博士論文

Ecological impacts of predator gigantism: experimental studies using cannibalistic salamanders

（捕食者の巨大化の生態学的影響：共食いするサンショウウオを用いた実験研究）

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Contents

Chapter 1

General Introduction

As many studies have repeatedly shown evolutionary changes in traits of the community members and changes in the composition and ecosystem functions of the community after introduction or removal of focal animal population, there is no doubt that animal populations play a key role in determining trait evolution and ecosystem dynamics (Reznick et al. 1990; Estes et al. 1995; Carpentar et al. 2001; Terborgh et al. 2001; Ripple & Beschta 2003; Croll et al. 2005; Sinclair et al. 2007; Sandy et al. 2008; reviewed by Estes et al. 2011). Thus, knowledge of how functions performed by the animal populations to community members such as predation pressures on prey and nutrient storage and release are determined provides insight into mechanism driving trait evolution and community dynamics. Because population functions are sum of functions performed by individuals constituting the population such as consumption of prey and nutrient storage and excretion, focusing on the individual functions is fruitful to reveal mechanisms determining ecological and evolutionary impacts of the animal populations.

While ecologist attempting to predict ecological and evolutionary impacts of animal population and to understand the underlying mechanisms have traditionally considered individual functions as primary determinant of population functions, they have assumed functional homogeneity of individuals within a species as represented by Lotka-Volterra model which is used as fundamental building block for theory of community ecology (Oksanen et al. 1981; Holt & Polis 1997; Abrams 2000; Leroux & Loreau 2010; Mougi & Kondoh 2012). However, as many empirical studies investigating the relationships between density of the populations and their functions consistently showed non-linear relationship between them (e.g., predation pressure, Sommer 1992; Hildrew et al. 2004; Brook & Bradshaw 2006; Sand et al. 2012; decomposition rate and nutrient release Klemmer et al. 2011), it is clear that individuals within a species are rarely functionally homogeneous. Even in same species, there are

considerable differences in phenotypes among individuals and individuals with distinctive phenotypes differentiate their functions (Polis 1984; Werner & Gilliam 1984; Woodward & Hildrew 2002; Bolnick et al. 2003; Aljetlawi et al. 2004). Because natural populations consist of individuals with different phenotypes and because phenotypic composition of populations varies among populations (Uchmanski 1985; Pfister & Stevens 2002), it is reasonable that incorporating phenotype of individuals as determinant of population functions to better predict ecological and evolutionary impacts of the animal populations (Bolnick et al. 2011; Miller & Rudolf 2011).

In particular, body size is one of most informative trait for identifying individual functions. First, size of functional traits increases with body size. Second, there are considerable differences in body size among individuals within a species. For example, body mass of adult cod is 10^9 fold larger than their hatchlings (Persson & De Roos 2002). Such considerable difference in body size among individuals should differentiate individual functions, and consequently, population functions differ depending on size composition of the population (Chalcraft & Resetarits 2004; Brose et al. 2006; Rudolf 2012; Rudolf & Rasmussen 2013, see also Moya-Larano 2011).

Individuals grow large during their ontogeny. The growth trajectory is determined by genetic and environmental factors (Pfennig 1990; Ballabeni 1995; Nicieza & Metcalfe 1997; Michimae 2006; Dahl et al. 2012). In particular, environmental factors could be general mechanisms in determining the size given that considerable differences in body size were observed among clonal individuals in same age (Cressler et al. 2014). In some cases, environmental factors could affect not only ordinary growth but also allometric growth as represented by inducible offence (Pfennig et al. 1990; Banerji & Morin 2009; Kishida et al. 2009a). Thus, driving force of temporal and spatial dynamics of size composition is

environment dependent growth. Therefore, incorporating environmental dependent growth into current ecological theories may improve our ability to explain dynamic nature of animal populations. Indeed, mathematical models considering the environmental dependent growth predict that it determines population functions by determining size composition of the population (Persson & De Roos 2013; Leeuman et al. 2013, 2014; Wollrab et al. 2013). In Eurasian perch which is common predatory fish in lake in Europe, Persson et al. (2003) found that the predictions considering environmental dependent growth of the perch was qualitatively more similar to trends in long term field data of density of population and its size composition of the perch and their prey community than the prediction without considering environmental dependent growth. While these studies suggest that growth should be key mechanisms in determining population functions in nature, surprisingly, there are no studies that empirically show such important roles of growth. In this thesis, to show importance of environmental dependent growth in determining population functions, I conducted empirical studies using cannibalistic animal species.

Cannibalism is known to a key interaction driving rapid growth of individuals and also demographic changes of animal populations (Fox 1975; Polis 1981; Claessen et al. 2004). In various taxa, individuals that succeeded to cannibalize (i.e., cannibals) grow extremely rapidly, becoming giants (e.g., ciliates, Kidder et al. 1940; rotifer, Gilbert 1973; planaria, Armstrong 1964; insects DeBlock & Stocks 2004; fish De Angelis et al. 1980; amphibians, Pfennig 1990). In some species, size of cannibal is several folds larger than that of non-cannibals even if they are in same age (Persson et al. 2003; Bystrom 2006). Thus, rapid growth due to cannibalism is a representative of environmental dependent growth. The extremely rapid growth of cannibals should increase their individual functions. On the other hand, cannibalism causes significant reduction in density of the population. Density reduction itself has potential to reduce

population function if one assumes functional homogeneity of individuals as most ecologists did. Thus, because of the contrasting effects of rapid growth of cannibals and density reduction on population function, depending on the relative relationship of the effects, net effects of cannibalism on their functions might be negative or positive. In particular, showing positive net effects of cannibalism on population functions emphasize importance of rapid growth of individuals as determinant of the functions.

In this thesis, I conducted three experiments to test how cannibalism in animal population alters their functions using cannibalistic salamander larvae (*Hynobius retardatus*) as model. The salamander larvae is ideal system because that is known to show cannibalism dependent rapid growth (Kishida et al. 2011) and because easy to manipulate occurrence of cannibalism. Consumption is a fundamental function of animal individual because any individual functions such as nutrient storage and excretion stem from the ingested nutrients. So, I first examined how cannibalism of salamander population alters predation pressures on their prey frog tadpoles (*Rana pirica*) (Chapter 2). In the study, I showed that cannibalism intensifies predation pressures on the frog tadpoles by emergence of the giant cannibals. Next, I focused on two population functions stem from the consumption of prey (Chapter 3 and 4). Strength of predation pressures on prey determines strength of selective pressures on defensive phenotypes of prey (Abrams 2000). The frog tadpoles exhibit inducible morphological defense and the salamander larvae is involved in the evolution and maintenance of the inducible defense in the frog tadpoles (Kishida & Nishimura 2004, 2006; Kishida et al. 2007). Thus, I examined whether cannibalism of salamander population alter selective pressures on induced defensive phenotypes of prey frog tadpoles (Chapter 3). Finally, I focused on nutrient storage and release of animal population (Chapter 4). In addition to showing effects of cannibalism on nutrient storage and release of the population, the study show ecological context that generates

intra-population variation in nutrient contents of individuals. I found that nutrient contents such as percentage of phosphorus of body and excretion differ between cannibals and non-cannibals within a cannibalistic population. This finding might contribute to understandings of ecosystem nutrient dynamics because researchers have assumed homogeneity of nutrient contents of body and excretion of individuals within a population (Daufresne & Loreau 2001; Hall 2009; Leroux & Loreau 2010).

Chapter 2

Predator cannibalism can intensify negative impacts on heterospecific prey

INTRODUCTION

Traditionally, ecologists attempting to predict population and community dynamics and to understand the underlying mechanisms have assumed homogeneity of individuals within a species or a population. The dynamics are modeled by using species specific trait values that represent the demographic parameters of the population (e.g., birth rate, death rate, and prey capture ability) (Oksanen et al. 1981, Holt & Polis 1997, Mougi & Kondoh 2012). However, natural populations consist of individuals with different phenotypes, and the degree of trait variation within a population varies among populations (reviewed by Uchmański 1985). Because conspecific individuals with distinctive phenotypes differentiate their ecological roles and niches (Werner & Gilliam 1984, Bolnick et al. 2003) and often intimately interact with each other (Polis 1981), intrapopulation phenotypic variation in key traits can have complex consequences for population dynamics, ones that cannot be predicted by models that assume homogeneous individuals (Bolnick et al. 2011, Miller and Rudolf 2011).

In particular, variation in size and development within predator and prey populations may be important determinants of community dynamics, because the outcomes of trophic interactions are determined by the physical performance balance between interacting predator and prey individuals. Size and development covary within stage-structured populations, and stage structure often differs among populations (Uchmański 1985). Therefore, manipulation of stage structure in a population may be a valuable approach for examining the mechanisms of spatial and temporal variation in the nature of trophic interactions.

Cannibalism is commonly observed in predators (Fox 1975, Polis 1981). In many cannibalistic interactions, stage structure is very important, because the occurrence of cannibalism depends strongly on the relative sizes and developmental stages of the interacting conspecifics (Sogard & Olla 1994, Qin & Fast 1996, Kishida et al. 2009b). Although the

outcome of cannibalistic interactions is expected to strongly influence the dynamics and structure of ecological communities (De Roos et al. 2003, Claessen et al. 2004, Rudolf 2007a,b, Miller & Rudolf 2011, Ohlberger et al. 2012), our understanding of the ecological impacts of cannibalism and their underlying mechanisms remains very limited. To better understand the ecological significance of predator cannibalism in community dynamics, one need to investigate (1) how cannibalism modifies predator population dynamics and the phenotypic characteristics of individual predators, and (2) how the modifications affect interspecific interactions such as the trophic relationships between predators and their heterospecific prey. For example, cannibalism is known to cause decreased predator density (reviewed by Fox 1975), to induce behavioral and morphological plasticity (Rudolf 2006, Banerji and Morin 2009, Crumrine 2010, Kishida et al. 2011), and to result in very large cannibal individuals (Kidder et al. 1940, Armstrong 1964, Gilbert 1973, DeAngelis et al. 1980, Pfennig 1990, Wissinger et al. 2004). Therefore, the specific impacts of these modifications on the predatory effects of the predators on their heterospecific prey need to be examined. In addition, one should explore (3) how such modified interactions lead to the phenotypic characteristics (e.g., behavior, morphology, and life history) of both predator and prey species in the subsequent period, because the phenotypic changes strongly influence ecological communities (Agrawal 2001, Werner & Peacor 2003, Miner et al. 2005).

Previous studies have shown that, in the short term (i.e., without considering predator reproduction), predator cannibalism weakens the predatory effects on the heterospecific prey (Crumrine 2005, 2010, Rudolf 2006, Law & Rosenheim 2011). The weakened impacts are caused through a reduction in both the predator population size (i.e., density-mediated effects; Persson et al. 2003, Crumine 2005, 2010) and the foraging activity of non-cannibals, because non-cannibal predators respond to the predation risk from cannibals by reducing their activity

level (i.e., a behavior-mediated effect; Rudolf 2006, Kishida et al. 2011). As a result of the accumulation of evidence for the positive impact of predator cannibalism on heterospecific prey (Persson et al. 2003, Crumrine 2005, 2010, Rudolf 2006, Law & Rosenheim 2011), current theoretical models designed to predict community-level consequences of cannibalism assume that this positive cannibalism effect is a typical ecological process (Rudolf 2007a,b, Ohlberger et al. 2012). Nevertheless, the net impact of predator cannibalism on heterospecific prey might not be always positive, because no studies have yet focused on one effect of cannibalism on the cannibalistic predator population itself, namely, the enhancement of the growth of the cannibals.

In various taxa, cannibals grow extremely rapidly, becoming giants (e.g., ciliates, Kidder et al. 1940; rotifers, Gilbert 1973; planaria, Armstrong 1964; insects, De Block & Stoks 2004, Wissinger et al. 2004; amphibians, Pfennig 1990, Wakahara 1995; and fish, DeAngelis et al. 1980; also see Polis 1981). This rapid growth occurs because cannibals benefit from the transfer of nutrients from their conspecific prey (i.e., non-cannibals), because conspecific prey are a rich nutrient source that is also easy to assimilate (Meffe & Crump 1987, Wildy et al. 1998), and also because the resulting reduction in the predator population size reduces resource competition among the survivors (Claessen et al. 2000, Persson et al. 2004, Huss et al. 2010, Kishida et al. 2011). In addition to the ordinary growth, allometric growth due to phenotypic plasticity, such as enlargement of the prey-capturing organ of individuals in response to cannibalistic interactions, may contribute to the occurrence of giants in a cannibalistic environment (Gilbert 1973, Pfennig 1990, Hoffman & Pfennig 1999, Wakahara 1995, Kishida et al. 2011). Because large predators can consume a wider variety of prey species, and because they have greater resource requirements than small predators (Werner & Gilliam 1984), the enhanced growth of individual predators can intensify the predatory effects on their

heterospecific prey. The net effect of predator cannibalism on the heterospecific prey might be negative if this growth-mediated negative effect of cannibalism overwhelms the density- and behavior-mediated positive effects. In interactions between gape-limited predators and their heterospecific prey, in which predation success is sensitive to changes in the size relationship, the expected outcome is possible because the rapid growth of cannibals seems to increase the likelihood of predation success on the relatively large prey.

If predator cannibalism intensifies predation pressure on heterospecific prey, the modified interaction might be reflected in changes in the characteristics of both the predator and prey populations (e.g., population size, behavior, and life history). Although past studies have evaluated the effects of cannibalism on the abundance of heterospecific prey (Persson et al. 2003, Rudolf 2006, Law & Rosenheim 2011), they did not examine how cannibalism affects the phenotypic characteristics of the two populations. I expect that, by exploring the consequences of cannibalism on the phenotypic characteristics of predator and prey populations, I will gain insight into the ecological consequences of cannibalism, because phenotypic traits such as behavior, life history, and morphology of key species influence both ecological interactions and trait evolution in a community (Agrawal 2001, Werner & Peacor 2003, Miner et al. 2005, Takatsu & Kishida 2013, Kishida et al. 2014).

In this study, I examined whether predator cannibalism, which is a possible consequence of the stage structure of the predator population, can intensify trophic interactions between the predators and their heterospecific prey, and I explored how the modified interactions affected the phenotypic characteristics of the predators and their heterospecific prey. To achieve these objectives, I studied the predator–prey relationship between larvae of a cannibalistic salamander (*Hynobius retardatus*) and their heterospecific prey, brown frog (*Rana pirica*) tadpoles. Larvae of both amphibian species frequently co-occur from spring to summer

in small ponds in Hokkaido, Japan (Kishida et al. 2009b). The salamander larvae are gape-limited carnivores that consume aquatic insects and amphibian larvae, including both frog tadpoles and conspecifics (Kishida et al. 2009b). However, the frog tadpoles generally hatch earlier than the salamander larvae (the difference between the average hatch timing of the frog tadpoles and the earliest hatch timing of the salamander larvae in a pond is from 5 to 16 days; Takatsu, K. personal observation); as a result, for several weeks after the salamander larvae hatch, they have difficulty consuming frog tadpoles because the size balance favors the prey (Nosaka et al. 2015). In such cases, while the salamander larvae are small, conspecific individuals can be major prey items. When cannibalism occurs, the salamander larvae that successfully consume their conspecifics grow much faster and plastically enlarge their gape (i.e., they become giants) (Wakahara 1995, Kishida et al. 2011). Based on the knowledge, I predicted that salamander larvae exert predation pressure on the frog tadpoles if the cannibalistic salamander giants become large enough to swallow the frog tadpoles.

In addition, I expect that the realized predator–prey interactions may modify characteristics of both amphibian populations. For example, frog (*Rana pirica*) tadpoles express a bulgy phenotype (characterized by enlarged bodies and tails) and reduce their activity level to protect themselves from being consumed by the salamander larvae (Kishida & Nishimura 2004, Kishida et al. 2009a). Because this plastic induction of morphological and behavioral defenses is risk sensitive (Kishida et al. 2009a) and because large salamander larvae cause greater mortality among less-defensive than among more-defensive frog tadpoles (Takatsu & Kishida 2013), I predict that frog tadpole populations subjected to intensive predation risk from salamander giants will include more individuals expressing a defensive phenotype than tadpole populations subjected to less predation risk. I also predict that salamander gigantism due to cannibalism will affect the life history of both amphibian species,

such as their size at metamorphosis and the time to metamorphosis, both of which are considered to be important for determining population fitness and community dynamics (Semlitsch et al. 1988, Schreiber & Rudolf 2008). To test this series of predictions, I conducted an experiment in which I manipulated the size structure of a cohort of salamander hatchlings.

MATERIALS AND METHODS

Experimental settings

Collection and keeping methods of experimental animals are described in Appendix A1. I used 86 semi-transparent polypropylene tanks (43.6 cm × 28.4 cm × 14.1 cm high), each filled with 5 L of aged tap water, for the experimental treatments. To create natural conditions, 2.5 g (dry weight) of oak leaf litter (*Quercus dentata*) was placed in each tank to provide refuges for the frog tadpoles. Two weeks after the frog tadpoles hatched, I assigned 45 frog tadpoles to each tank (day 1: body length 12.10 ± 0.66; body width, 7.85 ± 0.48; mean ± SD, N = 20; Gosner stage, 25–30 [Gosner 1960]).

I performed my experiment in two steps (i.e., the experimental design is illustrated in Appendix B). In the first step, I assigned early and late salamander hatchlings to treatments. I obtained the early and late salamander hatchlings (hatch time difference, 1 week) by manually controlling the water temperature experienced by the embryos (i.e., the detailed methods are described in Appendix A2). I assigned (1) 10 early and 10 late hatchlings (i.e., large-variation treatment) into each of 40 replicate tanks, and also assigned (2) 20 early hatchlings (i.e., small-variation 20-early-hatchling treatment) or (3) 20 late hatchlings (i.e., small-variation 20-late-hatchling treatment) into each of 10 tanks. Because the occurrence of cannibalism depends greatly on size and developmental asymmetry between interacting individuals (Kishida et al. 2011, 2015), I expected that the negative impacts of the salamander larvae on

the frog tadpoles would be larger in the large-variation treatment than in either of the two small-variation treatments. The differences in density of either hatch-timing group (i.e., early or late hatchlings) among the treatments, however, can have a confounding effect by causing a pattern similar to the predicted one. To preclude such a confounding effect, I assigned (4) 10 early hatchlings (i.e., small-variation 10-early-hatchling treatment) or (5) 10 late hatchlings (i.e., small-variation 10-late-hatchling treatment) to each of 10 tanks. In addition, I assigned (6) no salamander hatchlings to six tanks (i.e., no-salamander treatment). Because past studies with experimental settings similar to those of the present study have shown that the mortality of the frog tadpoles in the absence of predators is very low (e.g., Takatsu & Kishida 2013, Nosaka et al. 2015), I performed only six replicates of the no-salamander treatment to avoid excessive use of the animals. I defined the day on which the early hatchlings were assigned to the appropriate tanks as day 1 of the experiment, and I assigned the late hatchlings to the appropriate tanks one week later. This time difference (i.e., one week) between the assignment of early and late hatchlings is appropriate because it is within the natural variation of the hatching phenology (Takatsu, personal observation). These experimental densities and hatching phenology of frog tadpoles and salamander larvae are typical of those observed in their natural habitat (Michimae 2006).

To explicitly test whether the salamander cannibalistic giants imposed predation pressure on the prey frog tadpoles, on day 19, I performed a second experimental step by manipulating the large-variation treatment to create treatments in which giants were either present or absent (described below). I performed this manipulation on day 19 because, while from 1 to 3 salamander larvae became giants before day 19 as a result of cannibalistic interactions in most tanks of the large-variation treatment (Appendix C), trophic relationships between the salamander larvae and the frog tadpoles were not established until that day (see

Results). Hence, by removing the salamander giants at that time, I could clearly evaluate the effects of giants induced by cannibalism on the frog tadpoles. In the second experimental step, I established two additional treatments in 24 of the 40 tanks of the large-variation treatment (Appendix B). From each of 12 tanks, I removed the three individuals with the largest body length (i.e., large-variation giant-removal treatment), and from the other 12 tanks, I randomly removed three individuals (but not any of the three individuals with the largest body length) (i.e., large-variation non-giant-removal treatment). The remaining 16 of the original 40 tanks continued as large-variation treatment tanks.

Throughout the experiment, I added one piece of rabbit chow (dry weight: 0.2 g) and 20 frozen Chironomid larvae to all tanks every 2 days as alternative food for the frog tadpoles and the salamander larvae, respectively. The rearing water was changed every 2 days. The experiment was terminated 151 days after the beginning of the experiment.

Focal traits of the salamanders and frogs

(1) Mortality of salamander larvae and frog tadpoles

I counted the numbers of surviving frog tadpoles and salamander larvae on days 19, 31, 46, 61, 85, and 151. Mortality of the frog tadpoles or salamander larvae (i.e., the number of dead individuals, D) between the census at time t and the previous census at time ($t - 1$) was calculated as $D = (N_{t\text{-}1} - N_t - M_t)$, where $N_{t\text{-}1}$ is the number of surviving frog tadpoles or salamander larvae at time ($t - 1$), N_t is the number of frog tadpoles or salamander larvae surviving at time t, and M_t is the number of frog tadpoles or salamander larvae that had metamorphosed since time ($t - 1$). I removed metamorphs of both amphibian species from each treatment every day throughout the experiment. In the frogs, metamorphosis was defined as the first emergence of forelimbs (Gosner stage 42; Gosner 1960), and in the salamander, it was defined by the shrinkage of the tail fin and the loss of the external gills (stage 67;

Iwasawa and Yamashita 1991). The ventral sides of the frog and salamander metamorphs were digitally scanned and the snout–vent length measured on the scanned images was defined as body length at metamorphosis.

(2) Activity of the frog tadpoles

I examined the proportion of frog tadpoles moving in the tanks on days 13, 21, and 32 to determine whether the activity levels of the tadpoles differed among the treatments. I took three consecutive still images of the tanks using a relatively slow shutter speed (1/30 s) and counted the number of moving tadpoles (i.e., blurred images). I also took a still image of each tank on each of these three days after collecting the leaf litter from the tanks in order to count the surviving tadpoles in the tank. Then, I returned the leaf litter to the tanks. I defined the ratio of moving individuals to total individuals (i.e., the number of active individuals divided by the total number of surviving individuals) as the activity level. Because I counted moving tadpoles in three images per tank, I obtained three activity values. In the analyses, I used the median activity value in each tank.

(3) Morphology of the frog tadpoles and salamander larvae

On day 19, before predator–prey interactions between the salamander larvae and the frog tadpoles had become established, and on day 31, when a predator–prey relationship had been clearly established in most tanks of the large-variation and the large-variation non-giant-removal treatments (Fig. 1), I scanned the ventral sides of all surviving frog tadpoles. Then I digitally measured the snout–vent length (i.e., body length) and maximum body width on the scanned images projected onto a computer monitor to evaluate defensive morphology in the frog tadpoles.

In *R. pirica* tadpoles, a defensive morphology is one in which the ratio of body width to body length is relatively large. Because the body width of frog tadpoles covaries with their

body length, I adjusted for this effect by using analysis of covariance (ANCOVA) to compare the degree of defensive morphology in the frog tadpoles among the treatments. The ANCOVA results showed that differences among the treatments in the slopes of the regression lines of body width against body length were not significant (ANCOVA, interaction between body length and treatment $F_{7,1176} = 1.17$, $P = 0.32$). Therefore, I used size-adjusted body width of the frog tadpoles, calculated from the regression relationship of the relevant treatment, to compare the degree of defensive morphology among the treatments. When the size-adjusted body width in one treatment was larger than that in another treatment, I judged the degree of defensive morphology in the first treatment to be higher than that in the second treatment. The ventral sides of all surviving salamander larvae were also scanned on days 19 and 31, and the snout–vent length (i.e., body length) and gape width of the salamander larvae were digitally measured.

Statistical analysis

In preliminary analyses, differences in measured traits of the salamander larvae and the frog tadpoles among the four small-variation treatments were not significant (i.e., treatments 2–5 above; Appendix D). Therefore, I pooled the data of the four small-variation treatments (hereafter, the small-variation treatment) before performing further statistical analyses. In addition, differences in measured traits between the large-variation and the large-variation non-giant-removal treatments were not significant (Appendix E). Therefore, I also pooled these data (hereafter, the large-variation treatment) before further statistical analyses.

I used repeated-measures ANOVA to compare the activity of the frog tadpoles on days 13, 21, and 32. Because I found significant interaction between treatment and time (see Results), I used ANOVA followed by Tukey's HSD test to compare the activities of the frog tadpoles among treatments on each censes day. The remaining frog tadpole variables did not

meet the homoscedasticity assumption for parametric analysis. So Kruskal-Wallis tests were used to examine whether mortality during the three time periods between censuses (day 1 to 19, day 19 to 31, and day 31 to 151), the degree of defensive morphology (i.e., size adjusted body width) on day 19 and day 31, and the timing of and size at metamorphosis differed among the four treatments (i.e., large-variation, large-variation giant-removal, small-variation, and no-salamander treatments). When I found significant differences among the treatments, I performed pairwise comparisons using the Wilcoxon test and adjusted the statistical significance of each pairwise comparison by the sequential Bonferroni method (Holm 1979) based on a significant level of $\alpha = 0.05$.

To analyze larval salamander traits, I used the Kruskal-Wallis test, which was followed by the Wilcoxon tests for pairwise comparisons with sequential Bonferroni adjustment, to examine whether and how mortality during the three time periods between censuses (day 1 to 19, day 19 to 31, day 31 to 151) differed among the three salamander treatments (i.e., large-variation, large-variation giant-removal, and small-variation treatments). Because I hypothesized that salamander giants would occur in the large-variation treatment, morphology (i.e., gape width and body length) of the salamander larvae with the largest body length in each tank on days 19 and 31 was compared among the three treatments using the Kruskal-Wallis test followed by the Wilcoxon tests with sequential Bonferroni adjustment. My focus on a single individual in each tank on each of the two days is reasonable because salamander giants occur in very low proportions in populations (Kishida et al. 2011). I also expected that the salamander giants would metamorphose earlier and with larger size than salamander larvae that did not become giants, because they could effectively consume both conspecifics and heterospecific prey. Thus, I compared the size (i.e., body length) and timing at metamorphosis of the first salamander metamorph in each tank among the three salamander treatments. I used

the Kruskal-Wallis test followed by the Wilcoxon tests with sequential Bonferroni adjustment for the analyses of body length of the first salamander metamorph. I used a Kaplan-Meier analysis to examine whether the timing (day of appearance) of the first salamander metamorph in each tank differed among the three salamander treatments. If I found significant differences among the treatments, I conducted post hoc pairwise comparisons using a Kaplan-Meier analysis with sequential Bonferroni adjustment.

RESULTS

(1) Mortality of the frog tadpoles

There were no differences in the mortality of the frog tadpoles among the treatments until day 19 (Kruskal-Wallis test, $\chi^2_2 = 3.99$, $P = 0.135$). However, after I carried out the second step (i.e., removing salamander giants), I found significant differences in the mortality of the frog tadpoles (Kruskal-Wallis: from day 19 to day 31, $\chi^2_3 = 31.26$, $P < 0.0001$; after day 31, $\chi^2_3 = 30.45$, $P < 0.0001$). Mortality of the frog tadpoles in the large-variation treatment was highest among the four treatments from day 19 to the end of the experiment (Fig. 1 and Table F1, 2 in Appendix F). The mortality of frog tadpoles in the large-variation treatment was 3.5- and 2.8-fold greater than in the small-variation and the large-variation giant-removal treatments, respectively. There was no significant difference between the small-variation and large-variation giant-removal treatments, but the mortality of the tadpoles in these two treatments was significantly higher than the mortality in the no-salamander treatment during the same period (Fig. 1 and Table F1, 2 in Appendix F).

(2) Behavior, morphology, and life history characteristics of the frogs

Repeated-measures ANOVA of the activity of the frog tadpoles showed significant treatment effects ($F_{3,82} = 15.77$, $P < 0.0001$) and interactive effects between treatment and time

($F_{6,162}$ = 15.77, P = 0.002) (Fig. 2a). Subsequent ANOVA revealed significant differences in the activity of the frog tadpoles on day 21 ($F_{3,85}$ = 16.20, P < 0.0001) and day 32 ($F_{3,85}$ = 10.65, P < 0.0001). Tukey's post hoc tests showed that activity levels of the frog tadpoles in the large-variation and large-variation giant-removal treatments were lower than those in the small-variation and no-salamander treatments on day 21 (P < 0.05) (Fig. 2a), and on day 32, activity in the large-variation treatment was lower than in the other treatments (P < 0.05) (Fig. 2a), whereas there were no differences among the small-variation, large-variation giant-removal, and no-salamander treatments (Fig. 2a).

Differences in the degree of defensive morphology (i.e., size-adjusted body width) of the frog tadpoles among the treatments were not significant on day 19 (Kruskal-Wallis test, χ^2_2 = 3.9, P = 0.14) (Fig. 2b), but differences among the treatments were significant on day 31 (Kruskal-Wallis test, χ^2_3 = 47.82, P < 0.0001). Post hoc testing found significant differences in all pairwise comparisons (Table F3 in Appendix F). Tadpoles in the large-variation treatment had the highest degree of defensive morphology, followed by those in the large-variation giant-removal treatment, with those in the small-variation treatment exhibiting the lowest degree of defensive morphology (Fig. 2b).

I found significant differences in the timing of frog metamorphosis among the four treatments, using both tank mean data (Kruskal-Wallis test: χ^2_3 = 29.58, P < 0.0001) and median data (χ^2_3 = 26.25, P < 0.0001). Timing at metamorphosis in the large-variation treatment was significantly later than it was in any of the other treatments (Fig. 2c and Table F4, 5 in Appendix F), whereas the timing did not differ significantly between the small-variation and the large-variation giant-removal treatments (Fig. 2c and Table F4, 5 in Appendix F). The timing of metamorphosis in the small-variation and large-variation giant-removal treatments was significantly later than in the no-salamander treatment (Fig. 2c and Table F4, 5 in

Appendix F).

I found significant differences in size at metamorphosis of the frogs among the four treatments, using both tank mean data (Kruskal-Wallis test: $\chi^2_3 = 43.24$, $P < 0.0001$) and median data ($\chi^2_3 = 36.14$, $P < 0.0001$). The froglets (new metamorphs) in the large-variation treatment were significantly larger than those in any of the other treatments (Fig. 2d and Table F6, 7 in Appendix F). The froglets in the large-variation with giant-removal treatment were larger than those in the small-variation treatment, but there was no significant size difference between the small-variation and no-salamander treatments (Fig. 2d and Table F6, 7 in Appendix F).

(3) Mortality of the salamander larvae

Mortality of salamander larvae until day 19 in the large-variation treatment was 14.6-fold higher than that in the small-variation treatment (Wilcoxon-test: $\chi^2_1 = 47.20$, $P < 0.0001$). From day 19 to 31, mortality of salamander larvae differed significantly among the three salamander treatments (Kruskal-Wallis test: $\chi^2_2 = 26.47$, $P < 0.0001$); among the treatments, mortality was highest in the large-variation treatment, whereas mortality in the large-variation giant-removal treatment was similar to that in the small-variation treatment (Fig. 3 and Table G1 in Appendix G). After day 31, mortality did not significantly differ among the treatments (Kruskal-Wallis test: $\chi^2_2 = 2.57$, $P = 0.28$) (Fig. 3). These results indicate that cannibalism occurred frequently in the large-variation treatment before day 31 and the salamander giants surely cannibalized in that period.

(4) Morphology and life history of the salamander larvae

On day 19, the body length and gape width of the largest salamander larvae were respectively 1.2- (Wilcoxon test, $\chi^2_1 = 36.63$, $P < 0.0001$) and 1.4-fold (Wilcoxon test, $\chi^2_1 = 31.69$, $P < 0.0001$) larger in the large-variation treatment than in the small-variation treatment

(Fig. 4a,b). Kruskal-Wallis testing revealed significant differences in these traits among the treatments on day 31 (largest body length, $\chi^2_2 = 38.12$, $P < 0.0001$; largest gape width, $\chi^2_2 = 28.59$, $P < 0.0001$). On day 31, the largest body length among the salamander larvae in the large-variation treatment was 1.5-fold and 1.4-fold larger than it was in the small-variation and the large-variation giant-removal treatments, respectively (Fig. 4a and Table G2 in Appendix G). In addition, the largest gape width among the salamander larvae in the large-variation treatment was 1.5-fold larger than it was in both the small-variation and the large-variation giant-removal treatments (Fig. 4b and Table G3 in Appendix G). In contrast, these traits did not differ significantly between the small-variation and large-variation giant-removal treatments on day 31 (Fig. 4a,b and Table G2, 3 in Appendix G).

Kaplan-Meier analysis revealed that the earliest timing of metamorphosis of the salamanders was significantly different among the treatments ($\chi^2_2 = 26.19$, $P < 0.0001$). The timing of metamorphosis of the salamanders in the large-variation treatment was significantly earlier than that in any of the other treatments, whereas the timing of metamorphosis did not differ significantly between the small-variation and large-variation giant-removal treatments (Fig. 4c and Table G4 in Appendix G).

I found significant differences in size at metamorphosis of the first metamorphs among the treatments (Kruskal-Wallis test: $\chi^2_2 = 29.06$, $P < 0.0001$). The size at metamorphosis of the first metamorphs in the large-variation treatment was significantly larger than that in any of the other treatments, whereas the size at metamorphosis of the first metamorphs did not differ significantly between the small-variation and the large-variation giant-removal treatments (Fig. 4d Table G5 in Appendix G).

DISCUSSION

In this study, I clearly showed that cannibalism occurred more frequently in the treatments with large variation in the timing of hatching of salamander larvae than in those with small variation, and that the faster growth of cannibals strengthened predatory effect on frog tadpoles, the salamander's heterospecific prey. Before the second experimental step (i.e., before day 19), the salamander larvae did not prey on the frog tadpoles (Fig. 1), but cannibalistic giants occurred in the large-variation treatment (Fig. 4a, b, Appendix C) as a result of intensive cannibalism caused by the developmental asymmetry among the salamander hatchlings (Fig. 3). The presence of these giant salamander larvae intensified the salamander's predatory effect on the frog tadpoles (Fig. 1). Predator cannibalism, by creating salamander giants, also affected the trait characteristics of the frog tadpoles. In the treatments containing salamander giants, behavioral and morphological defenses were significantly more developed than they were in any of the other treatments (Fig. 2a,b). Moreover, the frog tadpoles metamorphosed later and their size at metamorphosis was larger (Fig. 2c,d). Because I found no statistically significant differences in any of the measured variables among the four initial small-variation treatments (Appendix D), my results clearly demonstrate that the variation in rather than mean timing of hatching of salamander larvae has more power to explain variations in the strength of predatory effect on and phenotypic characteristics of the frog tadpoles (i.e., morphological and behavioral defense and life history traits). This point is noteworthy because, although researchers have recently begun to recognize the importance of intraspecific variation in population and community dynamics as well as in ecosystem functioning (Bolnick et al. 2003, 2011, Miller & Rudolf 2011), most researches have focused on interpopulation differences in representative trait values (Post et al. 2008, Bassar et al. 2010, Walsh & Post 2011) rather than on individual trait variation within populations (i.e., intrapopulation variation) (Harmon et al. 2009, Pruitt & Ferrari 2011).

In general, predator cannibalism has three effects: the predator's population size is reduced; defensive behavior is induced in non-cannibals; and the cannibals grow rapidly (e.g., Fox 1975, Polis 1981, Miller & Rudolf 2011). The induction of defensive behavior in non-cannibals (Rudolf 2006) and the reduction in the population size of the predator both reduce predation pressure on the predator's heterospecific prey, thus having a positive impact on the prey population (Persson et al. 2003). My experimental results show, however, that the rapid growth of the cannibals can cause predator cannibalism to have a negative impact on the heterospecific prey population. The strength and sign of the net effect of predator cannibalism on the predator's heterospecific prey depend on the relative importance of these three effects. Here, I found that the net impact of cannibalism in the predatory salamander larvae on frog tadpoles, their heterospecific prey, was negative. Thus, under my experimental condition, the negative impact of predator cannibalism caused by the rapid growth of the cannibals overwhelms the possible positive impacts due to the reduced predator density and the defensive behavior of the non-cannibals. I expect that outcomes would be similar in other predator–prey systems where the likelihood of predation success is sensitive to the relative size relationship of the predators and their heterospecific prey (Cohen et al. 1993). Moreover, it is likely that in populations where predator cannibalism has been found to reduce predatory effects, predator growth probably does not influence the availability of heterospecific prey (Persson et al. 2003, Rudolf 2006). For example, Persson et al. (2003) reported strong, positive cascading impacts of cannibalism in predatory perch on the zooplankton community. In this system, cannibalism by large perch in the young-of-the-year cohort reduced the predation pressure of the cohort on zooplankton, because the increased growth of piscivorous large cannibals did not intensify the predation pressure on the zooplankton community. Because animals commonly broaden their diet as they grow (Werner & Gilliam 1984, Urban 2007), a negative net impact of predator

cannibalism on the predator's heterospecific prey is more likely in the case of interactions between predators and prey species of similar size.

Changes in the characteristics of predators can influence the expression of defensive traits in their prey through modification of plastic responses of prey individuals and selective mortality of prey phenotypes. For example, high-density and predaceous phenotypes of predators elicit the expression of more-defensive phenotypes in prey individuals (Pettersson & Bronmark 1997, Van Buskirk & Arioli 2002, Kishida et al. 2006) and impose strong selective mortality on less-defensive individuals (Pakes & Boulding 2010, Takatsu & Kishida 2013). In this study, even though salamander larval densities were lower in the large-variation and large-variation non-giant-removal treatments, the expression of defensive behavior and morphology was greater in the frog tadpoles in those treatments than in the four small-variation treatments. These results suggest that a focus on predator phenotypes rather than on predator density, and thus on factors that determine predator phenotypes such as cannibalism, can lead to a better understanding of the mechanisms responsible for interpopulation variation of prey defenses. This finding is particularly important because degree of prey defenses, in particular defensive behaviors, strongly determine outcomes of trophic interactions in natural communities (Agrawal 2001, Werner & Peacor 2003, Miner et al. 2005, Kishida et al. 2010).

Metamorphosis timing was later and size at metamorphosis was larger in the frog tadpoles in the treatments containing salamander giants than in those in the other treatments. To my knowledge, this finding is the first evidence that intraspecific trait variation in predators and the resultant predator cannibalism can affect the life history of their heterospecific prey. These life history modifications of the frogs could be adaptive plastic responses of individuals to relaxed resource competition due to a reduced population size or non-adaptive plastic responses due to the costs associated with expressing inducible defenses (Benard 2004, Relyea

2007). Alternatively, size selective predation of the salamander larvae on smaller tadpoles (Kishida et al. 2009b, Takatsu & Kishida 2013) may cause delayed metamorphosis in frogs. For example, if a trade-off exists between development and growth (Arendt 1997, Fujimoto et al. 2012), populations of frog tadpoles may consist of slowly developing but fast-growing individuals (i.e., less-developed but large frog tadpoles) or fast developing but slow-growing individuals (i.e., well-developed but small frog tadpoles). In this case, populations of frog tadpoles exposed to high predation pressure would contain a higher proportion of large frog tadpoles (i.e., fast-growing but slowly developing tadpoles). Regardless of the precise mechanism, the effects of salamander cannibalism on the metamorphosis of frogs likely have significant ecological consequences. For example, in the present study, although the mortality of the frog tadpoles in the large-variation treatment was consistently highest among the three salamander treatments (Fig. 1), that treatment did not have the fewest remaining frog tadpoles among the treatments (Fig. 5). Because the strong delaying effects on frog metamorphosis caused by the high predation risk from salamander giants (Fig. 2c) counteracted the mortality impact on the frog tadpole prey, more tadpoles remained in the large-variation treatment than in the small-variation treatment in the later experimental period (days 46 [$F_{1,66} = 4.97$, $P = 0.029$] and 61 [$F_{1,66} = 6.55$, $P = 0.012$]). *Rana pirica* tadpoles, which are omnivorous, may play important roles in the pond community, so a higher abundance of frog tadpoles due to predator cannibalism can affect other community members as well. In addition to such short-term population dynamics, salamander cannibalism may influence long-term population dynamics, because the metamorphosis timing and size at metamorphosis of individuals can influence their survival and reproductive success (Semlitsch et al. 1988).

The timing of metamorphosis of the salamander larvae that metamorphosed earliest in each tank (i.e., earliest timing at metamorphosis) of the treatments containing salamander

giants was 17 days and 12 days earlier than that in the small-variation treatment and the large-variation giant-removal treatment, respectively. The first metamorphs in the treatments containing salamander giants were 1.2-fold and 1.1-fold larger than in the small-variation treatment and the large-variation giant-removal treatment, respectively. I suggest that this enhanced growth and development was due to relaxed intraspecific competition and enhanced energy acquisition via consumption of both small conspecifics and frog tadpoles. To test this hypothesis, I conducted multiple regression analyses of the earliest timing of metamorphosis and size of the first metamorphs in the large-variation treatment, in which the numbers of cannibalized salamander larvae and consumed frog tadpoles before salamander metamorphosis were explanatory variables. I found a significant positive effect of the number of cannibalized salamander larvae ($F_{2,25} = 17.77$, $P = 0.0003$) and the number of consumed frog tadpoles ($F_{2,25} = 24.04$, $P < 0.0001$) on the earliest timing of salamander larval metamorphosis, and also a significant positive effect of the number of cannibalized salamanders ($F_{2,25} = 6.23$, $P = 0.012$) and the number of consumed frog tadpoles ($F_{2,25} = 7.52$, $P = 0.011$) on the size at metamorphosis of the first metamorphs. Hence, predator cannibalism affected the characteristics of the predators themselves not only through intraspecific interactions (i.e., changes in the energy flow due to cannibalism) but also through interspecific trophic interaction with their heterospecific prey.

In organisms with complex life cycles, environmental factors that affect the density, phenotype, or transition rate between life stages can have dramatic consequences for population and community dynamics across multiple life stages and habitats (Benard 2004, McCoy et al. 2009, Orrock et al. 2010). Hence, size and developmental variation in salamander larvae and the resultant cannibalism, by modifying population characteristics of both amphibian predator and prey species, may have a significant impact on other community

members in both aquatic and terrestrial ecosystems. On the other hand, other community members might modify the ecological consequences of the variation by influencing the occurrence of salamander cannibalism. For example, predation risk from a top predator, *Aeshna nigroflava* dragonfly larvae, strongly suppresses cannibalism in *Hynobius retardatus* larvae by reducing their activity, thus greatly reducing the likelihood of salamander gigantism (Kishida et al. 2011). Hence, salamander larvae in populations that otherwise have a large enough variation in size and development to induce cannibalism may not impose strong predation pressure on frog tadpoles if dragonfly larvae are present. Further studies of the interactions between community dynamics and salamander cannibalisms are required to deepen our understanding of the ecological consequences of intrapopulation trait variation.

Although there is growing recognition of the importance of intraspecific trait variation in ecological interactions (Bolnick et al. 2003, 2011, Miller & Rudolf 2011), the dynamic nature of key traits of individuals has received markedly little attention (Yang & Rudolf 2010, Rasmussen et al. 2014). As I demonstrated here, ecological interactions that are the outcomes of trait variation within populations may cause dramatic changes in the trait variation and, consequently, potentially can lead to further modifications of food web. Incorporating the interactive relationship between trait variation and ecological interactions into the recently developed trait-based approach would be fruitful for understanding how spatial and temporal variation in community composition is created.

Figure 1. Mean (± SE) number of dead frog tadpoles. Large-var, large-variation treatment; Large-var g-removal, large-variation giant-removal treatment; Small-var, small-variation treatment; and No-sal, no-salamander treatment. Treatments not sharing the same lowercase letter in each period had significantly different means at the $P < 0.05$ level following sequential Bonferroni adjustment.

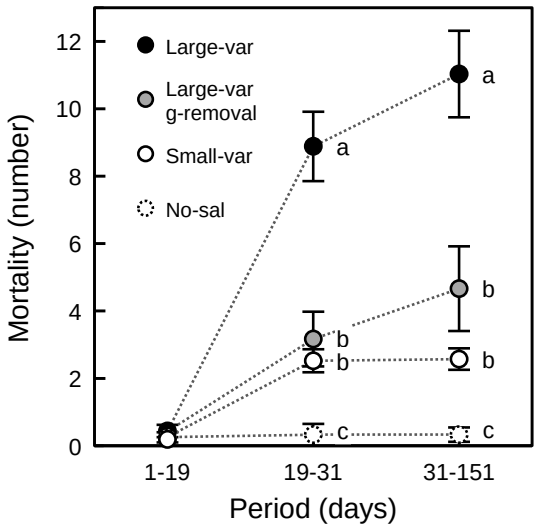


Figure 2. Mean (± SE) activity (a) and size-adjusted mean (± SE) body width (b) of frog tadpoles. Treatments on each day not sharing the same lowercase letter had significantly different means at the $P < 0.05$ level. Mean (± SE) metamorphosis timing (number of days after the start of the experiment) (c) and body length (size) at metamorphosis (d) in the frog tadpoles. Large-var, large-variation treatment; Large-var g-removal, large-variation giant-removal treatment; Small-var, small-variation treatment; and No-sal, no-salamander treatment. Treatments not sharing the same lowercase letter had significantly different means at the $P < 0.05$ level following sequential Bonferroni adjustment.

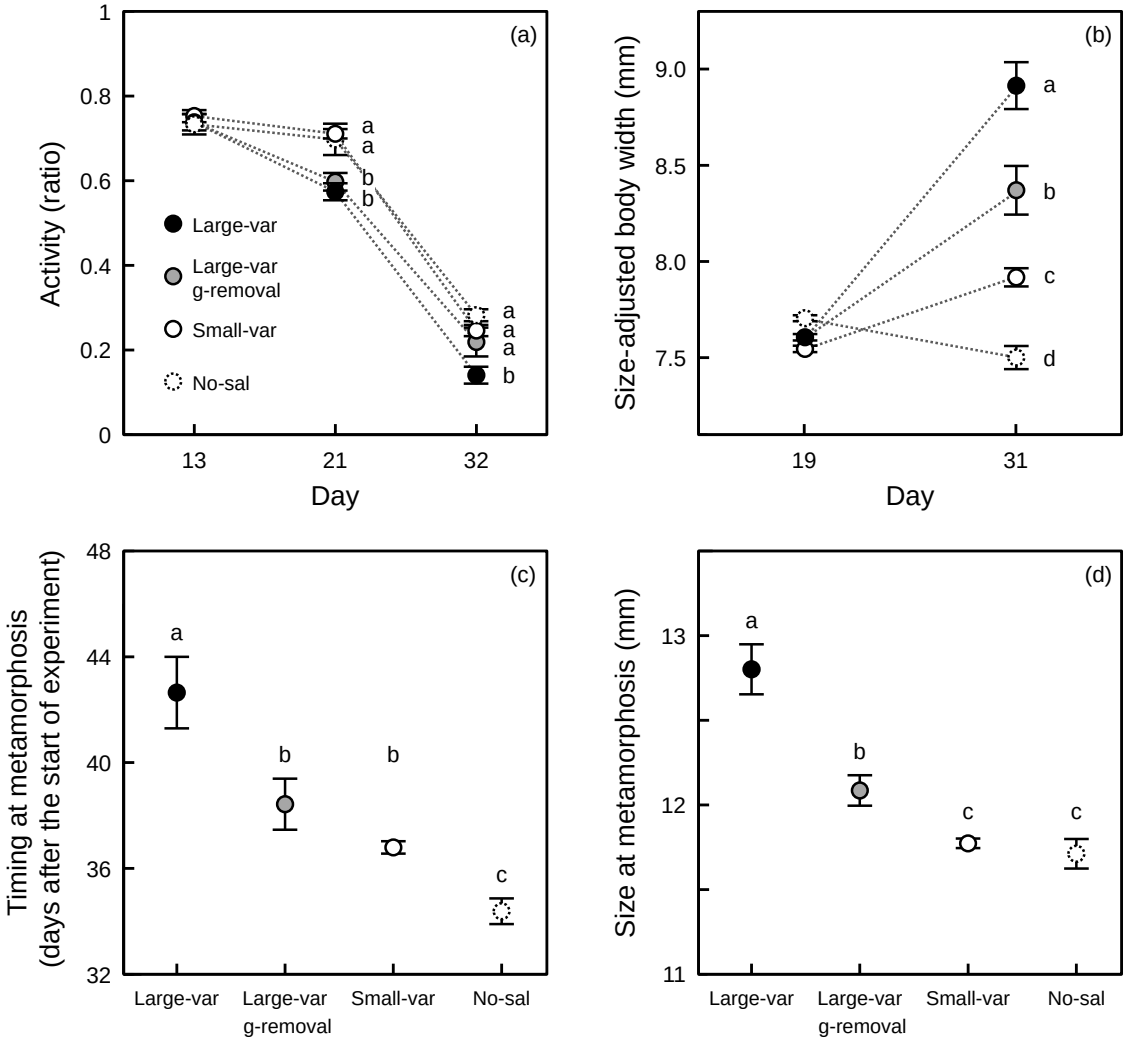


Figure 3. Mean (± SE) number of dead salamander larvae. Large-var, large-variation treatment; Large-var g-removal, large-variation giant-removal treatment; and Small-var, small-variation treatment. Treatments not sharing the same lowercase letter in each experimental period have significantly different means at the $P < 0.05$ level following sequential Bonferroni adjustment.

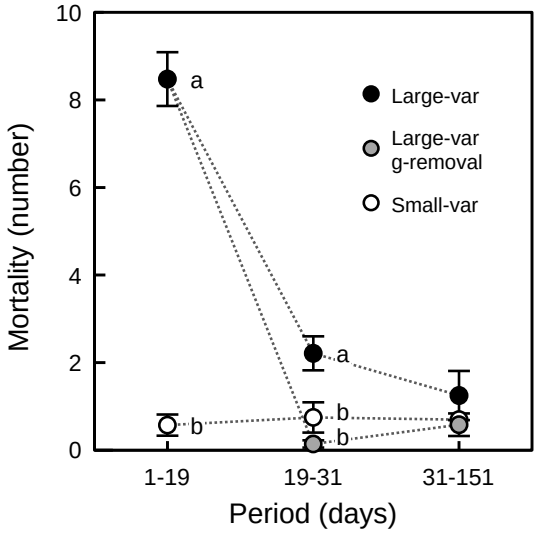


Figure 4. Mean (± SE) largest body length (a) and mean (± SE) largest gape width (b) of salamander larvae. Treatments not sharing the same lowercase letter on each day had significantly different means at the $P < 0.05$ level following sequential Bonferroni adjustment. (c) Kaplan-Meier plot of the earliest timing of metamorphosis in salamander larvae. Post hoc pairwise comparisons (Kaplan-Meier analyses with sequential Bonferroni adjustment) revealed that the earliest timing at metamorphosis of salamander larvae in the large-variation treatment was significantly earlier than it was in any of the other treatments. (d) Mean (± SE) body length (size) of the first salamander metamorphs. Large-var, large-variation treatment; Large-var g-removal, large-variation giant-removal treatment; and Small-var, small-variation treatment. Treatments not sharing the same lowercase letter in each period had significantly different means at the $P < 0.05$ level following sequential Bonferroni adjustment.

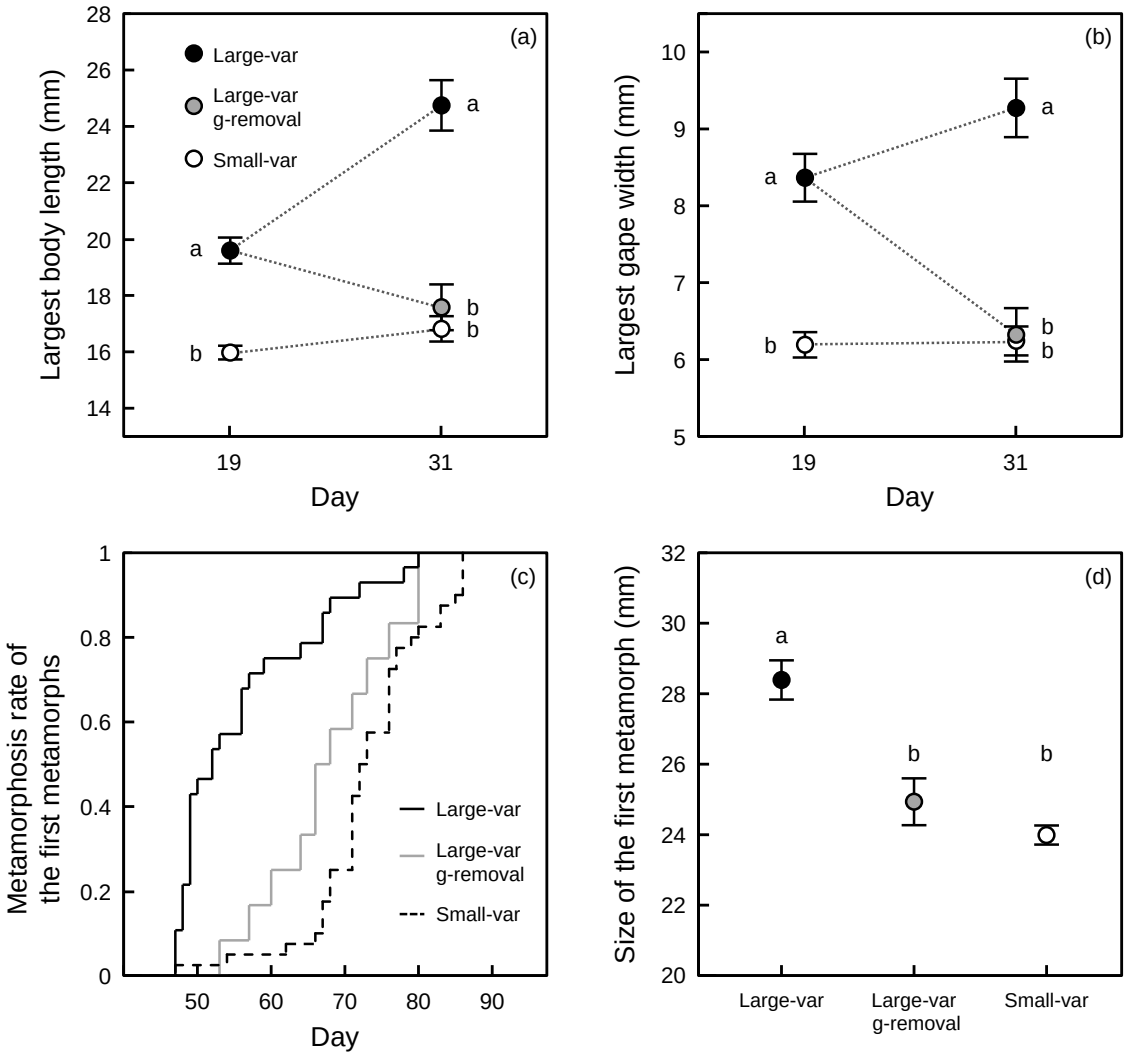
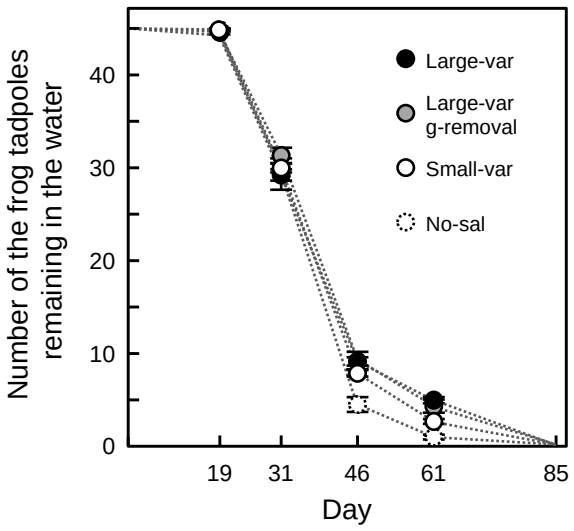

(a)
Large-var
Large-var g-removal
Small-var
Largest body length (mm)
Day
(b)
Largest gape width (mm)
Day
(c)
Metamorphosis rate of the first metamorphs
Large-var
Large-var g-removal
Small-var
Day
(d)
Size of the first metamorph (mm)
Large-var
Large-var g-removal
Small-var

Figure 5. Mean (± SE) number of frog tadpoles remaining in the tanks on experimental days 19, 31, 46, and 61. Large-var, large-variation treatment; Large-var g-removal, large-variation giant-removal treatment; Small-var, small-variation treatment; and No-sal, no-salamander treatment.



APPENDIX

Appendix A: Methods of collection, keeping, and manipulation of experimental animals

Appendix A1: Collection and keeping of eggs of *Hynobius retardatus* salamanders and *Rana pirica* frogs

Ninety egg clusters of *H. retardatus* salamanders and 10 egg masses of *R. pirica* frogs were collected from several ponds in the Teshio Experimental Forest of Hokkaido University, Hokkaido, Japan, in mid-May 2012. Each of the 10 frog egg masses was kept in a separate 22 L semi-transparent polypropylene tank (51.3 cm × 37.2 cm × 16.6 cm high) filled with 5 L of aged tap water, and the tanks were placed in my experimental room, which was maintained at 17 °C with a natural light–dark (14h/10h) regime. The eggs had started to hatch by late May. After the frog tadpoles hatched, I put eight pieces of rabbit chow (dry weight: 1.6 g) into each tank as food for the frog tadpoles every 2 days, and I also exchanged the water every 2 days. I cultured the hatchling frog tadpoles under the conditions described above two weeks (i.e., until the start of the experiment). Each of the 90 salamander egg clusters was placed separately in a colander (9 cm × 6.5 cm × 5 cm high), and the colanders were placed in 13 L semi-transparent polypropylene tanks (43.6 cm × 28.4 cm × 14.1 cm high; 20 colanders per tank) filled with 10 L of aged tap water. Then the tanks were placed in a refrigerator and maintained at 3 °C under natural light–dark (14h/10h) conditions.

Appendix A2: Method to manipulate timing of hatching of salamanders

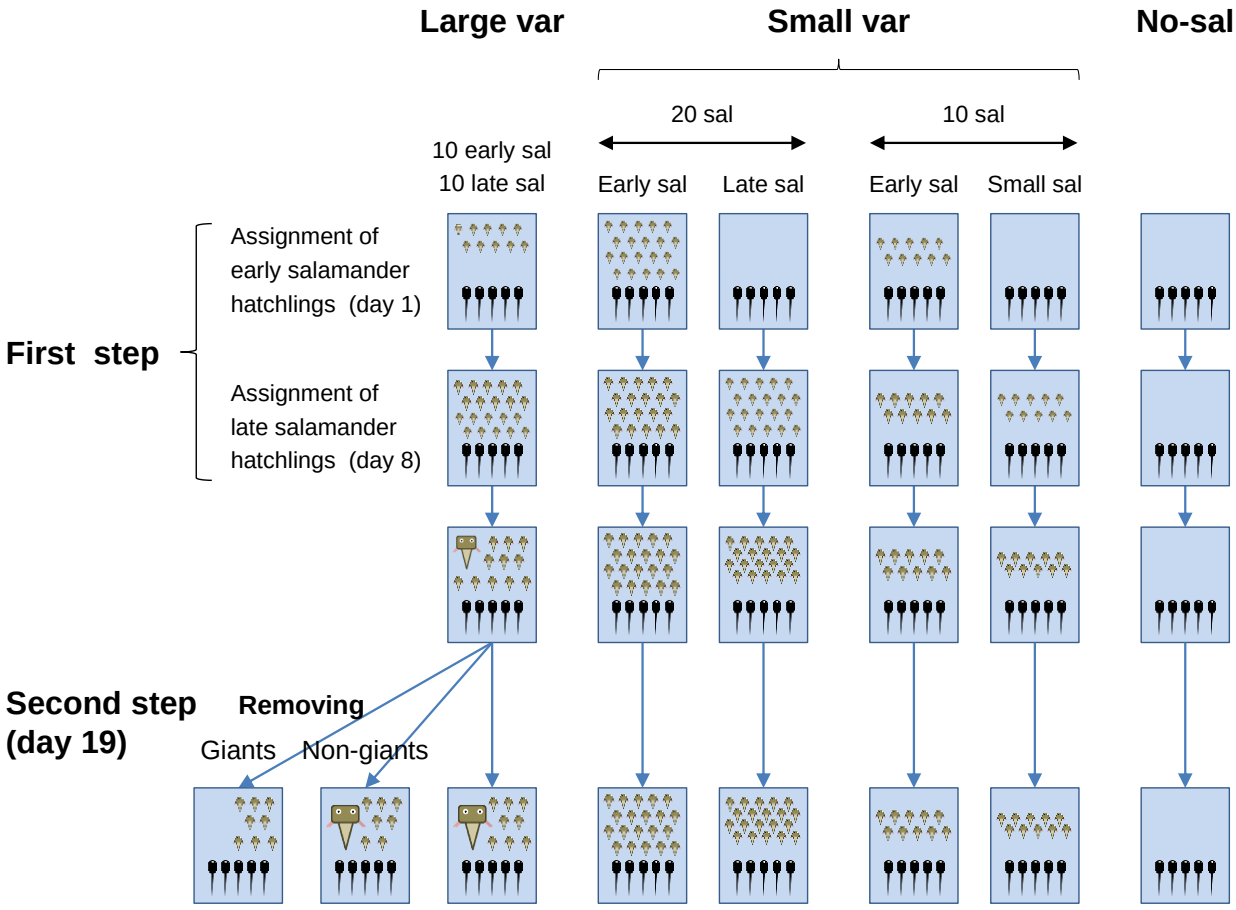
I obtained the early and late salamander hatchlings (hatch time difference, 1 week) by manually controlling the water temperature experienced by the embryos in a single egg as described below(i.e., full or half sibs). This method allowed us to preclude possible confounding genetic effects on cannibalism.

To obtain both early and late hatchlings from a single egg cluster for the large-variation treatment, each egg cluster was split in half before the larvae hatched. Then one half of the cluster was kept in an experimental room maintained at 17 °C to accelerate hatching, and the other half was kept in a refrigerator maintained at 3 °C to delay hatching by 1 week relative to the early-hatch group. The early-hatch group hatched two weeks after the frog tadpoles had hatched.

Appendix B: Diagram of the experimental design

Figure B1. Diagram of the experimental design. Large-var, large-variation treatment; Small-var, small-variation treatment; No-sal, no-salamander treatment; Sal, salamander hatchlings. On day 1 of the experiments, 45 frog tadpoles were assigned to each treatment.

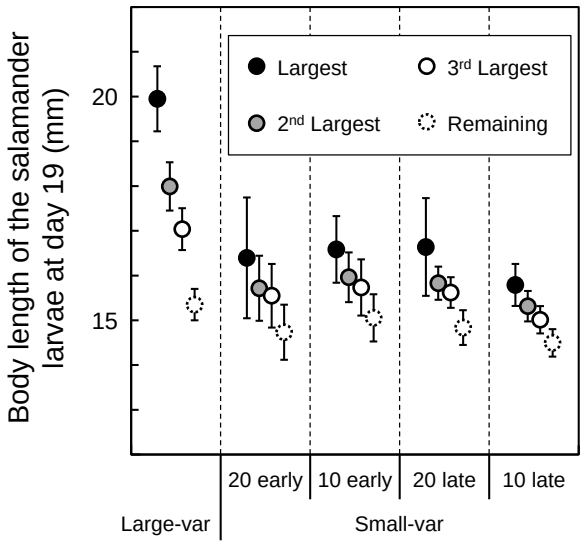
Figure B1



Appendix C. Size distribution of salamander larvae on day 19

Figure C1. Mean (± 95% confidence interval) body size of the largest (black circles), second largest (gray circles), third largest (white circles), and the remaining salamanders (dashed circles) in each treatment on day 19. Large-var, Large-variation treatment; Small-var, Small-variation treatment; Early, early hatchlings; late, late hatchlings.

Figure C1.



Appendix D. Summary of statistical analyses comparing the four small-variation treatments

I initially established a large-variation treatment and four small-variation treatments (i.e., small variation treatment with 20 early hatchlings, 20 late hatchlings, 10 early hatchlings, or 10 late hatchlings) at the start of the experiment. Because I expected that cannibalism would rarely occur in the four small-variation treatments, I conducted preliminary analyses of the data of those four treatments to determine whether the effects of the four treatments on the measured variables were similar. Therefore, I tested whether (1) frog tadpole mortality (Table D1), (2) behavior (i.e., frog tadpole activity; Table D2), morphology (i.e., degree of defensive morphology in frog tadpoles; Table D3), and life history (i.e., mean, Table D4, and median, Table D5, metamorphosis timing and size at metamorphosis of the frog tadpoles), (3) mortality of the salamander larvae (Table D6), or (4) morphology (i.e., largest body length, Table D7, and largest gape width, Table D8) and life history (i.e., earliest timing at metamorphosis, Table D9, and size at metamorphosis of the first metamorphs, Table D10) differed among the four small-variation treatments. I used repeated-measures ANOVA to compare activity of the frog tadpoles, performed a Kaplan-Meier analysis of the earliest timing of metamorphosis in the salamander larvae, and compared the other measurements by using Kruskal-Wallis tests. I did not find significant differences in any of these traits among the four small-variation treatments (Tables D1–10).

Table D1. Kruskal-Wallis test results for frog tadpole mortality.

Measurements		χ^2_3	*P*
Mortality of the frog tadpoles	Day 1 to day 19	0.76	0.86
	Day 19 to day 31	1.94	0.59
	Day 31 to day 150	7.16	0.067

Table D2. Repeated-measures ANOVA results for frog tadpole activity.

Factors	*d.f.*	*F*	*P*
Treatment	3	0.26	0.85
Time	2	296.65	<0.001
Treatment*Time	6	1.86	0.10

Table D3. Kruskal-Wallis test results for the degree of defensive morphology.

Measurements		χ^2_3	*P*
Degree of defensive morphology	day 19	2.64	0.45
	day 31	6.84	0.072

Table D4. Kruskal-Wallis test results for mean metamorphosis timing and mean size at metamorphosis in frog tadpoles.

Measurements	χ^2_3	*P*
Mean timing of metamorphosis	2.48	0.48
Mean size at metamorphosis	5.86	0.12

Table D5. Kruskal-Wallis test results for median metamorphosis timing and median size at metamorphosis of the frog tadpoles.

Measurements	χ^2_3	*P*
Median timing of metamorphosis	4.44	0.22
Median size at metamorphosis	6.46	0.091

Table D6. Kruskal-Wallis test results for mortality of salamander larvae.

Measurements		χ^2_3	*P*
Mortality of the salamander larvae	Day 1 to day 19	2.17	0.54
	Day 19 to day 31	2.11	0.55
	Day 31 to day 151	7.32	0.062

Table D7. Kruskal-Wallis test results for largest body length.

Measurements		χ^2_3	*P*
Largest body length	day 19	7.10	0.069
	day 31	0.31	0.96

Table D8. Kruskal-Wallis test results for largest gape width.

Measurements		χ^2_3	*P*
Largest gape width	day 19	4.67	0.20
	day 31	0.96	0.062

Table D9. Kaplan-Meier analysis results for the earliest timing of salamander metamorphosis

Measurements	χ^2_3	*P*
Earliest timing at metamorphosis	6.53	0.089

Table D10. Kruskal-Wallis test results for size at metamorphosis (i.e., body length) of the first metamorphs .

Measurements	χ^2_3	*P*
Size at metamorphosis of the first metamorphs	5.92	0.12

Appendix E. Summary of statistical analyses comparing the large-variation and large-variation non-giant-removal treatments

I established the large-variation giant-removal and large-variation non-giant-removal treatments as additional treatments in the second step. Because I expected that the occurrence of salamander giants would be key to establishing predator-prey interactions between the salamanders and the frog tadpoles, I conducted preliminary analyses of the data of the two treatments including salamander giants (large-variation and large-variation non-giant-removal treatments) to determine whether for any measured variables the results of the two treatments were similar. I tested whether (1) mortality of the frog tadpoles (Table E1), (2) behavior (i.e., activity, Table E2), morphology (i.e., degree of the defensive morphology, Table E3), or life history (i.e., mean, Table E4, and median, Table E5, metamorphosis timing and size at metamorphosis) of the frog tadpoles, or (3) mortality of the salamander larvae (Table E6), or (4) morphology (i.e., largest body length, Table E7, and largest gape width, Table E8) or life history (i.e., earliest timing of metamorphosis, Table E9, and size at metamorphosis of the first metamorphs, Table E10) of the salamander larvae differed between the two treatments. I used repeated-measures ANOVA to compare activity of the frog tadpoles, conducted a Kaplan-Meier analysis of the earliest timing of metamorphosis in the salamander larvae, and used Kruskal-Wallis tests to compare the other measurements. I did not find any significant differences in any traits between the two treatments (Tables E1–10).

Table E1. Kruskal-Wallis test results for mortality of frog tadpoles.

Measurements		χ^2_1	*P*
Mortality of frog tadpoles	Day 19 to day 31	0.49	0.49
	Day 31 to day 150	1.10	0.30

Table E2. Repeated-measures ANOVA results for frog tadpole activity.

Factors	*d.f.*	*F*	*P*
Treatment	1	3.41	0.076
Time	2	185.69	<0.001
Treatment*Time	2	1.88	0.17

Table E3. Kruskal-Wallis test results for the degree of the defensive morphology.

Measurements	χ^2_1	*P*
Degree of defensive morphology on day 31	0.0086	0.93

Table E4. Kruskal-Wallis test results for mean metamorphosis timing and mean size at metamorphosis of the frog tadpoles.

Measurements	χ^2_1	*P*
Mean timing of metamorphosis	0.078	0.78
Mean size at metamorphosis	0.054	0.82

Table E5. Kruskal-Wallis test results for median metamorphosis timing and median size at metamorphosis of the frog tadpoles.

Measurements	χ^2_1	*P*
Median timing of metamorphosis	0.75	0.39
Median size at metamorphosis	0.36	0.55

Table E6. Kruskal-Wallis test results for mortality of the salamander larvae.

Measurements		χ^2_1	*P*
Mortality of the salamander larvae	Day 19 to day 31	0.056	0.81
	Day 31 to day 151	0.051	0.82

Table E7. Kruskal-Wallis test results for largest body length.

Measurements	χ^2_1	*P*
Largest body length on day 31	0.0022	0.96

Table E8. Kruskal-Wallis test results for largest gape width.

Measurements	χ^2_1	*P*
Largest gape width on day 31	1.57	0.21

Table E9. Kaplan-Meier analysis results for earliest timing of metamorphosis in the salamander.

Measurements	χ^2_1	*P*
Earliest timing of metamorphosis	0.33	0.57

Table E10. Kruskal-Wallis test results for size at metamorphosis of the first metamorphs.

Measurements	χ^2_1	*P*
Size at metamorphosis of the first metamorphs	0.36	0.55

Appendix F. Summary of the results of post hoc statistical analyses of frog tadpole traits, comparing the four experimental treatments (i.e., large-variation, large-variation giant-removal, small-variation, and no-salamander treatments). Significant values are in bold (significant level was adjusted using sequential Bonfferoni methods with significant value P=0.05).

Table F1. Post hoc Wilcoxon test results for mortality of frog tadpoles on day 19-31.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	21.71	**<0.0001**
Large-variation vs. Large-variation giant-removal	9.11	**0.0025**
Large-variation vs. No-salamander	12.20	**0.0005**
Small-variation vs. Large-variation giant-removal	0.35	0.55
Small-variation vs. No-salamander	7.24	**0.0071**
Large-variation giant-removal vs. No-salamander	6.34	**0.012**

Table F2. Post hoc Wilcoxon test results for mortality of frog tadpoles on day 31-151.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	24.38	**<0.0001**
Large-variation vs. Large-variation giant-removal	6.69	**0.0097**
Large-variation vs. No-salamander	11.57	**0.0007**
Small-variation vs. Large-variation giant-removal	1.87	0.17
Small-variation vs. No-salamander	7.13	**0.0076**
Large-variation giant-removal vs. No-salamander	6.23	**0.013**

Table F3. Post hoc Wilcoxon test results for size adjusted body width of the frog tadpoles on day 31.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	33.86	**<0.0001**
Large-variation vs. Large-variation giant-removal	7.53	**0.0061**
Large-variation vs. No-salamander	13.72	**0.0002**
Small-variation vs. Large-variation giant-removal	8.85	**0.0029**
Small-variation vs. No-salamander	11.95	**0.0005**
Large-variation giant-removal vs. No-salamander	11.37	**0.0007**

Table F4. Post hoc Wilcoxon test results for mean timing of frog metamorphosis.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	19.35	**<0.0001**
Large-variation vs. Large-variation giant-removal	5.86	**0.016**
Large-variation vs. No-salamander	11.48	**0.0007**
Small-variation vs. Large-variation giant-removal	1.28	0.26
Small-variation vs. No-salamander	10.64	**0.0011**
Large-variation giant-removal vs. No-salamander	9.55	**0.0020**

Table F5. Post hoc Wilcoxon test results for median timing of frog metamorphosis.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	15.4	**<0.0001**
Large-variation vs. Large-variation giant-removal	3.93	**0.048**
Large-variation vs. No-salamander	13.27	**0.0003**
Small-variation vs. Large-variation giant-removal	1.86	0.17
Small-variation vs. No-salamander	7.86	**0.005**
Large-variation giant-removal vs. No-salamander	9.00	**0.0027**

Table F6. Post hoc Wilcoxon test results for mean size at metamorphosis of the frog.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	34.01	**<0.0001**
Large-variation vs. Large-variation giant-removal	8.37	**0.0038**
Large-variation vs. No-salamander	10.88	**0.0010**
Small-variation vs. Large-variation giant-removal	12.38	**0.0004**
Small-variation vs. No-salamander	0.61	0.43
Large-variation giant-removal vs. No-salamander	5.93	**0.015**

Table F7. Post hoc Wilcoxon test results for median size at metamorphosis of the frog.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	30.61	**<0.0001**
Large-variation vs. Large-variation giant-removal	5.93	**0.015**
Large-variation vs. No-salamander	10.29	**0.0013**
Small-variation vs. Large-variation giant-removal	6.57	**0.010**
Small-variation vs. No-salamander	0.56	0.45
Large-variation giant-removal vs. No-salamander	4.25	**0.039**

Appendix G. Summary of the results of post hoc statistical analyses of larval salamander traits, comparing the three salamander treatments (i.e., large-variation, large-variation giant-removal, and small-variation treatments). Significant values are in bold (significant level was adjusted using sequential Bonfferoni methods with significant value P=0.05).

Table G1. Post hoc Wilcoxon test results for mortality from day 19 to day 31.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	18.56	**<0.0001**
Large-variation vs. Large-variation giant-removal	15.15	**<0.0001**
Small-variation vs. Large-variation giant-removal	2.40	0.12

Table G2. Post hoc Wilcoxon test results for body length on day 31.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	35.33	**<0.0001**
Large-variation vs. Large-variation giant-removal	14.72	**0.0001**
Small-variation vs. Large-variation giant-removal	1.00	0.32

Table G3. Post hoc Wilcoxon test results for gape width on day 31.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	25.22	**<0.0001**
Large-variation vs. Large-variation giant-removal	14.05	**0.0002**
Small-variation vs. Large-variation giant-removal	0.0029	0.96

Table G4. Post hoc Kaplan-Meier analysis results for timing at metamorphosis.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	32.05	**<0.0001**
Large-variation vs. Large-variation giant-removal	6.64	**0.010**
Small-variation vs. Large-variation giant-removal	3.64	0.056

Table G5. Post hoc Wilcoxon test results for size at metamorphosis.

Treatment	χ^2_1	*P*
Large-variation vs. Small-variation	26.74	**<0.0001**
Large-variation vs. Large-variation giant-removal	9.24	**0.0024**
Small-variation vs. Large-variation giant-removal	1.30	0.25

Chapter 3

Giant cannibals drive selection for inducible defense in heterospecific prey

INTRODUCTION

The evolution of anti-predator traits is one of the best examples demonstrating the importance of species interactions for the evolution of life-history traits (Hoso *et al*., 2010; Kosloski & Allmon, 2015). The strength of the selection pressure on prey defenses is determined by the mortality imposed by the predator, which depends on both the predators' population size (i.e., density) and per-capita consumption rates (Abrams, 2000; Takatsu & Kishida, 2013). In general, decreasing predator density or per-capita consumption rate reduces the total number of predation events, but how changes in predator population influence prey evolution depends on how changes in the density and per-capita consumption rates of predators are correlated. Hence, exploring factors that modify predator demography and individual phenotype is imperative to better understand evolutionary processes of prey defenses (Turcotte, Corrin & Johnston, 2012; Walsh, 2013, see also terHorst *et al*., 2015). Despite considerable progress, little is known on how intra-specific interactions in predator populations influence selection pressure on prey defenses. Yet, intraspecific interactions among predators are likely to simultaneously affect both the demography and individual phenotypes of predators, and they can vary considerably across time and space and thus could lead to concurrent variation in selection pressure on prey defenses.

Cannibalism is a key interaction driving the demographic structure and even morphology of predator populations (Fox, 1975; Polis, 1981; Claessen, DeRoos & Persson, 2004), but its effect on the evolution of heterospecific prey is largely unknown (Rudolf, Sorrell & Pedersen, 2012). Predicting how cannibalism in predator populations affects prey evolution is challenging because it can have multiple and contrasting effects on the numerical and per-capita effects of the predator on its prey. Cannibalism in a predator can reduce predation rates on heterospecific prey via consumption of conspecific predators (i.e. numerical effects) or

changes in foraging behavior of conspecific victims or cannibals (i.e. per-capita effects) (Rudolf, 2006, 2007, 2012). These scenarios suggest that cannibalistic interactions in a predator should dampen the selection pressure on defensive phenotypes of heterospecific prey. In contrast, when interactions occur among growing predators and prey, cannibalism of predators can intensify predation rates on heterospecific prey if cannibalism allows predators to rapidly increase in growth and thereby improve per-capita predation ability of cannibalistic predators (Takatsu & Kishida, 2015). In this scenario, cannibalism could increase selection pressure favoring more defensive phenotypes of its heterospecific prey.

Here, I used two complementary experiments to test when and how cannibalism in a growing predator species alters selection for defensive phenotype of its heterospecific prey using a gape-limited predator-prey interaction between cannibalistic salamander larvae (*Hynobius retardatus* [Dunn]) and frog tadpoles (*Rana pirica* [Matsui]) as a model system. Specifically, I first examined whether cannibalism in salamander hatchlings produce selection pressures favoring the defensive ("bulgy") phenotype of tadpoles. In addition, I investigated whether expression of the defensive phenotype is associated with occurrence of salamander cannibalism. If salamander cannibalism has played a key role in evolution of the inducible defense, tadpoles should express more defensive (bulgy) phenotypes in the presence versus absence of cannibalism of the salamanders.

MATERIALS AND METHODS

Study system

Hynobius retardatus (Dunn) salamanders and *Rana pirica* (Matsui) frogs usually spawn in small temporary ponds in early spring in Hokkaido, Japan. Although salamander larvae are carnivores, the trophic relationship with frog tadpoles is not always established even

if adult salamanders and frogs lay their eggs in the same ponds. Strong predator-prey interactions can occur in the following two alternative scenarios. First scenario is that salamander hatchlings exhibit offensive phenotype, which is characterized by wider gape for effective consumption on frog tadpoles, in the presence of “small” frog tadpoles (Michimae & Wakahara, 2002; Takatsu & Kishida, 2013; Kishida et al., 2015). This scenario can occur when hatch timing of salamanders and frogs are very close (within 2 weeks) (Kishida, Trussell & Nishimura, 2009; Takatsu & Kishida, 2013; Nosaka, Katayama & Kishida, 2015). However, such hatching phenology is not common, because frog tadpoles typically hatch 3-4 weeks earlier than salamanders. In the typical hatching phenology, frog tadpoles are too large to consumption of salamander hatchlings (Nosaka, Katayama & Kishida, 2015). Strong predator-prey interaction can be established only when salamander larvae grow rapidly. Importantly, cannibalism among salamander hatchlings plays a critical role for salamanders to become substantial predators for tadpoles, because salamander hatchlings which successfully cannibalize conspecifics grow at a much faster rate and develop into “giants” with a much larger body size and offensive phenotype (i.e., wider gape width) (Wakahara, 1995; Kishida *et al*., 2011). The increase in size and change in morphology allows cannibalistic “giants” to consume the earlier-hatched tadpoles which are too large for their non-cannibalistic conspecifics (Takatsu & Kishida, 2015). This is the second scenario and, in the present study, I focus on this common case.

Importantly, in this system tadpoles exhibit inducible morphological defenses. When exposed to strong predation risk from salamander larvae, tadpoles develop an enlarged body and tail by thickening their epithelium tissue. This defensive “bulgy” phenotype makes it harder for the salamander larvae to swallow them (Kishida & Nishimura, 2004). Based on this relationship, I hypothesized that cannibalism in the salamander hatchlings could maintain (or

even drive evolution of) the inducible defensive (bulgy) phenotype of tadpoles.

Experiment summary

My study was composed of two experiments. Schematic diagram of the experiments is shown in Figure S1. In the first experiment (**Experiment 1: Establishment of cannibalistic and non-cannibalistic situations**), I controlled occurrence of cannibalism of salamander hatchlings. This allowed me (1) to establish different ecological situations with and without cannibalism in the predator and (2) to examine how these different cannibalism scenarios in the predator influence the expression of the defensive "bulgy" phenotype of tadpoles. Finally, I conducted the second experiment (**Experiment 2: Selection trials**) using the cannibalistic and non-cannibalistic situations established in the first experiment to (3) quantify how cannibalism in the predator alters the strength of selection pressure on the defensive bulgy phenotype by comparing relative survival of the tadpoles with more- and less-bulgy phenotypes among these situations. In the experiments, I used *R. pirica* tadpoles and *H. retardatus* larvae derived from eggs corrected from natural ponds. Methods of collection and husbandry of the amphibian eggs are described in Appendix S1.

Experiment 1: Establishment of cannibalistic and non-cannibalistic situations

I conducted the experiment in the laboratory using a semi-transparent polypropylene tank (43.6 cm × 28.4 cm × 14.1 cm high) filled with 5 L of aged tap water as an experimental unit. Just before assigning tadpoles into the experimental units, I mixed all of the tadpoles derived from 15 egg masses (i.e., two-week-old at stage 25-30 [Gosner, 1960], see Appendix S1). On May 15th, 2013, I haphazardly assigned 45 tadpoles from this mix to each of 46 tanks. Mean ± 1SD (N = 20; a subsample of the tadpoles used) snout-vent length (hereafter, body length) of the assigned tadpoles was 11.75 ± 0.72 mm.

Occurrence of cannibalism in salamander hatchlings depends greatly on size asymmetry

between interacting individuals (Kishida *et al*., 2015). Thus, to control occurrence of cannibalism, I manipulated population size structure (i.e., presence and absence of early or late hatchlings) in salamander hatchlings while keeping the total initial density of salamanders constant across the treatments. I obtained the early and late salamander hatchlings by manually controlling the water temperature experienced by the embryos from a single egg cluster. Difference in hatch timing between early- (May 14th) and late- (May 21st) hatchlings was seven days. The salamander hatchlings were assigned into the relevant treatments one day after they hatched. The method to obtain the early- and late- hatchlings is same as that described in Appendix A2 in Takatsu & Kishida (2015).

The experiment consisted of the following four treatments: (i) The "Cannibalism" treatment received 5 early and 15 late hatchlings, (ii) "No-cannibalism-early" treatment received 20 early hatchlings, and (iii) "No-cannibalism-late" treatment received 20 late hatchlings and (iv) "No-salamander treatment" received no salamander hatchlings to serve as a control treatment. I replicated treatment (i) 20 times, treatments without cannibals (ii and iii) 10 times and control treatments (iv) six times. I adopted the unbalanced replication design to avoid excessive use of the animals because my previous studies showed that mortality of tadpoles in the absence of predators was very low (e.g., Takatsu & Kishida, 2013; Nosaka, Katayama & Kishida, 2015) and variance of the demographic and trait level consequences was larger in the Cannibalism treatment than the No-cannibalism treatments (Takatsu & Kishida, 2015). Each replicate was randomly assigned to one of the 46 tanks. Densities of frog tadpoles (363 individuals m-2) and salamander hatchlings (162 individuals m-2) are within their natural ranges (Michimae, 2006).

I defined the day on which the early salamander hatchlings and frog tadpoles were assigned into the tanks as day one of the experiment, and assigned the late hatchlings into the

relevant tanks on day eight. At day eight, mean ± 1SD, (N = 20) body length of the early salamander hatchlings (stage 49-50 [Iwasawa & Yamashita, 1991]) in the Cannibalism and No-cannibalism-early treatment was 15.44 ± 0.76 and 15.24 ± 0.54, respectively, and body length of the late salamander hatchlings (stage 43-44 [Iwasawa & Yamashita, 1991]) in the Cannibalism and No-cannibalism-late treatments was 12.61 ± 0.58 and 12.25 ± 0.63, respectively. The timing of hatching of both frogs and salamanders is typical in natural habitats (Nosaka, Katayama & Kishida, 2015). Throughout the experiment, I added one piece of rabbit chow (dry weight: 0.2 g) and 20 frozen *Chironomid* larvae to all tanks every two days as alternative food for the tadpoles and the salamanders, respectively. Note that *Chironomid* larvae don't affect gigantism and expression of offensive phenotypes of salamanders (Kishida, Trussell & Nishimura, 2009). Throughout the experiments I exchanged the water every two days.

At day 31, I counted all surviving tadpoles and salamanders. Then I scanned ventral aspect of all surviving salamanders by using a computer scanner (CanoScan 8800F, Canon, Japan). At this point, most tanks only in the Cannibalism treatment had "giant" cannibals and salamanders had started to engage in cannibalism and consumption of tadpoles. I examined the effects of cannibalism treatments on mortality rate of tadpoles and salamanders (i.e., number of dead individuals during 30 days divided by the number of individual at the start of experiment) using generalized linear models with binomial error distribution, followed by pairwise comparisons. I adjusted significance levels ($\alpha = 0.05$) of post hoc tests using the sequential Bonferroni method (Holm, 1979). Using the scanned images, I measured gape width and body length of the all salamanders. To examine whether cannibalism of salamander hatchlings caused gigantism of the salamanders in the Cannibalism treatment, I compared body length and gape width of the salamander with largest body length within a tank among the treatments

using a Kruskal-Wallis test followed by Wilcoxon tests for pairwise comparisons. I focused on the largest individual within a tank because only a few salamanders become giant in cannibalistic population (Kishida *et al*., 2011; Takatsu & Kishida, 2015).

To examine whether expression of the bulgy phenotype was associated with salamander cannibalism, I compared proportion of tadpoles expressing the bulgy phenotype among the four treatments. Because the bulgy phenotype is characterized by the transparent, thickened epithelium tissues (Kishida & Nishimura, 2004), the ratio of width of transparent tissue to body width is a good index of the phenotype. I defined the tadpoles whose width of transparent tissue exceeds 10% of body width along the line of the greatest body width as tadpoles having bulgy phenotype. By using the scanned images of ventral side of tadpoles, I measured maximum body width and transparent tissue width of 10 tadpoles randomly selected from each tank and calculated the ratio of each tadpole. Then, I calculated proportion of tadpoles having bulgy phenotype in each tank. Kruskal-Wallis test was used to examine whether the proportion of tadpoles having bulgy phenotype was different among the four treatments. When I found significant difference among the treatments, I performed pairwise comparisons using the Wilcoxon test with sequential Bonferroni method.

Experiment 2: Selection trials

Thirty-one days after start of the experiment 1, I conducted selection trials to examine whether salamander cannibalism leads to selective regimes favoring defensive bulgy phenotype of tadpoles. I used the same four experimental treatments (i.e., Cannibalism, No-cannibalism-early, No-cannibalism-late, and No-salamander treatments) from the experiment 1 as possible selective environments. Because I was limited by the number of more- and less-bulgy tadpoles, I randomly selected nine replicate tanks from each of the three salamander treatments (i-iii) and I used all six replicate tanks from No-salamander control

treatment for conducting the Selection trials. To measure selection strength in each treatment, I used two types of tadpoles with different degree of defensive bulgy phenotype (more- and less-bulgy tadpoles [Fig. 2]) instead of the surviving tadpoles in the experiment 1 (see Figure S1). Details of method for preparation of the more- and less-bulgy tadpoles are described in Appendix S2.

I first removed all surviving tadpoles from each of the selected 33 tanks. Next, I assigned 10 more-bulgy and 10 less-bulgy tadpoles into each of the selected tanks. Before assigning tadpoles to tanks, I marked the tails of more- and less-bulgy tadpoles differently to track survival of each phenotype separately. In half of the replications of each treatment, I cut a small piece of the upper side of the tails of the more-bulgy tadpoles and the lower side of the tails of less-bulgy tadpoles while I did the opposite marking in the other half of the replicates. This tail-cutting method does not influence mortality of tadpoles (i.e., all tadpoles survived in the absence of salamanders). The marking method was important to discriminate between phenotypes during the experiment, because morphological differences between the more- and less-bulgy phenotypes disappeared due to stronger expression of the defensive phenotype in the less-bulgy tadpoles than more-bulgy tadpoles during the trial period. I added one piece of rabbit chow (dry weight: 0.2 g) and 20 *Chironomid* larvae into all tanks every two days as alternative food for the tadpoles and salamanders, respectively.

I counted the numbers of surviving tadpoles in the more- and less-bulgy tadpole category every day. The experiment was terminated six days after starting the trials, because the morphological (and thus functional) differences between more- and less-bulgy tadpoles almost disappeared at that time. I calculated the relative survival rate of the two groups by dividing the number of surviving more-bulgy tadpoles by the number of surviving less-bulgy tadpoles in each tank on each day. I used the Wilcoxon signed-rank test to determine whether the ratios

were larger or smaller than one in each day, because the data did not satisfy parametric test assumptions. When the ratio was significantly > 1, I inferred that the salamander selected for more-bulgy tadpoles, whereas when the ratio was significantly < 1, I inferred that they selected for less-bulgy tadpoles.

RESULTS

Experiment 1: Establishment of cannibalistic and non-cannibalistic situations

Survival and morphology of salamanders

I found significant differences in the mortality of the salamanders during 30 days among three salamander treatments (i.e., Cannibalism and two No-cannibalism treatments) ($\chi^2_2 = 21.98$, $P < 0.0001$). Salamander mortality in the Cannibalism treatment was 17.5 and 23.3 fold higher than in No-cannibalism-early or No-cannibalism-late treatments respectively (Fig. 1A). Only ~4% and ~3% of salamanders were dead in No-cannibalism-early and No-cannibalism-late treatments, respectively, and thus, there were no statistical difference between the two No-cannibalism treatments ($\chi^2_1 = 0.015$, $P > 0.90$). I found significant differences in body length (i.e., snout vent length; $\chi^2_2 = 27.89$, $P < 0.0001$) and gape width ($\chi^2_2 = 25.92$, $P < 0.0001$) of the largest salamander at day 30 among the three salamander treatments (Fig. 2). Body length of the largest salamander in the Cannibalism treatment was 41% and 48% larger than those in the No-cannibalism-early treatment and No-cannibalism-late treatment respectively. Furthermore, gape width of the largest salamander was 54% and 68% larger than those in the No-cannibalism-early treatment and No-cannibalism-late treatment respectively. There were no differences in these traits between the two No-cannibalism treatments (body length, $Z = -1.78$, $P = 0.076$; gape width, $Z = -1.70$, $P = 0.089$).

Survival and expression of bulgy phenotype of tadpoles

Salamander treatments significant affected mortality of tadpoles at day 30 (χ^2_3 = 12.67, P = 0.0054). Tadpole mortality in the Cannibalism treatment was 13.3- and 20- fold higher than in the No-cannibalism-early and No-cannibalism-late treatments respectively (Fig. 1B). Because only very few tadpoles died in the two No-cannibalism treatments and No-salamander treatment, I found no significant differences in any pairwise comparisons of these treatments (P > 0.61). Proportion of tadpoles having the bulgy phenotype was different among salamander treatments (χ^2_3 = 32.25, P < 0.0001). The proportion of the bulgy tadpoles in the Cannibalism treatment (83.5 ± 31.8 %, mean ± 1SD) was significantly higher than those in the other three treatments (pairwise comparisons, P < 0.002), and I found no significant difference among the remaining three treatments (Fig. 1C). In fact, no tadpoles expressed bulgy phenotype in the No-salamander treatment and only ~4 % of tadpoles expressed the bulgy phenotypes in the No-cannibalism-early and No-cannibalism-late treatments (Fig 1C).

Experiment 2: Selection trials

How did salamander cannibalism shape selection regimes?

In the two No-cannibalism and No-salamander treatments all but one tadpole survived the duration of the selection experiment (Fig. 3A). Hence, there were no differences in relative survival rates between more-bulgy tadpoles and less-bulgy tadpoles in these treatments. In contrast, 81.1 ± 11.6 % of the more-bulgy tadpole and 50.0 ± 18.7 % of the less-bulgy tadpoles survived to the end of the experiment in the Cannibalism treatment (Fig. 3A). Thus, proportions of surviving more-bulgy tadpoles was significantly (P < 0.012) higher (~62%) than those of surviving less-bulgy tadpoles (Fig. 3B) in the Cannibalism treatment. These results suggest that the defensive (bulgy) phenotype of frog tadpoles only has a higher fitness in the presence of “giant” cannibalistic salamander larvae.

DISCUSSION

Cannibalism in predators is increasingly recognized as a critical factor determining demography of their prey, but little is known of how cannibalism in a predator shapes the evolution of defensive traits of its prey (Rudolf, Sorrell & Pedersen, 2012). Using a combination of experiments, I demonstrate that cannibalism in a predator can determine the selection regime for inducible defense in its prey. Specifically, I found that only in the presence of cannibalism in the predatory salamander, (i) tadpoles were exposed to strong predation by "giant" cannibals big enough to consume prey, (ii) tadpoles only expressed the defensive (bulgy) phenotype in the presence of cannibalistic salamanders, (iii) proportion of surviving tadpoles with a defensive (bulgy) phenotype was only significantly higher in the presence of cannibalistic salamanders. To the best of my knowledge, these results provide the first evidence that the presence or absence of cannibalism in a predator can determine the selection pressure favoring defensive vs. normal phenotypes in their prey.

While the selection trial (experiment 2) was only six days, the selection pressure is expected to operate over most of the larval period of frog tadpoles in natural habitats. Once the salamanders consume tadpoles, the salamanders continue to impose strong predation pressure on tadpoles until they metamorphose and leave the aquatic habitat (Nosaka, Katayama & Kishida, 2015; Takatsu & Kishida, 2015). Even without cannibalism, salamanders can become predatory giants when the salamanders interacted with small tadpoles at their hatchling stage (Michimae & Wakahara, 2002; Kishida Trussell & Nishimura, 2009). However, such a scenario is only realized when salamanders hatch substantially before tadpoles or the two species hatch at the same time, both of which rarely occur under natural conditions (Nosaka, Katayama & Kishida, 2015). Therefore, I can conclude that cannibalism among the salamander hatchlings is a substantial factor causing the selection pressure favoring defensive phenotype of tadpoles.

My study generally suggests that paying attention to the relative importance of numerical and per-capita effect of ecological interaction isuseful to better understand how ecological interactions influence trait evolution. Although cannibalism in a predator can dampen predation pressure on its heterospecific prey via reduction of predator density (i.e. numerical effect) or foraging activity of cannibalistic victims on their heterospecific prey (i.e. per-capita effect of cannibalistic victim) (Rudolf, 2006, 2007, 2012), it can intensify the selective predation pressure via improvement of predation ability of cannibalistic predators by promoting their rapid growth (i.e., per-capita effect of cannibalistic predator). Hence, the net effect of predator cannibalism depends on the relative importance of the numerical and per-capita effects. Although the number of surviving salamanders in the Cannibalism treatment was equivalent to 31% of the number of salamanders in the No-cannibalism treatments at the beginning of the Selection trials, selective predation pressure on prey tadpoles emerged only in the Cannibalism treatment. This clearly indicates that the change in per-capita effect of cannibalistic predator was the more important factor shaping selection pressure on prey defense than the other two effects. The change in the per-capita effect was driven by the accelerated growth of cannibalistic predators; at the end of the induction experiment (i.e., at the beginning of the Selection trials), the body length and gape width of the largest salamanders were respectively 1.5-fold and 1.6-fold larger in the Cannibalism treatment than the No-cannibalism treatments. In the Cannibalism treatment, average gape width of the largest salamanders was 11% greater than average body width of less-bulgy tadpoles but 5 % smaller than average body width of more-bulgy tadpoles (Fig. 2). A salamander can consume a tadpole when its gape width is more than 10% larger than the tadpole's body width (Nosaka, Katayama & Kishida, 2015). Thus, giant cannibalistic salamanders could consume less-bulgy tadpoles, but were mostly unable to consume more-bulgy tadpoles. On the other hand, gape width of the largest salamanders in the

No-cannibalism treatments was at least 27% smaller than average body width of tadpoles regardless of their phenotype (Fig. 2). Thus, without the growth boost from cannibalism, salamander larvae were unable to consume either phenotype of tadpoles and consequently did not select for defensive phenotypes even though salamander density was three times higher without cannibalism. In fact, the reduction in predator density was necessary to increase the per-capita effect of predators on the prey. Because changes in predator density had little to no effect, the increase in per-capita consumption rates of giant cannibalistic predators resulted in a net increase in prey mortality and resulted in a selection regime that strongly favored expression of the defensive phenotype of its prey. These results emphasize that I cannot predict how changes in numerical effects in predator populations affect selection regimes on prey traits without accounting for concurrent changes in predator per-capita effects.

I saw clear differences in the expression of defensive phenotypes in the prey between treatments with vs. without salamander cannibalism (experiment 1). While very few tadpoles expressed a bulgy (defensive) phenotype in the two No-cannibalism treatments, the vast majority (84%) of tadpoles developed bulgy phenotype in the Cannibalism treatment. This suggests that the tadpoles did not simply express a defensive phenotype when salamanders were present, but only when predators engaged in significant levels of cannibalism. Because tadpoles develop bulgy phenotype in response to the close proximity of the salamander larvae (Kishida & Nishimura, 2004), the tadpoles might recognize the phenotypes of the salamanders by means of phenotype-specific aggressiveness (i.e., predation attempt by the salamanders) or non-diffusible chemicals of salamanders, perhaps even by direct contact (Kishida, Mizuta & Nishimura, 2006). Regardless of the precise mechanism, the risk-dependent expression of the bulgy phenotype of tadpoles suggests adaptive phenotypic plasticity as a cost saving strategy, because the defensive phenotype is likely costly to produce or maintain (Mori *et al*., 2009;

Kishida *et al*., 2010) and the emergence of predatory giant salamanders in salamander populations varies with abiotic and biotic conditions (Kishida, Trussel & Nishimura, 2009; Kishida *et al.*, 2011; Nosaka, Katayama & Kishida, 2015). For example, presence of the top predator, *Aeshna nigroflava* (Martin) dragonfly larvae, strongly suppresses cannibalisms of in *Hynobius retardatus* (Dunn) salamander hatchlings and thereby greatly reduces the likelihood of the emergence of giant cannibals (Kishida *et al*., 2011). This implies that variation in community structure can play a key role in evolution of the inducible bulgy phenotype by altering cannibalism within predator populations.

Cannibalism is widespread in a variety of predator species, and is known to play a prominent role in the dynamics of prey communities (Polis, 1981; Persson *et al*., 2003; Claessen, DeRoos & Persson, 2004; Persson, DeRoos & Bertolo, 2004; Woodward, Speirs & Hildrew, 2005; Rudolf, 2007). Contrary to my system, in many trophic systems between cannibalistic predator species and their heterospecific prey documented so far, cannibalism in predator populations weakens the consumption rates of heterospecific prey by reducing density and foraging activity of predators (Persson *et al*., 2003; Crumrine, 2005; Rudolf, 2006, 2008; Law & Rosenheim, 2011). In these systems, cannibalism of a predator species should reduce the selection pressure on defensive phenotypes in heterospecific prey. However, my study indicates that this effect may be reduced or even reversed in systems where cannibalism can alter the phenotype of predators by allowing cannibalistic individuals to grow rapidly and greatly improve their predatory ability. Given that cannibalism can strongly increase growth rates in many carnivorous insects, fishes and amphibian species (DeAngelis, Cox & Countant, 1980; Sogard & Olla, 1994; Fangan & Odell, 1996; Ziemba & Collins, 1999; Hardie & Hutchngs, 2014), my results suggest that cannibalism can be an important factor driving the evolution of defensive traits in heterospecific prey species in a wide range of terrestrial and

aquatic predatory prey systems. Therefore, future studies which examine how cannibalism affects phenotypic characteristics of predators and their per-capita effect on the prey promise to be a fruitful venue to gain a better understanding of the evolution of prey defenses.

Fig. 1. Proportions of (A) dead salamander larvae, (B) dead frog tadpoles, and (C) bulgy (defensive) frog tadpoles at the end of experiment 1. ‘Canni’, ‘No-canni’, and ‘No-sal’ are abbreviations of the Cannibalism, No-cannibalism, and No-salamander (i.e. control) treatments, respectively. ‘Early’ and ‘Late’ in the No-cannibalism treatment represent hatch timing of the salamanders used in the treatments. The thick horizontal bar indicates the median, the box contains 50% of the data, and the whisker indicates the range.

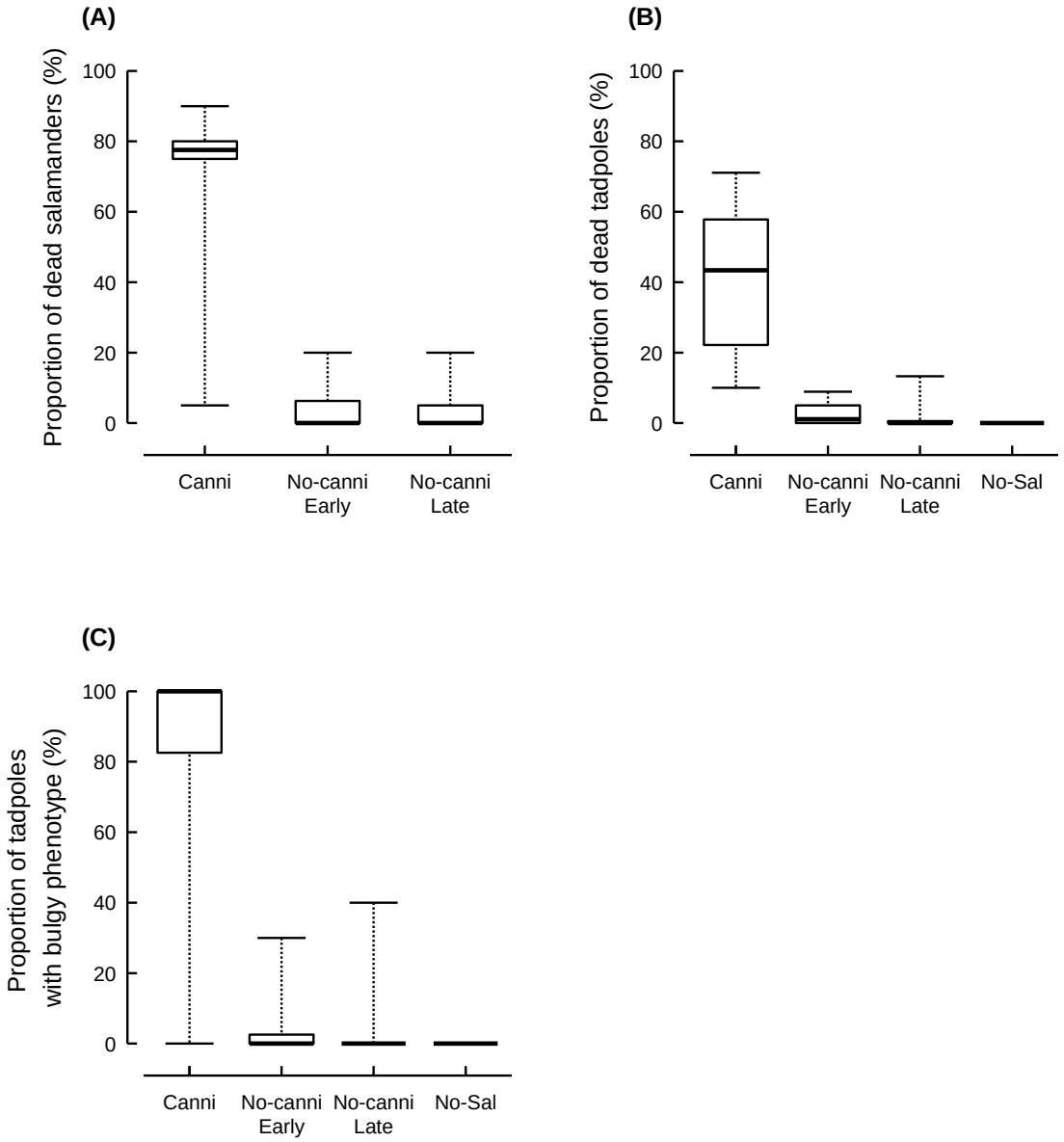


Fig. 2. Gape width of the largest salamanders within each tank (Left panel) and body width of tadpoles (Right panel) used in the Selection trials versus their respective body length. ‘Canni’, ‘No-canni Early, and ‘No-canni Late’ are abbreviations of the Cannibalism, No-cannibalism-early, and No-cannibalism-late treatments, respectively. ‘More-bulgy’, ‘Less-bulgy’, and ‘Non-bulgy’ represents the degree to which the defensive “bulgy” phenotype of tadpoles was expressed.

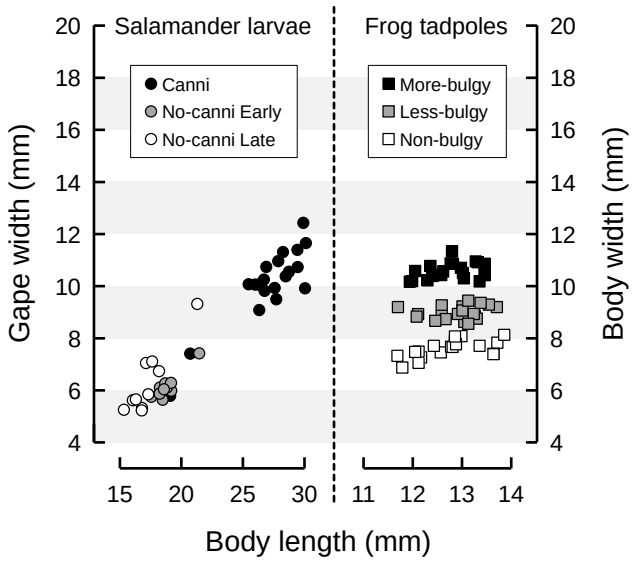
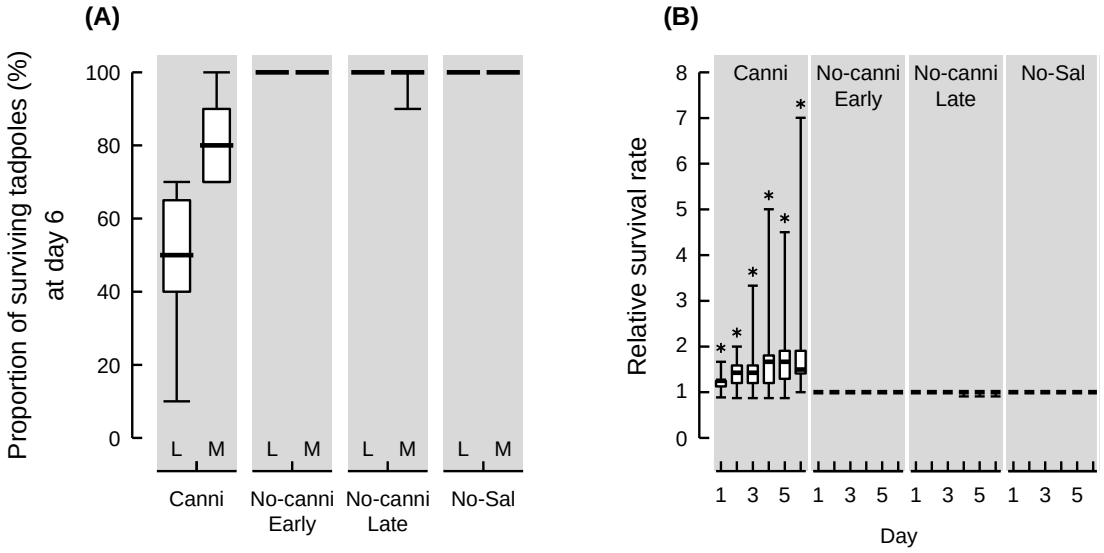


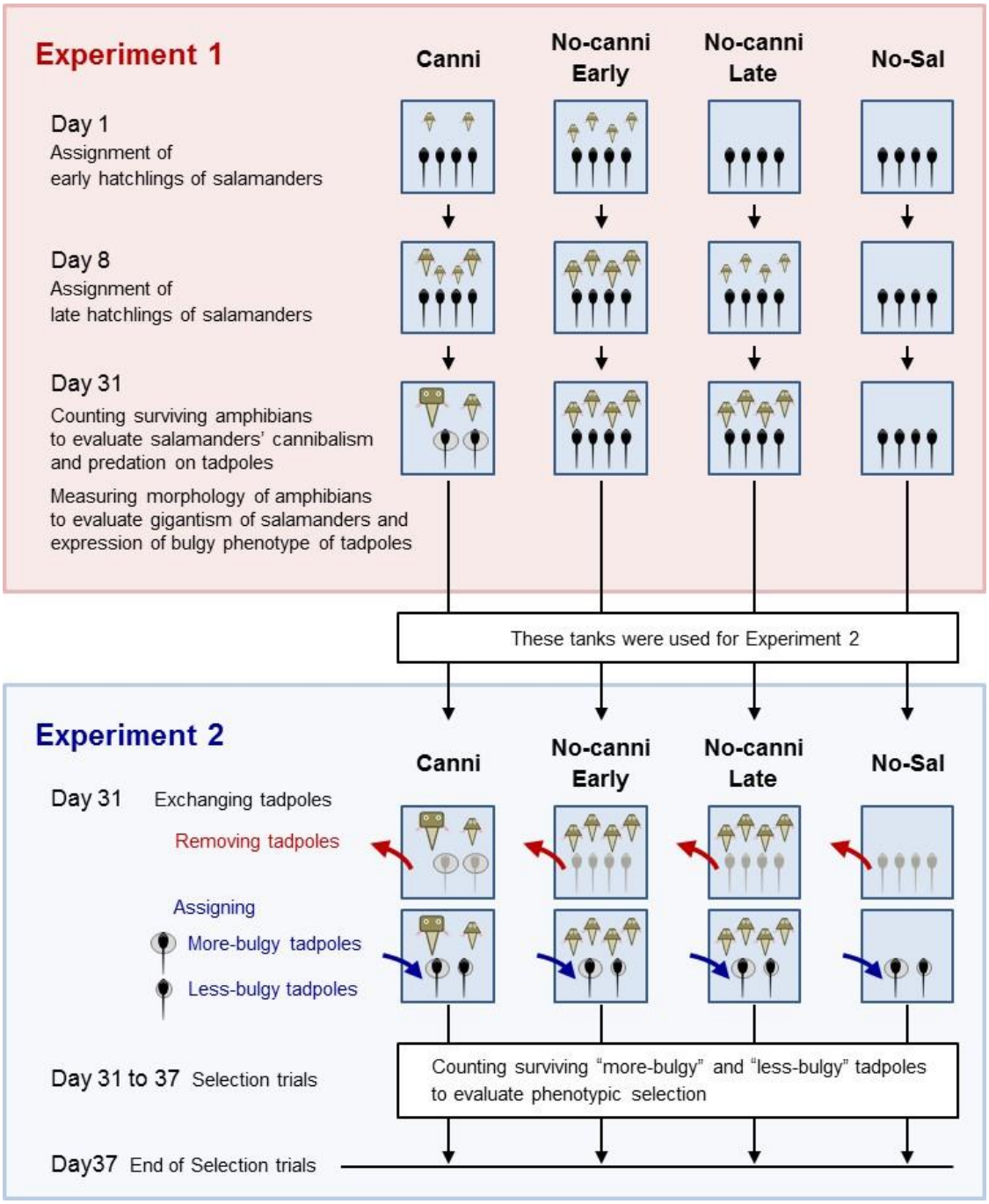
Fig. 3. (A) Proportion of surviving less-bulgy (L) and more-bulgy (M) tadpoles at the end of Selection trials after six days. (B) Relative survival rates of more-bulgy tadpoles to less-bulgy tadpoles during experimental period. The thick horizontal bar indicates the median, the box contains 50% of the data, and the whisker indicates the range. ‘Canni’, ‘No-canni’, and ‘No-sal’ are abbreviations of Cannibalism, No-cannibalism, and No-salamander treatments, respectively. ‘Early’ and ‘Late’ in the No-cannibalism treatment represent hatch timing of the salamanders used in the treatments. Asterisks indicate that the relative survival rates were significantly different from 1 ($P < 0.012$).



APPENDIX

Figure S1. Schematic diagram of the experiments

'Canni', 'No-canni Early, and 'No-canni Late' are abbreviations of the Cannibalism, No-cannibalism-early, and No-cannibalism-late treatments, respectively.



Appendix S1. Collection and husbandry methods of *Rana pirica* and *Hynobius retardatus* eggs

I collected 50 egg clusters of *H. retardatus* salamanders and 15 egg masses of *R. pirica* frogs from three ponds in the forests of Hokuto-shi, Hokkaido, Japan, in mid-April 2013. Each of the 15 frog egg masses was kept in a separate 22 L semi-transparent plastic tank made of polypropylenes (51.3 cm × 37.2 cm × 16.6 cm high) filled with 5 L of aged tap water. Tanks were placed in a controlled environmental room and maintained at 17 °C with a natural day/night (about 14h/10h) regime. Once tadpoles started to hatch (late April), I added eight pieces of rabbit chow (dry mass of 1.6 g; Yeaster, Tatsuno, Japan) into each tank to serve as tadpole food. Throughout the experiments I exchanged the water every two days. I kept the tadpoles in this condition for two weeks before starting experiment 1.

Of the 50 salamander egg clusters collected, 40 clusters were used for experiment 1 and 10 clusters for experiment 2. For experiment 1, each of 40 salamander egg clusters was placed separately in a fine mesh net. The nets were placed in 4 L semi-transparent polypropylene tanks (33.4 cm × 20 cm × 10 cm high; 5nets per tank) filled with 2 L of aged tap water. Tanks were then placed in a climate chamber and maintained at 3 °C under natural day/night (about 14h/10h) conditions to control their hatch timing. One week before the start of the experiment 1, when almost all of the embryos reached stage 38 - 39 (Iwasawa & Yamashita, 1991), I carried out a manipulation to these egg clusters to establish the three salamander treatments (i.e., Cannibalism, No-cannibalism-early, and No-cannibalism-late treatments. See below).

For experiment 2, each of 10 salamander egg clusters was kept in separate 13 L tank (43.6 cm × 28.4 cm × 14.1 cm high) filled with 5 L of aged tap water. Tanks were placed in the controlled environmental room and maintained at 17 °C with a natural day/night (about 14h/10h) regime until the salamanders hatched. The salamanders hatched in late April. These

salamander hatchlings were used to induce bulgy phenotype of the tadpoles as induction agents.

Appendix S2. Preparation of phenotypic variants of tadpoles as selection targets

The more- and less-bulgy tadpoles used in the Selection trials (Experiment 2) were induced using the following method. Because tadpoles readily develop bulgy phenotype in the close proximity of giant salamanders having enlarged gape (Kishida, Mizuta & Nishimura, 2006; Kishida, Trussell & Nishimura, 2009), I used giant salamanders to induce the bulgy phenotype of tadpoles. To prepare giant salamanders, I used 120 salamander hatchlings from 10 egg clusters which had been kept in the experimental room. The salamanders hatched in late April. I placed each of the 120 salamander hatchlings into a small plastic case (8.4 × 5.7 × 4.4 cm high) filled with 100 ml of aged tap water and containing 10 small tadpoles to induce the giant morph in salamanders. I added tadpoles every two days to replace consumed tadpoles and maintain the induction environment.

Two weeks after the tadpoles hatched (i.e., the day 1 of the experiment 1), I randomly selected the tadpoles from the same stock tanks used for experiment 1. I placed 40 tadpoles (body length 11.75 ± 0.72 mm; body width 7.38 ± 0.46 mm [mean ± 1SD, N = 20], stage 25-30 [Gosner, 1960]) into each of 60 semi-transparent polypropylene tanks (33.4 cm × 20 cm × 10 cm high) filled with 2 L of aged tap water. I placed two giant salamanders (gape width, 9.70 ± 0.78 mm; body length, 26.05 ± 1.83 mm [mean ± 1SD, N = 20], stage 50-53 [Iwasawa & Yamashita, 1991]) into 50 of the 60 tanks to induce the bulgy phenotype of the tadpoles. I did not place any salamanders into the remaining 10 tanks so that the tadpoles would retain the non-defensive phenotype.

After 31 days (i.e., at the start of the Selection trials), most of the surviving tadpoles exposed to predation risk by the giant salamanders expressed bulgy phenotype, but degree of bulginess varied among individuals. I collected the bulgy tadpoles from the tanks and visually divided them into two categorical groups, less- and more-bulgy, based on their degrees of bulginess.

Each group consisted of 330 tadpoles. The body length of all selected tadpoles was within the range of the surviving tadpoles in experiment 1 (11.17 mm – 13.73 mm). To confirm whether I successfully classified the tadpoles into the two phenotypic categories, I compared the morphology of the tadpoles between two groups. I randomly picked 20 tadpoles from each group (more- or less-bulgy) and scanned them ventrally to measure their body length and maximum body width. For reference, I applied the same method to measure 20 tadpoles randomly selected from the tanks without salamander larvae ("non-bulgy" group). I found no differences in body length among the groups (ANOVA, $F_{2,57}$ = 0.68, P = 0.51; mean body length ± 1SD: "more-bulgy" = 12.75 ± 0.49, "less-bulgy" = 12.9 ± 0.52, and "non-bulgy" = 12.71 ± 0.63 mm), but highly significant differences in body width ($F_{2,57}$ = 473.52, P < 0.0001; mean body width ± 1SD: "more-bulgy" = 10.58 ± 0.32, "less-bulgy" = 8.98 ± 0.26, and non-bulgy = 7.61 ± 0.34 mm) (Fig. 2). Results of Tukey–Kramer's post hoc tests for body width revealed that all three groups differed significantly from each other (P < 0.05). This supports my conclusions: (1) the tadpoles from tanks containing salamanders exhibited the bulgy phenotype, and (2) I had successfully categorized tadpoles into groups with more- or less-bulgy phenotypes. Of the three phenotypic categories, the more- and less-bulgy tadpoles were used as phenotypic variants of selection target in the Selection trials. The size ranges of both body length and body width of the tadpoles were similar to tadpoles in natural ponds (Kishida *et al*., 2009b).

Chapter 4

Differentiation in stoichiometry caused by cannibalistic dimorphism

INTRODUCTION

Traditionally, ecologists have assumed that individuals within a population are functionally uniform, and thus provide effects of the same type and strength on other community members. Although this simplification has substantially contributed to the development of seminal theories in community ecology (Schoener 1974; Harwell et al. 1977; DeAngelis 1980; Oksanen et al. 1981; Chapin et al. 2000; Loreau & Leroux 2010; Mougi & Kondoh 2012), it is questionable whether such simplification is appropriate for explain the spatial and temporal variability of community and ecosystem properties (Werner & Gilliam 1984; Hawlena & Schmitz 2010a; Karimi et al. 2010; Bolnick et al. 2011; Miller & Rudolf 2011). Recently, researchers have taken intraspecific variation into account to better understand mechanisms causing spatial and temporal variation in interaction strength and, in turn, to gain insights into processes involved in dynamics of community structure and ecosystem functions (De Roos et al. 2003; Grover 2003; Moya-Larano 2011; Nakazawa 2011; Schreiber et al. 2011).

Animals interact with other community members through the two alternative effects. First is the predatory effect on their prey, and the alternative is the resource providing effects to the predators, decomposers, and primary producers (reviewed by Vanni 2002). Because the both effects can be cascading to the other members (predatory effects, Schmitz et al. 2006; Dunham 2008; Wu et al. 2015; resource providing effects, Elser et al. 1988; Vanni et al. 2006; Knoll et al. 2009), identifying factors modifying these effects and their underlying mechanisms is inevitable to deepen our understandings of the dynamic nature of community and ecosystem properties. However, researchers have more focused on the predatory effects of animals compared to the resource providing effect so far. In fact, although past studies considering intraspecific variation of animals have repeatedly documented that individual differences in the predatory effect strongly affect top-down controls in the community (Post et al. 2008; Walsh &

Post 2011; Rudolf 2012; Rudolf & Rasmussen 2013), intraspecific variation in the resource providing effects has considerably less attention. Because resource providing effects of animals can dictate primary and secondary productions (Elser et al. 1988; Boersma & Elser 2006; Vanni et al. 2006; Knoll et al. 2009; Stephens et al. 2015), we should deepen our knowledge on its intraspecific variation.

Recently, several researchers found that animal individuals change compositions of nutrients stored in and excreted from their body (i.e., body and excretion stoichiometries) in response to environmental factors such as predator presence or absence (Hawlena & Schmitz 2010b; Vrede et al. 2011; Cotello & Michel 2013; Guariento et al. 2015). Such conditional changes of individuals are expected to determine the resource providing effects of animals to the community members (Leroux et al. 2012). Specifically, if stoichiometric responses vary among individuals within a population, it causes complicated patterns of the resource providing effects of individuals within and among populations. However, we have very little knowledge on how individuals differentiate body and excretion stoichiometries within a population in ecological scenarios and whether the individual responses translate into nutrients retained in and released from populations to which the individuals reside. To address this issue, I focus on polyphenism defined as a condition dependent emergence of distinct phenotypes within a population (West-Eberhard 1989), because the distinct phenotypes in a polyphenism differs in their trophic niches, morphology and life history which plausibly affect stoichiometries (Fagan et al. 2002; Hendrixon et al 2007; Gonzalez et al. 2011).

Intraspecific interaction such as cannibalism and competition is a representative factor causing polyphenism (Hoffman & Pfennig 1999; Moczek 2003; Svanback & Bolnick 2007). For example, in some cannibalistic fish and amphibian species, individuals differentiates their phenotypes after cannibalism occurs (reviewed by Fox 1975; Polis 1981). Specifically,

individuals that successfully consumed conspecifics grow rapidly and change their morph to effectively consume large prey items (i.e., cannibals) but the other individuals grow slowly and don't exhibit such morphological changes (i.e., non-cannibals) (DeAngelis et al. 1980; Pfennig 1990; Bystrom 2006; Takatsu & Kishida 2015). Since the ecological traits such as prey items, morphology, and life history can affect body and excretion stoichiometries (Fagan et al. 2002; Hendrixon et al 2007; Gonzalez et al. 2011), the individuals with distinct phenotypes within a cannibalistic population are expected to differ in their stoichiometries. And, if the cannibalistic polyphenism cause individual variation in stoichiometries, nutrients retained in and released from salamander populations are different between cannibalistic and non-cannibalistic populations. To test these predictions, I conducted empirical studies by using *Hynobius retardatus* salamander larvae which exhibit cannibalistic dimorphism as model organisms (Kishida et al. 2011; Takatsu & Kishida 2015; Takatsu et al. 2017).

In the *H. retardatus* salamander larvae, cannibalism causes dimorphism in various phenotypes like morphology, size, and prey items (Kishida et al., 2011; Takatsu & Kishida 2015; Takatsu et al. 2017). Adult salamanders lay their egg in lentic ponds in early spring and the embryos hatch several weeks later. One to two week after hatching, cannibalism occurs if enough size variation exists within a population. In the dimorphism of this species, cannibals are characterized by extremely larger body and wider gape compared to non-cannibals. The increases in size and change in morphology allow the cannibals to easily consume not only conspecifics but also large hetrospecific species (i.e., *Rana pirica* frog tadpoles) which is too large to consumption of small non-cannibals (Takatsu & Kishida 2015).

This study consisted of an experiment and a field investigation. First, I conducted a laboratory experiment, in which cannibalism among salamander hatchlings was controlled, to examine (1) whether cannibals and non-cannibals exhibit different body and excretion

stoichiometries and (2) whether the phenotype-specific stoichiometric responses explain nutrients retained in and released from salamander populations. Second, to confirm whether cannibalistic dimorphism is a mechanism of intra-population variation in stoichiometries in natural populations, I investigated association between phenotypes and stoichiometries of wild salamanders.

MATERIALS AND METHODS

Laboratory Experiment

Experimental setting

Collection and keeping methods of experimental animals are described in Appendix A. I used 62 semi-transparent polypropylene tanks (43.6 cm × 28.4 cm × 14.1 cm high), each filled with 5 L of aged tap water, for the experimental treatments. These experimental tanks were maintained in the experimental room at 17℃ with natural light-dark (14/10) regime. I assigned 35 two-weeks frog tadpoles to each tank (body length, 6.64 ± 0.42 mm; body width, 10.77 ± 0.55 mm; mean ± 1SD mm, N = 20; Gosner stage, 25-30 [Gosner 1960]). I established three treatments that manipulated presence and absence of early or late salamander hatchlings to control occurrence of cannibalistic dimorphism. I obtained early and late salamander hatchlings by controlling the water temperature experienced by the embryos from single egg clusters following the methods used in my previous study (described in Appendix A2 in Chapter 2). This procedure delayed hatchlings of the late group by seven days, compared to early group. To obtain populations in which salamanders cannibalize and thus cannibalistic dimorphism occurs, I assigned (1) 1 early and 24 late hatchlings (i.e., Cannibalism treatment) into each of 46 tanks. In this situation, one early hatchling in each replicate is predicted to successfully cannibalize late hatchlings and become giant cannibals because larger and more developed salamander

larvae consume smaller and less developed conspecifics (Kishida *et al*. 2009b, 2015) and conspecifics are a rich nutrient source (Meffe & Crump 1987; Wildy et al. 1998). Hence, I was able to discriminate cannibals and non-cannibals in each replicate and thus examine whether body and excretion stoichiometries of cannibals and non-cannibals differ. To obtain populations in which salamanders don't cannibalize and thus cannibalistic dimorphism doesn't occur, I also assigned (2) 25 early hatchlings (i.e., No-cannibalism early-hatchlings treatment) or (3) 25 late hatchlings (i.e., No-cannibalism late-hatchlings treatment) into each of 8 tanks. Because size variation among salamander hatchlings in each of the No-cannibalism treatments was very small, cannibalism rarely occurred and consequently cannibals rarely emerged in the treatments (see below). I defined the day on which the early hatchlings and frog tadpoles were assigned into the appropriate tanks as day one of the experiment, and I assigned the late hatchlings to the relevant tanks one week later (i.e., day 8). These experimental densities and hatching phenology of tadpoles and salamanders are within their ranges in natural habitats (Michimae 2006; Nosaka *et al*. 2015). I added one piece of rabbit chow (dry mass of 0.2g; Yeaster, Tatsuno, Japan) and 40 tubiflex to all tanks every two days as alternative food for the tadpoles and salamanders, respectively.

On day 30, I observed that body length of one salamander was far larger than remaining salamanders in the most replicates of Cannibalism treatment but such size variation did not occur in the most of replicates of No-cannibalism treatments. To make sure whether I successfully controlled emergence of cannibalistic dimorphism, I counted all surviving salamander and frog tadpoles on day 30 and measured morphological traits of salamanders. I scanned all of salamanders ventrally. On the scanned images projected onto a computer monitor, I measured snout-vent length (i.e., body length), maximum head width (i.e., head width), and gape width of all surviving salamanders. According to the previous studies

(Michimae & Wakahara 2002), I defined salamanders having gape width to head width ratio larger than 0.9 as expressing the offensive morphology.

Established trophic interactions and salamander dimorphism

About a half of the salamanders in the Cannibalism treatment survived during the 30-days experiment (mean ± 1SD survival rate = 48.2 ± 20.2%) while almost all of the salamanders in the two No-cannibalism treatments survived during the experiment (mean ± 1SD survival rate of salamanders in No-cannibalism early-hatchlings and No-cannibalism late-hatchlings were 92.0 ± 13.4% and 94.0 ± 6.8%, respectively). These results indicate that cannibalism frequently occurred in the Cannibalism treatment while it almost never occurred in the No-cannibalism treatments.

In 93% (i.e., 43 out of 46) of the replicates of Cannibalism treatment, in which cannibalism occurred (mortality of salamanders was ranged from 20 to 74%), salamander individual with largest body length was exceptionally large and the other individuals were in similar size (e.g., the salamanders with largest body length are 42% larger in body length compared to salamanders with second largest body length, and the difference in body length is comparable to 66% of difference in body length between salamanders with largest and smallest body length, Fig. 1). The largest salamander was only one individual which expressed offensive phenotype in each replicate of the Cannibalism treatment (but there were only a few exceptional tanks in which individuals with second largest body length also expressed offensive phenotype) (Fig 1). In these replicates, tadpoles suffered significant mortality (mean ± 1SD mortality of tadpoles = 20.0 ± 10.6 %). These results show that cannibalistic dimorphism in salamander larvae occurred (i.e., one salamander with the largest body length became a giant cannibal which consumed conspecifics and tadpoles and the remaining salamanders were small non-cannibals which rarely consumed amphibians) in the most of

replicates in the Cannibalism treatment as I expected. In 88 % of replicates in each of the two No-cannibalism treatments (i.e., 7 out of 8 replicates), populations constituted salamanders with similar body length (e.g., individuals with largest body length are only 5 % larger in body length compared to individuals with second largest body length, and the difference is comparable to only 17% of difference in body length between salamander with largest and smallest body length, Fig. 1). The salamanders with largest body length of the No-cannibalism treatments were 26 % smaller than those of the Cannibalism treatment (t_{55} = 11.28, $P < 0.0001$) and did not exhibit offensive phenotype (Fig 1). Mortality of tadpoles of No-cannibalism treatments was almost zero (mean ± 1SD mortalities of tadpoles in the No-cannibalism early-hatchings and No-cannibalism late-hatchlings were 5.3 ± 5.5 and 6.1 ± 7.2 %). These results confirmed that no giant cannibals emerged and salamanders' consumption on amphibian prey rarely occurred in most of the tanks of No-cannibalism treatments as I expected.

For the analyses of body and excretion stoichiometries, I used the salamanders in these replicates of Cannibalism (43 of 46 replicates) and two Non-cannibalism treatments (14 of 16 replicates) described above, in which I successfully controlled development of cannibalistic dimorphism. This selective method is relevant because the main objective of this experiment is to examine relationship between cannibalistic dimorphism and intra-population variation in body and excretion stoichiometries.

Measuring body and excretion stoichiometries

To compare body and excretion stoichiometries of cannibals and non-cannibals, I used the following 5 individuals from each replicate for the analyses; individuals with (1) largest, (2) 2nd largest, (3) 3rd largest, (4) median, and (5) smallest body length (hereafter I called them 1st largest, 2nd largest, 3rd largest, median-sized and the smallest salamanders, respectively). In stoichiometry analyses, instead of using all individuals, I used only the 5 individuals in each

replicate to reduce sacrificed salamanders. The selection of individuals is adequate to accomplish my objective, because cannibal and non-cannibals in each replicate in the Cannibalism treatment were in the selected individuals and body size of the selected individuals covers actual size variation in each tank. In particular, comparisons of stoichiometries among 1^{st}, 2^{nd}, 3^{rd} largest salamanders allowed me to clearly test whether variations in stoichiometries are explained by the focal phenotypes (i.e., cannibal or non-cannibal) or size order (i.e., individuals can change their stoichiometry depending on their size order).

I measured carbon (C), nitrogen (N), and phosphorus (P) stored in the body of salamanders because animal body can be a resource for predators and decomposers and these chemical elements are essential for their growth (Elser et al 2000; Sterner et al 2000). For excretion analyses, I measured dissolved N and P emitted from the individuals because dissolved nitrogen and phosphorus are particularly important for the growth of decomposer and primary producers (Tilman et al. 1982; Elser et al. 1988; Sterner 1990).

For the collection of excretion of salamanders, I placed the salamanders individually into small plastic cases (84 mm × 57 mm × 44 mm high) with 40 ml of aged tap water, immediately after photographed them by a scanner. In addition, I prepared five small plastic cases with 40 ml of aged tap water, which contained no salamander, as controls. I kept the small plastic cases in the experimental room maintained at 17℃ from 18:00 to 12:00 (i.e., 18h incubation). I did not feed them during the incubation. Just after the end of incubation, I removed the salamanders from the cases. Then, the incubation water was filtered through grass filters (ADVANTEC GC-50) to remove particles that might absorb dissolved N and P. As previous studies did (Tiegs et al. 2015), I defined amount of NH_4^+ and PO_4^{3-} in the water as index of dissolved N and P excreted from individual, respectively. I measured concentration of

NH_4^+ and PO_4^{3-} (mg × l^{-1}) by using Auto-Analyzer (BLTEC, Osaka, Japan). Although mean and range of concentrations of NH_4^+ (mg × l^{-1}) in the cases containing salamanders were 0.50 and 0.01 to 5.53, respectively; those of the controls was almost zero (i.e., mean, 0.00; range 0.03 -to 0.02 mg × l^{-1}). Hence, I considered the amount of NH_4^+ (mg) in the cases containing salamanders as dissolved N excreted from salamanders. Similarly, although mean and range of concentrations of PO_4^{3-} (mg × l^{-1}) in the treatments with salamanders were 0.071 and -0.006 to 1.059, those of the controls were almost zero (i.e., 0.002 and 0.000 to 0.005). Hence, I considered that amount of PO_4^{3-} (mg) contained in the cases containing salamanders as dissolved P excreted from salamanders. By dividing amount of the excreted N and P by body dry mass of individuals and by duration of incubation period (18 h), I calculated dissolved N and P excreted per unit body dry mass per hour (i.e., hereafter I called "mass specific N and P excretion rate") of each salamander;

Mass specific N excretion rate (μg × mg^{-1} × h^{-1}) = [Concentration of N (μg × l^{-1}) × 0.04] × dry mass $(mg)^{-1}$ × 18 $(h)^{-1}$,

Mass specific P excretion rate (μg × mg^{-1} × h^{-1}) = [Concentration of P (μg × l^{-1}) × 0.04] × dry mass $(mg)^{-1}$ × 18 $(h)^{-1}$,

where 0.04 is relative volume of the small plastic case (40 ml) to 1000 ml, and 18 (h) is incubation period.

After the incubation, the salamanders were kept for the analyses of body stoichiometry. The salamanders were euthanized by immersion in a 0.1% solution of MS-222 (ethyl m-amino-benzoate mehanesulfonate) by following to Gentz (2007). I removed their gut to prevent contamination from gut contents. Then the salamanders were dried at 50℃ for 5 days. Because weight of the salamanders did not change from day four to five after starting to dry, I considered that the samples were completely dried. I ground the dried salamanders to obtain

homogenized fine powder by using mortar and pestle. I divided the powder of each salamander into two. One of the subsamples was used for analyzing %C and %N contents by a CHNS analyzer (Perkin Elmer Series 2400 II, Norwalk, Connecticut, USA). The other subsample was used for analyzing %P contents of salamanders. The subsamples were ashed at 500℃ for 2 hours. %P contents were determined using the molybdate blue reduction method after dissolving the ashed samples with 5N HCl (Murphy & Riley 1962).

Estimation of nutrients stored in and excreted from salamander individuals and populations

I calculated nutrients stored in and excreted from each individual by multiplying individual values of body and excretion stoichiometries and individual dry mass as follows.

Nutrients stored in individual (mg) = dry mass (mg) × body stoichiometry (%)

Individual excretion rate ($\mu g \times h^{-1}$) = dry mass (mg) × mass specific excretion rate ($\mu g \times mg^{-1} \times h^{-1}$)

Nutrients retained in and released from a salamander population are sum of the nutrients stored in and excreted from the salamander individuals constituting the population. I estimated nutrients retained in and released from each salamander population using the stoichiometries and dry mass of the 5 individuals. Because only the 1st largest salamanders differed in body and excretion stoichiometries from the other salamanders (i.e., 2nd largest, 3rd largest, median-sized and the smallest salamanders) in the Cannibalism treatment (see Results), I estimated the nutrients retained in and released from salamander populations in this treatment by using values of stoichiometries and dry mass of the 1st largest salamander and mean values of the stoichiometries and dry mass of the other salamanders as follows.

Nutrients retained in populations of Cannibalism treatment (mg) = [dry mass (mg) of 1st largest salamander × body stoichiometry (%) of the 1st largest salamander] + {[mean dry mass (mg) of the other salamanders× (number of surviving salamanders - 1)] × mean body stoichiometry (%)

of the other salamanders}

Nutrients released from populations in Cannibalism treatment (μg × h^{-1}) = [dry mass (mg) of the 1st largest salamander × excretion stoichiometry (μg × dry mass mg^{-1} × h^{-1}) of the 1st largest salamander] + {[mean dry mass (mg) of the other salamanders × (number of surviving salamanders - 1)] × mean excretion stoichiometry (μg × dry mass mg^{-1} × h^{-1}) of the other salamanders }.

Because there was no correlation between size order and stoichiometries in No-cannibalism treatments, I estimated the nutrients retained in and released from salamander populations in the treatments by using mean stoichiometry values and mean dry mass in each replicate as follows;

Nutrients retained in populations in No-cannibalism treatments (mg) = [mean dry mass (mg) of salamanders × number of surviving salamanders] × mean body stoichiometry (%) of salamanders

Nutrients released from populations in No-cannibalism treatments (μg × h^{-1}) = [mean dry mass (mg) of salamanders × number of surviving salamanders] × mean excretion stoichiometry (μg × dry mass mg^{-1} × h^{-1}) of salamanders.

I also estimated total biomass of salamanders in each population of Cannibalism and No-cannibalism treatments because total biomass may determine both amounts of nutrients stored in and released from each population. Similar to the equations for estimating nutrients stored in and released from Cannibalistic and Non-cannibalistic populations described above, I took difference in body mass between cannibal and non-cannibals into account for the estimation of total biomass of populations.

Total biomass of population in the Cannibalism treatment (mg) = dry mass (mg) of 1st largest salamander + mean dry mass (mg) of the other salamanders × (number of surviving

salamanders - 1)

Total biomass of population in the No-cannibalism treatments (mg) = mean dry mass (mg) of salamanders × number of surviving salamanders

Statistical analysis

To examine relationship between cannibalistic dimorphism and intra-population variation in stoichiometries, I used multivariate analyses of variance (MANOVA) on each stoichiometry variable (body %C, %N, %P, mass specific N and P excretion rate, and P:N excretion ratio) of 1st largest, 2nd largest, 3rd largest, median, smallest salamanders. P:N excretion stoichometries were log (10) transformed to improve normality of the data. MANOVA is useful in my case because salamanders within a tank were not independent each other and this method allowed me to test significance of interactive effects between size order and treatments. If cannibalistic dimorphism generates intra-population variation in the stoichiometries, effects of size order on stoichiometries should differ between the treatments (i.e., significant interactive effects between size order and treatment) because only the 1st largest salamanders in the Cannibalism treatment were cannibalistic giants which are expected to have different stoichiometries from the other (i.e., non-cannibals). The same MANOVA model was used to examine the relationship between cannibalistic dimorphism and intra-population variation in dry mass. I also used the same MANOVA model on amount of each nutrients stored in (i.e., C, N, and P) and excreted from individuals (i.e., dissolved N and P excretion). In the MANOVA, I conducted Mauchley's test to determine whether my data satisfied the sphericity assumption. If this assumption was not satisfied, I evaluated statistical significance using Greenhouse-Geisser *F*-statistics (Quinn & Keough 2002). To examine effects of the treatments on nutrients retained in and released from salamander populations, I used analysis of variance (ANOVA) which

considering treatments as a fixed factor on total biomass of population, C, N, and P stored in population, dissolved N and P released from population, and P:N excretion ratio.

Before conducting of a series of statistical analyses, I pooled the data of the No-cannibalism-early and No-cannibalism-late treatments, because my preliminary analyses revealed that there were no differences in any measured variables (i.e., mortality of salamanders and tadpoles, body length, degree of offensive phenotype, body and excretion stoichiometry, nutrients stored in and released from population) between the two No-cannibalism treatments (Appendix B). This indicates that hatch timing of salamander itself had no impacts on any traits including stoichiometry of salamanders.

Field survey

My laboratory experiment clearly showed that cannibalistic dimorphism generates intra-population variation in body and excretion stoichiometries (see Result). Cannibals and Non-cannibals were differed in body %P, N and P excretion rates, and P:N ratio of excretion. To confirm whether cannibalistic dimorphism is a mechanism of intra-population variation in stoichiometries in nature, I investigated association between phenotypes and stoichiometries of salamanders inhabiting in two natural ponds (Kumagoe pond [45°03’02.58”N, 142°01’04.73”E] and Yamadori pond [45°04’19.77”N, 142°01’25.80”E]) in Teshio Experimental Forest of Hokkaido University.

Collection and measuring body and excretion stoichiometries of wild salamanders

On 25 June, 2014, I collected 32 salamanders from both Kumagoe and Yamadori ponds, by using dip nets. The surface area of Kumagoe and Yamadori ponds were 12 m^2 and 27 m^2, respectively. Both ponds were shaded by bamboo surrounding the ponds and bottom of the ponds are covered by soil. There were small aquatic invertebrates such as tubiflex and

Chironomid larvae and frog tadpoles as potential prey items for salamanders. In addition, these ponds contained a few larval dragonfly and diving beetles as potential predators of salamander larvae. According to Kishida & Nishimura (2006), I estimated density of salamanders in these ponds. Densities (mean ± SD) of salamanders in Kumagoe and Yamadori ponds were 33.3 ± 8.7 and 16.7 ± 7.6 individuals per m^2, respectively. After collection of the salamanders, I immediately transferred them to a laboratory room of Teshio Experimental Forest of Hokkaido University (44° 055'02.68"N, 142° 01'10.51"E) and photographed them ventrally using a scanner for morphological measurement. Then, I measured body and excretion stoichiometries in accordance with the protocol described in the section of the laboratory experiment (i.e., ***Measuring body and excretion stoichiometries***).

As shown in Fig. 2, size (phenotype) distribution of salamanders in both natural ponds does not exhibit clear dimorphic pattern. Because natural pond is spatially larger than experimental tanks, more than one salamanders become cannibals in the pond. In this natural situation, even if interactions among individuals cause dimorphic growth between cannibals and non-cannibals, size (phenotype) distribution of salamanders in pond population become continuous rather than discrete because of the differential growth of cannibals within populations (Michimae 2010). Importantly, phenotypic variation of salamanders differed between the two pond populations (Fig. 2). Standard deviations in body length (i.e., size) and ratio of gape width to head width (i.e., index of offensive phenotype) of salamanders in Kumagoe pond was 91% and 10% larger than those in Yamadori pond, respectively. While salamanders with smaller body length were similar between the ponds (e.g., mean ± SD body length of individuals in bottom 10 % in order of body length in Kumagoe and Yamadori ponds was 14.93 ± 1.10 and 13.02 ± 0.76 mm, respectively), salamanders with larger body length in Kumagoe pond were far larger than those in Yamadori pond (e.g., mean ± SD body length of

top 10 % individuals in order of body length in Kumagoe and Yamadori ponds was 27.95 ± 0.59 and 20.03 ± 0.35 mm, respectively) (Fig. 2). While almost all of salamanders with large body length (top 30%) exhibited typical offensive morph (i.e., ratio of gape width to head width is larger than 0.9) in Kumagoe pond, only a few salamanders with large body length (top 30%) exhibited such typical offensive morph. Therefore, I assumed that cannibalistic dimorphism was more developed in Kumagoe pond population than Yamadori pond population.

Statistical analysis

To examine the expected effects of phenotype on stoichiometries of wild salamanders, I used a regression analysis because natural population of salamander exhibited continuous phenotypic distribution described above. Because cannibals are characterized as large body length with offensive morph, I used a composite variable calculated from body length and degree of offensive morph (i.e., ratio of gape width to head width) as an indicator of the cannibalistic phenotypes. I conducted a principal-component analysis (PCA), using the correlation matrix, on body length and ratio of gape width to head width of the salamanders. Because the first principal component (i.e., PC1) explained 86.87% of the total variance and had high positive loadings for body length and ratio of gape width to head width (eigenvalue = 0.71, $\chi^2 = 49.06$, $p<0.0001$), I used PC1 as the variable of cannibalistic phenotypes.

Therefore, to test whether cannibalistic dimorphism generates variation in body and excretion stoichiometries in wild salamanders, I used a linear regression analysis considering PC1 as a fixed factor on each component of stoichiometries (i.e., body %C, %N, %P, mass specific N and P excretion rate, and P:N excretion ratio) for each population. Based on the results of aquarium experiment, here, I expected that stronger effects of PC1 on some stoichiometry traits (i.e., body %P, mass specific N and P excretion rate, and P:N excretion

ratio) should be detected in Kumagoe population than Yamadori population because phenotypic variation due to cannibalistic dimorphism in the former population was larger than the latter.

RESULTS

Laboratory experiment

Body and excretion stoichiometries

In MANOVA on %C and %N of 1st largest, 2nd largest, 3rd largest, median-sized, and smallest salamanders, which considers treatment as a fixed factor, I found no significant effects of treatment ($P > 0.54$), size order ($P > 0.52$) and their interaction ($P > 0.62$) (Table 1., Fig. 3a-b). On the other hand, MANOVA considering treatment as a fixed effect on body %P, mass specific N excretion rate, mass specific P excretion rate, and P:N excretion ratio revealed significant interactive effects of size order and treatment ($P < 0.044$) (Table 1).

To identify the relationship between size order and the stoichiometric variables (i.e., body %P, mass specific N excretion rate, mass specific P excretion rate, and P:N excretion ratio) in each treatment, I conducted MANOVA on each of the stoichiometry variables of 1st, 2nd, 3rd largest, median-sized, and smallest salamanders for each treatment. Although I found significant relationship between size order and each of the stoichiometry variables in the Cannibalism treatment ($P < 0.0094$), there was no significant relationship between size order and each of the stoichiometric variables in the No-cannibalism treatment ($P > 0.28$) (Table 2). This means that stoichiometries of individuals were different among size order only in the Cannibalism treatment. If the intra-population variation in the stoichiometries in the Cannibalism treatment was caused by phenotypes (cannibal or non-cannibal) rather than size order itself, the relationships between size order and the stoichiometries are no longer

significant when the 1st largest individuals (i.e., cannibals) are excluded from the analyses. So, I conducted MANOVA on each of the stoichiometry variables of the Cannibalism treatment after excluding the data of 1st largest salamanders. The MANOVA revealed no significant relationship between size order and the stoichiometries except body %P ($P > 0.23$) (Table 3). Although there was still marginal significant relationship between size order and body %P ($P = 0.034$) (Table. 3), the relationship was no longer significant when I excluded 7 replicates in which 2nd largest salamanders exhibited offensive morph ($F_{3, 105} = 2.38$, $P = 0.074$). In summary, a series of the analyses indicates that cannibalistic dimorphism generated intra-population variation in body %P, mass specific N excretion rate, mass specific P excretion rate, and P:N excretion ratio (Fig. 3c-f). Body %P, mass specific N excretion rate, mass specific P excretion rate, and P:N ratio of cannibals were 23%, 126%, 293%, and 26% higher than those of non-cannibals, respectively (Fig. 3c-f).

Nutrients stored in and excreted from individuals

In MANOVA on dry mass of 1st, 2nd , 3rd largest, median-sized, and smallest salamanders, which considers treatment as a fixed factor, I found significant effects of treatment ($P < 0.0001$), size order ($P < 0.0001$) and their interaction ($P < 0.0001$) (Table 4, Fig. 4). Significant interaction was caused by exceptionally heavy weight due to gigantism of the 1st largest salamanders in the Cannibalistic treatment. In fact, after excluding the 1st largest salamanders from the analyses, interaction term between treatment and size order ($P > 0.16$) and the treatment ($P > 0.24$) were no longer significant. Consequently, 1st largest salamanders in the Cannibalism treatment stored more nutrients (C, N, P) in their body (Table 4,5) (Fig 4b-d) and excreted more dissolved N and P than the others (Table 4,5) (Fig 4e-f). At least, C, N, P stored in body and N and P excretion rate of cannibals were 4, 4, 4.6, 6.5 and 11.7 fold larger than those of non-cannibals, respectively (Fig. 4b-f).

Nutrients retained in and released from populations

Biomass in the dimorphic Cannibalistic populations (mean ± 1SD = 139.06 ± 37.24 mg) was 38 % smaller than that in the monomorphic Non-cannibalistic populations (220.83 ± 68.95 mg) (t-test, $t_{55} = 5.69$, $P < 0.0001$) (Fig. 5a). C, N, and P retained in populations of Cannibalism treatment was 41%, 41% and 38% smaller than those stored in populations of Non-cannibalistic populations, respectively ($P < 0.0001$) (Fig. 5b-d). This indicated that nutrients retained in populations were strongly determined by total biomass of the salamander populations. In contrast, in spite of its smaller biomass, the dimorphic populations released the similar amount of dissolved N ($t_{55} = 6.17$, $P = 0.54$) and 55% greater amount of dissolved P ($t_{55} = 4.43$, $P = 0.020$) compared to non-dimorphic populations (Fig. 5e-f). As a result, P:N excretion ratio of populations in the Cannibalism treatment was 17% higher than that of populations in the No-cannibalism treatment ($t_{55} = 1.90$, $P = 0.031$) (Fig. 5g).

Field survey

Relationship between stoichiometries and phenotype

In the population in which dimorphism less developed (i.e., Yamadori pond population), I found no significant effect of PC1 on any stoichiometry variables except for body %N (Table 6). Body %N decreased with increasing PC1 value in the Yamadori pond population. The linear regression analyses revealed that body %P, mass specific N excretion rate, mass specific P excretion rate, and P:N ratio of excretion increased with increasing PC1 value in the population in which well-developed dimorphism was observed (i.e., Kumagoe pond population) (Table 6). These results suggest that cannibal individuals have higher body %P, mass specific N excretion rate, mass specific P excretion rate, and P:N ratio than non-cannibals, which are in line with the results in the laboratory experiment. In addition, similar to Yamadori pond, body %N decreased with increasing PC1 value (Table 6).

DISCUSSION

Although roles of animals in nutrient cycling had been considered to be negligibly small over the past several decades (Schleinger 1997), ecologists have increasingly recognized that animals can have strongly influence nutrient cycling (reviewed by Vanni 2002; Schmitz et al. 2010; Estes et al. 2011). To deepen our understanding of roles of animals in the nutrient flow as resource providers, it is required to investigate that (1) how individuals differentiate their body and excretion stoichiometries within a population and (2) how the individual variation in the stoichiometries affect nutrients retained in and released from animal populations (i.e., resource availability for top predators, decomposers, and primary producers). Here, by using cannibalistic salamander larvae as model organisms, I tested the hypothesis that cannibalistic dimorphism causes intra-population variation in body and excretion stoichiometries, and also examined whether the possible association between the dimorphism and stoichiometries determines nutrients retained in and released from salamander population.

Variation in body and excretion stoichiometries

In the both experimental and wild populations, cannibals consistently had higher body %P than non-cannibals (Fig. 3c). Much faster growth of cannibals compared to non-cannibals is a possible factor of the higher phosphorus content in the body of cannibals. For growth, organisms synthesize ribosomal RNA, which contains phosphorus richly, in their cell proliferations (Campana & Schwartz 1981). Previous studies showed that this biosynthesis mechanism explains inter-specific variation of body stoichiometry across the spectrum of growth speed (i.e., faster growth species have higher phosphorus contents than slower growthe species) (Elser et al. 2000, 2003; Vrede et al. 2004). The mechanism could also apply to intra-specific (population) variation in body stoichiometry. In my case, cannibals of

salamanders should synthesize ribosomal RNA more rapidly to achieve their exceptionally faster growth, compared to non-cannibals, so that cannibal body may contain higher amount of phosphorus per biomass. As an alternative mechanism, development of offensive morph, which is characterized as broadened gape and head (Kishida et al. 2009a; Takatsu & Kishida 2013), may explain phosphorous-rich body of cannibals. In general, bone is a primary storage of body phosphorus (McDowell 1992). Because skull bones account for a large proportion of bones in whole body (e.g., 20-30% in bird and mammal, Dumont 2010; 30% in fish, Bahuguna et al. 2013), changes in head and gape parts could strongly affect body phosphorus contents. To broaden their gape and head without impairing their function as capturing organ, cannibals need to enlarge their main bones without reduction in bone density, which in turn probably increase phosphorus contents in the body. Rapid growth of cannibals is commonly observed in various taxa (e.g., ciliate, Banerji & Morin 2009; insects, Wissinger et al. 2004; Fish, Bystrom 2006; Amphibian, Pfennig 1999), and in several species of them, cannibals exhibit different ecological morph from non-cannibals (Banerji & Morin 2009; Pfennig 1999) as like as *H. retardatus* salamanders do. Future studies investigating intra-population variation in body stoichiometry of the cannibalistic species are so effective to reveal the importance of growth and morphological changes as the determinant of body stoichiometry of animals.

Traditionally, researchers considered that body stoichiometry is a critical factor that strongly affect excretion stoichiometry. Because animals should restore the gained nutrients from the prey items into their body as many as possible, they should restore the gained nutrients at the same P:N ratio of their body and therefore they should excrete the residuals(Sterner & Elser 2002). Under this adaptationist perspective, one can expect that organisms with higher P:N body stoichiometry discharges the lower P:N excretions. In fact, as supporting the conventional theory, several comparative studies have documented negative

correlation between body stoichiometry and excretion stoichiometry across multiple species within taxa (Vanni et al. 2002; Alves et al. 2010). In contrast, the result of my study was opposed to the conventional theory. Although cannibals have P-richer body than non-cannibals, their excretion was P-richer than that of the non-cannibals. The phenotype-specific excretion stoichiometry may be caused by difference in body stoichiometry of the main prey items between cannibals and non-cannibals. In my experiment, while non-cannibals consumed relatively P-poor invertebrate (tubifex: mean ± SD of %C, N, P are 49.31 ± 0.33, 8.44 ± 0.17, 0.31 ± 0.01, respectively [N = 3]), cannibals mainly consume relatively P-rich vertebrates (salamander %C, N, P are 46.19 ± 2.88, 12.25 ± 0.87, 0.85 ± 0.23 [N = 172]; tadpole %C, N, P are 46.40 ± 0.97, 7.43 ± 0.19, 0.92 ± 0.36, respectively [N=3]). Thus, cannibals may not effectively utilize the nutrients contained in the amphibian prey due to poor assimilation so that their excretions contained higher ratio of phosphorous than those of non-cannibals. Regardless of precise mechanism, the result is meaningful because it suggests that body stoichiometry may not be a good predictor of excretion stoichiometry in the taxa in which species (populations or individuals) greatly differentiate prey items. I expect that diversity of prey items can be low in the previously studied taxa so that researchers detected negative correlation between phosphorous contents in body and excretion (Vanni et al. 2002; Alves et al. 2010). Past mathematical models pointed out that species-specific body stoichiometry is an important factor controlling community dynamics not only through affecting nutrient availability of their predator but also through determining excretion stoichiometry which regulates primary production (Daufresne & Loreau 2001a,b). Importantly, my results raises a question on the relevancy of assumption of the existing models. Therefore, I suggest that more efforts to investigate mechanism causing inter- and intra-specific variation in stoichiometry of animal species are needed to better understand the animal population impacts on nutrient dynamics.

I found that giant cannibals excrete P-richer nutrients than non-cannibals. This type of association between excretion stoichiometry and dimorphic phenotypes may be common among fish and salamander species exhibiting cannibalistic dimorphism, because these species generally shift their diets from P-poor invertebrates (i.e., small prey items such as zooplanktons and larval insects) to P-rich vertebrates (i.e., large prey items such as fishes and amphibians) through gigantism (fish, Post 2003, Bystrom 2006; amphibians, Pfennig 1990, Kishida et al. 2011). More generally, I suggest that positive correlation between body size and P:N ratio of excretion within population can be commonly observed in carnivore species exhibiting ontogenetic diet shift from invertebrate to vertebrate prey (Werner & Gilliam 1984). Because primary and secondary productions are limited by the amount of phosphorous in freshwater ecosystem (Elser et al. 2000), diet shift from small invertebrate prey to large vertebrate prey in the carnivorous fish and salamanders may play a prominent role to support bottom up trophic cascade in the phosphorous-limited world.

Inconsistent results between the experiment and field survey allows us to infer alternative factor affecting stoichiometry of the salamanders. Although there was no significant difference in body %N between cannibal and non-cannibals in the experiment, I found the statistically marginal trend that cannibals exhibit smaller body %N than non-cannibals in natural populations (Table 6). This phenotype and body nitrogen content relationship can be caused by phenotype-specific response of salamanders to the species living in the natural ponds, on which I did not focused. Predatory aquatic insects such as dragonfly and diving beetle are prospective agents to induce the possible phenotype-specific response. A recent study showed that body %N of *Hyla versicolor* frog tadpoles is higher in the presence of predatory larval diving beetle (*Dytiscus verticalis*) than the absence of the predators (Costello & Michel 2013). They argued that predator-induced increase in tail muscle as an inducible morphological defense

causes the increase nitrogen content of tadpole body in the presence of predators because muscle is comprised of N-rich structural proteins (McDowell 1992). In my system, inducible morphological defense may be a mechanism of higher N contents of body stoichiometry of the salamanders, because *H. retardatus* salamander enlarges their tail and gills in the presence of the predatory dragonfly larvae (*Aeshna nigroflava*) (Iwami et al. 2007; Kishida et al. 2009a) and small salamander larvae exhibit stronger response to the dragonfly than large salamander larvae (i.e., cannibals) in their behavioral defense (Kishida et al. 2011). Thus, in the natural ponds, stronger anti-predator responses of the non-cannibals to the potential insect predators might cause their higher body nitrogen contents of body stoichiometry relative to cannibals.

Variation in nutrients retained in and released from populations

In the experiment, cannibalism caused reduction in total biomass of the population due to density reduction. As traditional theoretical studies did, if one assumes homogeneity of body and excretion stoichiometries of individuals within a species (Daufresne & Loreau 2001a,b; Leroux & Loreau 2010), the reduction of total biomass due to cannibalism is predicted to only cause subsequent reduction in nutrients retained in and released from populations. However, in contrary to the prediction, I found that cannibalistic population more released nutrients into the environment than non-cannibalistic population due to emergence of cannibals that have higher excretion rates. This result emphasizes that without considering individual phenotype, one can't evaluate the resource providing effects of animal populations.

It is noted that my study evaluated effects of salamanders on nutrient distributions in each of cannibalistic and non-cannibalistic populations just at the time that cannibalistic dimorphism well developed in the cannibalistic populations. However, nutrients retained in and released from these populations should change through time and the way of changes possibly depends on the emergence of cannibalistic dimorphism. For example, although I showed

cannibalistic populations more released P-rich nutrients than non-cannibalistic populations, such differences are expected to be transient because structures of trophic interactions likely change differentially between the cannibalistic and non-cannibalistic scenarios (Takatsu & Kishida 2015). The greater amount of P-rich nutrients released from cannibalistic populations than non-cannibalistic populations, which was observed in this experiment, was the result of stronger consumption of giant cannibals on non-cannibals and tadpoles. Importantly, the consumption rate of the cannibals on amphibian prey greatly changes through time because availability of amphibian prey itself is not constant in this growing predator and prey interaction (Kishida et al. 2009b). In the cannibalistic scenario, rapid growth of cannibals results in high consumption rate of cannibals on amphibian prey in the initial period, but the resultant reduction in the density of amphibian prey results in the low consumption rate of cannibals on amphibian prey in the later period. This inevitable change in trophic interactions and expected early metamorphosis of giant cannibals (Takatsu & Kishida 2015) can cause dramatic changes in amount and quality of the released nutrients from the cannibalistic populations. In contrast, in the non-cannibalistic populations, amount and quality of nutrients released should be relatively constant because non-cannibals hardly consume amphibian prey and remains in pond as larvae longer time than cannibals (Nosaka et al. 2015, Takatsu & Kishida 2015). Comparison of temporal changes in retained and released nutrients between cannibalistic and non-cannibalistic populations is necessary to reveal whole picture of the impact of salamander populations to the dynamics of nutrient distribution in the pond ecosystem. In general, how changes in phenotypic composition of animal populations affects nutrient dynamics is open question that should be addressed in the future (Yang et al. 2008; Takimoto et al. 2009; Armstrong et al. 2016).

Fig. 1. (a) body size of salamanders and (b) morphology of salamanders. Canni and No-canni are abbreviations of the Cannibalism and No-cannibalism treatments, respectively. 1, 2, 3, M, and S represents size order of the salamanders. The thick horizontal bar indicates the median, the box contains 50% of the data, and the whisker indiates the range.

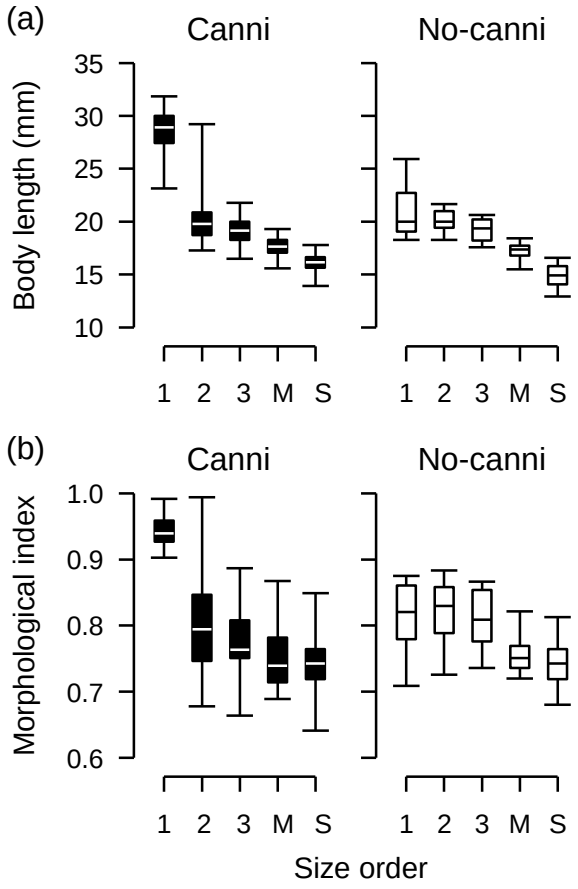


Fig 2. Body length (mm) and morphological index of salmanaers in Kumagoe pond (black circles) and Yamadori pond (white circles).

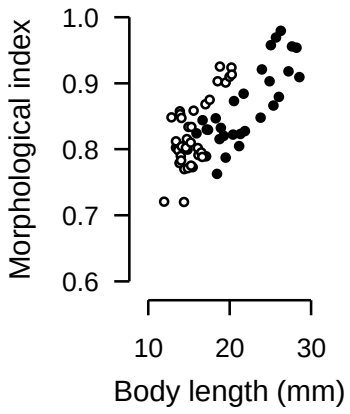


Fig. 3. Mean (± SE) body C contents (% of dry mass) (a), body N contents (b), and body P contents (c) of salamanders. Mean (± SE) mass specific N excretion rate (μg× mg^{-1}×h^{-1}) (d), mass specific P excretion rate (c) , and P:N excretion rate (f) of salamanders. Canni and No-canni are abbreviations of the Cannibalism and No-cannibalism treatments, respectively. 1, 2, 3, M, and S represents size order of the salamanders.

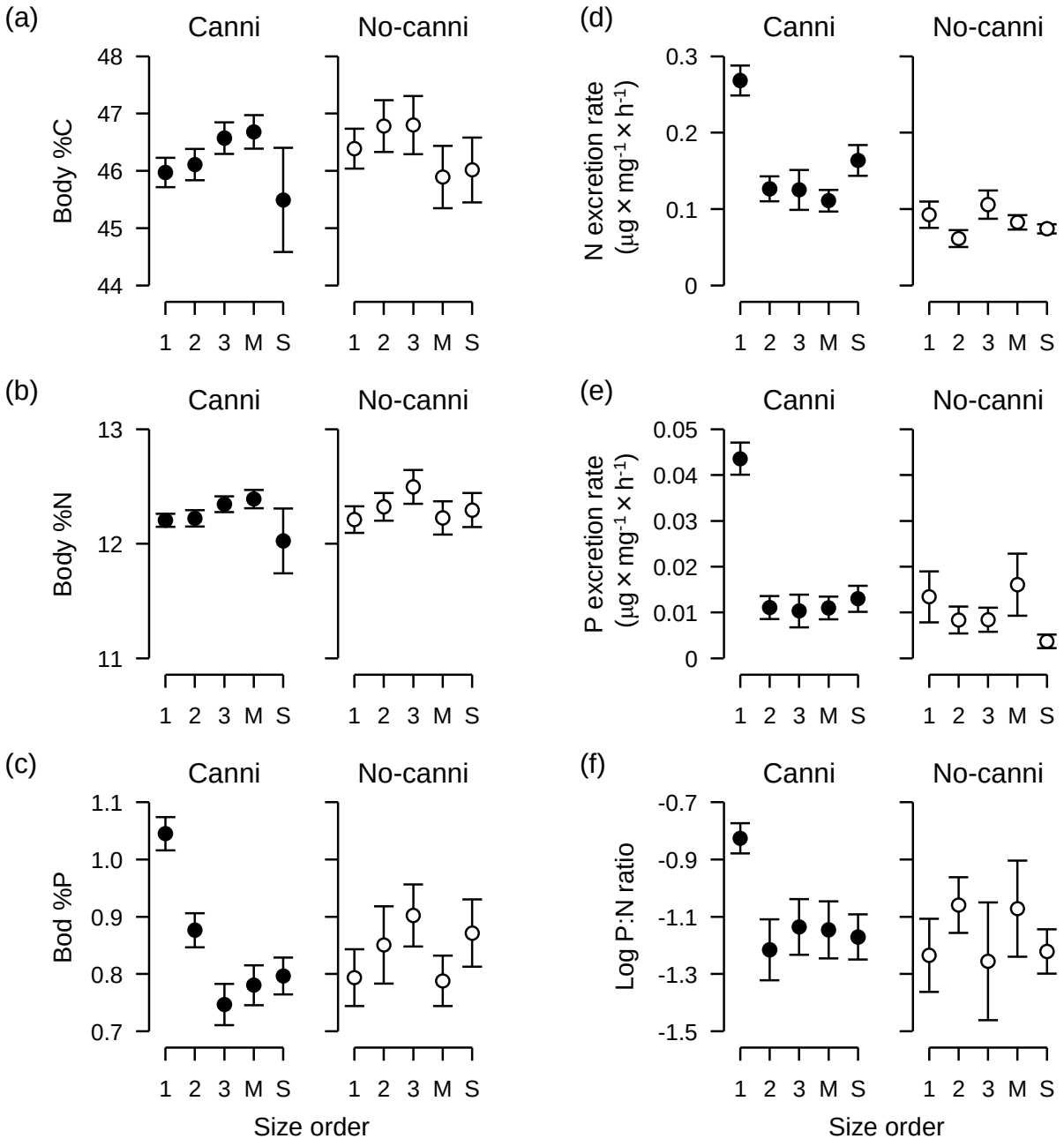


Fig 4. Mean (± SE) body dry mass (mg) (a), body C (mg) (b), body N (mg) (c), and body P (mg) (d) of salamanders. Mean (± SE) N excretion rate (μg×h^{-1}) (e) and P excretion rate (f) of salamanders. Canni and No-canni are abbreviations of the Cannibalism and No-cannibalism treatments, respectively. 1, 2, 3, M, and S represents size order of the salamanders.

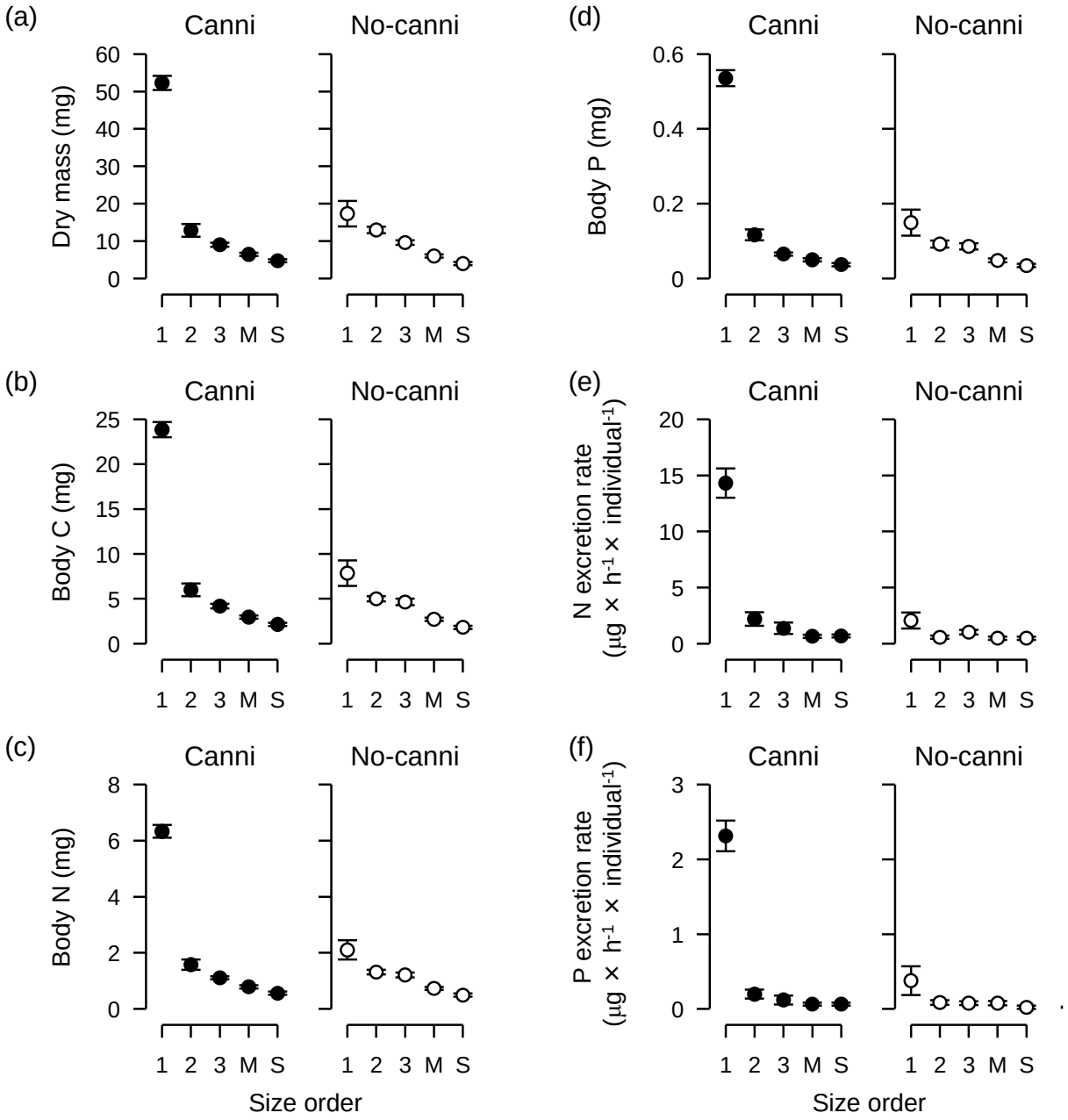


Fig 5. Mean (± SE) of total population biomass (mg) (a) and Carbon (mg) (b), Nitrongen (mg) (c), Phosphorus (mg) (d) stored in salamander populations. Mean (± SE) population excretion rate ($\mu g \times h^{-1}$) of Nitrogen (e) and Phosphorus (f). Mean (± SE) P:N excretion ratio of salamander population (g). C and NC are abbreviations of the Cannibalism and No-cannibalism treatments, respectively.

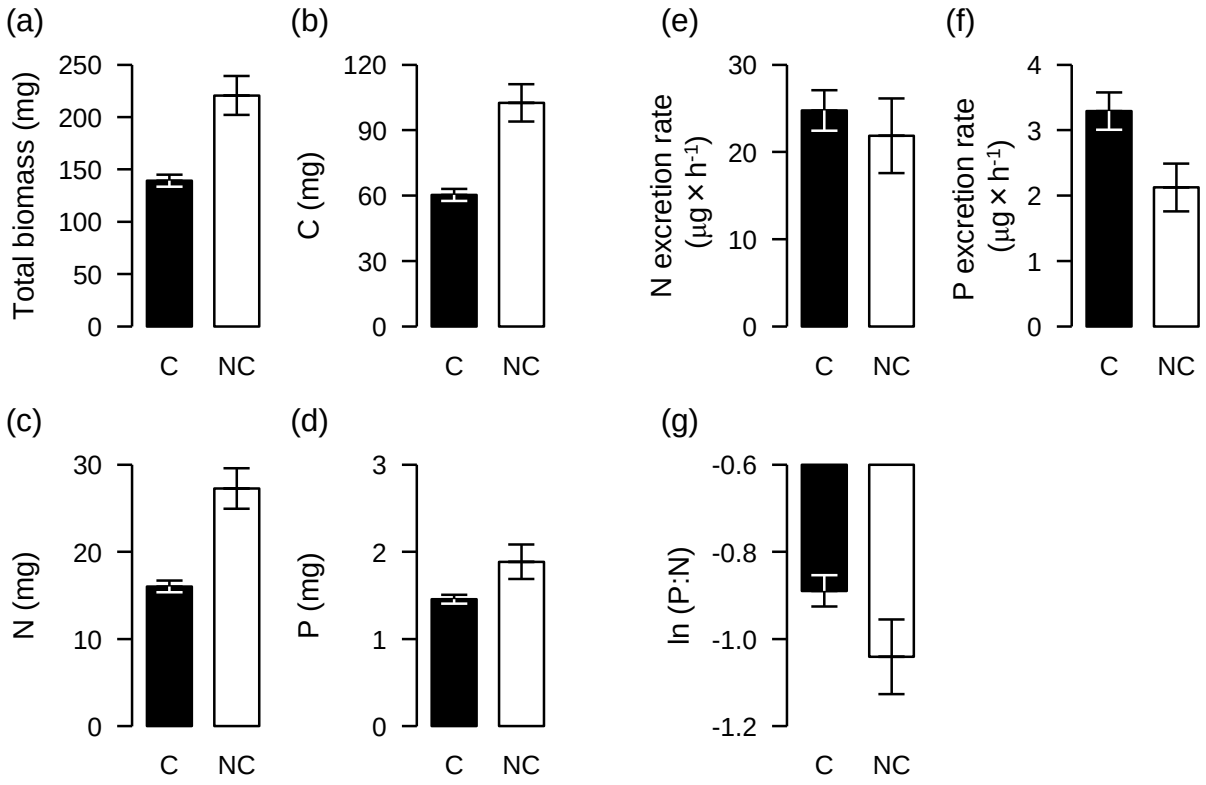


Table 1. Results of MANOVAs considering treatment on stoichiometries of 1st largest, 2nd largest, 3rd largest, median-sized, smallest salamanders. Asterisk represent (*) represent G-G adjusted probabilities for the significance levels associated with the respective *F*-statistics.

Stoichiometries	*Factor*	*d.f.*	*F*	*P*
Body %C	Treatment	1, 55	0.26	0.61
	Size order	1.80, 98.91	0.64	0.52*
	Treatment × Size order	1.80, 98.91	0.45	0.62*
Body %N	Treatment	1, 55	0.37	0.54
	Size order	1.60, 87.88	0.59	0.52*
	Treatment × Size order	1.60, 87.88	0.41	0.62*
Body %P	Treatment	1, 55	0.055	0.82
	Size order	4, 220	2.57	0.039
	Treatment × Size order	4, 220	5.95	0.0001
Mass specific	Treatment	1, 55	12.07	0.0010
N excretion rate	Size order	3.41, 187.34	4.39	0.0035*
	Treatment × Size order	3.41, 187.34	3.37	0.015*
Mass specific	Treatment	1, 55	7.39	0.0087
P excretion rate	Size order	3.30, 184.41	8.55	< 0.0001*
	Treatment × Size order	3.30, 184.41	5.50	0.0008*
N:P excretion rate	Treatment	1, 37	0.94	0.34
	Size order	4, 148	0.40	0.81
	Treatment × Size order	4, 148	2.50	0.044

Table 2. Results of MANOVA on each of stoichiometric variables in which I found significant interactive effects between size order and treatment (i.e., %P, mass specific N and P excretion rate, and P:N excretion ratio, see table 1) of 1st largest, 2nd largest, 3rd largest, median-sized, smallest salamanders for each treatment. Asterisk represent (*) represent G-G adjusted probabilities for the significance levels associated with the respective *F*-statistics.

a) Cannibalism treatment

Stoichiometry variable	*d.f.*	*F*	*P*
Body %P	4, 168	14.86	< 0.0001
Mass specific N excretion rate	3.34, 140.27	11.92	< 0.0001*
Mass specific P excretion rate	3.23, 135.75	24.24	< 0.0001*
N:P excretion rate	4, 104	3.54	0.0094

b) No-cannibalism treatment

Stoichiometry variable	*d.f.*	*F*	*P*
Body %P	4, 52	0.82	0.52
Mass specific N excretion rate	2.04, 26.50	1.35	0.28*
Mass specific P excretion rate	2.18, 28.29	1.18	0.33*
N:P excretion rate	4, 44	0.55	0.70

Table 3. Results of MANOVA on each of stoichiometric variables in which I found significant interactive effects between size order and treatment (i.e., %P, mass specific N and P excretion rate, and P:N excretion ratio) of 2nd largest, 3rd largest, median-sized, smallest salamanders for cannibalism treatment. Asterisk represent (*) represent G-G adjusted probabilities for the significance levels associated with the respective *F*-statistics.

Cannibalism treatment

Stoichiometry variable	*d.f.*	*F*	*P*
Body %P	3, 126	3.00	0.034
Mass specific N excretion rate	2.72, 114.25	1.46	0.23*
Mass specific P excretion rate	2.48, 104.12	0.20	0.87*
N:P excretion rate	3, 78	0.38	0.76

Table 4. Results of MANOVAs considering treatment on amount of nutrients stored in and released from individuals of 1st largest, 2nd largest, 3rd largest, median-sized, smallest salamanders. Asterisk represent (*) represent G-G adjusted probabilities for the significance levels associated with the respective *F*-statistics.

Nutrient	*Factor*	*d.f.*	*F*	*P*
Dry mass	Treatment	1, 55	38.93	< 0.0001
	Size order	1.84, 101.28	163.21	< 0.0001*
	Treatment × Size order	1.84, 101.28	62.20	< 0.0001*
Body C	Treatment	1, 55	36.69	< 0.0001
	Size order	1.84, 101.43	160.59	< 0.0001*
	Treatment × Size order	1.84, 101.43	61.34	< 0.0001*
Body N	Treatment	1, 55	38.79	< 0.0001
	Size order	1.81, 99.99	162.18	< 0.0001*
	Treatment × Size order	1.81, 99.99	62.79	< 0.0001*
Body P	Treatment	1, 55	48.28	< 0.0001
	Size order	1.71, 94.03	131.95	< 0.0001*
	Treatment × Size order	1.71, 94.03	61.61	< 0.0001*
N excretion rate	Treatment	1, 55	16.73	< 0.0001
	Size order	1.54, 84.80	33.85	< 0.0001*
	Treatment × Size order	1.54, 84.80	22.02	< 0.0001*
P excretion rate	Treatment	1, 55	23.39	< 0.0001
	Size order	1.25, 68.86	38.09	< 0.0001*
	Treatment × Size order	1.25, 68.86	21.27	< 0.0001*

Table 5. Results of MANOVAs considering treatment on amount of nutrients stored in and released from individuals of 2nd largest, 3rd largest, median-sized, smallest salamanders. Asterisk represent (*) represent G-G adjusted probabilities for the significance levels associated with the respective *F*-statistics. While I found significant effects of treatment and interaction between treatment and size order on all variables in the previous analysis (see table 4), the significant effects were no longer significant when I excluded 1st largest salamanders from the analysis. This suggests specificity of amount of nutrients stored in and released from cannibals.

Nutrient	*Factor*	*d.f.*	*F*	*P*
Dry mass	Treatment	1, 55	0.61	0.49
	Size order	1.16, 63.78	27.30	< 0.0001*
	Treatment × Size order	1.16, 63.78	0.95	0.35*
Body C	Treatment	1, 55	0.53	0.47
	Size order	1.17, 63.23	28.00	< 0.0001*
	Treatment × Size order	1.17, 63.23	0.92	0.36*
Body N	Treatment	1, 55	0.49	0.49
	Size order	1.19, 65.69	28.95	< 0.0001*
	Treatment × Size order	1.19, 65.69	1.00	0.34*
Body P	Treatment	1, 55	0.058	0.81
	Size order	1.29, 71.09	20.55	< 0.0001*
	Treatment × Size order	1.29, 71.09	1.98	0.16*
N excretion rate	Treatment	1, 55	1.41	0.24

	Size order	1.55, 85.31	2.06	0.14*
	Treatment × Size order	1.55, 85.31	1.15	0.31*
P excretion rate	Treatment	1, 55	0.92	0.34
	Size order	1.98, 109.08	1.55	0.22*
	Treatment × Size order	1.98, 109.08	0.53	0.59*

Table 6. Table of significance value for linear regression of the each of stoichiometry variables for each pond population against PC1. $P < 0.05$ are in boldface type. +: positive -: negative correlation

stoichiometry variable	*Kumagoe pond*	*Yamadori pond*
Body %C	0.41	0.39
Body %N	**0.049 (-)**	**0.048 (-)**
Body %P	**0.015 (+)**	0.60
Mass specific N excretion rate	**0.016 (+)**	0.24
Mass specific P excretion rate	**0.0001 (+)**	0.76
N:P excretion rate	**0.0030 (+)**	0.30

Appendix A. Collection and keeping methods of *Hynobius retardatus* and *Rana pirica*

I collected 62 egg clusters of *H. retardatus* salamander and 15 egg masses of *R. pirica* frog from a pond in the Experimental Forest of Hokkaido University, Hokkaido, Japan, in late-May 2014. Each of 15 frog egg masses was kept in a separate 22 L semi-transparent plastic tank made of polypropylenes (51.3 cm × 37.2 cm × 16.6 cm high) filled with 5 L of aged tad water. Tank were maintained in my experimental room at 17℃ with natural light-dark (14/10) regime. Once tadpoles started to hatch (late-May), I added eight piece of rabbit chow (dry mass of 1.6 g; Yeaster, Tatsuno, Japan) into each tank to serve as tadpole food. Throughout the experiment I exchanged the water every two days. I kept the tadpoles in this condition for two weeks before starting experiment.

Each of 62 salamander egg clusters was placed separately in a fine mesh net. The nets were placed in 4 L semi-transparent polypropylene tanks (33.4 cm × 20 cm × 10 cm high; 5nets per tank) filled with 2 L of aged tap water. Tanks were then placed in a climate chamber and maintained at 3℃ under natural light-dark (14/10) condition to control their hatch timing. One week before the start of the experiment 1, when almost all of the embryos reached stage 38 – 39 (Iwasawa & Yamashita 1991), I carried out a manipulation to these egg clusters to establish the three salamander treatments (i.e., Cannibalism, No-cannibalism early-hatchlings, No-cannibalism late-hatchlings). The method to obtain the early- and late- salamander hatchlings is same as that described in Appendix A2 in Chapter 2

Appendix B. Summary of statistical analyses comparing No-cannibalism early-hatchings and No-cannibalism late-hatchlings treatments

My previous study showed that one-week hatch timing difference in No-cannibalism treatments cause no effects on every measured traits (i.e., mortality of salamanders and tadpoles, body length, degree of offensive phenotype) (Takatsu & Kishida 2015). Given that diet and morphological traits are related to body and excretion stoichiometries as previous studies suggested (Costello & Michel 2013), I expected that hatch timing itself would have no impacts on every measured traits including body and excretion stoichiometry. I conducted preliminary analyses of the data of the two treatments to determine whether the effects of the two treatments were similar. I tested whether (1) mortality of salamanders, (2) mortality of frog tadpoles, (3) body length of salamanders, (4) degree of offensive phenotype of salamanders (i.e., gape width to head width ratio), (5-7) body stoichiometry of salamanders, (8-10) excretion stoichiometry of salamanders, and (11) total biomass, (12-14) nutrients stored in population, and (15-17) nutrients released from salamander population differed between the two treatments. To examine effect of treatment on mortality of salamander, mortality of tadpoles, total biomass, and nutrients stored in and released from salamander populations, I used analysis of variance (ANOVA) which considering treatments as a fixed factor on the each variables. I used multivariate analyses of variance (MANOVA) on each of body length, degree of offensive phenotype, body stoichiometry (%C, %N, and %P), and excretion stoichiometry (N and P excretion rate, and P:N excretion ratio) of 1^{st} largest, 2^{nd} largest, 3^{rd} largest, median, smallest salamanders. I did not find any significant effect of hatch timing on any variables between the two treatments (Table B1).

Table B1. Results of MANOVAs considering hatch timing (early or late) on each measured variables of 1st largest, 2nd largest, 3rd largest, median-sized, smallest salamanders in the No-cannibalism treatments. Asterisk (*) represent Greenhouse-Geiser adjusted probabilities for the significance levels associated with the respective *F*-statistics.

Variables	*Factor*	*d.f.*	*F*	*P*
(1) Mortality of salamanders	Treatment	1, 12	0.47	0.50
(2) Mortality of frog tadpoles	Treatment	1, 12	0.056	0.82
(3) Body length of salamanders	Treatment	1, 12	0.067	0.80
	Size order*	1.54, 18.47	80.0	< 0.0001*
	Treatment × Size order	1.54, 18.47	0.73	0.46*
(4) Degree of offensive phenotype of salamanders	Treatment	1, 12	0.18	0.67
	Size order	4, 48	12.52	< 0.0001
	Treatment × Size order	4, 48	1.40	0.25
(5) Body %C	Treatment	1, 12	0.55	0.47
	Size order	4, 48	0.67	0.62
	Treatment × Size order	4, 48	0.15	0.96
(6) Body %N	Treatment	1, 12	0.0096	0.92
	Size order	4, 48	0.62	0.65
	Treatment × Size order	4, 48	0.42	0.79

Variables	*Factor*	*d.f.*	*F*	*P*
(7) Body %P	Treatment	1, 12	4.27	0.061
	Size order	4, 48	0.78	0.54
	Treatment × Size order	4, 48	0.32	0.86
(8) N excretion rate	Treatment	1, 12	0.079	0.78
	Size order	1.97, 23.64	1.27	0.30*
	Treatment × Size order	1.97, 23.64	0.26	0.77*
(9) P excretion rate	Treatment	1, 12	0.0004	0.98
	Size order	2.33, 27.96	1.20	0.32*
	Treatment × Size order	2.33, 27.96	1.30	0.29*
(10) P:N excretion rate	Treatment	1, 10	0.39	0.55
	Size order	4, 40	0.51	0.73
	Treatment × Size order	4, 40	0.072	0.99

Variables	*Factor*	*d.f.*	*F*	*P*
(11) Total biomass of population	Treatment	1, 12	0.11	0.74
(12) C stored in population	Treatment	1, 12	0.090	0.77
(13) N stored in population	Treatment	1, 12	0.11	0.74
(14) P stored in population	Treatment	1, 12	0.68	0.43
(15) N released from population	Treatment	1, 12	0.21	0.66
(16) P released from population	Treatment	1, 12	0.025	0.88
(17) P:N excretion ratio	Treatment	1, 12	0.11	0.74

Chapter 5

General Discussion

Body size of animal individuals is increasingly recognized as most informative traits identifying individual functions such as prey consumption and nutrient storage and excretion (Woodward et al. 2005; Hall et al. 2007; Miller & Rudolf 2011), and consequently, population functions differ depending on size composition of the population (Chalcraft & Resetarius 2004; Brose et al. 2006; Rudolf 2012; Rudolf & Rasmussen 2013). While individual size is determined by both genetic and environmental factors, environment factors could be general mechanism in determining body size given that considerable differences in size were observed among clonal individuals in same age (Cressler et al. 2014). Indeed, mathematical models predict that considerable impacts of environmental dependent growth of individuals on population functions (Persson et al 2000; Claessen et al. 2002; Leeumen et al. 2013; Wollrab et al. 2013). One study showed that trends in long term field data of predatory fish population and their prey community qualitatively follow the prediction from the mathematical models (Persson et al. 2003). Although these studies suggest that importance of growth as important determinant of individual functions, there are no studies that empirically test size growth determines individual functions and population functions. In this thesis, by using cannibalistic salamander larvae (*Hynobius retardatus*), I clearly demonstrate that rapid growth due to cannibalism strengthens individual functions and emergence of the giant determine the population functions while the giants were only a few fraction of the animal populations.

Series of my experiments consistently emphasized importance of rapid growth of individuals in determining population functions. While cannibalism reduced density of the salamander population, cannibalism of salamander intensified predation pressures on their prey frog (*Rana pirica*) tadpoles by emergence of giant cannibals (Chapter 2). Due to the strong predation pressures from the giant cannibals, tadpoles coexisted with giant cannibals showed more defensive morphological and behavioral phenotype (i.e., bulgier head and moved less)

than that coexisted with non-cannibalistic salamander population. Salamander is considered to be strongly involved in the evolution of the bulgy defensive morphology of the tadpoles (Kishdia & Nishimura 2006; Kishida et al. 2007). I showed that salamanders can select for defensive phenotype of the bulgy defensive phenotype only when they cannibalize and become giants (Chapter 3). Rapid growth of cannibal and consumption also cause salamander population function performed in nutrient flow in aquatic ecosystem (Chapter 4). Due to the consumption of tadpoles which is largest prey and phosphorus rich prey in pond, giant cannibals excreted far more amount of phosphorus richer nutrient than small non-cannibals which consumed small aquatic invertebrates. Consequently, by emergence of the giant cannibals, populations where cannibalism occurred released more amounts of nutrients with higher phosphorus contents than populations where cannibalism did not occur. In addition, giant cannibals showed higher percentage of body phosphorus than small non-cannibals while it requires more detailed examination to understand the physiological mechanisms causing the difference in the body nutrient contents.. Consequently, by emergence of the giant cannibals, populations where cannibalism occurred stored less mount of nutrients with higher phosphorus contents than populations where cannibalism did not occur.

I suggest that salamander larvae can be keystone species only when they rapidly grow. Because tadpoles are often a large proportion of biomass in pond (Alford 1999), existence of tadpoles strongly affects the community members (Holomuski 1998; Iwai & Kagaya 2007; Iwai et al. 2012). Thus, effects of intensified predation pressures on frog tadpoles should propagate within pond ecosystem and also to terrestrial ecosystem after metamorphosis of the tadpoles. In addition to such top-down impacts of salamander populations, emergence of giant cannibals could also intensify bottom-up impacts of salamanders (i.e., fertilization). Primary and secondary productions are limited by the amount of phosphorus in most of the freshwater

ecosystem (Elser et al. 2000). Because salamanders and tadpoles larvae store terrestrial nutrients, consumption of tadpoles and salamander by giant cannibals and release of the terrestrial nutrients as excretion may play important role to support bottom up trophic cascade in oligotrophic pond ecosystem. Thus, due to emergence of giant cannibals, salamander may play key role in pond ecosystem although density significantly reduces; becoming keystone species.

Also in other species, such as fish and other amphibians, rapid growth of individuals may be a mechanism to be a keystone species. In those animal species, it is well studied that they shift their diet from small aquatic invertebrate prey to large vertebrate prey (fish, Post 2003, Bystrom 2006; amphibians, Pfennig 1990). In general, large vertebrate species are phosphorus richer than small invertebrate species (Persson et al. 2010) and larger animal species play key role in shaping community compositions (Schmitz et al. 2010; Estes et al. 2011). Indeed, there are many studies that show keystone effects of fish and amphibian species (fish, Thorp & Bergey 1981; Small et al. 2011; amphibians, Morin 1981, 1983; Resetarits & Fauth 1998).

There are studies that showing context dependency of impacts of potential keystone species on community members (Kurzava & Morin 1994; Fauth 1999). Investigating factors controlling the strength of cannibalism could be important to better explain variations in keystone effects. For example, community members affects occurrence of cannibalism (Rudolf 2008; Kishdia et al 2011). Predation risk from top predator, *Aeshna nigroflave* dragonfly larvae, strongly suppresses cannibalism in *Hynobius retardatus* salamander larvae (Kishida et al. 2011). In addition to other community members, as my experiments did, difference in hatch timing is common cause of cannibalism (e.g., Huss et al. 2010). For example, hatch timing is affected by reproductive timing of adults (Burt 2011). And abiotic factors such as water

temperature and oxygen concentration also affect hatch timing (Freda 1986; experimental manipulation in this thesis). This relationship between hatch timing and adult and several abiotic factors allows us to expect terrestrial environments could strongly influence population functions of salamander larvae.

Ecologists have focused on how functions of populations change with their density because it helps to predict functions of ecosystem where human induced declines in density of species occurs (Chapin et al. 1997; Crowl et al. 2001; Flecker et al. 2002; Hooper et al. 2005; Klemmer et al. 2011). Cannibalism is considered to be mechanisms that cause asymptotic relationship between density and population functions because strength of cannibalism which cause density reduction and behavioral interference increase with density (Hildrew et al. 2004; Wssinger et al 2004; Klemmer et al. 2011). In contrary, my studies suggest cannibalism can be mechanisms that cause disproportionate increase of population functions because increase of strength of cannibalism means that increase of the number of cannibal giants and increase the size of cannibals (Kishida et al. 2011). Thus, in species that show cannibalistic gigantism (reviewed by Fox 1975), reduction in the population density under threshold density that is needed to occurrence of cannibalism might cause significant decline in population functions. The possible contrast effects of cannibalism on relationships between density and population function emphasize importance of functions of each individuals constituting the population to understanding population functions.

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Acknowledgments

I am very grateful to Dr. O. Kishida for helping all of my experiment, revising my paper and encouraging me to write this paper. I also thank N. Katayama, A. Tezuka, M. Nosaka, A. Yamaguchi, K. Evangelia, N. Oyake, T. Sato, N. Sakai, H. Ohiwa, M. Taniguchi and staff members of Teshio Experimental Forest and Tomakomai Experimental Forest of Hokkaido University for their support in setting up the experiments and in rearing animals. I am also grateful to my parents for their support. I also thank Dr. T. Saitoh, Dr. S. Utsumi and Dr. T. Takada for constructive comments on my manuscript. This work was supported by JSPS KAKENHI Grant Number 253564.