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Maternal effects on phenotypic plasticity in larvae of the salamander *Hynobius retardatus*

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Abstract Maternal effects are widespread and influence a variety of traits, for example, life history strategies, mate choice and capacity to avoid predation. Therefore, maternal effects may also influence phenotypic plasticity of offspring, but few studies have addressed the relationship between maternal effects and phenotypic plasticity of offspring. We examined the relationship between a maternally influenced trait (egg size) and the phenotypic plasticity of the induction rate of the broad-headed morph in the salamander *Hynobius retardatus*. The relationship between egg size and the induction of the broad-headed morph was tested across experimental crowding conditions (densities of low conspecifics, high conspecifics, and high heterospecific anuran), using eggs and larvae from eight natural populations with different larval densities of conspecifics and heterospecifics. The broad-headed morph has a large mouth that enables it to consume either conspecifics or heterospecifics, and this ability gives survival advantages over the normal morph. We have determined that there is phenotypic plasticity in development, as shown by an increase in the frequency of broad-headed morph in response to an increase in the density of conspecifics and heterospecifics. This reaction norm differed between populations. We also determined that the frequency of the broad-headed morph is affected by egg size in which larger egg size resulted in expression of the broad-headed morph. Furthermore, we determined that selection acting on the propensity to develop the broad-headed morph has produced a change in egg size. Lastly, we found that an increase in egg size alters the reaction norm to favor development of the broad-headed morph. For example, an equal change in experimental density produces a greater change in the frequency of the broad-headed morph in larvae developing from large eggs than it does in larvae developing from small eggs. Population differences in plasticity might be the results of differences in egg

size between populations, which is caused by the adaptive integration of the plasticity and egg size. Phenotypic plasticity can not evolve independently of maternal effects.

Introduction

Maternal influences, known as maternal effects, often affect traits of adaptive importance, for example, life history strategies, mate choice, or predator avoidance, and have been reported in a wide range of taxa (Bernardo 1996; Mousseau and Fox 1998; Agrawal et al. 1999). An individual's phenotype is influenced by its own genotype and by the environment that it experiences itself, as well as by the genotype and environmental experience of other individuals, especially its mother (Mousseau and Fox 1998). The level of maternal investment can profoundly influence the development of embryos and the phenotypes and survival of the hatchlings, especially in animals that do not practice parental care (Fleming and Gross 1990; Sinervo and Doughty 1996; Fox et al. 1997, Einum and Fleming 1999), and the evolutionary fitness of both offspring and parents (Smith and Fretwell 1974; McGinley et al. 1987; Fox et al. 1997).

Egg size can be important for the fitness of cannibalistic oviparous amphibians because larger individuals, which develop from larger eggs, can catch and consume larger prey items (e.g., conspecific and heterospecific amphibian larvae), and have higher survivorship than smaller individuals that developed from smaller eggs (Michimae 2007). In the salamander *Hynobius retardatus*, egg size can diverge between populations in response to differences in the density of conspecific and heterospecific anuran larvae (*Rana pirica*) in natural ponds, with eggs being larger when larval density is higher (Michimae 2007). Maternal investment strongly influences the size of eggs and the potential for efficient cannibalism during in the larval period (Michimae 2007).

H. retardatus larvae can exhibit a broad-headed morph that is induced from a normal morph during early development in some individuals under some environmental

conditions. This alternative morph is particularly common in larvae of the tiger

salamander *Ambystoma tigrinum*, in which it has been called a cannibal morph (Lannoo

and Bachmann 1984). Crowding, either among conspecifics or with heterospecific

anuran larvae, induces this alternative morph in many individuals in *H. retardatus*

larvae (Michimae and Wakahara 2002) and *A. tigrinum* larvae (Whiteman et al. 2003).

This morph is behaviorally aggressive and has a large mouth that enables it to consume

either conspecifics or heterospecific anuran larvae (Stephen et al 1992). This ability

gives it a feeding advantage over the normal morph in these conditions and results in

growth and survival advantages, but these advantages may vary ontogenetically (Denoel

et al. 2007). Broad-headed morphs that have been consuming both conspecific larvae

and typical prey items (freshwater oligochaetes) are much larger at metamorphosis than

normal morphs (Wakahara 1997), but those fed only conspecific larvae metamorphose

much earlier and at a smaller size than normal larvae fed only typical prey items

(Michimae and Wakahara 2002). The fast development rates that result from

cannibalism may allow larvae to metamorphose before the pond in which they were

spawned dries up, reducing mortality due to desiccation (Lannoo and Bachmann 1984).

Even if induction of the broad-headed morph increases the likelihood of survival during

the larval stage, there is a trade-off associated with the morph (accelerated development

can result in a smaller size at metamorphosis) which can negatively affect

fitness-related traits expressed later in life (e.g., Altwegg and Reyer 2002).

   Variable egg size is a representative example of a developmental modification,

because egg size affects early embryonic development epigenetically and, consequently,

the phenotype of the larva (Gilbert 2003). There is phenotypic plasticity in the

development of *H. retardatus* larvae, as shown by an increase in the frequency of

broad-headed morphs in response to the density of conspecifics and heterospecific anuran larvae (Michimae and Wakahara 2002). A positive relationship between plasticity in the frequency of the broad headed morph and egg size would provide necessary evidence for adaptive integration of plasticity and egg size and support the idea of coadaptation (Doughty 2002). However, natural selection can act on morphological plasticity and trophic polyphenism (Moran 1992), driving evolution of different reaction norms among populations (Pfennig & Murphy, 2000; Laurila et al., 2002; Trussell & Nicklin, 2002). Plasticity in the frequency of the broad-headed morph among natural populations may be caused by the adaptive response of the plasticity itself, which would support the idea that plasticity and egg size are optimized independently. Here we hypothesize that egg size (a maternal effect) and plasticity of expression of the broad-headed morph in larval *H. retardatus* (an offspring trait) are phenotypically correlated during the larval stage, because in early development maternal effects are large and closely related to many traits (Gilbert 2003). If a phenotypic correlation between these two traits does exist, then we can evaluate and discuss the adaptive value of the maternal effect in relation to this plasticity. In the present study, we used larvae from eight natural populations of the salamander *H. retardatus* from eight different ponds (Michimae 2006). Larval salamanders in each pond have a different probability of encountering conspecifics or *R. pirica* larvae. The eight ponds represent different points along an environmental continuum of conspecific and heterospecific larval density.

Methods

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Study Populations

Since our focus was on the adaptive aspect of egg size and the polyphenic feeding morph in natural environments, we did not choose the *Hynobius retardatus* larval habitats randomly. We chose eight discrete larval habitats (ponds) in Hokkaido, Japan, characterized by different densities of conspecific and heterospecific (anuran *Rana pirica*) larvae. The number of *H. retardatus* egg clutches in each pond and the number of eggs in each collected clutch were counted to estimate the conspecific larval density in each habitat. The annual density of conspecific larvae in each pond was estimated by multiplying the mean clutch size (see Egg size and Clutch size) by the estimated density of clutches in the pond. The number of clutches of *R. pirica* eggs in the eight *H. retardatus* habitats was also counted to estimate the larval densities of *R. pirica* in each habitat. The annual density of *R. pirica* larvae in each pond was estimated by multiplying the assumed value of 800 eggs per clutch by the estimated density of clutches in the pond. We know both the end of the oviposition period of *H. retardatus* and *R. pirica* and also the total number of egg clutches of *H. retardatus* and *R. pirica* in each pond because we visited all eight ponds two or three times, about every 14 to 20 days, during the breeding season of each pond (Table 1).

 Each *H. retardatus* larval habitat used in this study had been previously investigated over two or three years, so the density of conspecific and heterospecific larvae in each pond was already known (Michimae 2006). The mean annual density of conspecific and *R. pirica* larvae in each pond was calculated by dividing the sum of the annual total larval densities by the number of years that clutches were collected. The respective mean densities of conspecific larvae (individuals/m^2 ± SD), heterospecific larvae

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(individuals/m^2 ± SD), and conspecific plus heterospecific larvae (individuals/m^2 ± SD) was calculated for each pond: Konuma (13.5 ± 1.5, 0, 13.5 ± 1.5), Nopporo (77.7 ± 3.8, 53.3 ± 9.2, 131.0 ± 8.5), Okusawa (62.3 ± 17.8, 93.3 ± 18.9, 155.6 ± 1.0), Toyoha (112.8 ± 4.0, 280.0 ± 56.6, 392.8 ± 52.6), Kamitobetsu (323.2 ± 45.8, 100.0 ± 141.4, 423.2 ± 95.7), Erimo (415.7 ± 38.0, 977.8 ± 113.0, 1393.5 ± 85.4), Atsuta (1469.3 ± 128.3, 1240.0 ± 56.6, 2709.3 ± 71.7), and Tomaru (2132.9 ± 202.9, 1844 ± 453.8, 3799.9 ± 405.9) (Table 1 in Michimae 2006). We used mean larval densities in the statistical analyses described below. The detailed abiotic and biotic features (surface area, depth, density of egg clutches, and larval density of *H. retardatus* and *R. pirica*) and the geographic locations of the ponds are described in Table 1 and Fig. 1, respectively, of Michimae (2006).

Some studies have reported that egg mass counts are highly correlated with numbers of larvae in a natural pond, and that the number of egg masses is an accurate indicator of larval density, especially initial larval density (Matsushima and Kawata 2005) if embryonic survival is known. We consider the initial larval density in natural ponds to be especially important for induction of the broad-headed morph because it is usually induced only in larvae within the first week after hatching (Nishihara-Takahashi 1999) and because hatching dates of *H. retardatus* clutches in the same pond do not vary extremely (personal observation).

Fertilized egg clutches of *H. retardatus* were collected during the breeding season in 2004 (early April to late May) from each of the eight ponds: Atsuta (16 clutches), Erimo (30), Kamitobetsu (13), Konuma (24), Nopporo (30), Okusawa (31), Tomaru (29), and Toyoha (26). Fertilized egg clutches of *R. pirica* were also collected during their breeding season (early April to late May) in 2004 from several ponds at different

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altitudes in the vicinity of Sapporo. All *R. pirica* clutches were then transferred to the laboratory and placed in plastic tanks, where they were kept at room temperature (20–21 °C).

Egg size and clutch size

The egg clutches from each population of *H. retardatus* were numbered. Then the number of eggs in each clutch was counted, and five randomly selected eggs from each clutch were dejellied and their diameters measured to the nearest 0.01 mm under a dissecting microscope. The eggs were measured at the blastula developmental stage or earlier to minimize the effect of increasing egg size with further development. Then the representative egg size (mean egg volume) of each clutch was calculated by assuming that the eggs were spherical (sum of five egg volumes/five).

  After counting the number of eggs and measuring diameters of eggs in each clutch, each clutch was placed in a stock tank filled with 1.6 L of dechlorinated tap water at 4 °C. Because the developmental stages of the embryos were very different among the populations, the hatching day of the embryos was controlled by placing the embryos at either 4 °C or room temperature (20–21 °C), to slow or speed development, respectively.

Induction experiment of the broad-headed morph

To investigate the frequency of the broad-headed morph in different proximate environments, we conducted an induction experiment. After the eggs hatched, we

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assigned nine of the clutches from each of the eight populations (72 in all) to three different experimental treatments consisting of three levels of crowding: low conspecific density, high conspecific density, and high heterospecific density. Thirty randomly selected larvae from each of these 72 clutches were divided equally among the three different crowding treatments. The 10 larvae in each treatment were all from the same clutch (i.e., full sibs). Therefore, for each population we replicated each treatment nine times (nine clutches of full sibs for each population). The low-conspecific-density treatment consisted of only 10 *H. retardatus* larvae in a large tank (22 × 15 × 12.5 cm deep) with 1.6 L of dechlorinated tap water. The high-conspecific-density treatment consisted of 10 *H. retardatus* larvae in a small tank (8 × 8 × 8 cm deep) with 0.3 L of dechlorinated tap water. The high-heterospecific-density treatment consisted of 10 *H. retardatus* larvae and 40 *R. pirica* larvae in a large tank with 1.6 L of dechlorinated tap water. The numbers of *R. pirica* larvae were counted every morning because they were continuously eaten by *H. retardatus* larvae. Additional heterospecific larvae were added to each tank in the treatment group as necessary to replace those that had been eaten by the *H. retardatus* larvae since the previous morning so that each tank of this treatment always contained 40 *R. pirica* larvae.

The larvae used for the experiment were offered frozen Chironomidae from 9:00 A.M. to 12:00 noon every other day. Larvae were always given 3 h to eat the food, and any food remaining in their tanks was removed after the feeding period. The water was exchanged every other day during the experiments. All experiments were conducted at room temperature in the laboratory (20–21 °C) with a natural light/dark schedule.

We counted the numbers of broad-headed and normal morphs among the 10 larvae in

each tank of the three experimental treatments every morning during the experimental

period (2 weeks). We began the experiments at approximately the same time for each

population, but at slightly different times among populations because we had difficulty

synchronizing hatching among all eight populations. All experiments were begun

between 24 and 31 May 2004. The morphological characteristics by which the

broad-headed and normal morphs were distinguished were described in our previous

study (Michimae and Wakahara 2002). Briefly, we calculated the ratio of head width at

the level of the eyes (HWE) to the largest head width (LHW). HWE/LHW $\geq$ 0.86

defined a broad-headed morph, and a ratio <0.86 a normal morph. For each clutch, the

frequency of the broad-headed morph in each tank was recorded along with the mean

egg size of the clutch, measured previously.

   Induction of broad-headed morph was observed during the first week after hatching,

but cannibalism was infrequent during this period. Since we counted the number of

broad-headed morphs in each tank every day, we could accurately determine the

number of each morph in the tanks even if some of the larvae had died by cannibalism.

The frequency of the broad-headed morph was expressed as the ratio of the number of

broad-headed morphs to the initial number of *H. retardatus* larvae in the tank.

Statistical analyses

To test whether there was a possible relationship between the mean larval density of the

pond in which a female lived and the egg size she produced, we applied a non-linear

regression model (power fit model) to the relationship.

   The effect of egg size is particularly pronounced during early larval growth and

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development and morphogenesis (Mousseau and Fox 1998). Egg size, therefore, is a property of the egg that may be an explanatory factor for the frequency of the broad-headed morph. It would be ideal to use egg clutches collected under well-controlled conditions (e.g., in a laboratory), because egg size in natural environments might be affected by unknown and uncontrolled environmental factors and the conditions of the female and male parents. However, it is extremely difficult to breed wild adult salamanders in the laboratory because they refuse to eat. Thus, we used size of eggs collected from natural ponds for the following analysis. We conducted analysis of covariance (ANCOVA) with standardized log10- transformed mean egg size of the clutch as a covariate, an arcsine square root transformed frequency of the broad-headed morph in each tank as a dependent variable, and two factors, population as a confounding factor that might affect the frequency of the broad-headed morph and experimental treatment.

  Eight populations used for ANCOVA were quite distinct in the space of arcsine square root transformed frequency of the broad-headed morph in each tank / log10- transformed mean egg size of the clutch. This violates one of the assumptions of the ANCOVA analysis. We therefore standardized the log10- transformed mean egg size of the clutch to a zero mean per population in order to properly test the lack of the various interactions between covariate and factors.

Results

Phenotypic plasticity in development and population differences in phenotypic plasticity

Experimental treatment and source population significantly affected the frequency of the broad-headed morph in the larvae of *H. retardatus* (Table 2). The effects of experimental treatment on the frequency of the broad-headed morph indicate that the morph is plastic. However, the ANCOVA also revealed a significant interaction between experimental treatment and source population (Table 2), indicating that the relationship between experimental treatment and frequency of the broad-headed morph differed among populations. The interaction between experimental treatment and source population shows population differences in the phenotypic plasticity of the broad-headed morph.

  The relationship between the experimental treatment and the expression of the broad-headed morph is shown in Fig. 1 for each source population. Although the frequency of the broad-headed morph increased with an increase in crowding, larvae derived from ponds with high larval density exhibited a greater change in the frequency of the broad-headed morph compared with larvae derived from ponds with low larval density (Table 2, Fig. 1).

Effects of egg size on frequency of expression of broad-headed morph

Egg size also significantly affected the frequency of the broad-headed morph in the larvae of *H. retardatus* (Table 2). The main effects of egg size indicate that the maternal investment in a larger egg size has a positive effect on the frequency of the broad-headed morph. (Fig. 3).

Relationship between natural larval density and egg size

The power fit model was Y (egg size) = 10.171 * X (larval density) ^ 0.099 (Fig. 2; regression coefficient 10.171, $t = 7.746$, d.f. = 6, $P < 0.001$; regression coefficient 0.099, $t = 2.054$, d.f. = 6, $P = 0.085$). The sigmoidal curve relating the mean larval density with egg size suggests that the mean egg size increased with the mean larval density in the ponds, although the regression coefficient (0.099) indicated a very strong trend. The pattern of differences in egg size among source populations is also consistent with the idea that selection acting on the propensity to produce the broad-headed morph has produced a change in egg size.

Maternal effects on phenotypic plasticity

ANCOVA revealed a significant interaction between experimental treatment and egg size (Table 2), indicating that the effects of egg size on the frequency of the broad-headed morph differed between experimental treatments. The interaction between egg size and experimental treatment demonstrates maternal effects on phenotypic plasticity itself.

  The relationship between the expression of the broad-headed morph and the mean egg size of the clutch from each source population is shown in Fig. 3 for each crowding treatment. The slopes through all eight populations were 0.539 (low conspecific density treatment), 0.829 (high conspecific density treatment) and 1.292 (high heterospecific density treatment). Although the frequency of the broad-headed morph increased with changes in the crowding treatment, larvae developing from large eggs exhibited a greater change in the frequency of the broad-headed morph compared with larvae developing from small eggs (Fig. 3).

Discussion

Phenotypic plasticity in development and population differences in phenotypic plasticity

In this study we found an important interaction between source population and experimental treatment on the frequency of the broad-headed morph, regardless of the main effects of experimental treatment or population itself, indicating that different reaction norms apparently existed among the eight populations from discrete habitats, which were subject to different pressures (Table 2, Fig. 1). Michimae (2006) previously found significantly different frequencies of the broad-headed morph among the eight source populations, which probably resulted from different developmental responses to experimental crowding treatments (low conspecific, high conspecific, and high heterospecific densities) among the populations. The frequency of the broad-headed morph in all treatments increased with an increase in the density of conspecific and heterospecific larvae in the natural habitat. Population differences in the expression of the broad-headed morph may be caused by the population-specific reaction norms (Fig. 1). Larvae derived from ponds with high larval density exhibited a greater change in the frequency of the broad-headed morph compared with larvae derived from ponds with low larval density (Fig. 1). Several studies recently have reported different reaction norms in populations occupying habitats with different levels of selection (Pfennig & Murphy, 2000; Laurila et al., 2002; Trussell & Nicklin, 2002). Different plastic responses, or different reaction norms between populations, provide good evidence that those different responses are adaptive (Laurila et al., 2002). In this study developmental plasticity that ranges from highly plastic response to non-plastic response at individual

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level can be a target of natural selection (Moran 1992) and at clutch (family) level we

can find different developmental plasticity in the different phenotypic plasticity of the

frequency of the broad headed morph. Therefore the different reaction norms between

populations (different phenotypic plasticity at population level) represented an adaptive

response to the selection pressures, which target plasticity itself (Fig. 1).

Population differences in egg size

Egg size and larval body size in amphibians are likely important components of fitness

because they are positively correlated with size at metamorphosis (Berven 1990; Scott

1994), which in turn correlates positively with the probability of further survival

(Altwegg and Reyer 2002), size at maturity (Smith 1987), and fecundity (Semlitsch et al.

1988), although the opposite pattern, in which a small egg size confers a survival

advantage, has also been reported (Kaplan and Phillips 2006). In the cannibalistic

salamanders *Hynobius retardatus* (Michimae 2007) and *Ambystoma opacum* (Scott

1990), the potential for size-dependent cannibalism may be greatly increased by

variation in the initial larval body size. Production of larger eggs in these species may

result in higher maternal fitness, despite a reduction in clutch size, because larvae from

small eggs are suitable prey items for larger larvae (Michimae 2007). When larval

density is higher, selection may favor larger eggs and individuals (Fig. 2). In various

taxa, mothers in poor condition produce larger (presumably better provisioned) eggs

and better quality offspring that show greater survivorship than mothers in good

condition (Fleming and Gross 1990; Sinervo and Doughty 1996; Fox et al. 1997; Einum

and Fleming 1999). This maternal effect on offspring size and quality is considered an

adaptive reproductive strategy (Bernardo 1996; Mousseau and Fox 1998) and is observed in insects (Fox et al. 1997), amphibians (Kaplan 1998), and other groups (Price 1998).

Maternal effects on phenotypic plasticity

We found main effect of egg size, suggesting changes in egg size affect the overall propensity to develop the broad-headed morph (Table 2). However, we also found an important interaction between egg size and experimental treatment on the frequency of the broad-headed morph (Table 2), suggesting that changes in egg size alter the reaction norm of the frequency of the broad-headed morph, such that, an equal change in experimental treatment produces a greater change in the frequency of the broad-headed morph in larvae developing from large eggs than it does in larvae developing from small eggs (Fig. 3). These results are interesting because it has recently been shown that maternal effects are widespread and influence a large variety of traits (Mousseau and Fox 1998) and because in some invertebrates and vertebrates maternal effects have been observed to be closely related to phenotypic plasticity (Royle et al. 2001; Mitchell and Read 2005; Nussey et al. 2005). Furthermore, maternal effects are most important during early development (Mousseau and Fox 1998), and the broad-headed morph is usually induced in larvae only within the first week after hatching (Nishihara-Takahashi 1999).

  Female salamanders might respond to crowding by producing larger (and presumably fewer) eggs, giving their offspring an advantage by making them more likely to change to the broad-headed morph and increasing their survival under harsh conditions. It is

possible that during egg production salamander females assess their local crowding

levels using either behavioral or chemical cues in the pond where they will deposit their

eggs and that populations have evolved mean differences in egg size. In the seed beetle

Stator limbatus, when host plants are switched, reproducing females adjust their egg

size to the new host through plasticity in maternal allocation of resources (Fox at al.

1997). Although the females may use a chemical cue to discriminate among hosts

before oviposition, the cue is not known. Hence, in the seed beetle, an adaptive change

in the maternal allocation of energy to offspring occurs because egg survivorship

depends on both egg size and the host plant.

In this study, although we found the interaction between egg size and experimental

treatment in results of ANCOVA, the three-factor interaction of egg size, source

population and experimental treatment was not found, indicating that the maternal

effects on phenotypic plasticity of the frequency of the broad-headed morph did not

differ between populations (Table 2 and Fig.3). The absence of variation in a

phenotypic combination or correlation suggests that it has been evolutionarily stabilized

(Lande 1979), although gene flow also can limit the evolution of variation in a

phenotypic combination. The observed correlation pattern, that larvae from large eggs

size have a greater increase in the frequency of the broad-headed morph than larvae

from small eggs, may indicate that the combination of these two traits, egg size and

phenotypic plasticity of expression of the broad-headed morph in the larval stage, are

adaptive. The population differences in plasticity that we detected (Fig. 1) might reflect

population differences in egg size caused by the adaptive integration of the plasticity

and egg size (Doughty 2002).

We infer that evolutionary differences in phenotypic plasticity of the morph induction

were caused not only by the adaptive response of the plasticity itself but also by the adaptive integration of the plasticity and egg size (Table 2, Figs. 1 and 3). Phenotypic plasticity has not evolved independently of maternal effects.

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Figure legends

Figure 1 Frequency (mean ± SD) of the broad-headed morph of the salamander

Hynobius retardatus in eight populations at three crowding treatments: low conspecific,

high conspecific, and high heterospecific larval density (n=9 per treatment). The eight

salamander populations studies are Okusawa (◯), Erimo (□), Atsuta (△), Konuma

(◇), Kamitobetsu (●), Tomaru (■), Toyoha (▲) and Nopporo (◆). Frequency data

are arcsine square-root transformed

Figure 2 The relationship between mean egg size and larval density in the eight

populations. See Fig.1 for symbol explanations. The power fit model is Y (egg size) =

10.171($t = 7.746$, d.f. = 6, $P < 0.001$)* X (larval density) ^ 0.099 ($t = 2.054$, d.f. = 6, $P =$

0.085)

Figure 3 The relationship between frequency of the broad-headed morph and

standardized log10-transformed mean egg size in the low conspecific density treatment

(◯; Y=0.539X+0.345, dotted line), high conspecific density treatment (△;

Y=0.829X+0.265, dashed line), and high heterospecific density treatment (□;

Y=1.292X+0.608, solid line). Frequency data are arcsine square-root transformed. We

standardized the log10-transformed egg size of the clutch to a zero mean per population

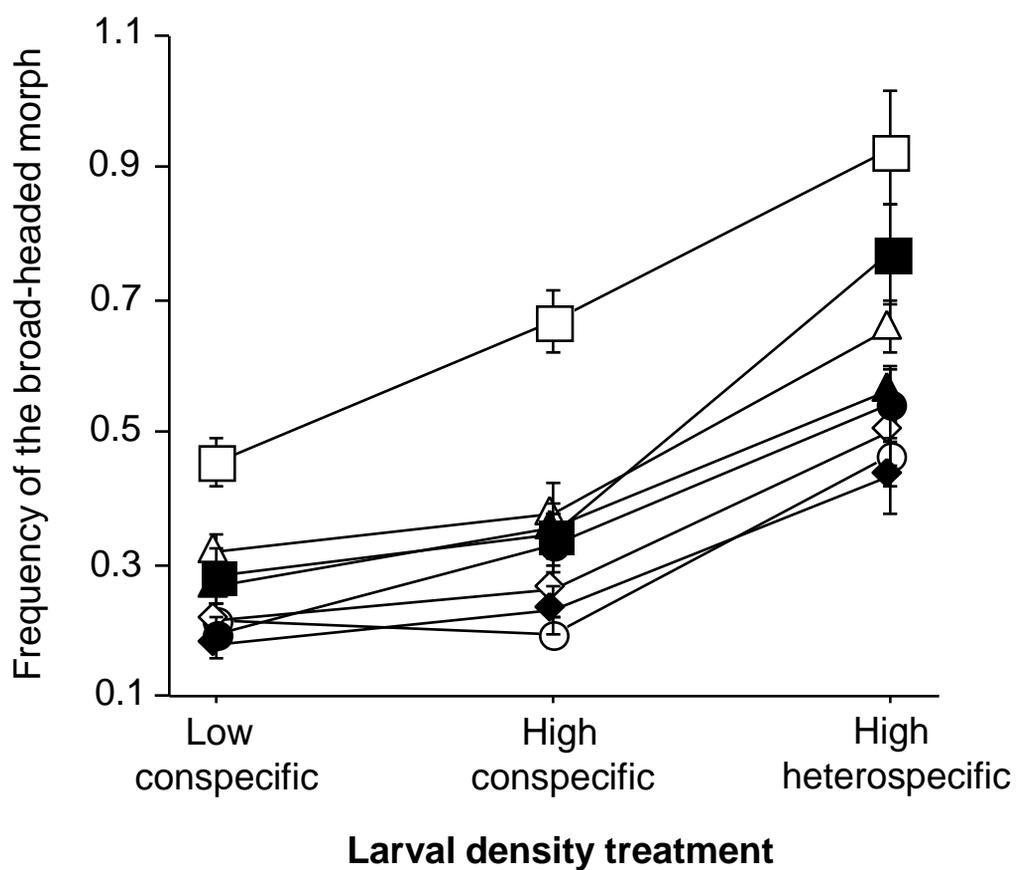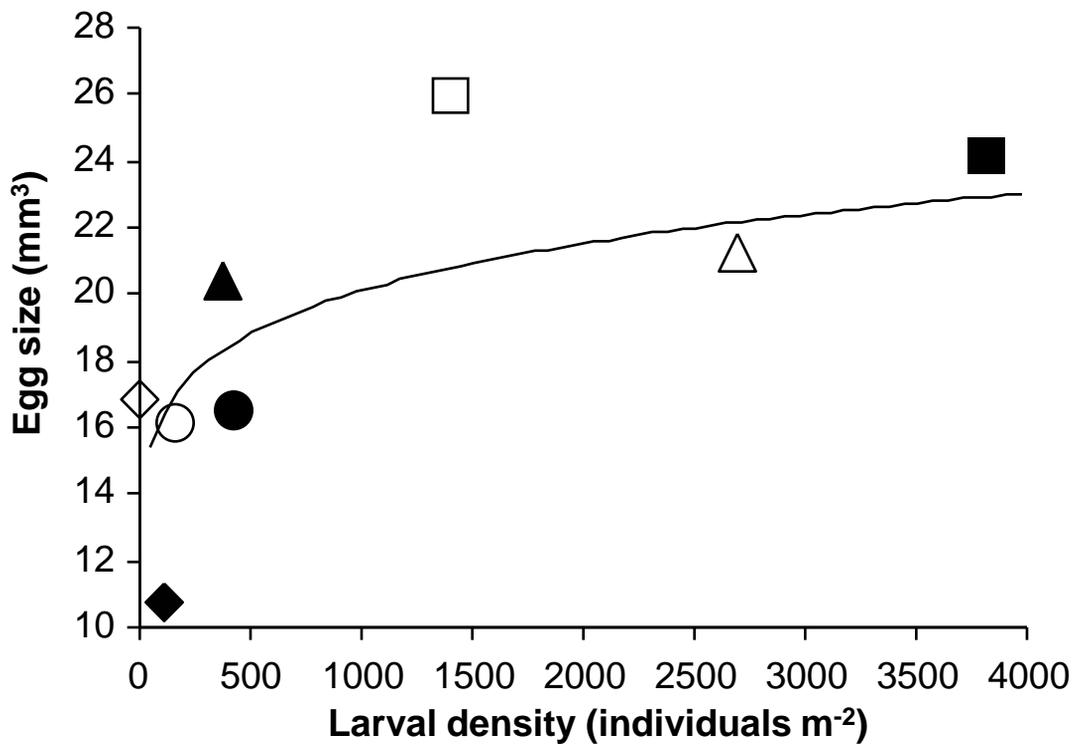(see methods for further explanation)
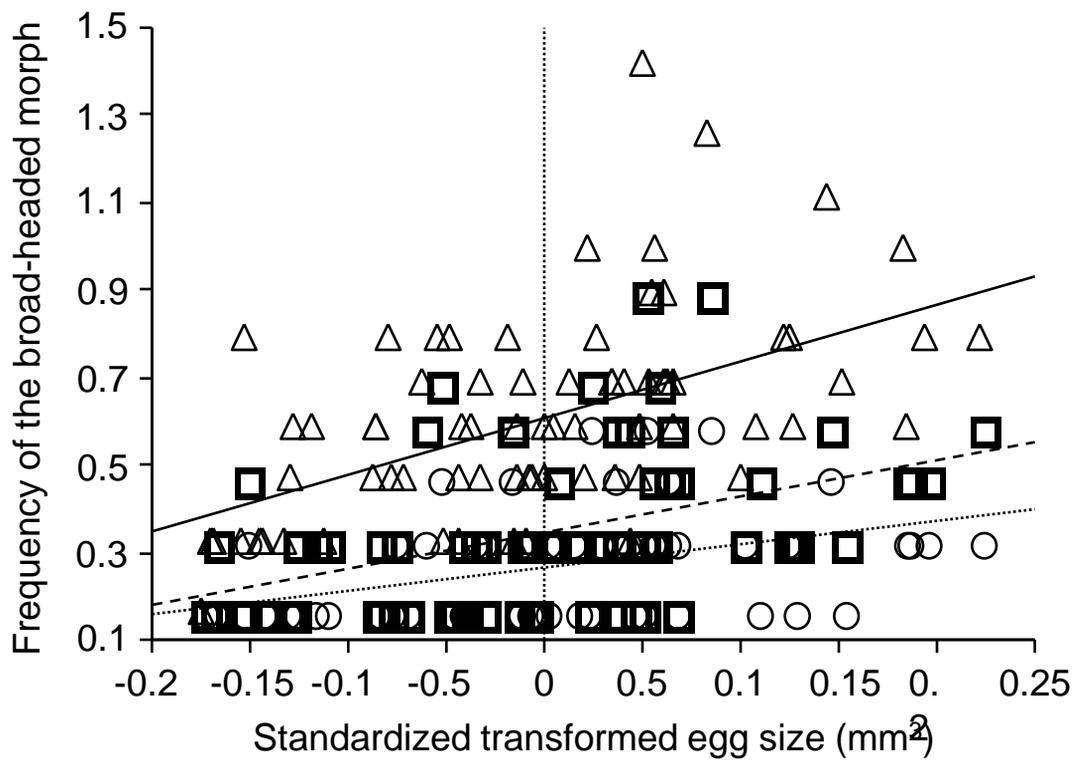
Figure 1

Figure 2

Figure 3

Table 1 Sampling dates and locations of the eight discrete sites (ponds) where *Hynobius retardatus* samples were collected in 2004.

Sampling site	Sampling date			Latitude	Longitude	Altitude (m)
Erimo	4/19	4/27	5/14	42.1252	143.2529	118.91
Konuma	5/12	6/3		43.1916	140.6099	311.15
Nopporo	4/8	4/14	5/4	43.0651	141.5039	63.92
Tomaru	4/22	5/12	6/3	43.1907	140.5151	356.14
Atsuta	4/23	5/1	5/13	43.4445	141.4144	59.65
Kamitobetsu	4/23	5/13		43.2333	141.4819	24.67
Okusawa	5/5	5/18		43.1447	140.9605	236.39
Toyoha	5/5	5/18		42.9811	141.1599	305.67

Table 2. Results from ANCOVA of the effect of experimental treatment and source population on frequency of the broad-headed morph, with egg size as covariate. Data were arcsine square root transformed and covariate was standardized after log10 transformed, before analysis, respectively.

Source	MS	d.f.	*F*	*P*
Experimental treatment (T)	2.319	2	245.734	<0.0001
Source population (P)	0.469	7	49.724	<0.0001
Egg size (E)	0.948	1	100.410	<0.0001
T*P	0.028	14	2.918	0.0006
T*E	0.054	2	5.759	0.0038
P*E	0.012	7	1.268	0.2690
T*E*P	0.003	14	0.340	0.9876
Error	0.009	168		