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Differentiated phenotypic plasticity in larvae of the cannibalistic salamander

Hynobius retardatus

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Abstract Alternative phenotypes in natural populations can arise from either genetic polymorphism or an environmentally induced phenotype, that is, polyphenism. Evolutionary models of polyphenism developed by theoretical studies predict that polyphenism is favored when there are environment-dependent fitness trade-offs between alternatives and that the threshold frequency for a facultative switch between alternative phenotypes is adjusted in accordance with different selection regimes. The broad-headed (alternative) larval morph of *Hynobius retardatus*, which is induced by crowding with conspecifics or heterospecific anuran (*Rana pirica*) larvae, is a representative example of cannibalistic polyphenism. Morph induction by such proximate factors must reflect evolutionary (conditional frequency-dependent) processes. To clarify the role of frequency-dependent processes in polyphenism, I investigated the occurrence rate of the broad-headed morph under experimental crowding conditions (low conspecific, high conspecific, and high heterospecific densities) using larvae from eight natural populations with different larval densities of conspecifics and heterospecifics, and found interpopulational differences in the expression of the morph. Thus, there is a larval density-dependent equilibrium frequency of the morph in each pond, suggesting that the local switch point for morph induction was modified by selection to produce evolved differences between ponds. The evolution of such interpond differences has three necessary conditions: (1) There are pond-dependent fitness trade-offs between alternatives, (2) The maintenance of the morph is costly, and (3) The presence of conspecific or, especially, heterospecific larvae provides a reliable cue to the receiver.

Keywords Broad-headed morph · Cannibalistic polyphenism · Phenotypic plasticity ·

Reaction norm · Salamander.

Introduction

Phenotypic plasticity is the ability of an individual organism to alter its phenotype in response to a change in environmental conditions. Phenotypic plasticity in many animals is adaptive (West-Eberhard 2003) and various degrees and timings of plasticity among populations reveal adaptation to a wide range of environments (Hjelm et al. 2001; Dahl and Peckarsky 2002; Trussell and Nicklin 2002). Several studies have compared phenotypic responses in populations occupying habitats with different levels of selection, conditions that promote the evolution of phenotypic plasticity (Leips et al. 2000; Dahl and Peckarsky 2002; Trussell and Nicklin 2002). Different plastic responses or different reaction norms among populations in different ecological settings provide good evidence that those different responses are adaptive (Laurila et al. 2002).

Polyphenism is the expression of two or more alternative phenotypes from a single genotype in response to different environmental stimuli (West-Eberhard 2003). Species or populations that exhibit polyphenism are ideal for studying the evolutionary causes of phenotypic plasticity because the occurrence of alternatives is easily discerned, making it possible to determine the fitness consequences of different phenotypes (West-Eberhard 2003). In particular, a species exhibiting cannibalistic polyphenism is an excellent model system for performing such evolutionary studies because the factors triggering the alternative morphology (in this case, the cannibal morph) can be easily manipulated (Elgar and Crespi 1992).

In larvae of the salamander *Hynobius retardatus*, crowding with conspecifics or with heterospecific anuran larvae (*Rana pirica*) is the major proximate factor that induces the broad-headed morph (Michimae and Wakahara 2002). The specific morphology and aggressive behavior of the morph are advantageous for the consumption of large prey such as conspecific larvae or heterospecific anuran larvae, similar to cannibal morphology in the tiger salamander (*Ambystoma tigrinum*) larvae (Loeb et al. 1994; Whiteman et al. 2003). Because *H. retardatus* usually spawns during early spring in ponds created by melting snow, the typical food (macro benthos such as Oligochaeta and Chironomidae) of newly hatched larvae is not always available to the newly hatched larvae. Thus, *H. retardatus* larvae are sometimes exposed to a scarcity of typical prey during the early larval stage. In the absence of typical prey, it is particularly adaptive for newly hatched larvae to develop a broad-headed morphology so that they can consume frequently encountered large prey (Ohdachi 1994; Nishihara 1996; Michimae and Wakahara 2002). However, broad-headed morphs that fed only on conspecific larvae, metamorphose much earlier so they are much smaller at metamorphosis than typical larvae that fed only on typical prey items. On the other hand, those that fed only on heterospecific larvae, which are less nutritious than salamander larvae, metamorphose much later, but they are also smaller at metamorphosis than typical larvae that fed only on typical prey items (Michimae and Wakahara 2002). Therefore, although this phenotypic plasticity can be adaptive if it increases survival during the larval life stage, there is a trade-off associated with the broad-headed morph (accelerated development results in smaller size at

metamorphosis), which can affect traits expressed later in life, resulting in negative fitness consequences. In most species, this body size disadvantage at metamorphosis is retained through the age at first reproduction, thus compromising reproductive fitness (Goater 1994; Scott 1994; Altwegg and Reyer 2003).

Although the genetic and developmental mechanisms producing alternative morphs are not well known, theoretical studies predict that conditional frequency-dependent selection targets and adjusts the threshold frequency for a facultative switch between alternatives (Lively 1986, 1999; Hazel et al. 2004). From the viewpoint of the evolution of cannibalistic polyphenism, expression of the broad-headed morph in *H. retardatus* larvae is expected to be maintained at different densities of conspecific and heterospecific larvae. In natural habitats, the ability of the broad-headed morph to utilize other larvae as food may contribute more to a high survival rate and substantially to fitness when larval densities are higher. If the relative contribution of the broad-headed morph to fitness varies among populations with heterogeneous larval densities, then populations can be expected to diverge with respect to the expression of the broad-headed morph in response to the heterogeneous environmental conditions. Nishihara (1996) reported from field observations that broad-headed *H. retardatus* morphs were more frequent in a pond with high larval density than in one with low larval density. This observation may imply that local differences in larval densities led to morph induction or that an evolutionary change in the threshold for switching between morphs had occurred, or both. I used controlled rearing experiments of larvae originating from different ponds combined with field observations of larval density in

natural ponds to discriminate among these hypotheses. I studied the occurrence rate of the broad-headed morph of *H. retardatus* larvae across several experimental conditions. The larvae used in the experiments were from eight different ponds, each of which occupied a different point along the environmental continuum with respect to the densities of conspecific and heterospecific larvae; these densities are the selection agent that causes the propensity to express the broad-headed morph to diverge. Despite the theoretical importance of the role of frequency-dependent processes in polyphenism, empirical studies supporting their role are still limited. The specific aims of this study were to determine whether different degrees of expression of the alternative morph (different occurrence rates of the broad-headed morph) and therefore different phenotypic plasticity with respect to the expression of the alternative morph, evolve in populations occupying discrete habitats subject to different pressures by the selection agent.

Methods

Field study

I conducted a 3-year field survey during each breeding season (early April to late May) from 2002 to 2004 in four ponds, Erimo, Konuma, Nopporo, and Tomaru and a 2-year field survey during the breeding seasons of 2003 and 2004 in four additional ponds, Atsuta, Kamitobetsu, Okusawa, and Toyoha. All ponds are in Hokkaido, Japan and are

located far apart from one other (Fig. 1). Although Konuma and Tomaru are only 5 km apart by straight-line distance, they are effectively separated by two branches of the large Furubira River (Fig. 1 inset). Thus, it is reasonable to assume that genetic interactions cannot occur among the populations of the eight ponds.

The number of *H. retardatus* egg clutches in each salamander habitat and the number of eggs in each collected clutch were counted to estimate the conspecific larval density in each habitat. The respective number of egg clutches collected in 2002, 2003, and 2004 was described in Table 1. The annual density of conspecific larvae in each pond was estimated by multiplying the mean clutch size by the estimated density of clutches in the pond. Then, the mean density of conspecific larvae in each pond was estimated. The number of egg clutches of *R. pirica* in the eight *H. retardatus* habitats was also counted to estimate the heterospecific larval densities in each habitat. The annual density of heterospecific 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. Then, the mean heterospecific larval density was estimated. The mean density of conspecific and heterospecific larvae in each pond was calculated by dividing the sum of the annual total larval densities by the number of year when the egg clutches are corrected. Abiotic and biotic features (location, surface area, depth, number of egg clutches collected, density of egg clutches, and density of *H. retardatus* and *R. pirica* larvae) of the eight ponds are summarized in Table 1.

Induction experiment

The induction experiment was performed by using nine egg clutches of *H. retardatus*, from each of the eight ponds: these were collected from Erimo, Konuma, Nopporo, and Tomaru in 2002 and from Atsuta, Kamitobetsu, Okusawa, and Toyoha in 2003. Each collected clutch was placed in a stock tank filled with 1.6 l of dechlorinated tap water at room temperature (20–21 °C) until hatching. Fertilized egg clutches of *R. pirica* were also collected during the breeding season (early April to late May) in 2002 and 2003 from several ponds at different altitudes near Sapporo. The collected *R. pirica* clutches were transferred to the laboratory and placed in large plastic tanks, which were kept in the laboratory at room temperature, until use.

After the collected *H. retardatus* clutches hatched, I assigned larvae from each population to one of the three different experimental treatments: low conspecific density, high conspecific density, and high heterospecific density. Thirty randomly selected larvae from each of the nine clutches from each of the eight populations were divided equally among the three different crowding treatments. The low-conspecific-density treatment consisted of only ten *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 ten *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 ten *H. retardatus* larvae and 40 *R. pirica* larvae in a large tank with 1.6 l of dechlorinated tap water. To ensure that each tank always contained 40 *R. pirica* larvae, the numbers of *R. pirica* larvae, which were continuously eaten by *H. retardatus* larvae, were counted every morning. Then, additional heterospecific larvae

were added to each tank in the treatment group as necessary to replace those that were eaten by the *H. retardatus* larvae since the previous morning.

The experimental larvae were fed every other day by offering frozen Chironomidae from 9:00 A.M. to 12:00 noon. They were always given enough food to eat within 3 h and any food remaining in their tanks was removed after the feeding period. The rearing water was also exchanged every other day during the experiments. All experiments were conducted in the laboratory at room temperature and with a natural light/dark schedule.

I counted the numbers of broad-headed and typical morphs among the ten *H. retardatus* larvae in each tank every morning during the experimental period (2 weeks). The broad-headed and typical morphs were distinguished as described previously (Michimae and Wakahara 2001). Briefly, the head width of larvae at the level of the eyes (HWE) and the head width at its widest point (LHW) were measured by calipers to the nearest 0.05 mm and the HWE to LHW ratio was calculated. In broad-headed morphs, the ratio of HWE to LHW is ≥ 0.86 and in typical morphs, the ratio is < 0.86 . The occurrence rate of the broad-headed morph in each tank, expressed as the ratio of the number of broad-headed morphs to the initial number of *H. retardatus* larvae in the tank, was recorded.

Induction of broad-headed morphs was frequently observed during the first week after hatching, but cannibalism was infrequent during this period. The mortality rate (salamander larvae per tank) for each treatment (mean \pm SD) during the first week was for the low-conspecific-density treatment, 0.279 ± 0.081 , $n=68$; for the

high-conspecific-density treatment, 0.485 ± 0.095 , $n=68$; and for the high-heterospecific-density treatment, 0.162 ± 0.058 , $n=68$. Because I counted the number of broad-headed morphs in each tank every day, I was able to accurately determine the number of each morph in the tanks even if larvae had died by cannibalism. Thus, eaten *H. retardatus* larvae were not replaced.

Statistical analysis

I conducted a two way analysis of variance (ANOVA) for two factors after performing an angular transformation of the data (the occurrence rates of the morphs): one factor was population, which was ranked by the mean larval density of conspecifics and heterospecifics in the original habitat and the other was experimental treatment (crowding factor). To determine significant factors in the analyses, I used Fisher's protected least significant difference (PLSD) test to determine whether there were differences in the responses among levels of the factors.

I also used a multiple logistic regression model to look for a possible association between the response variable (mean occurrence of the broad-headed morph in each population) and two independent variables: the experimental treatment (categorical variable, low or high conspecific, or high heterospecific density) and the mean larval density of conspecifics and heterospecifics (continuous variable) in the natural ponds. A final parsimonious model was determined by comparing the deviance (likelihood ratio test statistic) to evaluate the fit of the models, which consisted of the various

combinations of and interactions among the independent variables. Then, to find the effects of each independent variable on the occurrence rate of the broad-headed morph in the final model, the odds ratios, adjusted for the other independent variables, were compared among treatment groups (categorical) or for units (continuous) by the Wald χ^2 test.

Although I originally prepared nine egg clutches from each population for the experiment and the statistical analysis, one clutch from the Erimo and three clutches from the Kamitobetsu populations were removed from the statistical analysis because some samples were lost as the result of an accident in the laboratory.

Results

Reaction norms in three crowding treatments

Population and crowding factors significantly affected the rate of occurrence of the broad-headed morph in the larvae of *H. retardatus* (crowding, $F_{2,180}=142.752$, $P<0.0001$; population, $F_{7,120}=6.460$, $P<0.0001$), but the interaction of crowding and population factors was not significant (crowding by population, $F_{14,180}=1.226$, $P=0.26$). The occurrence rates of the morph for the three crowding treatments (the population factor was pooled because its effect was additive) are shown in Fig. 2. Differences between low- and high-conspecific-density treatments ($P=0.0054$) and between low-conspecific- and high-heterospecific-density treatments ($P<0.0001$) and also that

between high-conspecific- and high-heterospecific-density treatments ($P < 0.0001$) were significant (Fig. 2). Thus, the induction rate of the morph was higher when conspecific density was high than when conspecific density was low, and it was also higher when heterospecific density was high compared with both low and high conspecific densities (Fig. 2).

Relationship between larval densities in natural habitats and the occurrence rate of broad-headed morph

The most parsimonious logistic regression for the occurrence of the broad-headed morph was acquired by a stepwise reduction of the number of explanatory terms from the full model and by comparisons of the deviances between the models with more explanatory terms and those with fewer (Table 2). The final model included larval density of conspecifics and heterospecifics in the natural habitats and crowding treatment as explanatory variables but no interactive effect of the two variables, larval density, and crowding treatment (Table 2). The relationship between larval density and the expression of the broad-headed morph is plotted in Fig. 3 for each crowding treatment (a, low conspecific density; b, high conspecific density; and c, high heterospecific density). The occurrence rate of the morph in all treatments increased with the density of conspecific and heterospecific larvae. It also increased with changes in the crowding treatment from low conspecific to high conspecific density or from low conspecific to high heterospecific density as indicated by the positive logistic

regression (Table 3 and Fig. 3).

Discussion

Responses to conspecific and heterospecific larvae

Previous studies of larval salamanders (*H. retardatus* and *A. tigrinum*) reported that cannibals are triggered only by crowding with conspecific larvae (Collins and Cheek 1983; Michimae and Wakahara 2001). Recently, some studies, however, have examined the effects of heterospecific amphibian larvae on the production of cannibals (Hoffman and Pfennig 1999; Michimae and Wakahara 2002; Whiteman et al. 2003). Cannibals in *A. tigrinum* are indirectly induced via increased body size variation within a population of larvae as a result of differential consumption of heterospecific anuran tadpoles (Whiteman et al. 2003). In contrast, in this study, the broad-headed morph in *H. retardatus* was shown to be induced directly by the presence of heterospecific tadpoles because even *H. retardatus* larvae that exhibited little plastic response to the conspecific cue showed a high occurrence of the broad-headed morph in response to the heterospecific cue (Fig. 2). The results reported here suggest that *H. retardatus* larvae choose to eat heterospecific larvae over conspecifics whenever possible, either to avoid cannibalism or because heterospecific larvae are easier to manage (Michimae and Wakahara 2002). In other words, the morphs evolved to eat large, tough prey, including both conspecific and heterospecific larvae, which is contrary to the case described by Whiteman et al. (2003) in which *A. tigrinum* larvae with the cannibal

morphology prefer to eat conspecifics rather than heterospecific prey items. Because *H. retardatus* larvae can and may prefer to eat heterospecific *R. pirica* larvae in habitats where they are present, the major costs of cannibalism (decreasing inclusive fitness, Walls and Roudebush 1991; Elgar and Crespi 1992; possible pathogen transmission, Pfennig 2000; and injury or death by retaliation, Elgar and Crespi 1992) are presumably reduced in such habitats. Therefore, selection favors high occurrence rates of the morph in the presence of heterospecific larvae owing to the advantages of possessing the morph.

I previously showed that hydraulic vibration originating from the flapping tails of heterospecific *R. pirica* larvae is a key cue for the induction of the broad-headed morph (Michimae et al. 2005). Therefore, I prefer to call the plastic response in *H. retardatus* larvae associated with heterospecific larval cues “cannibalistic polyphenism” or “broad-headed morph” because it is not a true cannibalistic morph (Michimae and Wakahara 2002).

Differentiated occurrence rate of the broad-headed morph

Frequency-dependent selection plays a major role in many evolutionary models (Maynard Smith 1998; West-Eberhard 2003). With negatively frequency-dependent fitness, it is possible for natural selection to maintain alternative genotypes (genetic polymorphism). The alternatives developed are generally irreversible and selection adjusts the ratio of alternative genotypes in the population to an equilibrium value at

which the fitness of the different genotypes is equal and natural selection will not alter their frequencies. Theoretical studies (Lively 1986, 1999; Moran 1992) have also developed conditional frequency-dependent models to investigate the conditions necessary for the evolution of environmental induction of alternative morphs, that is, polyphenism, and these models predict that polyphenism may be favored when there are environment-dependent fitness trade-offs between alternatives and reliable environmental cues are available that enable the organism to predict the future habitats.

This study satisfies these conditions. First, there are clear larval density-dependent fitness trade-offs between morphs (Michimae and Wakahara 2002). In environments with higher densities of conspecific larvae or heterospecific anuran larvae, the broad-headed morph is more advantageous than the typical morph because broad-headed morphs can consume frequently encountered large prey, such as conspecific and heterospecific larvae, and thus grow more rapidly when their usual food is in short supply. In environments with lower larval densities, the typical morph is more advantageous because their larger body size enhances postmetamorphic survival. In addition, a larva with an induced broad-headed morphology reverts to the typical morph when placed by itself, suggesting that the maintenance of the morph is costly (Michimae and Wakahara 2002). Second, the presence of conspecific or heterospecific larvae is a direct indicator of larval density to the newly hatched larva (Michimae et al. 2005). Thus, the occurrence rate of the broad-headed morph is dependent on the larval density of conspecific or heterospecific larvae (Fig. 2).

Thus, it is reasonable to infer the evolutionary maintenance of the broad-headed morph and interpopulational differentiation of the morph's expression from the results of these induction experiments. Under common experimental conditions, the observed interpopulational differences in the expression of the morph correspond to the gradient of larval density in the natural ponds (Table 2 and Fig. 3). Therefore, there is a larval density-dependent equilibrium frequency of the morph in each pond, suggesting that the local switch point for morph induction was adjusted by selection to produce evolved differences among the ponds (Lively 1986, 1999; Pfennig 1992). It is also possible that the mix of morphologies is controlled both genetically and environmentally (Lively 1986,1999). In such a population, development in some members of the population would be strictly genetically determined: Those individuals would always develop one of the two morphologies regardless of the environmental conditions (unconditional strategist), whereas the remaining members would retain the capacity to develop both morphologies (conditional strategist). The ratio of conditional to unconditional strategists would be determined by natural selection in accordance with the environmental heterogeneities. In *H. retardatus*, the apparent match between the rate of occurrence of alternative feeding morphologies and the selection pressure (conspecific and heterospecific larval density in the natural habitat) suggests that the interpopulational variation in the occurrence rate of the broad-headed morph represents an adaptive response to selection pressure.

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Figure legends

Fig. 1 Map of sampling sites (open circles) in Hokkaido, Japan used in this study

Fig. 2 The reaction norm (represented by the occurrence rate of the morph; means \pm SEs) in three distinct crowding treatments: low and high densities of conspecifics and high density of heterospecifics. Differences between low- and high-conspecific-density treatments ($P=0.0054$) and between low-conspecific- and high-heterospecific-density treatments ($P<0.0001$), and also that between high-conspecific- and high-heterospecific-density treatments ($P<0.0001$), were significant (Fisher's protected least significant difference test)

Fig. 3 The relationship between mean larval density of conspecifics and heterospecifics (individuals/m². see Table 1) and the occurrence rate of the broad-headed morph in the a low-conspecific-density, b high-conspecific-density, and c high-heterospecific-density treatments

Table 1 Features of the eight discrete sites where *Hynobius retardatus* samples were taken (Erimo, Konuma, Nopporo, and Tomaru in 2002, 2003, and 2004; Atsuta, Kamitobetsu, Okusawa and Toyoha in 2003 and 2004).

sampling site	surface area (m ²)	maximum depth (m)	Number of egg clutches collected 2002	Number of egg clutches collected 2003	Number of egg clutches collected 2004	mean (1 SD) clutch density <i>Hynobius</i> (egg clutches/m ²)	estimated mean (1 SD) density of conspecific larvae (individuals/m ²)
Erimo	150	1.0	9	30	30	6.89 (1.018)	415.733 (38.041)
Konuma	3200	2.0	9	30	24	0.223 (0.025)	13.533 (1.501)
Nopporo	50	0.3	9	30	30	0.933 (0.05)	77.667 (3.786)
Tomaru	12	0.5	9	30	29	33.2 (4.204)	2132.933 (202.933)
Atsuta	10	0.3		9	16	19.5 (0.707)	1469.3 (128.269)
Kamitobetsu	4	0.9		9	13	4.5 (0.707)	323.15 (45.75)
Okusawa	300	1.0		9	31	0.835 (0.165)	62.285 (17.84)
Toyoha	40	0.7		9	26	1.59 (0.085)	112.835 (4.009)

mean (1 SD) clutch density <i>Rana</i> (egg clutches/m ²)	estimated mean (1 SD) density of <i>Rana</i> larvae (individuals/m ²) ^b	estimated mean (1 SD) density of conspecific and heterospecific larvae (individuals/m ²)
1.223 (0.136)	977.767 (113.020)	1393.5 (85.424)
0	0	13.533 (1.501)
0.067 (0.012)	53.333 (9.238)	131.0 (8.544)
2.583 (0.144)	1844.443 (453.792)	3799.943 (405.918)
1.55 (0.071)	1240.0 (56.569)	2709.3 (71.701)
0.125 (0.177)	100.0 (141.421)	423.15 (95.672)
0.1165 (0.023)	93.335 (18.859)	155.62 (1.018)
0.35 (0.071)	280.0 (56.569)	392.835 (52.559)

Table 2 Comparisons of multiple logistic regression models that consists of the various combinations and interactions between the independent variables (T crowding treatment; L larval density of conspecifics and heterospecifics)

Model	Log-Likelihood	<i>df</i>	variable evaluated	Deviance (<i>df</i>)	<i>P</i>
Constant + T + L + T*L	869.9957	5			
Constant + T + L	870.7519	3	T*L	1.509 (2)	0.4714
Constant + T	875.0626	2	L	8.6214 (1)	<0.005
Constant + L	999.3032	1	T	258.6714 (2)	<0.0001

Constant + T + L model was selected as the final model

Table 3 Results of the multiple logistic regression analyses on two independent variables adjusted for a second variable in the final model

Variable	Regression coefficient	Wald χ^2	<i>P</i>
Constant	-1.7964	408.73	<0.0001
Larval density (adjusted for crowding treatment)	0.0001	8.75	0.0031
Crowding treatment (adjusted for larval density)			
Low conspecific density	-0.9268	70.22	<0.0001
High conspecific density	-0.3195	10.94	0.0009
High heterospecific density	1.2463	227.28	<0.0001

Figure 1

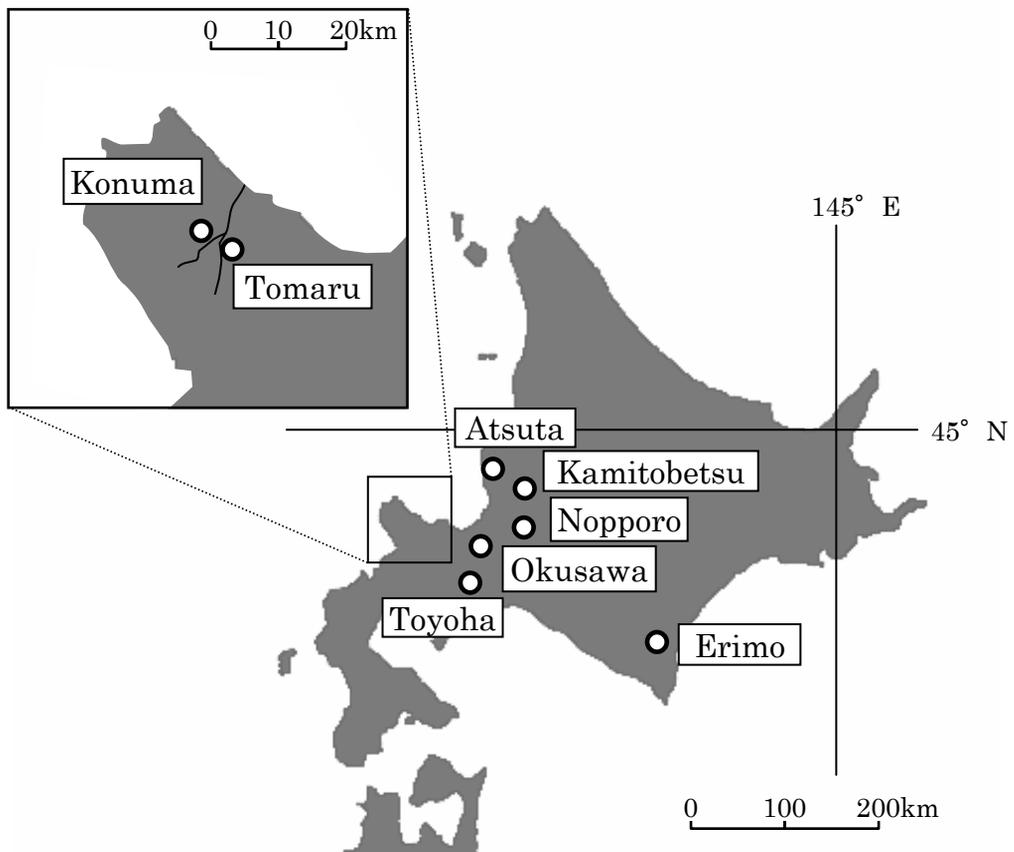


Figure 2

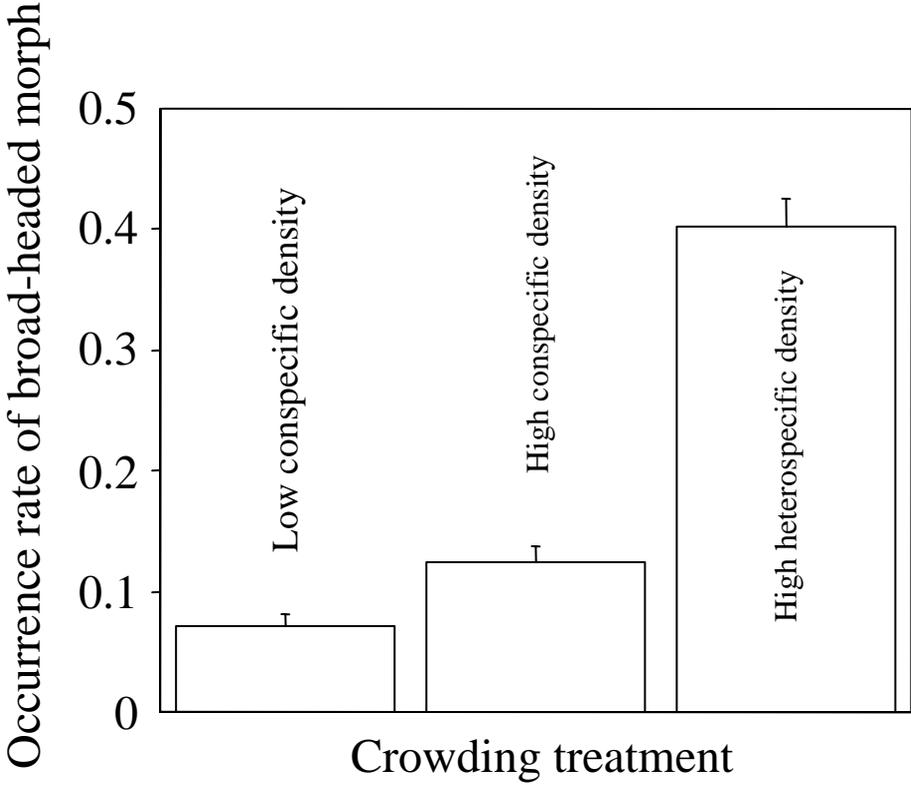


Figure 3

