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이학석사학위논문

**Aggression in larval *Hynobiusleechii*: Kin
discrimination of polymorphic larvae and
relatedness as a factor in pathogen transmission**

***Hynobiusleechii* 유생의 공격성 :
다형성을 지닌 유생의 친족 식별과
병원체 전염 인자로서의 관련성**

2014 년 8 월

서울대학교대학원

생명과학부

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**Aggression in larval *Hynobiusleechii*: Kin discrimination of
polymorphic larvae and relatedness as a factor in pathogen
transmission**

A Thesis presented by **Samantha Wojciechowski**

Supervised by
Professor **Dr. Bruce Waldman**

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Abstract

Aggression in larval *Hynobiusleechii*: Kin discrimination of polymorphic larvae and relatedness as a factor in pathogen transmission

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Larvae of some amphibians can develop into two distinct morphological types, a large-headed morph adapted to cannibalize the other, small-headed morph. Cannibalism confers nutritional benefits that can accelerate development, essential for survival in transient environments, but incurs potential inclusive fitness costs. Selective cannibalism of non-kin thus should be favored, but siblings share immunological defenses. Thus subjects may be more vulnerable to pathogens of non-siblings, to which they are naïve. I investigated kin discrimination and pathogen transmission among Korean salamander (*Hynobiusleechii*) larvae. I placed large-headed morph larvae into a circular arena together with two small headed-morph larvae, one sibling and one non-sibling, recorded behavioral interactions among them, and scored the aggressiveness of their interactions. In a second experiment, I fed large-headed morph larvae either a sibling or non-siblings small-headed morph larva that I had previously infected with the pathogenic bacterium *Aeromonashydrophila*. Two days after ingestion, I euthanized the

cannibals and determined by qPCR whether the bacterium had infected their hearts. Large-headed morph larvae behaved more aggressively toward siblings than non-siblings but only late in development. Some large-headed morph larvae that ate non-siblings had higher infection loads than those that ate siblings, but infection loads varied more widely when non-siblings were cannibalized. Cannibalizing non-siblings thus may increase the risk of contracting disease. However, large-headed morph larvae do not discriminate between siblings and non-siblings until late in development when inclusive fitness costs of cannibalizing siblings may be diminishing.

Keyword: Kin recognition, cannibalism, pathogen transmission

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1. Introduction:

Cannibalism is present in many forms of life, from lower eukaryotes to birds and primates (Elgar and Crespi, 1992). Cannibalism also occurs frequently in amphibians, most commonly in larvae (Crump, 1992). Some larval amphibians, such as those of the spadefoot toad *Scaphiopus bombifrons* and the tiger salamander *Ambystoma tigrinum* exhibit developmental plasticity so that even among siblings of the same clutch, some develop into a “cannibal” or large-headed morph that appears specialized to prey upon the smaller, more “typical” or small-headed morph (Pfennig and Collins, 1993; Pfennig et al., 1993). Large-headed morphs have a broader, larger head along with specialized feeding features such as enlarged vomerine teeth (Pedersen, 1991; Wakahara, 1995), whereas small-headed morphs have a narrower head, lack enlarged teeth and eat small invertebrates and zooplankton.

Larval cannibalism in amphibians can confer benefits on the cannibals. Cannibals grow more quickly, gain more mass (Wildy et al., 1998) and metamorphose sooner than small-headed morph larvae (Michimae and Wakahara, 2002). As vernal ponds begin to dry up, faster development is strongly selected (Lannoo and Bachmann, 1984). Cannibals also reproductively mature sooner, potentially raising their lifetime reproductive success (Lannoo et al., 1989). But cannibalism also incurs costs. Cannibals may be injured when struggling with similarly sized prey (Pfennig et al., 1998), they may incur increased risk of disease by eating conspecifics (Pfennig et al., 1998), and they may suffer decreased inclusive fitness by killing kin (Walls and Rousebush, 1991). However, kin cannibalism may evolve in certain circumstances such as when the cannibalized individuals have little

chance of metamorphosing themselves before their pond dries. Then, they would increase their inclusive fitness by offering themselves to their larger siblings (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 Arizona 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) in experimental tests prefer to attack and eat non-kin but the Korean salamander *H. leechei* prefers to eat their kin (Park et al., 2005). Behavioral analyses showed that large-headed marbled salamander (*A. opacum*) larvae were less aggressive toward their kin than non-kin (Walls and Rouse, 1991) but preferred to eat their siblings even when non-siblings were available as food (Walls and Blaustein, 1995). Although the tendency of amphibians to avoid cannibalizing kin is often 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 transmission.

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). 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 mediates adaptive immune system function (Villinger and Waldman, 2008). 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.

If one cannibalizes an infected relative, however, the cannibal might incur an elevated risk of contracting disease. As siblings are immunogenetically similar, their immune systems are likely to have common vulnerabilities for pathogens or parasites to exploit. Eating a sick sibling exposes one to potentially dangerous parasites or pathogens for one’s genotype. 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 a 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 siblings (Pfennig et al., 1999). Across taxa, the few data available suggest that sometimes pathogens are more readily transmitted among more closely related animals, but sometimes not, and effects may vary by pathogen (Dharmarajan et al., 2012; Shykoff and Schmid-Hempel, 1991). Possibly the role of cannibalism in transmitting diseases may have been overstated, for cannibalism is unlikely to be a primary mode of pathogen transmission (Rudolf and Antonovics, 2007).

Among the urodeles, hynobiid salamanders represent the most archaic lineage and their

development of large-headed morphs as larvae suggests that this developmental polymorphism may be an ancestral character. I studied agonistic behavior and cannibalism in larvae of the polymorphic Korean salamander, *Hynobius leechii*. Most hynobiids hatch in streams and ponds when food is not plentiful (Michimae and Wakahara, 2002). The development of a distinct large-headed morph previously was documented in this species (Park et al., 2005) and may be dependent on availability of food resources including larval anurans (Michimae and Wakahara, 2002). I investigated the abilities of small-headed and large-headed morphs to recognize kin, compared levels of agonistic behavior of the two morphs, and asked whether aggressive behaviors are more likely to be directed toward kin or non-kin. I also tested how the infectivity and virulence of the bacterial pathogen, *Aeromonas hydrophila*, varies in relation to the genetic relatedness of cannibals to their victims.

2. 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 21st 2013. Site 1 (37° 27.401 N, 126° 57.414 E), a pond, and site 2 (37° 27.874 N, 126° 57.756 E), a pool in a lentic stream, 1 km apart on northern side of the mountain. Ten egg clutches were taken from each site for the first experiment, and 32 were taken from each breeding site for the second experiment.

Experiment 1: Do cannibals discriminate between siblings and non-siblings?

Larvae were reared in 52cm x 36cm x 20cm polypropylene tanks filled with 20L of UV-treated dechlorinated and filtered water at 14°C, and were kept on a 12:12 LD cycle. Clutches were kept separate until the larvae developed sufficiently that they could be easily handled, about 2 weeks after hatching (stage 50 – the loss of balancers and the beginning of forelimb toe differentiation; Iwasawa and Yamashita, 1991). Then, to mark individuals, I injected into the tail of each larva a 3mm line of acrylic paint (Alpha Color, Seoul, South Korea) by syringe with a 23-gauge hypodermic needle. Subjects 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. This allowed me to identify individuals but also control for possible effects of marking, such as preferences towards a specific color. Water was changed every 4 days and larvae were fed live *Tubifex* worms once

a day ad libitum.

One day after marking I placed all marked larvae from different breeding ponds into 52cm x 36cm x 20cm blue polypropylene plastic containers. Each clutch was paired with a clutch from the other breeding site to ensure there were no paternal half-siblings in each experiment; i.e. individuals were tested against full siblings and non-siblings. Individuals from different clutches were allowed to acclimate to one another for 24 h to remove any “dear enemy” effects (Jaeger, 1981), and during this period I did not feed the larvae. Subjects tested together thus all were familiar with each other from the time they were marked until testing.

To test their responses to one another, I placed three larvae—one large-headed morph, one of its small-headed morph siblings, and one small-headed morph non-sibling—into a clear, plastic, circular arena filled with 1 L of filtered water in a dark room. I recorded larval behavior with a camcorder (Sony DCR-SR82) with night vision capabilities. The infrared light of the camcorder cast a weak red hue (<2.5 lux) in the visible spectrum onto the apparatus. As the larvae normally are nocturnally active, I ran the trials between 8 pm and 6 am. We identified 34 larvae as large-headed morphs by their head shape. We conducted experiments on these large-headed subjects with 120 small-headed larvae as stimuli. Each large-headed morph was tested twice with small-headed subjects having different combinations of color markings. The experiment was repeated three times during development, during stage 50 (2 weeks from hatching; the beginning of forelimb differentiation), stage 56 (5 weeks from hatching; the beginning of hind limb differentiation), and stage 62 (8 weeks from hatching; all limbs fully

developed), using different subjects each time.

An observer, “blind” to treatment conditions, watched recordings on a 35 x 27.5 cm color monitor and recorded the time and sequence of key behaviors of all larvae in the arena using Observer 3.0 software (Noldus Information Technology, Wageningen, The Netherlands). After a 5-min acclimation period, behaviors were scored as: moving away, looking away, looking toward, moving toward, and bite (Table 1). The observer scored “moving away” when the focal subject made a quick movement in the opposite direction of the other larva, “looking away” when the focal subject turned its head in the opposite direction of the other larva, “looking 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 determine whether a focal subject bit another larvae, the observer and I confirmed that the subjects opened their mouths by reviewing the video at 1/6th speed using VLC Media Player.

Table 1: Larval salamander ethogram.

Rank	Behavior	Description
1	Move Away (MA)	Larva curls its body and springs away from another larva.
2	Look Away (LA)	Larvae turns only its head, orienting it away from conspecific
3	Look Toward (LT)	Larva turns only its head, oriented it toward conspecific
4	Move Toward (MT)	Larva moves forward on a course that, if continued, will put in contact with another larva.
5	Bite (B)	Larva opens its mouth and lunges at conspecific, either successfully or unsuccessfully closing its mouth on the other larva.

I assigned approach/withdrawal scores to each behavior on an ordinal scale: 1 Move Away, 2 Look Away, 3 Look Toward, 4 Move Toward, 5 Bite. Using these values I calculated mean approach/withdrawal indices (AWI) for each subject in response to siblings and non-siblings. I analyzed the AWI indexes according to morphotype, stage, and kinship using a two-way ANOVA with kinship, stage, clutch, and their interactions as independent variables. Analyses were conducted with the GLM procedure in SAS 9.4.

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

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

I 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 an LB agar plated with ampicillin to prevent competing bacteria from growing (Palumbo et al., 1985).

I reared larvae from stage 13, late gastrulation (Iwasawa and Yamashita, 1991), in 52 x 36 x 20 cm blue polypropylene tanks, as in the previous experiment, except divided in half by 1 x 1 mm black fiberglass screen mesh. I placed one clutch from each breeding site on each side of the mesh.

To infect small-headed larvae that were to be presented to large-headed subjects, I cut 3 mm from the tips of their tails and placed the small-headed larvae into individual rectangular 6 x 6 x 9 cm polyethylene terephthalate lidded containers in 50 ml of filtered water. Liquid cultures of *A. hydrophila* were diluted with TBS to 10^8 mM. For inoculation, 1 ml of the diluted *A. hydrophila* culture was pipetted into containers holding the larvae. Larvae were left in this solution overnight and fed to the large-headed morphs the next day. This method was tested using 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.

I placed one large-headed morph into each covered 1,000 mL beaker with 500 mL of filtered water. I ran 32 replicates, each consisting of two large-headed morphs from a clutch, each fed an infected small-headed morph but one a sibling and the other a non-sibling from the same tank as the large-headed morph. From each clutch, I took two large-headed morphs and did not feed them for two days prior to starting the experiment. I checked the beakers every day to determine whether the large-headed morph was alone with a bloated belly indicating that the large-headed morph cannibalized the small-headed morph. Two days after the large-headed morph ingested the small-headed morph, I euthanized the large-headed morphs by freezing at -80°C for later

DNA extraction. If a large-headed morph failed to cannibalize the small-headed morph within 1 week, I euthanized and preserved the large-headed morph.

I dissected out the heart and digestive organs of each subject and placed them into separate 1.5 ml microfuge tubes. To ensure no contamination of the internal organs by external contaminants, I 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 of the dissection. DNA extraction was done using the Qiagen DNeasy blood and tissue kit.

I screened for the presence of *A. hydrophila* and quantified infection load using an Illumina Eco Real-Time PCR thermocycler, testing each sample in triplicate. Amplification reactions contained 2 ng of DNA, 0.25 mM of each primer, and 1X QuantiSpeed SYBR buffer in 10 μ L.

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 sec and 62°C for 1 min, and a melt cycle consisting of 95°C for 15 sec followed by 55°C for 15 sec and 95°C for 15 sec. Positives in the digestive organs were taken as a sign of exposure, while a positive in the heart indicated infection. I pooled samples from multiple plates as all reactions were completed the same day using the same standard dilutions.

I used the mean of each individual's three replicate heart qPCR results to statistically test whether cannibals of siblings and non-siblings had different infection loads (t-test, two-tailed). Normality assumptions were met, and a modified formula for unequal variances used. I also tested whether large-headed morphs fed siblings had different rates of clinical signs than those fed non-siblings

higher rates of clinical signs (binomial test).

3. Results

Do cannibals discriminate between siblings and non-siblings?

Large-headed morphs approached and tended to be aggressive more often toward their small-headed siblings (AWI: 2.51 ± 0.10 , $\bar{x} \pm \text{SE}$) than non-siblings ($\bar{x} \pm \text{SE}$ 2.26 ± 0.10 ; $F_{1,129} = 5.46$, $p = 0.02$). Large-headed morphs significantly varied their behaviors by developmental stage ($F_{2,129} = 3.95$, $p = 0.03$); however there was no interaction effect between relatedness and developmental stage ($F_{2,129} = 2.05$, $p = 0.14$). There was no significant effect of clutch on the large-headed morphs' behavior ($F_{19,129} = 1.52$, $p = 0.12$), or on the interaction between relatedness and clutch ($F_{19,129} = 0.65$, $p = 0.85$). However, the AWI of a clutch was dependent on the clutch's developmental stage ($F_{38,129} = 2.13$, $p = 0.01$).

Figure 1: Large-headed morph behavior

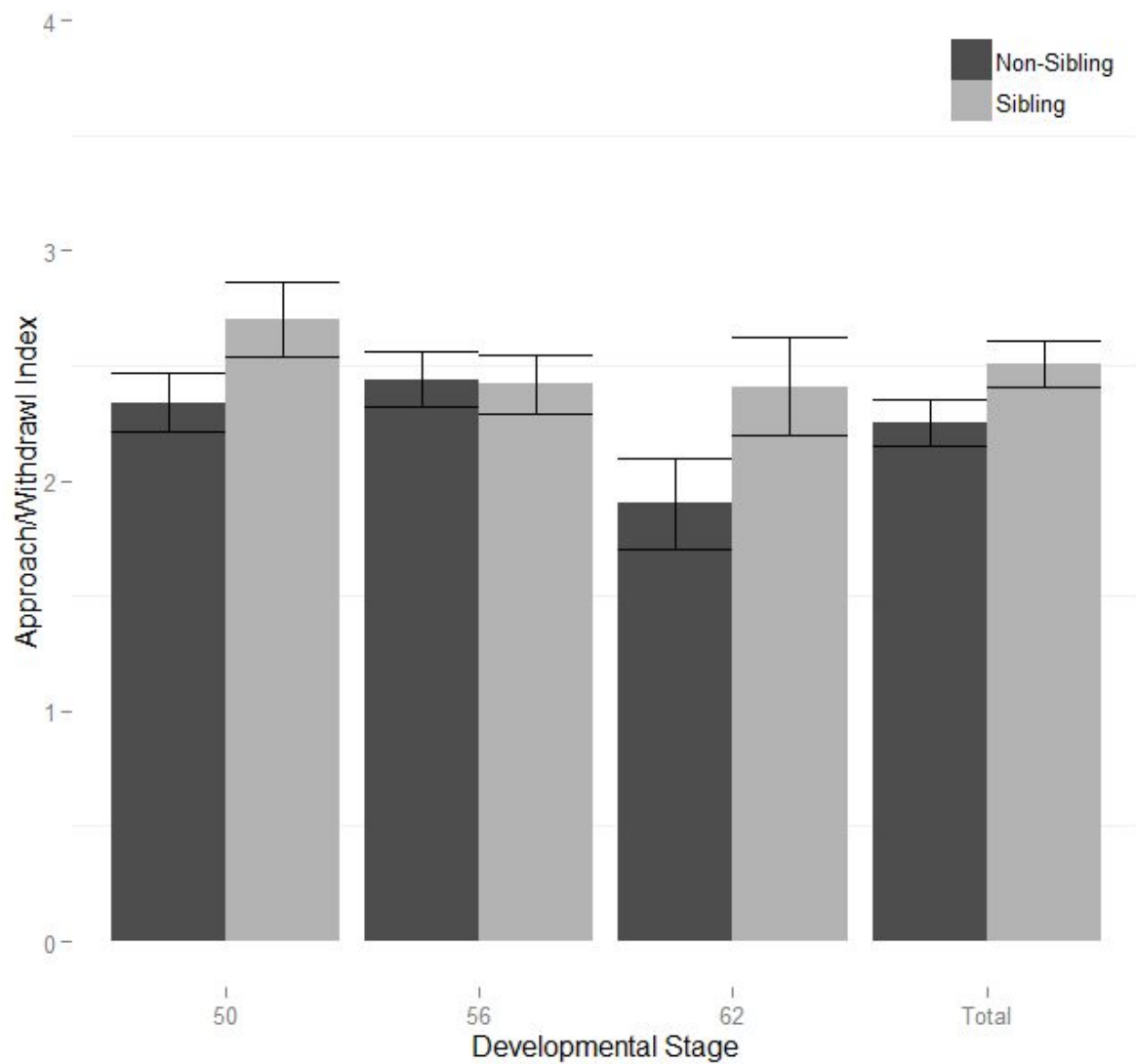


Fig 1: The mean aggression level of large-headed morphs \pm SE for weeks 2, 5, and 8. Dark bars represent behaviors directed at non-siblings, and light bars behaviors towards siblings. Overall, the mean AWI towards siblings was greater than that directed at non-siblings. Week 8 is different from Weeks 2 and 5 in that the mean AWI is reduced much more in non-siblings than it had been previously.

Small-headed morphs did not differ in their approach/withdrawal tendencies toward their large-headed siblings (AWI: 2.00 ± 0.10) and non-siblings (2.04 ± 0.10) ($F_{1,239} = 0.30$, $p = 0.58$). Their behaviors did not vary by developmental stage ($F_{2,239} = 2.06$, $p = 0.13$), nor did these kinship effects significantly vary with developmental stage ($F_{2,239} = 2.35$, $p = 0.10$). Small-headed morphs's behavior did significantly differ by clutch ($F_{19,239} = 2.37$, $p = 0.002$). There was no interaction between clutch and relatedness ($F_{19,239} = 0.94$, $p = 0.54$), but there was an interaction between clutch and developmental stage ($F_{38,239} = 2.14$, $p = 0.001$).

At the latest developmental stage, stage 62, both large-headed and small headed morphs had a higher AWI towards siblings, although this was only significant in large-headed morphs. Large-headed morphs at stage 62 also had a greater difference in AWI between siblings and non-siblings. This difference is attributable to a reduction of aggression towards non-siblings and not an increase in aggression towards siblings. A similar trend is present in small-headed morphs, with a decreased AWI towards non-siblings, but similar AWI towards siblings.

Figure 2: Small-headed morph behavior

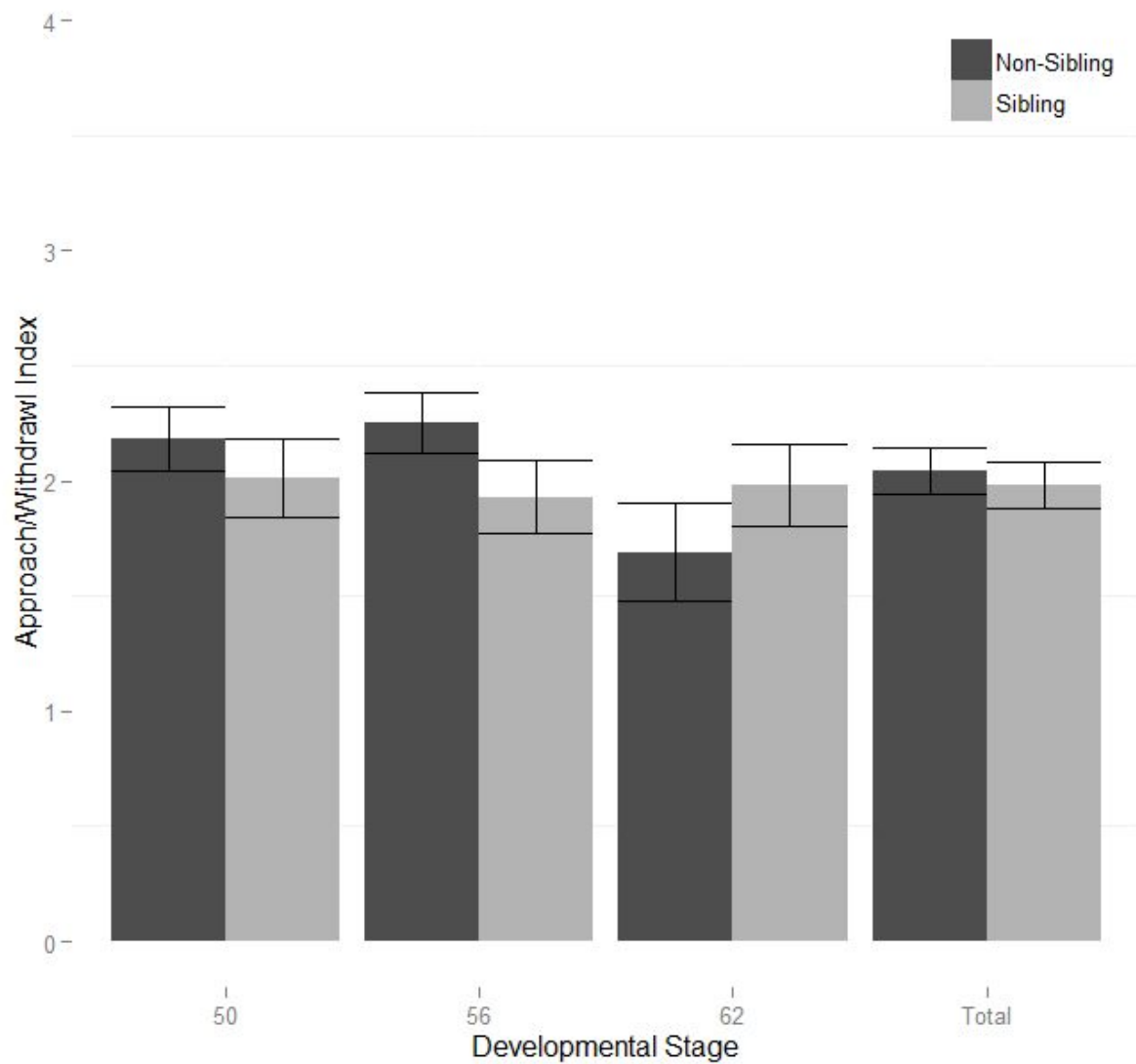


Fig 2: The mean aggression level of small-headed morphs \pm SE for weeks 2, 5 and 8. Dark bars represent behaviors directed at non-siblings, and light bars behaviors towards siblings. There were no differences in small-headed behavior towards siblings or non-siblings at any point in the experiment. However, developmental stage 62 is close to being significantly different from the others. At developmental stage 62, small-headed morph behavior shifted to

having a higher AWI towards siblings than non-siblings, as opposed to the similar AWI towards siblings and non-siblings previously.

Table 2: Results of the ANOVA for the effects of kinship and month on *H. leechii* behavior.

Variables	Df	Mean square	F	p
Large-headed morph				
Relatedness	1	2.16	5.46	0.02
Developmental Stage	2	1.56	3.95	0.03
Clutch	19	0.60	1.52	0.12
Relatedness x Developmental Stage	2	0.80	2.05	0.14
Relatedness x Clutch	19	0.26	0.65	0.85
Developmental Stage x Clutch	38	0.84	2.13	0.0006
Error	48	0.39		
Small-headed morph				
Relatedness	1	0.26	0.30	0.58
Developmental Stage	2	1.77	2.06	0.13
Clutch	19	2.03	2.37	0.002
Relatedness x Developmental Stage	2	2.02	2.35	0.10
Relatedness x Clutch	19	0.81	0.94	0.54
Developmental Stage x Clutch	38	1.84	2.14	0.0006
Error	239	0.86		

Are cannibals more likely to be infected by siblings or non-siblings?

Of the 64 large-headed morphs, 43 cannibalized the small-headed morph offered to them, 20 cannibalized siblings and 23 cannibalized non-siblings. I used these 43 large-headed morphs in my analysis. Thirteen large-headed morphs displayed clinical signs of “red leg”, typically caused by *A. hydrophila* infection, 4 from the sibling treatment and 9 from the non-sibling treatment. Fewer large-headed morphs that ate siblings displayed clinical signs than those that ate non-siblings; however, this was not significant ($p = 0.13$, binomial test). All 43 large-headed morphs tested positive in both the heart and intestine. Mean infection load did not differ between cannibals that ate siblings (1933.2 ± 184.5 ; $\bar{x} \pm \text{SE}$) and non-siblings (1855.5 ± 253.1) ($t_{41} = 0.24$, $p = 0.81$). However, the range of infection load for cannibals that ate siblings (3,000 copies) was smaller than those that ate non-siblings (4,389 copies), and variances of infection load were twice as high in the non-sibling treatment as in the sibling treatment (Bartlett’s test, $\chi^2 = 2.84$, $p = 0.09$).

Figure 3: Infection load on cannibal morphs

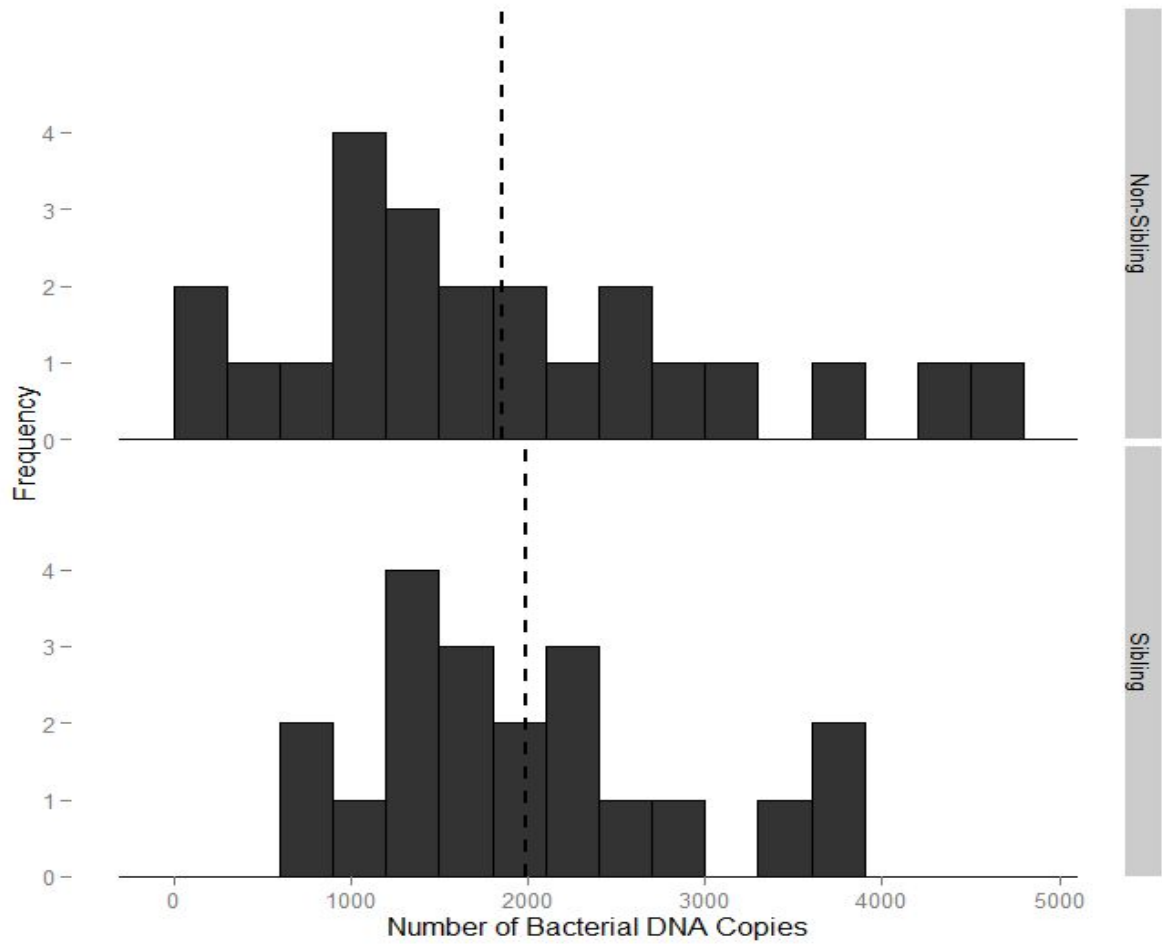


Fig 3: Histogram displaying distribution and mean number of DNA copies of *A. hydrophila* in hearts of cannibal morphs. Mean represented by dashed line. Large-headed morphs that cannibalized siblings have a tighter curve around the mean than those that cannibalized non-siblings.

4. Discussion

Can larval salamanders discriminate kin?

Larval *H. leechii* large-headed morphs were able to discriminate between siblings and non-siblings from the earliest trial, two weeks after hatching. This discrimination occurred even though they were familiar with both siblings and non-siblings, and continued throughout the larval period. In contrast, small-headed morphs displayed no significant behavioral differences between sibling and non-sibling large-headed morphs at any time in the experiment. In *H. leechii*, large-headed morphs appear to be kinship discriminators, while small-headed morphs either cannot or do not discriminate.

During the three-month experiment, large-headed morphs behaved on average more aggressively towards their siblings. This overt aggression towards siblings concurs with studies documenting preferential cannibalism of siblings both in *Ambystoma opacum* (Walls and Blaustein, 1995) and *H. leechii* (Park et al., 2005). Unlike *A. opacum*, *H. leechii* displays more aggressive behavior towards siblings and cannibalizes them preferentially. However, results on *A. opacum* are puzzling as larvae displayed less aggression toward siblings than non-siblings (Walls and Roudebush, 1991), yet preferentially cannibalized siblings (Walls and Blaustein, 1995). Walls and Roudebush (1991) did not test large-headed morph individuals as I did here and instead tested similarly sized larvae. Walls and Blaustein (1995) and Park et al. (2005) tested large-headed morphs as I did. Larval

salamanders of other species, when of equal size, tend to be less aggressive toward relatives than non-relatives (Markman et al., 2009). Size differences affect levels of aggression among salamander larvae (Brumkow and Collins, 1998), with smaller larvae having different behavioral patterns than larger larvae. The two previous studies on large-headed morph behavior (Walls and Blaustein, 1995, Park et al., 2005) along with my study, collectively demonstrate that large-headed morphs preferentially cannibalize siblings.

In this study, large-headed morphs tended to shift their behavioral patterns to be more aggressive towards kin at the end of the larval phase, suggesting that *H. leechii* changes its behavior patterns before completing the transition from an aquatic to a terrestrial lifestyle. In the closely related species *H. retardatus*, cannibals experienced increased growth only within the first 20 days of hatching at the apparent cost of reduced growth later in development (Michimae and Wakahara, 2002). The change in *H. leechii*'s aggression towards non-siblings coincides with the change in the growth curve of cannibals, so increased growth from cannibalizing siblings is unlikely the reason for the increased aggression. Larval marbled salamanders, *A. opacum*, were more aggressive toward non-siblings than siblings (Walls and Roudebush, 1991), but 8 months after metamorphosis were more aggressive towards siblings than to non-siblings (Walls, 1991). This behavioral shift may reflect a shift from larval kin cooperation to inbreeding avoidance as adults (Walls, 1991). As they approach metamorphosis, larvae may be under increased pressure to emerge from ephemeral habitat in which their smaller siblings are very likely to perish in any case. Moreover, as immune system function is suppressed during metamorphosis (Ussing and Rosenkilde, 1995), larvae may be more at risk from pathogens transmitted from kin. Thus, kin discrimination may have increased adaptive value as larvae mature.

Are non-siblings better transmitters of diseases?

All large-headed morphs that cannibalized siblings tested positive for *A. hydrophila*, the pathogenic bacteria I used to infect small-headed victims. Large-headed morphs that cannibalized non-siblings also all tested positive for *A. hydrophila*, and no large-headed morph avoided disease transmittance when cannibalizing a non-sibling. Observations that disease transmission is more likely when preying on related species (Pfennig et al. 1998; Pfennig, 2000) led Pfennig (1997) to conjecture that sibling cannibalism posed more risk than feeding on non-relatives. Since siblings share MHC alleles, and therefore adaptive immune responses to particular pathogens, disease certainly might be more readily transmitted among siblings (Barribeau et al. 2012). However, my study provides no support for this hypothesis.

Large-headed morphs that cannibalized non-siblings displayed clinical signs more frequently, and showed more variability in infection loads, than those that cannibalized siblings. Indeed, large-headed morphs with the highest infection load were in the non-sibling treatment, suggesting that cannibalizing non-siblings might be riskier than cannibalizing siblings. Testing the hypothesis that large-headed morphs prefer to cannibalize non-siblings to avoid pathogen transmission, Pfennig et al. (1998) found, contrary to their predictions, that subjects that cannibalized siblings were more likely to survive. Results of this study, consistent with those presented by Pfennig et al. (1998), suggest that non-relatives are more infectious than non-relatives, at least for bacterial diseases transmitted by cannibalistic salamanders. Both studies support the hypothesis that kin

might cannibalize siblings to avoid pathogens because individuals with different MHC are more likely to transmit pathogens (Lewis, 1998).

Nonetheless, enhanced pathogen transmission among relatives has been clearly documented in other taxa (Skykoff and Schmid-Hempel, 1991; Dharmarajan et al., 2012). Pathogen transmission through cannibalism has been amply demonstrated across a range of species (Pfennig and Hoffman, 1995; Le Clec'h et al., 2013; Pizzatto and Shine, 2011), but prior to my work, only one study tested whether disease spreads more readily through kin cannibalism (Pfennig, 1998). Dharmarajan et al. (2012) argued that because pathogens usually are shared among relatives living in close proximity, they likely would share defenses against indirectly transmitted pathogens from relatives but not those from non-relatives. Cannibalism provides a conduit for direct transmission, not just for a single pathogen, but for a microbial community for which complex interactions make effects on hosts more difficult to predict. Possibly individuals that cannibalize their close kin may overcome the victim's total pathogen load more readily than those that cannibalize non-kin.

5. Conclusion

I found that *H. leechii* large-headed morphs approach and act more aggressively toward siblings, possibly leading to preferential cannibalism of their close relatives. Large-headed and small-headed morphs were better able to discriminate kin as they developed and approached metamorphosis. Larvae that ate their siblings were less likely to show signs of clinical infection and had less variable infection loads than those that ate non-siblings. Older larvae close to metamorphosis are better equipped to discriminate kin and avoid cannibalizing risky, high pathogen transmitting non-kin, and are less likely to benefit from increased growth by cannibalizing. Potentially it will be more important for any late stage cannibalizing large-headed morphs to clear infections quickly and efficiently, which will be more likely by cannibalizing kin.

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초록

***Hynobiusleechii* 유생의 공격성 :**
다형성을 지닌 유생의 친족 식별과
병원체 전염 인자로서의 관련성

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어떤양서류의유생은두가지의구분되는형태로발달할수있는데하나는동족포식에유리한큰머리를가진형태이고또다른하나는작은머리를가진형태이다.

동족포식은일시적인환경에서의생존에필수적인발달을촉진시키는영양적인이익을주지만잠재적인적응비용을감수해야한다. 따라서친족을제외한선택적인동족포식은선호되어야하지만,같은부모의유생들은면역적방어를공유한다.

그러므로그들은다른부모의유생들의병원체에더취약하고더무방비할것이다.

나는한국도롱뇽[Korean salamander (*Hynobiusleechii*)] 유생의친족식별과병원체전염을조사했다.

나는큰머리유생을같은부모로부터태어난유생한마리와다른부모로부터태어난작은머리유생한마리와함께원형의구역에넣고그들간의행동적상호작용과그들의상호작용의공격성을기록했다.

두번째실험에서,

나는큰머리의유생에게이전에병원성세균 *Aeromonashydrophila* 으로감염시켰던같은부모로부터태

어난작은머리유생이나다른부모로부터 태어난작은머리유생을먹이로주었다.

섭취후이틀뒤,나는동족포식했던큰머리유생을안락사시키고세균이그들의심장을감염시켰는지 qPCR 로확인했다.

큰머리유생은발달후기에만다른부모로부터 태어난유생보다같은부모로부터 태어난유생에대하여 더공격적으로행동했다.다른부모로부터 태어난유생을먹은몇몇큰머리유생은같은부모로부터 태어난유생을먹은집단보다더많이감염되었다.

따라서다른부모로부터 태어난유생을동족포식하는것은질병에전염될위험성을증가시키는것같다. 하지만,큰머리유생들은같은부모로부터 태어난유생을동족포식하는적응비용이감소하게되는발달후기가되기전에는,같은부모로부터 태어난유생과아닌유생을식별하지않는다.

주요어: 혈연인식, 동종포식, 병원균저염

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