

Kin discrimination in polyphenic salamander larvae: trade-offs between inclusive fitness and pathogen transmission

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Abstract Some larval amphibians can develop into two distinct morphological types: a small-headed “normal” morph and a rarer broad-headed morph with specialized adaptations to feed on the normal morph. Cannibalism confers nutritional benefits that accelerate development, essential for survival in transient environments, but incurs potential inclusive fitness costs. Selective cannibalism of non-kin thus should be favored. However, subjects may be more vulnerable to contracting disease from non-siblings to which they lack immunological defenses. We investigated kin discrimination and pathogen transmission among Korean salamander (*Hynobius leechii*) larvae. We placed broad-headed morph larvae into a circular arena together with two normal morph larvae, one their sibling and the other a non-sibling. To test for kin discrimination, we recorded all behavioral interactions among them. To study pathogen transmission, we fed broad-headed larvae either a sibling or non-sibling normal larva that we previously had infected with the bacterium *Aeromonas hydrophila*. Two days after ingestion, we determined whether the bacterium had systemically infected cannibals by quantitative PCR (qPCR). Broad-headed larvae discriminated between sibling and non-sibling normal larvae, directing aggressive behaviors mostly toward siblings. Infection loads varied more widely among broad-headed larvae that cannibalized non-siblings than those that cannibalized siblings, but the

highest infection loads were recorded after ingestion of non-siblings. Cannibalizing non-siblings thus may increase the risk of contracting disease. Broad-headed larvae discriminate most strongly between siblings and non-siblings late in development, when inclusive fitness costs of cannibalizing relatives diminish and vulnerability to novel pathogens transmitted by non-relatives rises.

Keywords Agonistic behavior · Cannibalism · Infectious disease · Interference competition · Kin recognition · Salamander larvae

Introduction

Many forms of life, from lower eukaryotes to birds and primates, practice cannibalism (Elgar and Crespi 1992). Among amphibians, cannibalism occurs most commonly in aquatic larvae (Crump 1992). Some larval amphibians exhibit developmental plasticity that facilitates adaptive responses to uncertain environments (Newman 1992; Pfennig 1992). For example, spadefoot toad (*Spea bombifrons*) and tiger salamander (*Ambystoma tigrinum*) larvae can develop into two morphs, even from the same clutch, with the larger one showing specializations to prey upon the other, smaller one (Pfennig and Collins 1993; Pfennig et al. 1993, 1994). Larger, broad-headed morph larvae have a wider head along with specialized feeding features such as enlarged vomerine teeth, whereas the more typical normal morph larvae have a narrower head, smaller teeth and prey on small invertebrates and zooplankton (Pedersen 1991; Wakahara 1995).

Larval cannibalism in amphibians can confer benefits on the cannibals, which grow faster, gain more mass (Wildy et al. 1998), and metamorphose sooner (Michimae and Wakahara

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2002) than normal morph larvae. As vernal ponds begin to dry up, more rapid development is strongly selected and cannibals may be favored (Lannoo and Bachmann 1984). After metamorphosis, cannibals reproductively mature sooner, potentially raising their lifetime reproductive success (Lannoo et al. 1989). However, cannibalism also incurs costs. Cannibals may be injured when struggling with prey, they risk contracting infectious disease by eating conspecifics (Pfennig et al. 1998), and they may suffer decreased inclusive fitness if they eat their kin (Walls and Roudebush 1991; Pfennig et al. 1993, 1994). Nonetheless, kin cannibalism may evolve in certain circumstances, for example, when the cannibalized individuals have little chance of metamorphosing themselves before their pond dries. Then, they would increase their inclusive fitness by effectively sacrificing themselves to their larger siblings if, as a consequence, the larger siblings are more likely to survive (Waldman 1982).

Cannibalistic salamander larvae sometimes, but not always, discriminate among potential prey based on genetic relatedness. When they do discriminate, sometimes they appear to avoid cannibalizing kin, but other times they preferentially eat their siblings. Larvae of the tiger salamander *Ambystoma tigrinum* (Pfennig et al. 1994), the Hokkaido salamander *Hynobius retardatus* (Wakahara 1997), and the fire salamander *Salamandra atra* (Markman et al. 2009; Sadeh 2012) prefer to attack and eat non-kin in experimental tests. By contrast, larvae of the Korean salamander *Hynobius leechii* prefer to cannibalize their kin (Park et al. 2005). Marbled salamander (*Ambystoma opacum*) larvae are less aggressive toward their kin than non-kin (Walls and Roudebush 1991) but prefer to eat their siblings even when non-siblings are available as food (Walls and Blaustein 1995). Although the tendency of amphibians to avoid cannibalizing kin often is presented as an example of the adaptive value of kin discrimination (e.g., Davies et al. 2012), the relationship between kinship and cannibalism in salamanders appears complex. Whether discriminative cannibalism is directed toward kin or non-kin might be determined by the balance of inclusive fitness costs and risks of disease contagion.

The risk of pathogen transmission may be the most serious cost incurred by cannibals (Pfennig et al. 1991). If a cannibal eats an infected animal, the cannibal may contract the disease afflicting its meal. Even should it survive, the cannibal may suffer reduced growth because of the immunological cost of clearing the pathogen or maintaining tolerance of it (Roy and Kirchner 2000; Schmid-Hempel 2011). Pathogen avoidance may be a key element favoring the evolution of altruism (Lewis 1998), especially as kin discrimination often occurs by detecting signals of the major histocompatibility complex (MHC) that mediate adaptive immune system function (Villinger and Waldman 2012). Close relatives share immune defenses, vulnerabilities to novel pathogens, and possibly similar microbial communities (Barribeau et al. 2012). Thus,

associating with—or even cannibalizing—one's kin may present less of an immunological challenge than interacting with non-relatives that may harbor novel pathogens or parasites.

Conversely, a cannibal that eats an infected relative might incur an elevated risk of contracting disease. As siblings are immunogenetically similar, their immune systems are likely to have common vulnerabilities that pathogens or parasites can exploit. Eating a sick sibling exposes one to parasites or pathogens that, given one's immunome, are potentially dangerous to it. Thus, Pfennig (1997) argued that an individual that cannibalizes an infected relative is likely to be more at risk than if it were to cannibalize an infected non-relative. Curiously, his experimental findings do not support this view (Pfennig et al. 1998) as salamanders fed sick non-siblings died in higher numbers than those fed sick siblings (Pfennig et al. 1999). Across taxa, the few data available suggest that sometimes pathogens are more readily transmitted among close relatives, but sometimes not, and effects may vary by pathogen (Shykoff and Schmid-Hempel 1991; Dharmarajan et al. 2012). Possibly, the role of cannibalism in transmitting diseases has been overstated, as cannibalism is unlikely to be a primary mode of pathogen transmission (Rudolf and Antonovics 2007).

Among the extant Caudata, hynobiid salamanders represent the most basal lineage (Pyron and Wiens 2011), so the existence of a broad-headed morph in this taxon suggests that developmental polyphenism may be an ancestral character. We studied agonistic behavior and cannibalism in larvae of the polyphenic Korean salamander, *H. leechii*. The development of a distinct broad-headed morph previously was documented in this species (Park et al. 2005) and may be dependent on the availability of food resources, including larval anurans (Michimae and Wakahara 2002). We investigated the abilities of normal and broad-headed larvae to recognize kin, compared levels of agonistic behavior between the two morphs, and asked whether aggressive behaviors are more likely to be directed toward kin or non-kin. We also tested how the infectivity and virulence of the bacterial pathogen *A. hydrophila* vary in relation to the genetic relatedness of cannibals to their victims.

Materials and methods

Study area and population

Salamander embryos were collected from two ponds on Gwanak Mountain, near the Seoul National University campus, on the evening of March 21, 2013. Site 1 (37° 27.401 N, 126° 57.414 E), a spring-fed pond, and site 2 (37° 27.874 N, 126° 57.756 E), a pool in a lentic stream, are 1 km apart on the northern side of the mountain. Ten egg clutches were taken

from each site for the first experiment and 16 egg clutches were taken from each site for the second experiment.

Experiment 1: do larval *H. leechii* discriminate between siblings and non-siblings?

Animals and rearing conditions Larvae were reared in $52 \times 36 \times 20$ cm polypropylene containers filled with 20 L of UV-treated dechlorinated, filtered (5μ) water at 14°C and were kept on a 12/12 LD cycle. Water was changed every 4 days and larvae were fed live *Tubifex* worms once a day ad libitum. Clutches were kept separate until the larvae developed sufficiently that they could be easily handled, about 2 weeks after hatching (stage 50, corresponding to the loss of balancers and the beginning of forelimb toe differentiation; Iwasawa and Yamashita 1991). Then, to mark individuals, we injected into the tail of each larva a 3-mm line of acrylic paint (Alpha Color, Seoul, South Korea) by syringe with a 23-gauge hypodermic needle. Larvae thus were marked with red, yellow, orange, green, blue, or white lines. Each clutch was labeled with two corresponding colors, half one color and half the other.

One day after they were marked, we placed 40 larvae, 20 from each of two clutches, into additional polypropylene containers of the same size as those used above. Each clutch was paired with a clutch from the other breeding site as a control against the possibility of multiple paternity of clutches within sites. Individuals from different clutches were allowed to acclimate to one another for 24 h, so subjects tested together all were familiar with one other from the time they were marked until testing. We inspected containers daily for any missing larvae, but no cannibalism occurred. Nonetheless, between two and five broad-headed morph larvae developed in each group. These broad-headed larvae subsequently were tested. Twenty-four hours prior to testing, we stopped feeding all larvae.

Testing procedure To test their responses to one another, we placed three larvae—one broad-headed morph larva, one of its normal morph siblings, and one normal morph non-sibling—into a circular arena filled with 1 L of filtered water in a dark room. Larval head shape is clearly dimorphic in this species (Park et al. 2005). To control for possible effects of marking, such as preferences for a specific color, we tested broad-headed larvae twice by introducing them to one sibling and one non-sibling normal larva that were marked with different colors assigned to their clutch in each trial. The second test was run 48 h after the first.

We recorded larval behavior using a camcorder (Sony DCR-SR82) with night vision capabilities. The infrared light of the camcorder cast a weak red hue (<2.5 lx) in the visible spectrum onto the circular arena. As the larvae normally are nocturnally active, we ran the trials between 8 p.m. and 6 a.m.

The experiment was repeated three times during development: at stage 50 (2 weeks after hatching; the beginning of forelimb toe differentiation, $n=20$), stage 56 (5 weeks after hatching; the beginning of hind limb differentiation, $n=20$), and stage 62 (8 weeks after hatching; all limbs fully developed, $n=20$). Snout-vent lengths of broad-headed larvae were 10.3 ± 0.1 , 13.1 ± 0.1 , and 14.7 ± 0.1 mm, and normal larvae were 8.4 ± 0.1 , 11.5 ± 0.1 , and 13.3 ± 0.1 mm, respectively, at these stages ($\bar{x} \pm$ standard error of the mean (SE)).

An observer, blind to treatment conditions, viewed recordings on a 35.0×27.5 cm color monitor and recorded the time and sequence of behaviors of broad-headed larvae toward normal larvae, and normal larvae toward broad-headed larvae. After a 5-min acclimation period, behaviors were scored as follows: moving away, looking away, looking toward, moving toward, and biting. The observer scored *move away* when the focal subject made a quick movement away from the other larva, *look away* when the focal subject turned its head in the opposite direction of the other larva, *look toward* when the focal subject turned its head in the direction of the other larva, *move toward* when the focal subject either swam or walked anteriorly toward the other larva, and *bite* when the focal subject lunged with an open mouth toward the other larva. To confirm that a focal subject bit another larva, we reviewed the video at one-sixth speed to verify that the individual had opened its mouth.

We rated each behavior by intensity of withdrawal or approach on an ordinal scale to generate an approach/withdrawal index (AWI). Moving away was scored as -1.5 , looking away as -0.5 , looking toward as $+0.5$, moving toward as $+1.5$, and biting as $+2.5$. Positive scores thus indicate some level of approach and aggression whereas negative scores indicate withdrawal behavior. In a study of *Ambystoma talpoideum* and *Ambystoma maculatum* larvae, Walls and Semlitsch (1991) demonstrated that victors of agonistic encounters more frequently displayed *moving toward* and *looking toward* behaviors, while losers more frequently displayed *moving away*.

Broad-headed morph behavior The testing procedure allowed us to present two normal larvae simultaneously to each broad-headed larva. The broad-headed larva was a sibling of one but not the other normal larva. Forty-eight hours after the initial test, the same broad-headed larva was tested a second time with another sibling and non-sibling normal larva, color-coded differently than in the first test. Order of presentation of the different sibling group color assignments was randomly determined.

Over all tests, we compared the numbers of broad-headed larvae that first bit sibling and non-sibling normal larvae and how quickly they bit sibling or non-sibling normal larvae. If a broad-headed larva bit a normal larva in the first trial, we used that response for analysis. If a broad-headed larva first bit a normal larva in the second trial, then we used that response for

analysis. We compared proportions by a binomial test and times by a two-sample *t* test.

Using the behavioral scores, we calculated AWI scores for each broad-headed larva's responses to sibling and non-sibling normal larvae at each developmental stage. Over the two trials at each developmental stage, we thus calculated each subject's mean response to its siblings, based on two trials, and its mean response to non-siblings, based on two trials. No differences were apparent in preliminary analyses of broad-headed individuals' behaviors by breeding site, so we pooled data from the two sites. For each broad-headed larva, we compared its mean AWI scores directed toward sibling and non-sibling normal larvae by paired *t* test. We ran this analysis separately at each developmental stage.

We further examined the intensity of agonistic behaviors displayed by comparing the maximally aggressive behavior, based on the AWI, demonstrated by each broad-headed larva toward siblings and non-siblings at each developmental stage. Statistical departure from random expectations at each stage was assessed by a resampling chi-square test based on Monte Carlo simulations ($n=2000$). Effects of relatedness, developmental stage, and their interactions were further evaluated by log-linear analysis.

Normal morph behavior The testing procedure allowed us to present the same normal larvae both to sibling and non-sibling broad-headed larvae. Normal larvae from two different clutches were tested in pairs, simultaneously presented first to one broad-headed larva and then 48 h later to another. The broad-headed larvae used in the sequential tests were chosen from the same two clutches as the normal larvae; thus, in every test, each normal larva was related to one or the other of the broad-headed larvae to which it was presented. Order of presentation was randomized. Normal larval individuals were held together in 1-L containers during the period between tests.

Based on blindly scored behaviors, we examined how the normal larvae responded to the broad-headed larvae using mean AWI scores as described above. We obtained two AWI scores for each individual normal larva, one with its sibling and one with a non-sibling broad-headed larva. As normal larvae were tested in pairs, their responses cannot be considered independent. Therefore, for each pair, we calculated from these scores mean responses directed to sibling and non-sibling broad-headed larvae. We then compared these means by paired *t* test.

We also compared each pair of normal larvae's maximally aggressive behavior directed toward sibling and non-sibling broad-headed larvae at each developmental stage. Departure from randomness was assessed by a resampling chi-square test based on Monte Carlo simulations ($n=2000$). Effects of relatedness, developmental stage, and their interaction were evaluated with a log-linear model as described above.

Behavioral analyses were conducted with Observer v. 3.0 (Noldus Information Technology, Wageningen, The Netherlands). For *t* tests, AWI scores were first transformed to natural logarithms so that they would meet normality assumptions. Monte Carlo bootstrapping was done with the `chisq.test` function in R v. 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria), and log-linear models were run in the CATMOD procedure of SAS v. 9.2 (SAS Institute, Cary, NC, USA). All statistical inferences were based on two-tailed distributions.

Experiment 2: are cannibals more likely to be infected by siblings or non-siblings?

Aeromonas hydrophila is an opportunistic bacterial pathogen commonly found infecting amphibians, as either a primary or secondary cause of disease. Clinical signs of red leg, associated with this bacterium, include reddening of legs and abdomen due to the dilatation of blood capillaries, bleeding, lethargy, weight loss, fluid accumulation, and skin ulcerations (Green 2010).

Animals and infection protocols We isolated *A. hydrophila* from the heart of an adult *Xenopus laevis* that had died of disease in a captive colony at Seoul National University shortly before beginning our experimental inoculations. The species identity of the bacterial isolate was confirmed by species-specific PCR (Cascón et al. 1996). The culture was maintained at 4 °C on Luria-Bertani (LB) agar plates with ampicillin to prevent competition from bacteria (Palumbo et al. 1985).

We reared larvae from stage 13, late gastrulation (Iwasawa and Yamashita 1991), in 52×36×20 cm polypropylene containers, as in the previous experiment, except that containers were divided into halves by 1×1 mm gray fiberglass screen mesh. We placed one clutch from each breeding site on either side of the mesh divider, so 70 larvae in total (35 from each clutch) were in each container. Between four and six larvae in each container developed into the broad-headed morph. Only one of these larvae cannibalized a normal larva in its container prior to testing. This larva was placed into a separate container (25.0×7.5×6.2 cm, with 3.5-cm slits, 0.5 cm wide, covered with 2×2 mm mesh) which was suspended within its previous container. This individual was excluded from subsequent testing.

To infect normal larvae that were to be presented to broad-headed subjects, we cut 3 mm from the tips of their tails and placed the normal larvae into individual rectangular 6×6×9 cm polyethylene terephthalate (PET) lidded containers in 50 mL filtered water. Liquid cultures of *A. hydrophila* were diluted with Tris-buffered saline (TBS) to 10⁸ mM. For inoculation, we pipetted 1 mL of the diluted *A. hydrophila* culture into containers holding the normal larvae. Individuals were left in this solution overnight and were presented to the

broad-headed larvae the next day. This method was determined after first testing slightly older larvae, with one group exposed to 10^8 mM *A. hydrophila* in LB broth and another exposed to *A. hydrophila* diluted to 10^8 mM with TBS.

Presentation protocol Two days prior to starting the experiment, we withheld food from broad-headed subjects. We placed subjects individually into covered 1-L polypropylene beakers filled with 500 mL filtered water. We ran 32 replicates, each consisting of two broad-headed larvae from a clutch, one presented with an infected sibling and the other an infected non-sibling. Although broad-headed larvae were larger in size than the normal larvae with which they were tested, all were at the same developmental stage (56). We checked the beakers every day to determine whether the broad-headed larva was alone with a bloated abdomen indicating that it had cannibalized the normal larva. Two days after a broad-headed larva ingested the normal larva, we euthanized the subject by freezing at -80 °C for later DNA extraction. If a broad-headed larva failed to cannibalize the normal larva within 1 week, we euthanized and preserved it.

Infection analyses We dissected out the heart and digestive organs of each subject and placed them into separate 1.5-mL microfuge tubes. We used a new sterile Petri dish for each subject, rinsed the subject with distilled water prior to dissection, skinned the body of the subject, and sterilized dissection tools between each step to prevent contamination. DNA was extracted using the Qiagen DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). We screened for the presence of *A. hydrophila* and quantified infection load by determining the numbers of copies of the pathogen's lipase gene (see below) by quantitative PCR (qPCR) using an Illumina Eco Real-Time PCR system (Illumina, San Diego, CA, USA). We tested each sample in triplicate, together with a standard of known bacterial DNA concentration, to calculate infection load. Amplification reactions contained 2 ng of DNA, 0.25 mM of each primer, and $1\times$ QuantiSpeed SYBR buffer (PhileKorea Technology, Seoul, South Korea) in 10 μ L total volume.

Primers were developed within the lipase gene described in Cascón et al. (1996) and did not amplify *H. leechii* DNA (5'-TATACCGGCACCGTCAAGCT-3' and 5'-ATCACTTCGCTGTCAGAGGC-3'). The PCR program included incubation at 50 °C for 2 min, activation at 95 °C for 10 min, 35 cycles of 95 °C for 10 s and 62 °C for 1 min, and a melt cycle consisting of 95 °C for 15 s followed by 55 °C for 15 s and 95 °C for 15 s. Positives in the digestive organs were taken as a sign of exposure to the pathogen from the infected normal larva, while positives in the heart indicated systemic infection by the pathogen. We pooled samples from multiple plates as all reactions

were completed on the same day using the same standard dilutions.

We used the mean of each individual's three replicate heart qPCR results to test whether cannibals of siblings and non-siblings had different infection loads (two-sample *t* test). Normality assumptions were met, and a modified formula for unequal variances used (Satterthwaite approximation; Littell and Strup 2002). We also tested whether broad-headed larvae that were fed siblings differed in incidence of clinical signs from those fed non-siblings using the binomial test. Some broad-headed larvae did not cannibalize the normal larva presented to them. These were used to test the possibility of infection through exposure to the bacteria in water. Statistical analyses were conducted in SAS v. 9.2. All statistical inferences were based on two-tailed distributions.

Results

Experiment 1: do larval *H. leechii* discriminate between siblings and non-siblings?

Broad-headed larvae Twenty-six different broad-headed larvae bit a normal larva during the experiment; 12 bit siblings and 14 bit non-siblings ($p=0.85$, binomial test). However, broad-headed larvae bit siblings sooner (latency to bite, 435 ± 114 s, $\bar{x} \pm \text{SE}$) than they did non-siblings (841 ± 139 s; $t_{24}=2.20$, $p=0.04$). Broad-headed larvae bit mostly siblings at stage 50, bit siblings and non-siblings about equally at stage 56, but bit only siblings at stage 62 (Fig. 1).

Based on their mean AWI scores, broad-headed larvae behaved more aggressively toward siblings early in development (stage 50, $t_{19}=2.80$, $p=0.01$). However, they were not aggressive and did not appear to discriminate between siblings and non-siblings midway through development (stage 56, $t_{19}=0.28$, $p=0.78$). As they approached metamorphosis, they actively avoided non-siblings (stage 62, $t_{19}=3.18$, $p=0.005$; Fig. 2).

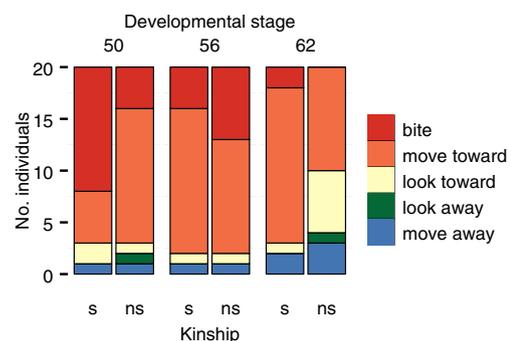


Fig. 1 Number of broad-headed larvae displaying each maximally aggressive behavior, broken down by their relatedness to the small-headed larva (s=sibling, ns=non-sibling) and developmental stage (see text)

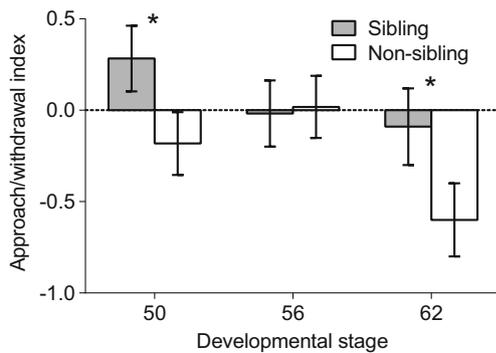


Fig. 2 Mean approach/withdrawal index (AWI) scores of broad-headed morph larvae as a function of developmental stage. Shaded bars represent behaviors directed at siblings and open bars behaviors toward non-siblings. Error bars denote \pm SE; $p < 0.05$, t test, two-tailed

Analyses of maximal levels of aggression reveal similar patterns. Broad-headed larvae showed higher maximally aggressive behaviors toward siblings at stage 50 ($\chi^2 = 8.89$, d.f. = 4, $p = 0.03$), but not at stage 56 ($\chi^2 = 1.18$, d.f. = 4, $p = 0.85$) nor stage 62 ($\chi^2 = 7.77$, d.f. = 4, $p = 0.07$). Maximally aggressive behaviors varied significantly with stage ($\chi^2 = 21.24$, d.f. = 8, $p = 0.006$), but by this measure, kinship discrimination did not significantly vary by stage ($\chi^2 = 0.07$, d.f. = 4, $p = 0.96$; Fig. 1).

Normal morph larvae Normal larvae were less aggressive than broad-headed larvae, as shown by their negative AWI scores (Fig. 3). At all developmental stages, normal larvae did not discriminate between sibling and non-sibling broad-headed larvae based on mean AWI scores (stage 50, $t_{19} = 1.16$, $p = 0.26$; stage 56, $t_{19} = 0.54$, $p = 0.59$; stage 62, $t_{19} = 0.37$, $p = 0.72$). However, like broad-headed larvae, normal larvae tended to withdraw from non-sibling broad-headed larvae more as they approached metamorphosis.

Analyses of maximal levels of aggression reveal a pattern quite different from that for broad-headed larvae, especially

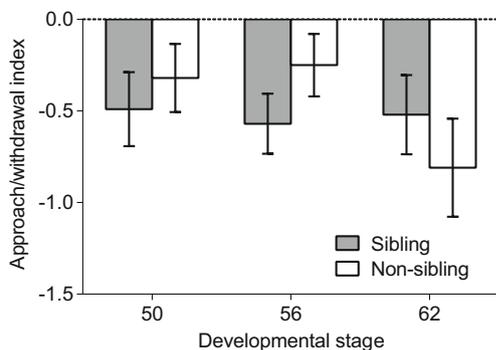


Fig. 3 Mean approach/withdrawal index (AWI) of normal morph larvae as a function of developmental stage. Shaded bars represent behaviors directed at siblings and open bars behaviors toward non-siblings. Error bars denote \pm SE

early in development. Normal larvae showed more aggressive behaviors toward non-sibling than sibling broad-headed larvae at stage 50 ($\chi^2 = 10.33$, d.f. = 4, $p = 0.02$), but not at stage 56 ($\chi^2 = 4.37$, d.f. = 4, $p = 0.43$) nor stage 62 ($\chi^2 = 4.65$, d.f. = 4, $p = 0.18$). Overall, maximally aggressive behaviors did not vary significantly with stage ($\chi^2 = 13.38$, d.f. = 8, $p = 0.10$) nor kinship ($\chi^2 = 0.07$, d.f. = 4, $p = 0.98$). Unlike broad-headed larvae, normal larvae bit mostly non-siblings at stage 50 but showed little tendency to bite either siblings or non-siblings thereafter (Fig. 4).

Experiment 2: are cannibals more likely to be infected by siblings or non-siblings?

Of the 64 broad-headed larvae tested, 43 cannibalized the normal larva offered to them: 20 cannibalized siblings and 23 cannibalized non-siblings ($p = 0.76$; binomial test). During the experiment, none of the normal morph larvae presented obvious clinical signs of *A. hydrophila* infection. After cannibalism, 13 broad-headed larvae displayed internal bleeding, a clinical sign of red leg typically associated with *A. hydrophila* infection. Fewer broad-headed larvae that ate siblings (4) displayed clinical signs than those that ate non-siblings (9); however, this difference is not significant ($p = 0.27$; binomial test).

All broad-headed larvae that ate infected subjects tested positive for infection in their heart and digestive tissues. By contrast, none of the subjects that failed to cannibalize showed signs of infection in their heart tissue. The mean infection load of cannibals that ate siblings (1933.2 ± 184.5 ; $\bar{x} \pm$ SE) did not significantly differ from that of those that ate non-siblings (1855.5 ± 253.1 ; $t_{41} = 0.24$, $p = 0.81$). However, the range of infection load for cannibals that ate siblings (3000 copies) was smaller than that for those that ate non-siblings (4389 copies). Variances of infection load were approximately twice as high in the non-sibling treatment as in the sibling treatment (Fig. 5).

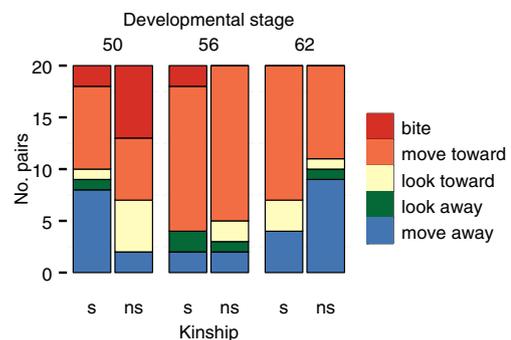


Fig. 4 Number of normal morph larvae pairs displaying each maximally aggressive behavior, broken down by their relatedness to the broad-headed larva (s=sibling, ns=non-sibling) and developmental stage (see text)

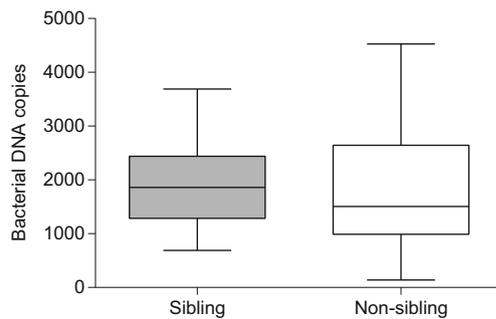


Fig. 5 Infection load of *A. hydrophila* in hearts of broad-headed morph larvae. Boxplots display median, interquartile range (*box*), and range (*whiskers*). Variances in infection load were approximately twice as high in the non-sibling as in the sibling treatment

Discussion

Can larval salamanders discriminate kin?

Broad-headed *H. leechii* larvae were able to discriminate between siblings and non-siblings from the earliest trial, 2 weeks after hatching. Tendencies to discriminate subsequently waned as larvae matured but reappeared as they approached metamorphosis. Discrimination between siblings and non-siblings occurred despite subjects having been reared together prior to testing. Broad-headed larvae directed biting, the most aggressive behavior, mostly toward their normal siblings (Fig. 1). By contrast, normal larvae only discriminated between sibling and non-sibling broad-headed larvae early in larval development, biting non-siblings and withdrawing from siblings (Fig. 4). Presumably, normal larvae bite broad-headed non-siblings to deter possible attacks. Otherwise, we found little evidence to suggest that normal larvae initiate encounters with broad-headed larvae.

The overt aggression that broad-headed morph *H. leechii* demonstrated toward their normal siblings in our study concurs with previous reports of preferential cannibalism of siblings both in *A. opacum* (Walls and Blaustein 1995) and *H. leechii* (Park et al. 2005). These results stand in stark contrast to studies of other larval salamanders that found aggression toward, or preferential cannibalism of, non-siblings (Pfennig et al. 1994; Wakahara 1997; Markman et al. 2009; Sadeh 2012). However, repeated studies on the same species sometimes yield apparently contradictory findings. *A. opacum* larvae are less aggressive toward siblings than non-siblings when both are similar in size (Walls and Roudebush 1991), yet preferentially cannibalize smaller siblings (Walls and Blaustein 1995). Many factors, including larval stage (this study), larval density (Kishida et al. 2015), presence of other species (Pomeroy 1981; Pfennig 1990; Michimae and Wakahara 2002), size differences among individuals (Brunkow and Collins 1998), and possibly the risk of disease transmission (Pfennig et al. 1991; see below), affect the social dynamics of salamander cannibalism.

Why were broad-headed larvae aggressive toward their siblings during the early larval period? Perhaps normal larvae, because of their more docile response to attack by their larger siblings, are easier prey. In the closely related species *H. retardatus*, cannibalism confers a strong growth advantage but only during the first 20 days after hatching (Michimae and Wakahara 2002). Our findings are consistent with these results. When broad-headed larvae were less likely to benefit from increased growth by cannibalism, their aggressive behaviors waned.

Broad-headed larvae also discriminated between siblings and non-siblings late in development, before completing the transition from an aquatic to a terrestrial lifestyle. As they approach metamorphosis, large larvae may be under intense selection to emerge from ephemeral habitat in which smaller larvae are very likely to perish. At this stage, broad-headed larvae appeared to actively avoid all conspecific larvae but especially non-siblings. Any cannibalism that occurred would likely be of siblings, perhaps representing altruistic kin self-sacrifice (Waldman 1982). Moreover, as immune system function is suppressed during metamorphosis (Ussing and Rosenkilde 1995), larvae then may be more at risk from pathogens transmitted by other individuals. As we discuss below, siblings may constitute less of an immunological challenge and thus be preferred as prey especially during this stage. Kin discrimination then may have increased adaptive value as larvae mature.

Very little is known about the natural history of *H. leechii*. After larvae metamorphose, they disperse into forests and can be found under leaf litter, rocks, and logs. In the spring, they return to ponds and streams where males may establish territories or follow females to fertilize deposited egg sacs. Male *H. leechii*, when breeding, use chemical cues to detect ovulating females (Park and Sung 2006). In turn, females sense the chemical cues emitted by males' tail wagging displays (Kim et al. 2009). Under typical breeding conditions for this species, relatives are likely to encounter one another. Mating with close relatives may result in inbreeding depression (Waldman and McKinnon 1993). Walls (1991) found that 8 months after metamorphosis, *A. opacum* were more aggressive toward siblings than non-siblings. Further studies of kin discrimination by *H. leechii* after metamorphosis are needed. As *H. leechii* use chemical cues during mating, possibly they have been selected to discriminate among potential mates based on kinship (Waldman 2005).

Are non-siblings more dangerous as transmitters of disease?

Aeromonas hydrophila was only transmitted through cannibalism of the infected normal morph larvae, and not through

the water. All broad-headed subjects that cannibalized normal larvae, whether siblings or non-siblings, tested positive for systemic *A. hydrophila* infection. Infection loads did not significantly differ between cannibals that ate siblings and those that ate non-siblings. However, broad-headed larvae that cannibalized non-siblings displayed more variability in infection load than those that cannibalized siblings. We only examined disease transmission with larvae midway through development (stage 56), so results might differ earlier or later in development.

In our study, the highest infection loads were present in cannibals that ate non-siblings, raising the possibility that non-relatives sometimes may be more infectious than relatives. Nonetheless, enhanced pathogen transmission among relatives has been clearly documented in other taxa (Shykoff and Schmid-Hempel 1991; Dharmarajan et al. 2012). Pathogen transmission through cannibalism has been amply demonstrated across a range of species (Pfennig et al. 1998; Pizzatto and Shine 2011; Le Clec'h et al. 2013), but prior to our work, only one study tested whether disease spreads more readily through kin cannibalism (Pfennig et al. 1999). Results of that study and ours, which demonstrate the same trend, are consistent with the hypothesis that kin cannibalize siblings to reduce their risk of becoming infected by non-relatives that harbor pathogens to which they lack resistance (Lewis 1998).

Salamanders, like other amphibians, currently are in the midst of major population declines in part attributable to infectious disease. Although the source population for our study showed no signs of disease, finding unhealthy salamanders in the wild is not unusual (e.g., Pfennig et al. 1991), and we have found clinical signs of disease in other *H. leechii* populations that we monitor. Previous research has shown that *H. leechii* larvae, when given a choice, cannibalize unhealthy conspecifics in preference to healthy ones (Park et al. 2005). Our experiment demonstrates that diseases are transmissible by cannibalism and that genetic relatedness between cannibal and victim may influence susceptibility to infection.

Cannibalism provides a conduit for direct transmission, not just for a single pathogen, but for a microbial community whose complex interactions make effects on hosts difficult to predict. Genetic relatives share microbial assemblages and immune-system genes that confer resistance against, and susceptibilities to, particular pathogens (Barribeau et al. 2012). Although the dynamics of pathogen transmission under various genetic backgrounds and social environments have been modeled, surprisingly few empirical studies have attempted to elucidate the importance of such factors on the spread of infectious disease through populations (Kubinak et al. 2012). Possibly, individuals that cannibalize their close kin may overcome the victim's total pathogen load more readily than those that cannibalize non-kin.

Conclusion

Cannibalistic salamander larvae readily discriminate between siblings and non-siblings as potential prey. Interactions appear most aggressive early in development, which should allow cannibalistic broad-headed larvae to accrue maximal growth benefits. While broad-headed larvae direct aggressive behaviors particularly toward their siblings, this may in part reflect some acquiescence of normal larvae to their siblings' attacks. The strongest kin discrimination, however, was apparent in broad-headed larvae late in development.

Metamorphosis is a complex process during which most bodily functions, including the immune system, are reorganized. During this period of immunosuppression, non-siblings appear to pose a more significant immunological threat than siblings to broad-headed larvae. In natural conditions, the ephemeral habitat in which salamanders live tends to vanish around this time. As the likelihood that larvae survive to metamorphosis decreases, so too does the inclusive fitness cost associated with cannibalism. How kinship influences discriminative cannibalism in amphibian larvae thus appears to be a more complex problem than previously recognized.

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Ethical standards The research was approved by the Seoul National University Institutional Animal Care and Use Committee (permit SNU-130115-1). Salamanders were collected under a permit issued by Gwanak-gu, Seoul Metropolitan City.

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