted the associated measurements of body size

14   before calculating the mean body size.

15   This study was approved by the Hokkaido University Animal Committee, and the

16   animals were maintained in accordance with the Hokkaido University Guidelines for the

17   Care and Use of Laboratory Animals.

18

1 **Results**

2 **Time to metamorphosis**

3 Table 2 summarizes the results of variable selection in the Cox proportional hazards

4 model for time to metamorphosis. The final model comprised the two experimental

5 factors (water level and food type), one environmental factor (pond permanency), two

6 interactions (water level by pond permanency and food type by pond permanency), and

7 the identities of the egg clutches.

8 The two interactions indicate that the effects of the two experimental treatments

9 on time to completion of metamorphosis differed between the types of pond

10 permanency (permanent vs. ephemeral). For permanent environments, the estimated

11 hazard ratio of low water level to high water level was 11.3 (95% CI, 8.1–15.7), and the

12 estimated hazard ratio of conspecific larvae to Chironomidae was 7.5 (95% CI,

13 5.4–10.5) (Fig. 1a). For ephemeral environments, the estimated hazard ratio of low

14 water level to high water level was 58.5 (95% CI, 35.1–97.5), and the estimated hazard

15 ratio of conspecific larvae to Chironomidae was 16.6 (95% CI, 10.0–27.6) (Fig. 1b).

16 The hazard ratios and the lower limit of the associated 95% CIs were more than 1,

17 indicating that the time to metamorphosis for both permanent and ephemeral

18 environments was shortened both by a decrease in the water level from high to low and

1 by feeding larvae the conspecific larvae instead of Chironomidae (Fig. 1). The variance

2 of the random effect for clutches was estimated to be 0.134, which shows weak

3 within-clutch correlation (Kendall rank correlation coefficient = 0.062, $P < 0.0001$).

4

Two plastic responses in metamorphic timing at the population level

6 There was no interaction between the two experimental factors on time to the

7 completion of metamorphosis (Table 2). Therefore, we calculated two plastic responses

8 in metamorphic timing to water level and food type at the population level, as described

9 in the Analyses section. Correlation analysis showed a significant relationship between

10 the two plastic responses in metamorphic timing to water level and food type at the

11 population level ($R = 0.775$, $n = 9$, $P = 0.0115$; Fig. 2a).

12

Two plastic responses in body size at the population level

14 Based on MANCOVA results, we found no higher interactions, including the

15 interaction between the two experimental factors on body size (SVL and body mass) at

16 metamorphosis (results not shown). We found only two significant experimental factors

17 on body size: water level (Walks' lambda = 0.064, $F_{2,30} = 219.197$, $P < 0.0001$) and food

18 type (Walks' lambda = 0.255, $F_{2,30} = 43.857$, $P < 0.0001$). Thus, we calculated

1   phenotypic plasticity in body size in response to water level and food type at the

2   population level, as described in the Analyses section. Correlation analysis showed a

3   significant relationship between plastic responses in SVL to water level and food type

4   ($R = -0.721$, $n = 9$, $P = 0.0258$; Fig. 2b), but not in body mass to water level and food

5   type ($R = -0.468$, $n = 9$, $P = 0.2140$; Fig. 2c), likely because of the use of a small

6   sample size for this analysis.

7

8   **Relationships between body size and age at metamorphosis**

9   Correlation analysis showed significant relationships both between SVL at

10   metamorphosis and age at metamorphosis ($R = 0.882$, $n = 36$, $P < 0.0001$; Fig. 3a), and

11   between body mass at metamorphosis and age at metamorphosis ($R = 0.901$, $n = 36$, $P <$

12   0.0001; Fig. 3b), indicating a trade-off between body size and the developmental rate.

13

14   **Discussion**

15   The salamander species *H. retardatus* experiences greater variability in the risk for

16   desiccation in ephemeral ponds than in permanent ponds (Iwasaki & Wakahara, 1999).

17   In this study, we experimentally shortened the duration of the larval period of *H.*

18   *retardatus* by raising the larvae at a low water level, indicating that this species exhibits

1   phenotypic plasticity in metamorphic timing in response to water level (Fig. 1). This

2   decrease in the duration of the larval period was dependent on the permanency of the

3   source pond (ephemeral or permanent) of the larvae, but not on the conspecific larval

4   density of the source pond; thus, the interaction between water level and pond

5   permanency was included in our final model (Table 2). This interaction shows that, in

6   this species, there is geographical variation in the plastic response in metamorphic

7   timing in response to water level (Fig. 1). These results imply that when populations

8   experience a drying pond, individuals from ephemeral habitats may alter their

9   physiological and developmental traits more than individuals from permanent habitats.

10   The acceleration of metamorphosis offers clear advantages to many pond animals,

11   which might face catastrophic mortality if their habitat were to dry up (Laurila &

12   Kujasalo, 1999; Altwegg & Reyer, 2003). However, this acceleration also has an

13   associated cost because of the trade-off between developmental rate and body size at

14   metamorphosis (Fig. 3). Therefore, in animals living in ephemeral ponds, evolution may

15   favor individuals with highly plastic developmental systems. At the other extreme,

16   however, theoretical study predicts that homogeneous environments can lead to the

17   evolution of specialists (Moran, 1992). Evolution may favor the development of

18   specialist phenotypes with limited phenotypic plasticity in animals living in permanent

1   water bodies, thus reducing the fitness costs of being phenotypically plastic (Relyea,

2   2002). In this study, individuals from permanent ponds displayed less plasticity in

3   response to water level (Fig. 1a), as indicated by the larger estimated hazard ratio (low

4   water level to high water level) for larvae from ephemeral ponds (58.5) than for larvae

5   from permanent ponds (11.3). Nevertheless, larvae from permanent ponds still produced

6   a plastic response in metamorphic timing in response to water level (Fig. 1a). These

7   results may suggest that individuals from permanent ponds do not experience selective

8   forces strong enough to produce specialist phenotypes. Furthermore, there may have

9   been alternating selection over time that has periodically selected against specialist

10   phenotypes (Relyea, 2002).

11   It is interesting that although the duration of the larval period of *H. retardatus* was

12   shortened by feeding the larvae conspecific larvae instead of Chironomidae, this

13   decrease in the larval period due to cannibalism depended not on the larval density of

14   the ponds from which the larvae were derived but instead on the pond's permanency.

15   This was indicated by the exclusion of the interaction between food type and pond

16   conspecific larval density from the final model, and the inclusion of the interaction

17   between food type and pond permanency (Table 2). These results imply that the

18   selective factor favoring the evolution of phenotypic plasticity in metamorphic timing in

1 response to food type may be pond permanency, even though the frequency of

2 cannibalism itself depends on the pond conspecific larval density (Michimae, 2006).

3 One question that remains is why plasticity in response to food type interacts with the

4 selective factor pond permanency instead of conspecific larval density.

5   Conspecifics are assumed to provide a better balance of nutrients than

6 heterospecific foods, with the result that cannibalism can greatly enhance an

7 individual's growth or rate of development (Elgar & Crespi, 1992). However, in this

8 study, *H. retardatus* larvae fed only conspecific larvae metamorphosed much earlier but

9 at a smaller size than those fed only Chironomidae (Fig. 3). These results suggest that

10 cannibalism in *H. retardatus* larvae may have increased the rate of development but did

11 not increase the growth rate (Fig. 3). The fast development associated with cannibalism

12 might result in amphibian larvae metamorphosing before the ephemeral ponds they

13 inhabit dry up. Therefore, in the case of amphibians, cannibalism may also be an

14 important mechanism by which the larvae reach the necessary developmental stage

15 before the pond in which they were spawned dries up, thus reducing larval mortality due

16 to desiccation (Lannoo & Bachmann, 1984). In particular, *H. retardatus* larvae spawned

17 in ephemeral ponds must metamorphose as soon as possible after the ponds begin

18 drying up (Iwasaki & Wakahara, 1999); therefore, such larvae may have secondarily

1 utilized their cannibalism-mediated developmental plasticity to cope with the risk of

2 desiccation. Thus, it follows that the cannibalism-mediated developmental plasticity that

3 evolved to enhance the rate of development and conditions also depends on the pond

4 permanency. However, the lack of dependence of the cannibalism-mediated

5 developmental plasticity on the pond conspecific larval density still requires

6 explanation.

7   The plastic responses in metamorphic timing to experimental changes in water

8 level and food type were correlated (Fig. 2a), which indicates that these two plastic

9 responses may have evolved correlatively. Unfortunately, our present data cannot

10 resolve which plastic response evolved first in *H. retardatus*, the one to water level or

11 that to food type, but the plastic response in metamorphic timing to water level appears

12 to be a relatively ancient trait. The ability to accelerate metamorphosis in response to a

13 reduction in water level is present in most amphibian species and is widely conserved in

14 the genus *Hynobius* (Misawa & Matsui, 1997; Mori & Natuhara, 2004). However,

15 plastic response in metamorphic timing to food type, especially cannibalism, might

16 represent an apomorphic trait found only in the species *H. retardatus*, because

17 cannibalism-mediated developmental plasticity, particularly cannibalism-mediated

18 acceleration of metamorphosis, has been reported in no other species in the genus

22

1   *Hynobius*. Thus, in *H. retardatus*, phenotypic plasticity in metamorphic timing may

2   have been initially selected in response to risk of habitat desiccation, as indicated by a

3   water level reduction, which is the most reliable and informative cue for pond drying.

4   Once the initial plasticity evolved in this ecological context, the evolution of a response

5   to additional ecological factors (e.g., food type), utilizing pre-existing developmental

6   pathways, might have been relatively easy. There is some evidence showing how

7   environmental factors modulate the HPT axis under desiccation stress and the

8   availability of alternative food types (Pfennig, 1992; Denver, 1997; Ledon-Rettig *et al.*,

9   2010). Western spadefoot toad (*Scaphiopus hammondii*) tadpoles respond to decreasing

10   water levels by increasing production of corticotropin-releasing hormone (CRH, Denver,

11   1997; Denver, 1998). CRH stimulates not only thyroid hormone production in tadpoles,

12   but also production of the stress hormone corticosterone in *Spea multiplicata*, which

13   accelerates metamorphosis by converting thyroxine to the more potent triiodothyronine

14   (Rose, 2005). Carnivore morphs in southern spadefoot toad (*Scaphiopus multiplicatus*)

15   tadpoles are induced directly by thyroxine in conspecific larvae, which accelerates their

16   development (Pfennig, 1992). In short, the acceleration to a reduction in the water level

17   may involve an endogenous sensory and hormonal response that leads to activation of

18   the developmental pathways. Experimental changes in food type may involve activation

23

1   of the developmental pathways by exogenous hormones obtained by eating conspecific

2   larvae. The two plastic responses in metamorphic timing might use the same

3   downstream developmental pathways. In addition, plastic responses in SVL to

4   experimental changes in water level or food type were negatively correlated (Fig. 2b),

5   indicating that plastic responses in SVL may have a shared downstream developmental

6   pathway. Although the reduction of the larval period caused by being reared in low

7   water level or eating conspecifics also caused the larvae to have a smaller body size at

8   metamorphosis, plastic responses in body size to water level or food type might be

9   directly and partially regulated by the developmental pathways overlapping with the

10   HPT axis (Rose, 2005). However, the upstream regulation of the stress response to

11   water level and the regulation of eating conspecific larvae or not may be different from

12   each other. Nevertheless, the plastic responses in metamorphic timing to experimental

13   changes in water level and food type were correlated (Fig. 2a). Although the impact of

14   the upstream regulation or the decision-making process on the evolution of plastic

15   responses in metamorphic timing is not known, phenotypically plastic responses

16   triggered by different environmental cues might evolve correlatively because of

17   constraints imposed by their shared downstream developmental pathways. The main

18   selective factor, pond permanency, might have favored the evolution of phenotypic

1   plasticity in metamorphic timing, with the degree of expressed plasticity dependent on

2   the ecological context. As a result, the phenotypic plasticity in metamorphic timing does

3   not depend on pond conspecific larval density.

4   Furthermore, a correlation between evolutionary changes in two traits can exist if

5   the selection factors giving rise to the traits are correlated. Thus, the correlation we

6   found between the two different plastic responses in metamorphic timing might be due

7   to their selections factors being correlated or even the same. For example, pond

8   permanency may be strongly correlated with pond conspecific larval density in ponds.

9   For amphibians, informative cues of environmental drying include an increase in

10   conspecific density, a decrease in swimming volume, a decrease in food, and changes in

11   the chemical and physical properties of the water (Denver et al., 1998). Furthermore, an

12   increase in conspecific larval density caused by an ephemeral pond drying up may be a

13   reliable cue of pond drying to a larva (Newman, 1998).

14   It is possible that the correlation between the plastic responses in metamorphic

15   timing is due to an adaptive response to desiccation risk in ephemeral ponds, favoring

16   the evolution of the plasticity based on one or more of the environmental cues described

17   above. However, our results related to metamorphic plasticity in *H. retardatus* may not

18   be congruent with this scenario. Although desiccation of ephemeral ponds can lead to

25

an increase in conspecific larval density, conspecific larval density in permanent ponds

is not necessarily low, as indicated by the high conspecific density in the permanent

pond Tomaru examined in this study, as well as in other permanent ponds. Phenotypic

plasticity in metamorphic timing in response to food type, therefore, may not depend on

the conspecific larval density of the source pond but instead on the pond permanency,

because the two plastic responses are correlated as a constraint caused by their sharing

the same downstream developmental pathway (Rose, 2005).

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Table 1 Features of the 9 discrete sites (ponds) where Hynobius retardatus samples were collected.

Sampling site	Latitude	Longitude	Monitoring period for pond permanency (year)	Pond permanency	Monitoring period for larval density (year) except for 2005	Annual density of conspecific larvae (individuals/n					Mean density of conspecific larvae (individuals/m2 ± SD)
						2002	2003	2004	2006	2007	
Erimo	42.1252	143.2529	9 (from 2000 to 2009)	ephemeral	5 (from 2002 to 2007)	412.7	455.2	379.3	452.5	556.4	451.2 ± 66.6
Konuma	43.1916	140.6099	7 (from 2002 to 2009)	permanent	5 (from 2002 to 2007)	12.0	15.0	13.6	17.1	18.8	15.3 ± 2.7
Nopporo	43.0651	141.5039	9 (from 2000 to 2009)	ephemeral	5 (from 2002 to 2007)	82.0	75.0	76.0	73.4	82.9	77.9 ± 4.3
Tomaru	43.1907	140.5151	7 (from 2002 to 2009)	permanent	5 (from 2002 to 2007)	2034.6	1997.9	2366.3	2243.5	2484.0	2225.3 ± 209.3
Atsuta	43.4445	141.4144	6 (from 2003 to 2009)	ephemeral	4 (from 2003 to 2007)		1560.0	1378.6	1579.2	1347.1	1466.2 ± 120.3
Kamitobetsu	43.2333	141.4819	6 (from 2003 to 2009)	ephemeral	4 (from 2003 to 2007)		290.8	355.5	200.9	165.7	253.2 ± 86.2
Okusawa	43.1447	140.9605	9 (from 2000 to 2009)	ephemeral	4 (from 2003 to 2007)		74.9	49.7	56.0	91.3	68.0 ± 18.9
Toyoha	42.9811	141.1599	9 (from 2000 to 2009)	ephemeral	4 (from 2003 to 2007)		110.0	115.7	111.3	135.6	118.1 ± 11.9
Asari	43.0524	141.0412	18 (from 1992 to 2009)	permanent	2 (2006 and 2007)				149.8	135.6	142.7 ± 10.1

1

Table 2 Values of −2logL for models fitted to the data of time to metamorphosis. Experimental treatments were water level (w) and food type (f), and covariates were pond permanency (p; as a categorical covariate) and pond conspecific larval density (as a continuous covariate). Identities for egg clutch were included as a random effect, accounting for the within-clutch correlation.

Variables in model	-2log$L$
None	9870.017
Pond permanency	9858.272
Pond conspecific larval density	9870.016
Pond permanency + pond conspecific larval density	9857.400
Pond permanency + water level	9332.887
Pond permanency + food type	9622.808
Pond permanency + water level + food type	8761.745
Pond permanency + water level + food type + w*f	8761.739
Pond permanency + water level + food type + w*p	8677.528
Pond permanency + water level + food type + f*p	8737.330
Pond permanency + water level + food type + w*p+f*p	8648.875
Pond permanency + water level + food type + w*p+f*p+clutc	8466.763

1

2 NOTE: First, 2 environmental factors were selected, ignoring the treatment effects. Pond

3 permanency led to the reduction in −2logL by 12.745 (P = 0.0036), while pond conspecific larval

4 density led to the reduction in −2logL by 0.001 (P = 0.97). Second, we built a Cox model based on

5 the 2 environmental factors. When pond permanency was omitted, −2logL increased by 12.616 (P =

6 0.00038), whereas when conspecific larval density was omitted, −2logL increased by 0.872 (P =

7 0.35). Hence, larval density was not considered further for inclusion. Third, we considered the

8 effects of the 2 experimental factors. In this way, any differences between the 2 groups that arose as

9 a result of differences between the distributions of the covariates were adjusted. When water level

10 was added to the model containing pond permanency, the −2logL value was reduced by 524.385 (P <

11 0.0001). When food type was added, the reduction was 234.464 (P < 0.0001). Finally, the

12 interactions among the 3 significant variables were considered. The interaction of water level by

13 food type was not significant, whereas the other two were significant. Therefore, the final model

14 comprised the 2 experimental factors (water level and food type), 1 environmental factor (pond

15 permanency), and 2 interactions (water level by pond permanency and food type by pond

16 permanency).

Table 3 MANCOVA results for the effects of experimental factors (water level and food type) and environmental factors (pond permanency and pond conspecific larval density as covariate) on body size (snout-vent length and body mass).

MANCOVA

Factor	Walks' lambda	d.f.	*F*	*P*
Water level	0.064	2, 30	219.197	<0.0001
Food type	0.255	2, 30	43.857	<0.0001
Pond permanency	0.936	2, 30	1.030	0.3694
Pond conspecific density	0.958	2, 30	0.656	0.5262

1

Figure 1

(a) Permanent

(b) Ephemeral

Time to metamorphosis (day)

Figure 2

Figure 3

(a)

(b)

Median time to metamorphosis (day)
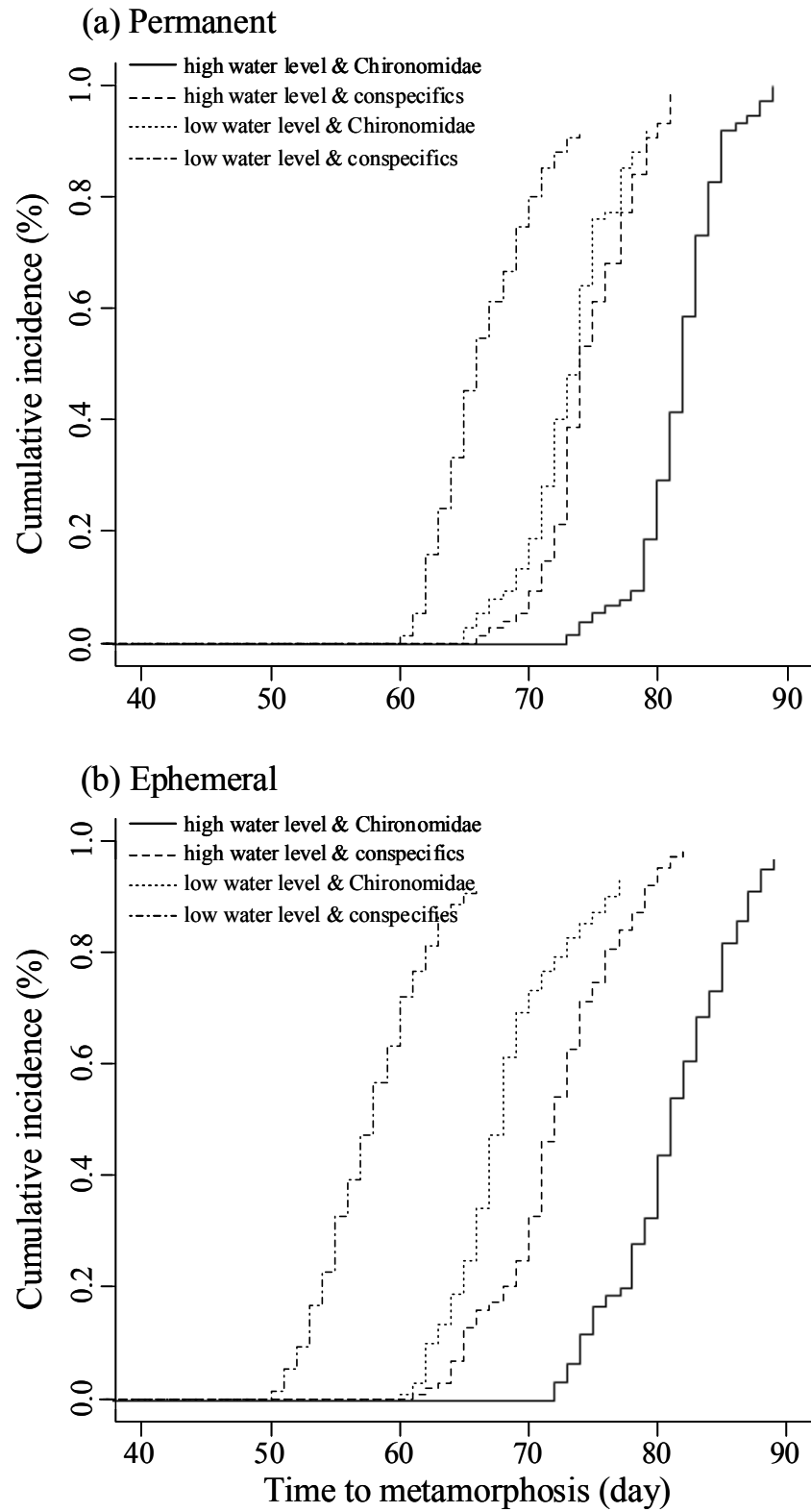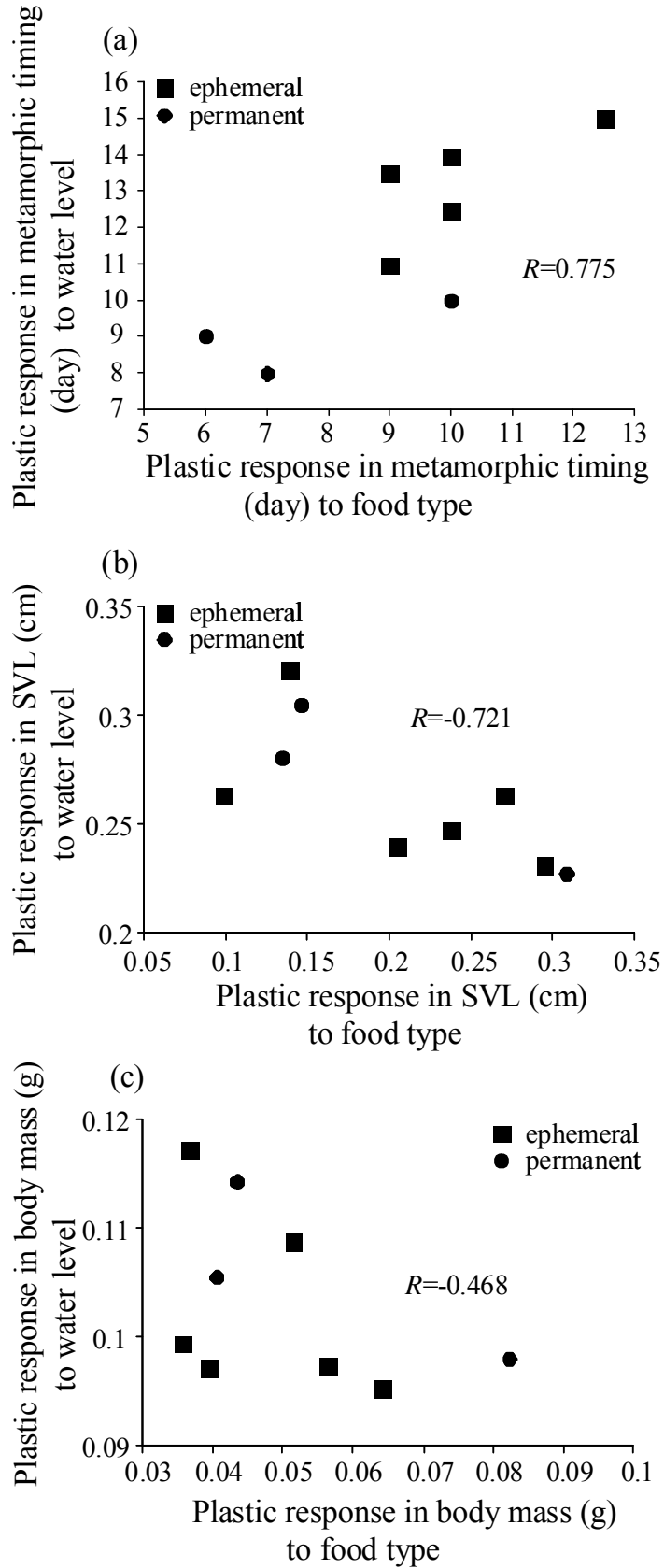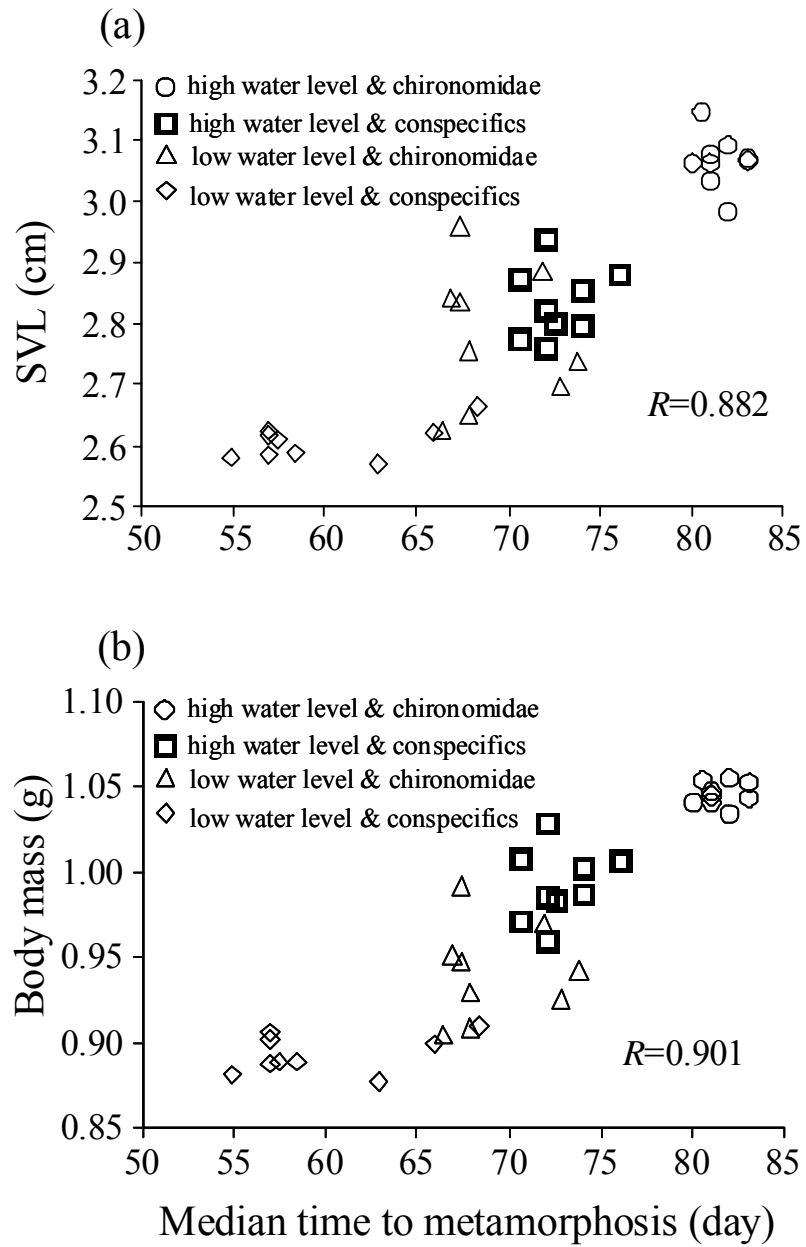
1 **Figure legends**

2

3 **Fig. 1** Cumulative incidence functions (%) for time to metamorphosis with each

4 experimental treatment of larvae from permanent ponds (a) or ephemeral ponds (b).

5

6 **Fig. 2** Results of correlation tests between two plastic responses in metamorphic timing

7 (a; days), snout-vent length (A; cm), and body mass (B: g) to water level and food type

8 at the population level. Two points (Kamitobetsu and Nopporo) in Fig. 2a are

9 overlapping. Body mass was transformed by taking its cube root before analysis.

10

11 **Fig. 3** Results of correlation tests between snout-vent length (SVL) at metamorphosis

12 and age at metamorphosis (a), and between body mass at metamorphosis and age at

13 metamorphosis (b). Body mass was transformed by taking its cube root before analysis.

14

15