

## ADDING INFECTION TO INJURY: SYNERGISTIC EFFECTS OF PREDATION AND PARASITISM ON AMPHIBIAN MALFORMATIONS

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**Abstract.** We explored the importance of interactions between parasite infection and predation in driving an emerging phenomenon of conservation importance: amphibian limb malformations. We suggest that injury resulting from intraspecific predation in combination with trematode infection contributes to the frequency and severity of malformations in salamanders. By integrating field surveys and experiments, we evaluated the individual and combined effects of conspecific attack and parasite (*Ribeiroia ondatrae*) infection on limb development of long-toed salamanders (*Ambystoma macrodactylum*). In the absence of *Ribeiroia*, abnormalities involved missing digits, feet, or limbs and were similar to those produced by cannibalistic attack in experimental trials. At field sites that supported *Ribeiroia*, malformations were dominated by extra limbs and digits. Correspondingly, laboratory exposure of larval salamanders to *Ribeiroia* cercariae over a 30-day period induced high frequencies of malformations, including extra digits, extra limbs, cutaneous fusion, and micromelia. However, salamander limbs exposed to both injury and infection exhibited 3–5 times more abnormalities than those exposed to either factor alone. Infection also caused significant delays in limb regeneration and time-to-metamorphosis. Taken together, these results help to explain malformation patterns observed in natural salamander populations while emphasizing the importance of interactions between parasitism and predation in driving disease.

**Key words:** *Ambystoma macrodactylum*; amphibian limb abnormalities; deformities; disease; long-toed salamander; malformations; parasitism; predation; *Ribeiroia cercariae*; *Ribeiroia ondatrae*; synergism; trematode infection.

### INTRODUCTION

Ecologists are increasingly recognizing the importance of reciprocal interactions between parasitism and predation in controlling disease levels (Packer et al. 2003, Ostfeld and Holt 2004). Often these interactions are antagonistic; many predators selectively remove infected prey from a population (e.g., Hudson et al. 1992, Duffy et al. 2005), potentially reducing the period of time during which infection can spread to new susceptible hosts. However, interactions between predators and parasites can be complex, particularly if sublethal predator effects, indirect parasite transmission, pathogen evolution, or time delays in predator–prey–parasite dynamics are considered (Choo et al. 2003, Joly and Messier 2004). In some cases, predation is likely to enhance disease, particularly if predators directly or indirectly cause an increase in infection. Predators that consume infected prey often acquire their parasites. Although common among multi-host parasites, this phenomenon can also occur for a variety of viruses,

bacteria, and prions. Predation also can cause increases in infection by changing the behavior or habitat use of prey in ways that expose them to parasites (e.g., Thiemann and Wassersug 2000). Decaestecker et al. (2002), for example, found that predatory fish caused *Daphnia* to spend more time at or near pond sediments, exposing them to higher concentrations of parasite spores. Similarly, predation-related injury can lead to increased secondary infection and associated pathology (e.g., Walls and Jaeger 1987).

Here we explore the importance of interactions between parasitism and predation in driving an emerging phenomenon of conservation importance: amphibian limb malformations. Although predation-related injury and parasite infection have each been implicated in recent accounts of limb abnormalities (see Blaustein and Johnson 2003), no study has examined the combined contribution of these factors. Field surveys and experiments have recently established trematode infection (*Ribeiroia ondatrae*) as a widespread cause of amphibian limb malformations, including missing, malformed, and extra limbs or digits (Sessions and Ruth 1990, Johnson et al. 1999, 2002, Schotthoefler et al. 2003). Deformities often occur at high frequencies (>20%) and are expected to impair locomotion and

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survival, potentially contributing to localized population declines (Blaustein and Johnson 2003). Similarly, injury resulting from predation by aquatic insects, leeches, crayfish, small fishes, and other amphibians can be important in amphibian populations (e.g., Brodie and Formanowicz 1983, Fauth 1990, Figiel and Semlitsch 1991, Wildly et al. 2001). Although not well studied, these injuries sometimes occur at high frequencies and frequently affect the limbs (Licht 1974, Bohl 1997, Johnson et al. 2001a, b). Johnson et al. (2001b), for example, found that 15–80% of California newt larvae exhibited severe limb injuries, including missing feet and limbs. The authors speculated that predation by mosquitofish was responsible (see Gamradt and Kats 1996).

Larval salamanders are an excellent model system in which to examine interactions between parasitism and predation. In addition to predation by fish and invertebrates, salamanders often experience intraspecific predation (e.g., Collins and Cheek 1983, Crump 1992). Although cannibalistic interactions can cause death, unsuccessful predation attempts frequently lead to partial or complete losses of digits, limbs, or tails (Walls and Jaeger 1987, Semlitsch and Reichling 1989, Wildy et al. 1998, 2001). Some salamander populations also exhibit high frequencies of limb malformations (Bishop 1947, Worthington 1974, Sessions and Ruth 1990), many of which have been linked to *Ribeiroia* infection (e.g., Johnson et al. 2002, 2003). Finally, the capacity of salamanders to regenerate lost or injured limbs may enhance their vulnerability to parasite-induced malformations. In anurans, *Ribeiroia* infection causes malformations only if it occurs during a narrow time window corresponding to early limb development (Schotthoefer et al. 2003). However, injury followed by regeneration may effectively “reset the clock,” as infection of regenerating limbs should have the same capacity to alter development and induce malformations (Sessions and Ruth 1990).

We combined field studies and laboratory experiments to investigate the importance of interactions between intraspecific predation and parasitism in driving salamander limb malformations. We focused on the long-toed salamander (*Ambystoma macrodactylum*), a widely distributed salamander in the western United States that is cannibalistic (e.g., Walls et al. 1993, Wildy et al. 1998, 2001) and, in certain populations, afflicted with high levels of limb malformations (e.g., Sessions and Ruth 1990, Johnson et al. 2002, 2003). Under experimental conditions, we (1) quantified the types and frequency of limb injuries resulting from cannibalistic attack, and (2) evaluated the individual and combined effects of limb injury and *Ribeiroia* exposure on limb development, survivorship, and time-to-metamorphosis of long-toed salamanders (see Plate 1). Finally, we compared laboratory-induced anomalies to those observed in natural *A. macrodactylum* populations with and without *Ribeiroia* infection.

## MATERIALS AND METHODS

### *Field surveys*

We collected data on parasite-associated malformations in larval salamanders from sites in Oregon, USA (Spyglass Pond, Washington County) and Montana (Jette Pond, Lake County and Toolman Marsh, Sanders County) between July and August of 1999. At Jette Pond, we surveyed breeding *Ambystoma macrodactylum* each spring between 1998 and 2004 (see Johnson et al. [2003] for history of Jette). Additional data on malformations associated with *Ribeiroia* infection in *A. macrodactylum croceum* (an endangered subspecies of *A. macrodactylum*) were obtained from a site in Aptos, California (Seascape, Santa Cruz County), USA, surveyed between 1986 and 1987 (Sessions and Ruth 1990). To document abnormality patterns in the absence of *Ribeiroia*, we examined larval *A. macrodactylum* from the Potholes site (Deschutes County) in the Cascade Mountain range of Oregon in September 2002 and 2004. This high-altitude (1951 m a.s.l.) pond does not support *Ribeiroia*. Collected larvae were examined with a stereo dissecting microscope for abnormalities. Detailed information on sampling methods and malformation categorization is provided elsewhere (Johnson et al. 2002). Given the small number of sampled wetlands and variability in sampling methodologies, field data are offered to evaluate the plausibility of our hypotheses, rather than as a definitive test of the combined role of parasitism and injury in nature.

### *Predation experiments*

To establish the nature of injuries resulting from attempted cannibalism, we conducted 48-hour trials with individual or size-matched pairs of long-toed salamanders. Animals were collected from the Potholes site and inspected for morphological abnormalities. We selected 60 normal animals (mean size 24.7 mm) and placed 20 pairs of size-matched individuals in 1.5-L containers without food. We maintained 20 additional animals individually as a control treatment. Injuries were characterized visually at 24 and 48 hours.

### *Combined effects of injury and infection*

To investigate the individual and combined effects of *Ribeiroia* infection and predation, we exposed larval *A. macrodactylum* to limb injury and three levels of cercarial infection over a 30-day period. Sixty morphologically normal larval salamanders (mean size 20.4 mm) collected from the Potholes site were isolated in 1.5-L containers filled with 1.0 L of commercial spring water. To standardize limb injuries among animals, we simulated the effects of conspecific attack by amputating one forelimb and one hind limb on opposite sides of each animal (e.g., left forelimb and right hind limb). We anesthetized salamanders using MS-222 and removed 95% of each limb using sterilized iridectomy scissors (following methods in developmental biology, e.g., del

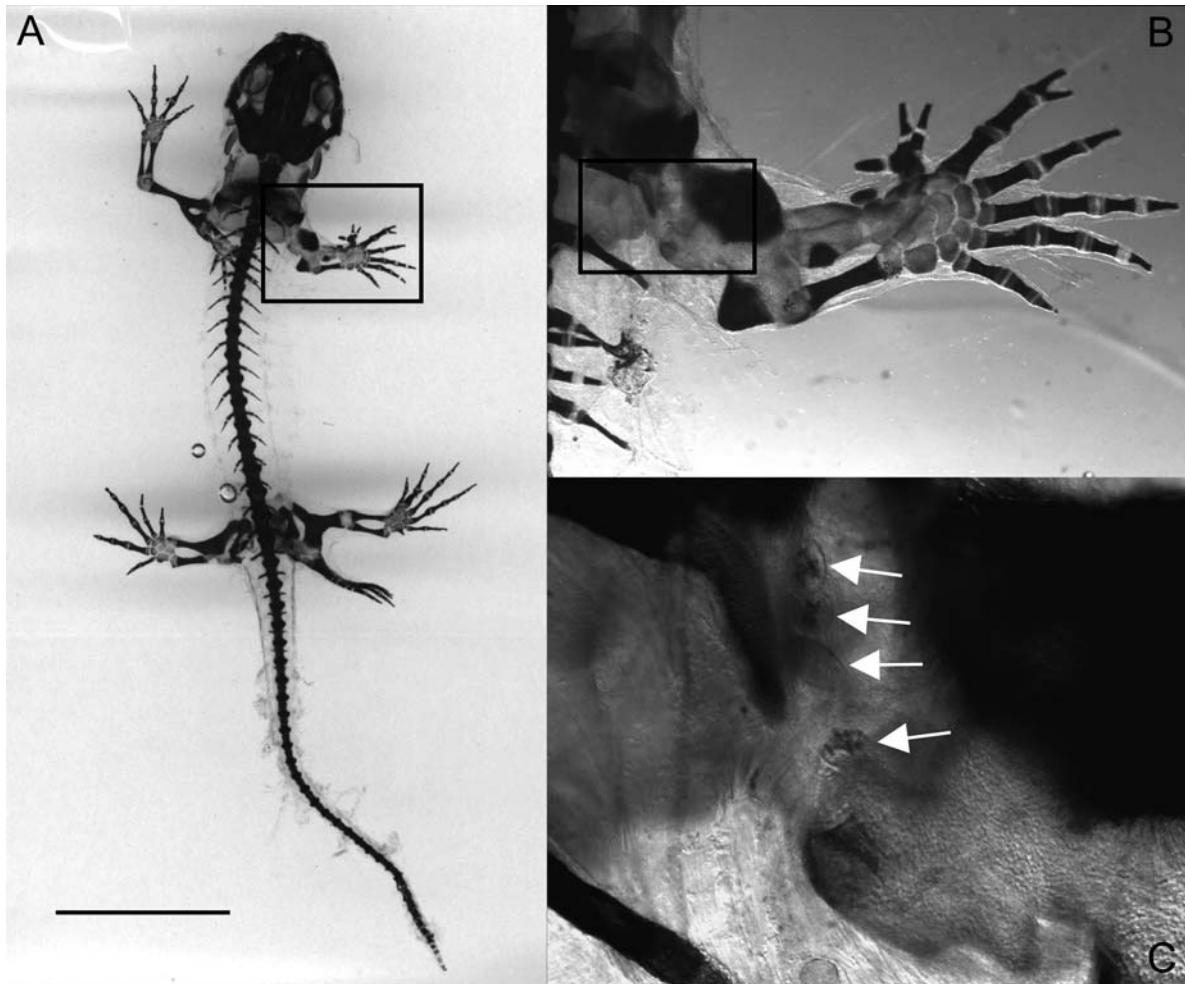


PLATE 1. Cleared and stained salamander from the parasite exposure experiment illustrating the extent of bone malformations. (A) Whole-body image of an individual from the heavy exposure treatment with six limbs and 36 digits. Scale bar represents 10 mm. (B) Magnified view of the right forelimb. (C) Magnified view of the base of the limb with *Ribeiroia* metacercariae indicated (by arrows). Photographs and clearing and staining courtesy of Stanley Sessions.

Rincon and Scadding [2002]). Minimal bleeding occurred and all animals recovered full activity within 10 minutes. We chose to simulate the effects of conspecific attack to ensure greater control in the types and numbers of injuries per animal. In this manner, regenerating limbs revealed the combined effects of injury and parasite infection, whereas uninjured limbs served as the within-subject control, evaluating the effects of parasite infection alone. Although animals missing two limbs are uncommon in nature, this design allowed simultaneous comparisons of the effects of parasite infection on injured and uninjured fore- and hind limbs, respectively, while minimizing the number of experimental animals.

We randomly assigned salamanders to one of three parasite exposure treatments: unexposed (0 cercariae), light (500 cercariae), and heavy (1000 cercariae), with 20 animals per treatment. Although high, these exposure levels are within the range of what is observed under field

conditions (D. R. Sutherland and P. T. J. Johnson, *unpublished data*), and were used to ensure a strong malformation response. *Ribeiroia* cercariae were obtained from infected snails (*Planorbella trivolvis*) collected in the field and shed individually in 50-mL vials. Following limb injury, larval salamanders were exposed to 0, 50, or 100 cercariae every third day over a 30-day period (10 exposures). During each exposure, salamanders were transferred to 100-mL containers containing the appropriate number of cercariae. After five hours, containers were examined to ensure all cercariae had encysted (i.e., no dead or inactive cercariae) and salamanders were returned to 1.5-L containers. Animals in the uninfected treatment were exposed to water from uninfected snails.

We fed salamanders *Tubifex* worms every other day and replaced water and containers weekly. Salamanders were maintained at 25°C on a 16:8 h light:dark cycle. Every two weeks, we estimated regeneration progress as length of the injured limb relative to the corresponding

TABLE 1. Composition of morphological abnormalities in *Ambystoma macrodactylum* from field sites and experiments, showing numbers of each abnormality type (*n*; in parentheses) and percentage relative to the total number of abnormalities.

Abnormality type	Relative percentage of all abnormalities (with <i>n</i> ), by site					
	Potholes	Seascape †	Spyglass	Toolman	Jette Pond	
					Larvae	Adults
<b>Forelimb</b>						
Missing digit	13.9 (27)	2.1 (36)	0	0	0	5.8 (4)
Missing limb or foot	29.7 (59)	0.2 (3)	33.3 (2)	23.2 (9)	0	1.4 (1)
Fused digit	0	0.1 (2)	0	0	0	4.3 (3)
Superficial extra digit <sup>B</sup>	n/a	n/a	n/a	n/a	n/a	n/a
Extra digits <sup>C</sup>	0.5 (1)	19.8 (339)	33.3 (2)	2.6 (1)	11.1 (4)	0
Extra limb or foot <sup>E, F</sup>	0	0.1 (1)	0	2.6 (1)	0	10.1 (7)
Femoral projection	0	n/a	0	13.2 (5)	0	2.9 (2)
Skin webbing <sup>D</sup>	0	n/a	0	0	0	0
Micromelia	0	n/a	0	0	0	0
Other malformation‡	0	0.2 (4)	0	0	0	4.4 (3)
<b>Hind limb</b>						
Missing digit	18.5 (36)	4.3 (72)	16.7 (1)	7.9 (3)	2.8 (1)	7.2 (5)
Missing limb or foot	37.4 (73)	18.2 (311)	0	25.3 (10)	0	0
Fused digit	0	0.6 (11)	0	0	0	0
Superficial extra digit	n/a	n/a	n/a	n/a	n/a	n/a
Extra digit <sup>H</sup>	0	29.7 (508)	16.7 (1)	2.6 (1)	19.4 (7)	10.1 (7)
Extra limb or foot <sup>L</sup>	0	23.4 (399)	0	0	0	14.5 (10)
Femoral projection <sup>K</sup>	0	n/a	0	7.9 (3)	22.2 (8)	0
Skin webbing	0	n/a	0	0	22.2 (8)	14.5 (10)
Micromelia <sup>I</sup>	0	n/a	n/a	13.2 (5)	19.4 (7)	20.3 (14)
Other malformation‡	0	1.3 (22)	0	0	2.7 (1)	4.3 (3)
No. abnormalities	195	1708	6	38	36	69
No. abnormal	141	1686	6	28	15	45
No. inspected	403	5926	25	183	43	477
No. abnormalities per abnormal animal	1.38	1.01	1.00	1.36	2.4	1.53

Notes: Infection by *Ribeiroia* occurred at all sites except Potholes. For the experimental infections, salamanders in the control treatment exhibited no malformations and are therefore omitted. Mean numbers of abnormalities per abnormal amphibian, an index of the severity of abnormalities, are presented in the final row. Letter superscripts correspond to images in Fig. 2.

† Data from Sessions and Ruth (1990) for *A. macrodactylum croceum* ("n/a" indicates that data were unavailable).

‡ Includes brachydactyly, clinodactyly, anteversion and limb hyperextension.

unmanipulated limb. Regeneration was considered complete when the observer could no longer differentiate between injured and uninjured limbs. Within 24 hours of metamorphosis, we measured (snout-vent length), weighed, and photographed each animal. A subset of salamanders ( $N = 5$ ) from each treatment was dissected to estimate actual infection intensity and to quantify parasite distribution.

## RESULTS

### Field surveys

At the Potholes site, the frequency of abnormalities in larval *A. macrodactylum* averaged 35% ( $N = 403$  salamanders). Abnormalities were dominated by missing digits, missing feet, and missing limbs (Table 1). In many cases, injured limbs appeared to be regenerating. Only one individual exhibited an extra digit. The frequencies of fore- and hind limb abnormalities were comparable (45% and 55%, respectively). At field sites that supported *Ribeiroia* infection, we observed a wider diversity of malformations, including extra digits, extra feet, extra limbs, cutaneous fusion, and micromelia (Table 1). Malformations were more frequent among hind limbs than forelimbs (66% vs. 34%). Malformation

frequencies in larval salamanders were 14.2% (Toolman,  $N = 183$ ), 24% (Spyglass,  $N = 25$ ), and 35% (Jette,  $N = 43$ ). Malformations in adults ranged from 4.7% to 12.5% ( $N = 477$ ). Comparable levels and types of malformations were observed by Sessions and Ruth (1990) at Seascape Pond (4.6% to 39.2%; Table 1).

### Predation experiments

After 48 hours, eight of 20 experimental pairs (40%) exhibited severe limb or digit damage in one or both animals, whereas no abnormalities were observed among 20 control animals (Fisher's exact test,  $P < 0.003$ ). Despite size-matching, one individual was consumed (and subsequently regurgitated) in the experimental treatment. In three cases, both paired animals suffered limb injuries; only one animal was injured in the remaining five cases. We observed a total of 17 limb injuries among 11 salamanders: 10 forelimb and 7 hind limb abnormalities. Abnormalities were dominated by missing digits, missing feet, and completely missing limbs. One individual was missing its right arm, left hand, and digits from both hind limbs. The other animal in the pair was missing its left foot.

TABLE 1. Extended.

Experimental infection: percentage of all abnormalities (with <i>n</i> )			
Light (500), no injury	Light (500), injury	Heavy (1000), no injury	Heavy (1000), injury
0	0	0	1.4 (1)
0	0	0	0
0	0	0	1.4 (1)
26.7 (4)	2.0 (1)	21.5 (6)	0
0	10.2 (5)	0	16.7 (12)
13.3 (2)	10.2 (5)	35.7 (10)	8.3 (6)
6.7 (1)	4.1 (2)	0	2.8 (2)
0	14.3 (7)	0	4.2 (3)
0	26.5 (13)	0	23.6 (17)
0	0	0	0
0	2.0 (1)	0	0
0	0	0	0
0	2.0 (1)	0	0
40 (6)	2.0 (1)	17.9 (5)	1.4 (1)
0	12.2 (6)	0	20.8 (15)
6.7 (1)	0	14.3 (4)	1.4 (1)
6.7 (1)	0	10.7 (3)	1.4 (1)
0	0	0	0
0	10.2 (5)	0	13.9 (10)
0	4.1 (2)	0	2.8 (2)
15	49	28	72
	17		20
	19		20
	3.76		5.00

*Combined effects of injury and infection*

We observed severe limb malformations in 89% and 100% of metamorphosing salamanders in the light and heavy parasite exposures, respectively (Fig. 1A). No malformations were observed among animals exposed only to injury. Because our data involved a binary response variable (limb malformed vs. limb normal) and multiple, within-subjects variables (limb position [fore- vs. hind] and limb treatment [injured vs. uninjured]), we used generalized estimating equations (GEE), an extension of generalized linear models, to account for correlation among observations from the same individual (Horton and Lipsitz 1999). In this instance, GEE is analogous to repeated-measures ANOVA for a binomially distributed response variable. We assumed an unstructured covariance matrix but varied the structure with no change to our results. We found a significant main effect for *Ribeiroia* infection (Robust  $z = 4.67$ ,  $P < 0.0001$ ), but not for limb position or limb treatment ( $P > 0.05$ ). However, *Ribeiroia* infection interacted significantly with limb treatment (Robust  $z = -2.15$ ,  $P < 0.05$ ). Following *Ribeiroia* exposure, injured limbs were almost twice as likely to become deformed and exhibited 3–5 times more deformities than did uninjured limbs (Fig. 1B; see Plate 1).

No parasite-associated mortality was observed; one animal from the light exposure treatment was lost due to handling error. However, *Ribeiroia* exposure signifi-

cantly delayed both time-to-limb regeneration and time-to-metamorphosis. We found that salamanders in the light and heavy *Ribeiroia* exposures regenerated significantly more slowly than did uninfected animals (repeated-measures ANOVA,  $F_{2,56} = 147.435$ ;  $P < 0.0001$ ; Fig. 2A). Salamanders in the heavy treatment were also significantly delayed in time-to-metamorphosis relative to those in the uninfected and light exposure treatments (Kaplan-Meier survival analysis; log rank = 6.86,  $P < 0.01$ ); the latter two groups were not significantly different (Fig. 2B). Median emergence times (and 95% ci) for uninfected, light, and heavy exposure treatments were 64 (63–65), 70 (64–76), and 72 (70–74) days post-injury. Parasite exposure had a significant positive effect on both length and mass of salamanders at metamorphosis (MANOVA Wilks' lambda = 0.759;  $F_{4,108} = 3.916$ ;  $P < 0.005$ ), but these effects were eliminated when time-to-metamorphosis was incorporated as a covariate, indicating that the influence of infection on salamander size was mediated through effects on metamorphosis time.

Dissections of metamorphosed salamanders recovered an average of  $43.6\% \pm 3.3\%$  of the parasites to which the animals were exposed (mean  $\pm$  SE; range 28.9–58.1%). The majority of metacercariae were found in and around the resorbed gills, nares, eyes, and mandible ( $77.7\% \pm 1.58\%$ ). Only 21.2% ( $\pm 1.6\%$ ) of recovered parasites were found around the limbs. Metacercariae were evenly distributed between fore- and hind limbs, and between injured and uninjured limbs. *Ribeiroia* metacercariae often occurred in close association with malformations and supernumerary structures. Within parasite exposure treatments, extra digits, extra limbs, and incompletely developed limbs (micromelia) were the most common malformations (Fig. 2, Table 1). Deformities became more abundant and severe with increasing parasite exposure (Fig. 3, Table 1). Truncation of one or both long bones was commonly observed among injured limbs (Fig. 2C, I). Polydactyly was the predominant malformation and ranged from a split digit (minor polydactyly) to eight complete extra digits (Fig. 2C, H, I). Among uninjured limbs, extra digits were almost exclusively superficial (Fig. 2B), originating from the base of an existing digit but devoid of corresponding metacarpals. However, polymelia occurred at comparable frequencies in both injured and uninjured limbs. Extra limbs were typically smaller and posterior or dorsal to the primary limb (Fig. 2E, F, L). One heavily parasitized individual had seven limbs and 37 digits (rather than the expected four limbs and 18 digits; Fig. 3F). Among salamanders exposed only to injury, all limbs regenerated completely and no abnormalities were observed (Fig. 3A).

DISCUSSION

Parasitism and predation interact through a diversity of mechanisms, particularly when the sublethal effects of predators are considered (e.g., Thiemann and Wassersug

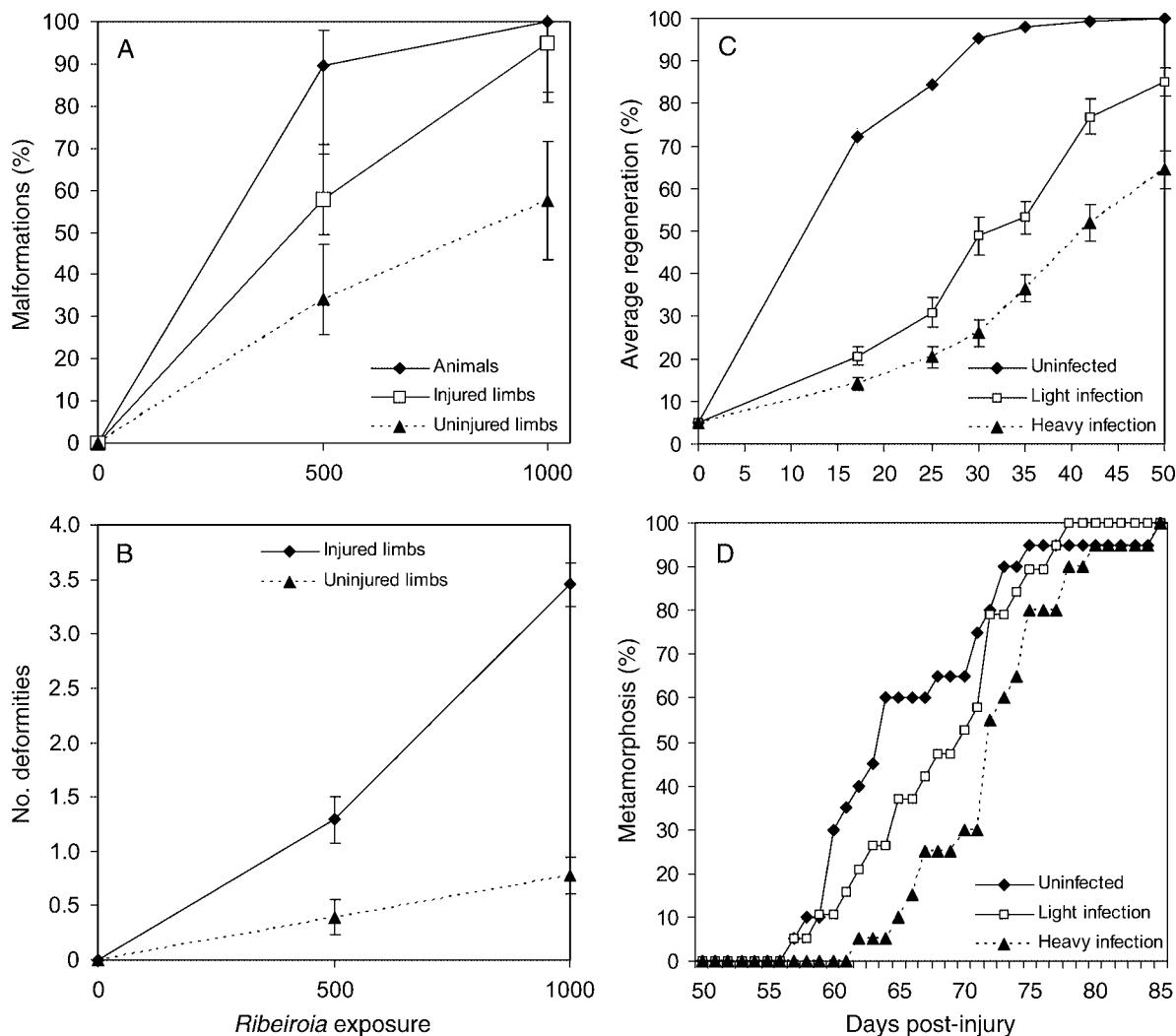


FIG. 1. (A) Percentage of salamander malformations (mean and 95% CI) by animal and by limb (injured and uninjured). (B) Number of deformities (mean  $\pm$  SE) among injured and uninjured limbs as a function of parasite exposure. (C) Regeneration progress (mean  $\pm$  SE) of injured limbs and (D) time to metamorphosis, among infection treatments.

2000, Decaestecker et al. 2002, Ostfeld and Holt 2004). Our results illustrate how interactions between predation and parasitism can enhance disease pathology (i.e., amphibian limb malformations). In the absence of *Ribeiroia* infection, long-toed salamanders exhibited primarily missing limbs and digits. Experiments involving paired and isolated salamanders demonstrated that conspecific attack (i.e., attempted cannibalism) was sufficient to explain these abnormalities, consistent with previous studies on agonism among larval salamanders (e.g., Semlitsch and Reichling 1989, Wildy et al. 1998, 2001). In aquatic environments that supported *Ribeiroia*, however, long-toed salamanders often exhibited extra digits, extra limbs, and improperly or incompletely developed limbs. Although parasite exposure experiments indicated that *Ribeiroia* infection can cause identical limb malformations in salamanders, limbs subjected to a combination of infection and injury

exhibited a 300% increase in malformations relative to limbs exposed only to parasites. Often these deformities were more severe than those in uninjured limbs, involving substantial deviations in morphological structure. Thus, although trematode infection is the likely proximate cause of many observed malformations, the frequency and severity of those malformations can be critically influenced by predation-related injury.

The importance of injury in enhancing parasite-induced malformations in natural amphibian populations remains conjectural. Although *Ribeiroia* infection and limb injury are often common in salamander populations (e.g., Johnson et al. 2001b, 2002) and probably interact, field experiments that manipulate predation and parasite exposure are needed to assess their individual and combined importance. Information about the levels of infection and injury necessary to induce this interaction (and the proximity in their

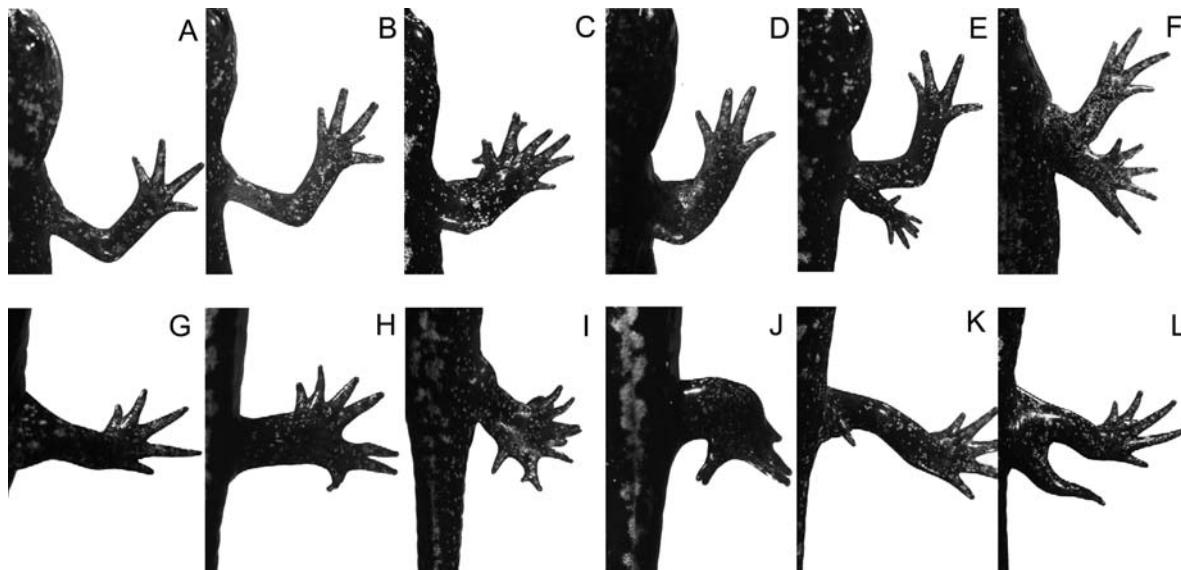


FIG. 2. Representative malformations among salamander limbs exposed to injury and *Ribeiroia* infection. (A). Normal forelimb; (B) forelimb with superficial polydactyly; (C) forelimb with severe polydactyly (nine digits) and micromelia (truncation of long bones); (D) forelimb cutaneous fusion; (E) auxiliary supernumerary forelimb; (F) complete supernumerary forelimb; (G) normal hind limb; (H) hind limb with seven digits (polydactyly); (I) hind limb with 10 digits and micromelia; (J) anteversion of hind limb; (K) femoral projection from hind limb; and (L) supernumerary hind limb lacking digits.

timing) would be particularly valuable. *Ribeiroia* infection intensities used in this study were high, but well within observations from natural amphibian populations. On average, we recovered 208 and 455 parasites from salamanders in the light and heavy exposures, respectively, whereas the upper value from nature is 960 metacercariae in a single amphibian, with numerous populations averaging  $>200$  parasites per individual (D. R. Sutherland and P. T. J. Johnson, *unpublished data*).

Although particular types of malformations (e.g., micromelia, polydactyly) were associated with limbs exposed experimentally to injury and infection, we do not suggest that the presence of these abnormalities in naturally occurring salamander populations is necessarily indicative of injury followed by infection. Field experiments in combination with more information on the effects of *Ribeiroia* infection during initial limb development (rather than during regeneration) are

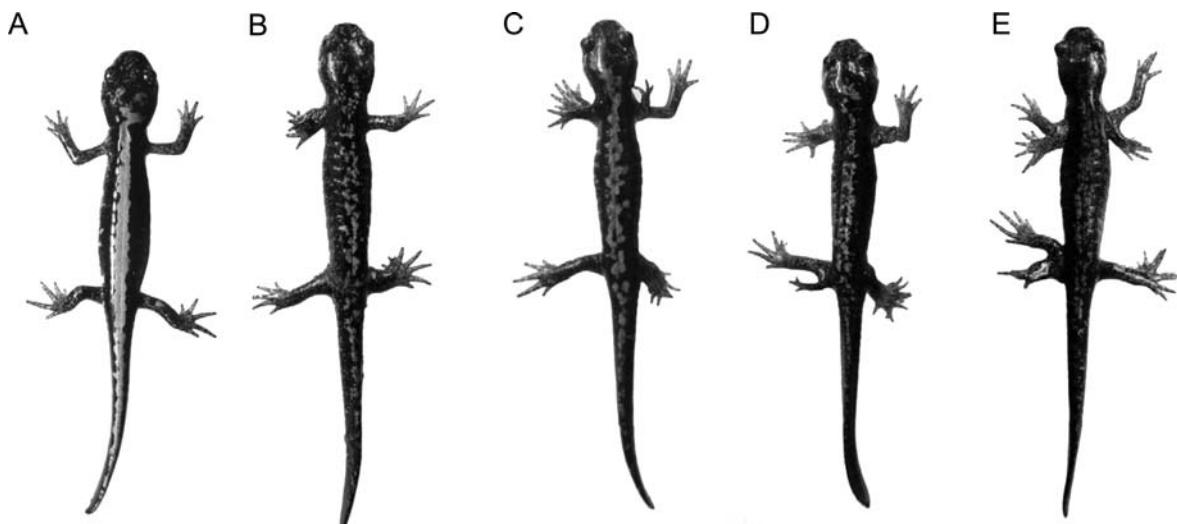


FIG. 3. Gradient in malformation severity among long-toed salamanders. Injured limbs are left forelimb and right hind limb. (A) Normal limbs; (B) polydactyly and micromelia in injured limbs only; (C) polymely and polydactyly in left forelimb, micromelia in right hind limb, and auxiliary limb in right forelimb (uninjured); (D) severe polydactyly and micromelia in injured limbs with minor polymely in left hind limb (uninjured); (E) polymely and/or polydactyly in all limbs.

needed to evaluate whether abnormality type(s) can be diagnostic of the etiologic agent(s).

Injury probably exacerbates malformation levels by extending the period of time during which limbs are vulnerable to developmental disturbance. Actively developing limbs are highly susceptible to external interference such as invading trematode cercariae (Schotthoefler et al. 2003). In anurans, this temporal window is restricted to the period of initial limb growth (Bowerman and Johnson 2003). However, the regenerative capacity of caudates effectively “restarts” this window. Injured limbs offer *Ribeiroia* a second opportunity to interfere with limb growth and cause improper development. Correspondingly, infected animals regenerated significantly more slowly than did uninfected salamanders, and micromelia was among the most common malformations following exposure to infection and injury. Surprisingly, however, *Ribeiroia* infection also induced extra limbs and digits among non-regenerating limbs (Fig. 3D, E). These auxiliary limbs and digits frequently were smaller and more superficial than abnormalities observed among regenerating limbs (e.g., Fig. 2F vs. E). The occurrence of malformations in the absence of pre-existing injuries is consistent with the idea of invading cercariae themselves causing injury and cellular rearrangement in limb tissue (Sessions et al. 1999). In heavy infections, injuries may be severe enough to induce regeneration and formation of supernumerary limb structures (Sessions and Ruth 1990, Sessions et al. 1999).

Although limb malformations have been observed in salamander populations for 125 years, the cause(s) of these anomalies have rarely been determined (e.g., Kingsley 1880, Winslow 1904, Sealander 1944, Bishop 1947, Worthington 1974, Brown 1981). Our study is the first to demonstrate experimentally that *Ribeiroia* infection can induce many of the malformations observed in wild salamander populations, including extra and malformed limbs and digits (see also Sessions and Ruth 1990). Our results also provide possible insights as to why malformations are often more common in anurans than in caudates, even within a single site (Sessions and Ruth 1990, Johnson et al. 2002, 2003). Although we used 10–20 times more parasites in this study than in previous experiments with larval frogs (Johnson et al. 1999, Schotthoefler et al. 2003), fewer than half of the parasites were recovered as metacercariae and only 10% were found around the limb regions, suggesting that more parasites may be required to induce limb malformations in salamanders. In studies with anurans, ~90–95% of *Ribeiroia* metacercariae are found around the limbs (e.g., Johnson et al. 2002; D. R. Sutherland, unpublished data). Thus, the conditions favoring malformations in salamanders, namely, high levels of *Ribeiroia* infection and predation-induced limb injuries, may only occur in select years, possibly contributing to the episodic nature of salamander malformations (Johnson et al. 2003).

The consequences of *Ribeiroia* infection and malformations for salamander populations are still poorly understood. Although we observed no mortality, the effects of *Ribeiroia* are stage dependent (Schotthoefler et al. 2003); recently hatched *A. macrodactylum* died within 12 hours of exposure to eight cercariae (P. T. J. Johnson and E. R. Preu, unpublished data). Limb malformations also impair amphibian fitness, and long-toed salamanders exhibited a decline in malformation frequency with life history stage suggestive of elevated mortality among malformed individuals. At Seascape Pond, for example, the frequency of malformations declined from 40% in larvae to 4.6% in adults (Sessions and Ruth 1990). Correspondingly, malformations in adult *A. macrodactylum* in this study rarely exceeded 5%, even when they were considerably higher among larvae.

*Ribeiroia* infection caused significant delays in both limb regeneration and time-to-metamorphosis, each of which could further contribute to reduced salamander survival. Although most predation-related limb injuries regenerate, resources devoted to regeneration of injuries are costly to survival (Semlitsch and Reichling 1989), particularly when combined with infection. Walls and Jaeger (1987), for example, reported that larvae with injuries suffered increased mortality from *Saprolegnia* infection. Although we observed high levels (>30%) of limb loss among natural populations of *A. macrodactylum*, we cannot exclude the possibility that some of these injuries occurred as larvae were transported to the laboratory (but note that many injuries showed clear signs of regeneration). Moreover, although it is common in larval salamanders, intraspecific predation is not the only source of injury; predators such as aquatic insects, leeches, or fish could also be contributing to injuries observed in wild populations (see Johnson et al. 2001a, b).

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