CHAPTER 4

Digenetic Trematodes and their Relationship to Amphibian Declines and Deformities

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I. INTRODUCTION

EMERGING diseases in amphibians have recently become the focus of intense research, especially in connection with anthropogenic habitat change and amphibian declines. Of particular concern are apparently newly emerging diseases sometimes involving widespread pathogens previously thought to be rare and/or harmless (Daszak *et al.* 2003; Stuart *et al.* 2004). One such case is chytridiomycosis, thought to be emerging and directly involved in amphibian declines, especially in the Neotropics (Berger *et al.* 1998; Daszak *et al.* 2003; Lips *et al.* 2006). The purpose of the present chapter, however, is to focus on another example of an apparently emerging amphibian disease, digenetic trematode infections (Johnson *et al.* 2003; Skelly *et al.* 2006). Certain trematode infections can cause grotesque limb deformities, kidney damage, debility, and mortality (Fried *et al.* 1997; Johnson and Sutherland 2003; Schotthoefer *et al.* 2003a). After a brief review of the biology and diversity of digenetic trematodes, environmental factors that affect their interaction with amphibians are examined, especially focusing on anthropogenic environmental perturbations. Then a particular trematode (*Ribeioria ondatrae*) is treated and an evaluation made of evidence that the deformities produced by this trematode are part of an adaptive component of the parasite's life history (the "handicapped frog hypothesis"; Sessions 2003). Also discussed is the co-evolution of the parasite with its several hosts in a complex life cycle. This provides an opportunity to review what is known about amphibian immune

defence mechanisms and corresponding infective adaptations utilized by the parasites. Evaluation is made of the evidence that trematodiasis is an emerging disease, followed by an assessment of what makes certain amphibian species particularly susceptible to trematodes and what makes certain trematode species particularly deadly to amphibians. The possible connection between parasite disease and amphibian decline is addressed by examining the incidence of trematode disease and its relationship to other environmental factors, including natural factors and anthropogenic influences such as habitat fragmentation, nutrient input, and pesticide pollution, within a population context. Finally comments are offered concerning future research directions for the study of amphibian trematodes.

II. DIGENETIC TREMATODE BIOLOGY

Trematodes (or flukes) represent a class in the phylum Platyhelminthes (flatworms), characterized by a dorso-ventrally flattened body in which the internal organs are embedded in parenchyma and the animal has one or two hold-fast organs ("suckers"). The class includes at least 200 families and 18,000 – 28,000 species (Cribb *et al.* 2001; Poulin and Morand 2004). The digenetic trematodes (subclass Digenea), characterized by the presence of both an oral and a ventral sucker or acetabulum (Fig. 1), constitute the vast majority of trematode diversity. Digenetic trematodes are obligate parasites of molluscs and vertebrates. The digestive system of trematodes includes an anterior mouth, muscular pharynx, oesophagus, and usually two intestinal ceca (Fig. 1). Most digenetic trematodes are hermaphroditic (monoecious) but some (e.g. schistosomatids) are dioecious and sexually dimorphic. Various organs easily visible inside the adult trematode include the digestive system (oesophagus and ceca), the uterus, ovary, and ovarian follicle, the testes and seminal vesicles, and the excretory vesicle (Fig. 1).

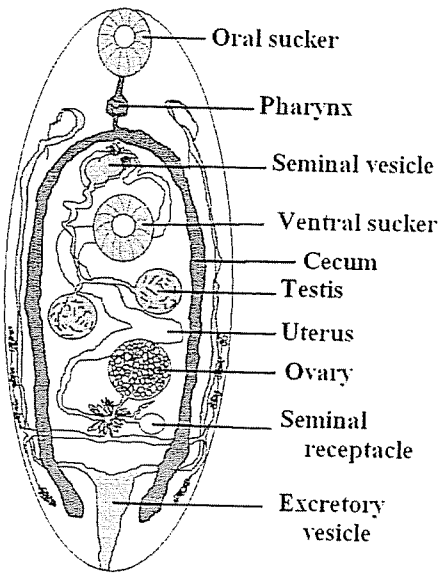


Fig. 1. Structure of a generalized adult digenetic trematode. After Barnes (1968).

Digenetic trematodes usually have complex life cycles, often involving two or more intermediate hosts in addition to a definitive host (i.e., the host for the reproductive adult parasite) (Noble and Noble 1982; Fig. 2). All trematodes have a mollusc, usually a gastropod, as the first intermediate host, but secondary intermediate hosts can include amphibians, fish, insects, or gastropods. The definitive host is generally a vertebrate, often a bird or a mammal. Adult trematodes usually reside in the digestive tract of the definitive host.

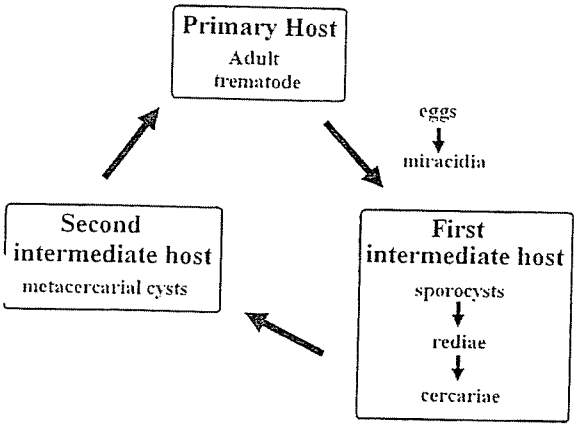


Fig. 2. Generalized life cycle of a digenetic trematode (see text). The definitive ("primary") host is usually a vertebrate predator, and the first intermediate host is usually a mollusc (snail). The second intermediate host is prey for the primary host.

Typically trematode eggs are released from the definitive host into the environment, often in the host's faeces, although the life cycle varies among species. The eggs then hatch and release miracidia, a swimming infectious stage that seeks out a molluscan first intermediate host or, alternatively, are eaten by and hatch inside a mollusc. Once inside a mollusc, the trematode undergoes "polyembryonic amplification". This is a kind of asexual reproduction resembling Russian dolls, in which each stage produces and gives birth to numerous embryos of the subsequent stage (Fig. 3). First, a miracidium develops into a sporocyst. This stage produces either cercariae (the final infective stage) or an additional amplifying stage called a redia, which in turn produces cercariae. Sporocysts and rediae can also give birth to additional sporocysts or rediae, so that hundreds or thousands of

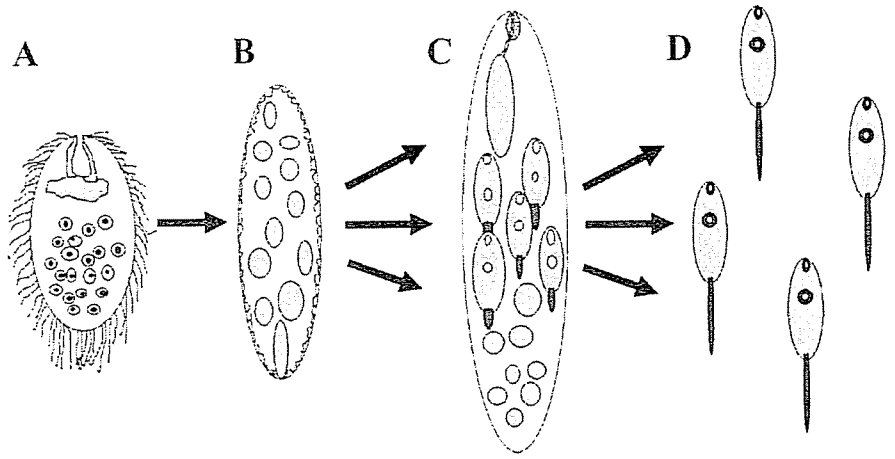
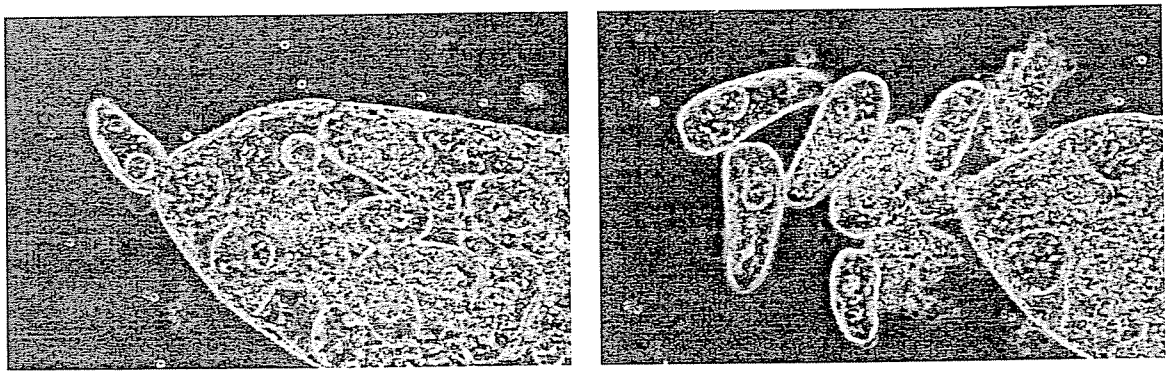


Fig. 3. Embryonic amplification in a digenetic trematode. A: miracidium containing germ cells. B: sporocyst containing immature rediae (germ balls). C: Redia containing immature cercariae. D: mature cercariae.

Fig. 4. Sporocyst (removed from snail) giving birth to rediae.



cercariae can be produced from a single infection. It is an amazing and educational spectacle to open up one of these infected snails under a dissecting microscope and view the various stages in the trematode's life cycle (Fig. 4).

Mature cercariae emerge from the molluscan first intermediate host into the environment, a process that can be harmful or even lethal to the mollusc (Noble and Noble 1982). Once outside, the trematodes are challenged by the problem of getting to the intestine of a definitive host. Most trematodes resolve this issue by utilizing a second intermediate host that is prey for the definitive host. If the definitive host is a bird, for example, the second intermediate host might be a fish or amphibian that is a prey species

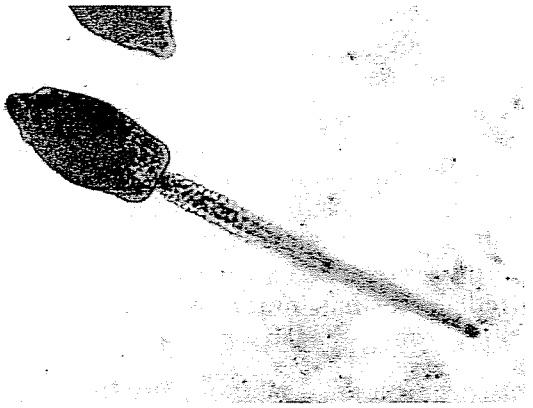


Fig. 5. Ribeiroia cercaria (hematoxylin).

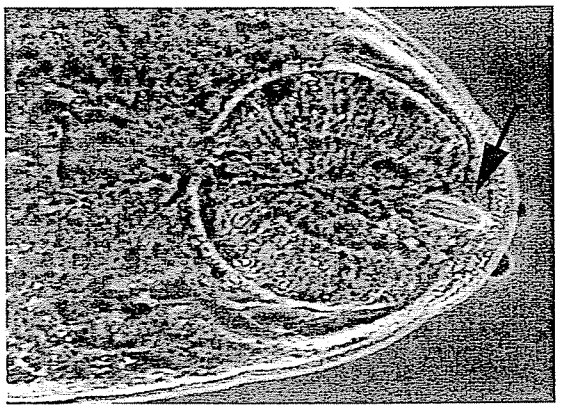


Fig. 6. Stylet on the oral sucker of an armatae cercaria (phase contrast).

of the bird. Trematodes with amphibian definitive hosts frequently infect invertebrate intermediate hosts. Cercariae actively swim to their second intermediate host using a muscular tail (Fig. 5). Once they infect a second intermediate host, the cercariae drop their tails and form cysts.

Some cercariae form cysts on the surface of the second intermediate host, while others penetrate the skin and form cysts in the host's tissues. Cercariae that penetrate the tissues of their hosts are usually equipped with a sharp stylet on the oral sucker (Fig. 6) that allows them to poke a hole in the integument through which the worm then squeezes. Cercariae can form a cyst within a minute or two of contacting the host; this encysted stage is called a metacercaria (Fig. 7). Metacercariae usually induce a reaction in which the host forms a connective tissue capsule around the cyst, and then remains relatively inactive. The trematode's life cycle is completed if, and when, the infected secondary host is eaten by the definitive host, at which time the metacercariae excyst and mature inside the definitive host.

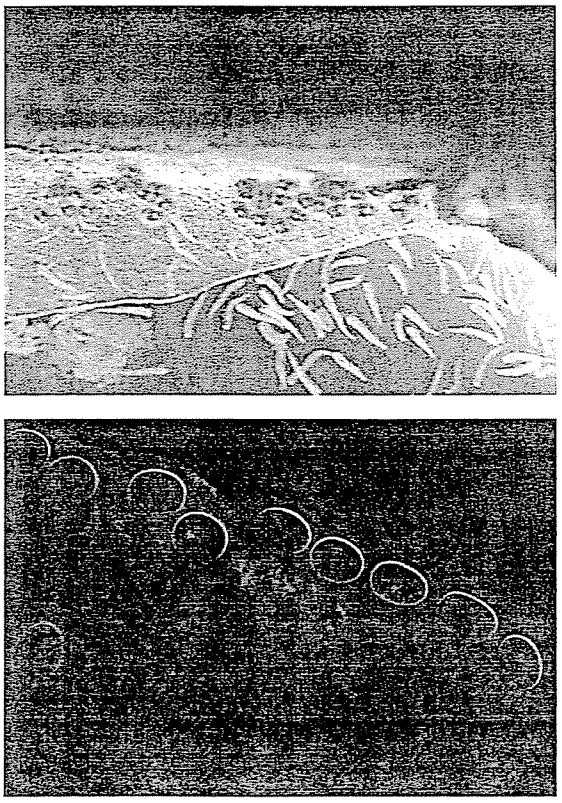
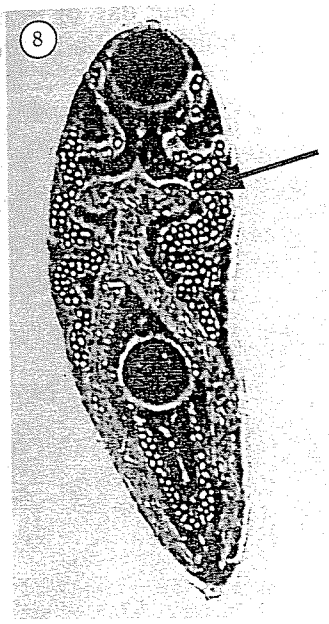


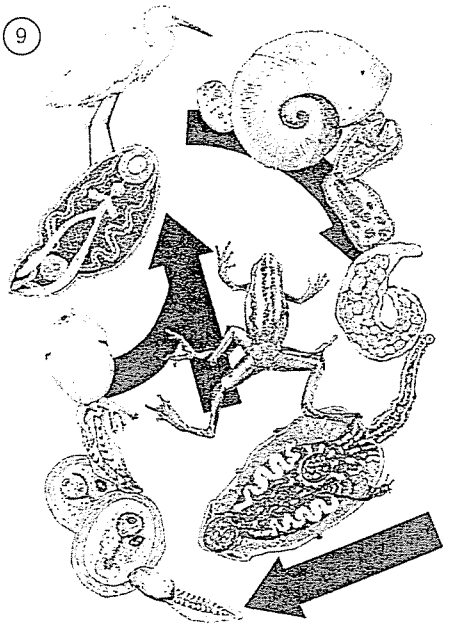
Fig. 7. Top: *Ribeiroia cercariae* immediately after encysting in the tail and hind limb region of a tadpole. Their dropped tails can be seen below the tadpole's tail fin. Bottom: Metacercarial cysts of *Echinostoma trivolvis* embedded in the kidney of a *Rana* tadpole.

III. TREMATODES AND DEFORMED AMPHIBIANS

The occurrence of "deformed" (or "malformed") amphibians became a major environmental issue by the late 1990s, fueled by the fear that the abnormalities were caused by UV-irradiation, chemical pollution, or a combination of environmental factors. The causal role of trematodes, identified as *Ribeiroia* sp. by Sessions *et al.* (1999), in the development of frog and salamander limb deformities has since been confirmed by numerous studies (Sessions and Ruth 1990; Johnson *et al.* 1999; 2001a,b; Sessions *et al.* 1999; Stopper *et al.* 2002). The genus

Ribeiroia is identifiable by unique projections from the oesophagus known as diverticulae (Fig. 8). Like other digenetic trematodes, *Ribeiroia*, identified as *R. odontrae* by Johnson *et al.* (1999), has a complex life cycle involving several hosts (Fig. 9). The preferred definitive host is thought to be a predatory aquatic bird (e.g., heron) that feeds on fish and amphibians. The first intermediate hosts are planorbid snails; amphibians serve as the second intermediate hosts. Experiments have revealed that *Ribeiroia* infections can cause tadpoles to grow extra limbs and a wide variety of other debilitating deformities or malformations (Fig. 10). These deformities are now believed to represent an adaptive host-modification strategy of the parasite whereby its chances of survival, i.e. to be captured and eaten by a primary host, are increased. This idea is known as the "handicapped frog hypothesis" (Sessions 2003).





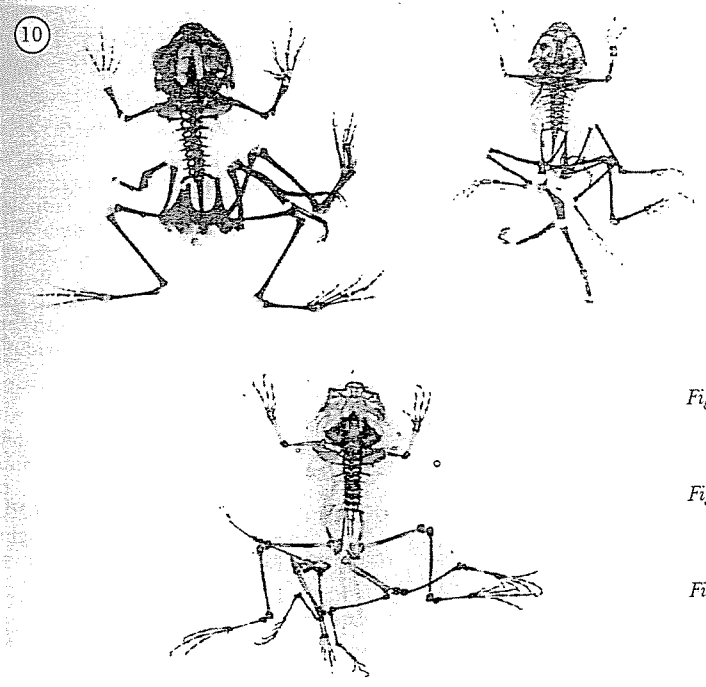


Fig. 8. Ribeiroia anatomy, showing diagnostic oesophageal diverticulae (arrow).

Fig. 9. Life cycle of *Ribeiroia ondatrae* (drawing by B. Ballengée).

Fig. 10. Some examples of cleared and stained parasite-induced deformities in Pacific treefrogs (*Hyla* [*Pseudacris*] *regilla*).

A. The "Handicapped Frog Hypothesis"

Survival of encysted trematodes depends on the secondary host being eaten by a primary host, so any mechanism that "handicaps" the secondary host and increases its probability of being consumed by a definitive host will be adaptive for the parasite. The process by which *Ribeiroia* cercariae attack their amphibian hosts and cause them to develop deformed limbs has been observed (Stopper *et al.*, 2002). When *Ribeiroia* cercariae encounter a frog tadpole, they attach and crawl along the surface of the body. A few individuals migrate into the mouth or the spiracle, but the majority crawl towards the cloacal vent and form cysts in the crease between the body and tail, where the hind-limb buds are located (Fig. 7). The cercariae do not penetrate the skin at first but instead form mectacercarial cysts on the surface of the skin. Over the next few hours, however, the cysts gradually sink into the tissues, which become badly inflamed and swollen, and the cysts become completely embedded in the tissues in and around the limb buds within one day of infection. This process can completely scramble the cells of the developing hind-limb buds (Stopper *et al.*

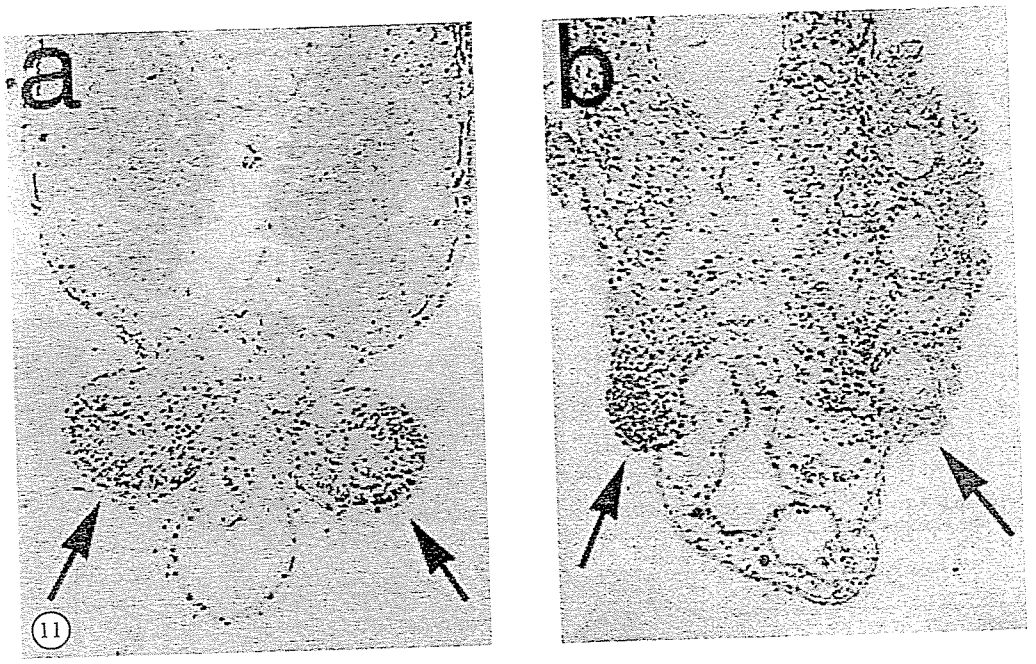


Fig. 11. Histological preparations of the hind limb region of young *Rana pipiens* tadpoles showing (a) normal limb buds and (b) limb buds infected with *Ribeiroia* cysts. BrdU-labelled cells are visualized with immunocytochemistry to show patterns of dividing limb-bud cells.

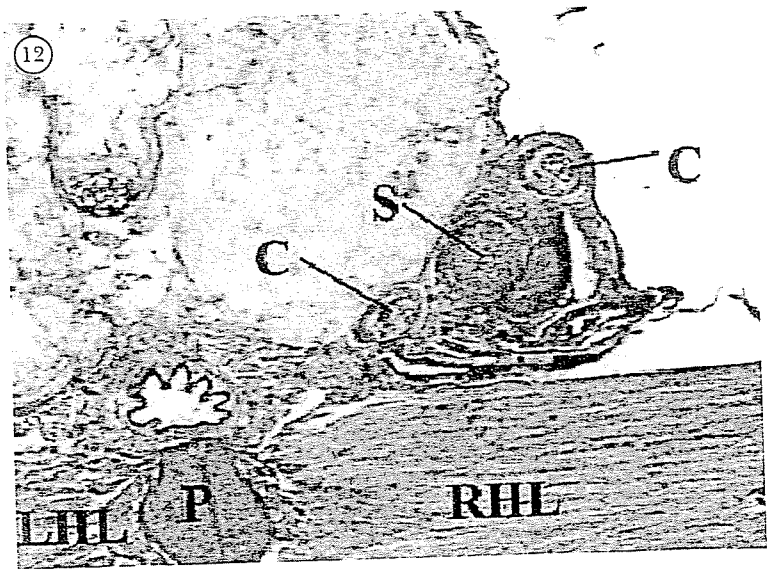


Fig. 12. Trematode (presumably *Ribeiroia*) cysts (C) and supernumerary limb (S) in *Hyla* (*Pseudacris*) *regilla*. LHL and RHL = left and right hind limbs, respectively; P = pelvis.

2002) (Fig. 11), resulting in a dizzying array of deformities or malformations (Fig. 10). The cysts remain closely associated with the deformed structures throughout the rest of development (Fig. 12).

Consistent with this hypothesis, *Riberoia*-induced deformities are concentrated in the hind limbs (Fig. 10), interfering with locomotion without causing unnecessary damage to the rest of the frog's body. Presumably this interference makes frogs more vulnerable to predation. Once the deformed tadpoles have resorbed their tails they are quite helpless and seldom live long, even in captivity.

Something is now known about the developmental mechanism by which the deformities are produced. Virtually all cases of polymelia (extra limbs) show mirror-image morphologies in which a limb with right-handed morphology grows adjacent to a limb with left-handed

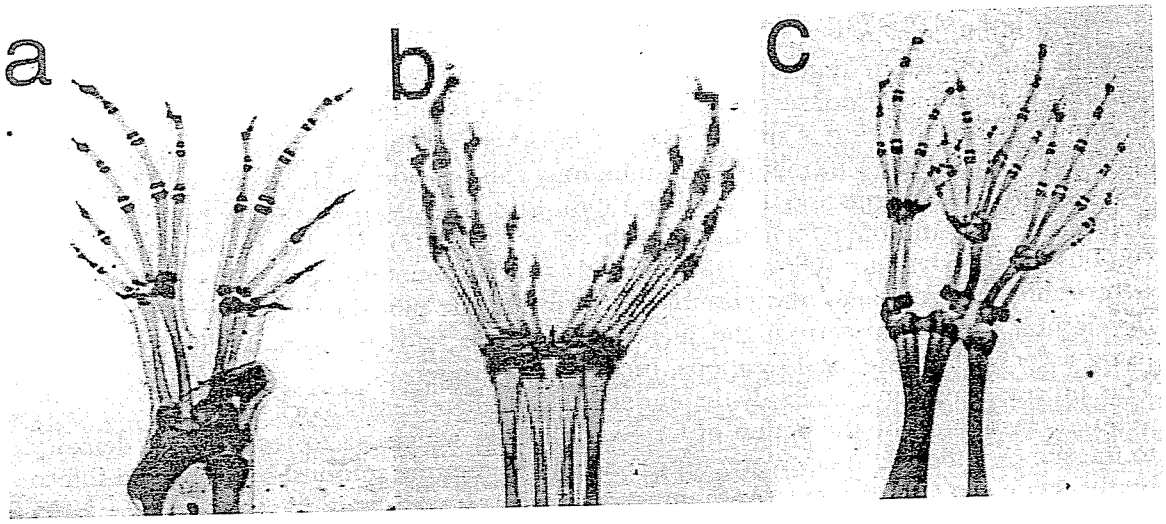


Fig. 13. Some examples of mirror-image limb duplications among parasite-induced limb deformities in Pacific treefrogs: a = anterior mirror-image duplication (AMID); b = posterior mirror-image duplication (PMID); c = mirror-image triplication (MIT).

morphology, and a series of three limbs in a row are always right-left-right or left-right-left (Fig. 13). The evidence of mirror-image symmetry is apparent even in partial duplications. These results are the hallmark of a well-known developmental mechanism called intercalation, a mitotic cellular growth response to the disruption of normal positional relationships of differentiating limb-bud cells (French *et al.* 1976; Sessions and Ruth 1990; Stopper *et al.* 2002). Intercalation resulting in identical deformities to those caused by trematode cysts, including mirror-image duplications and bony triangles (also called "bony bridges") (Meteyer 2000), can be induced simply by surgically removing the distal piece of an early tadpole hind-limb bud, rotating it 180 degrees and grafting it back on the stump (Stopper *et al.* 2002). This is significant because bony triangles were previously thought to be an indicator of retinoid teratogenicity (Gardiner and Hoppe 1999). Thus, intercalation can readily account for most of the observed parasite-induced limb deformities seen in natural populations of amphibians. The role of intercalation is thus supported by both theory and by empirical evidence.

B. Co-evolution and Limb Deformities

Parasites must accomplish two things to successfully produce handicapped frogs: (1) they must attack tadpoles at a stage in the tadpole's development when the limbs are capable of being deformed, and (2) they must be able to thwart, or at least survive, attack by the host's immune system, especially in the intermediate host, where there is direct contact between parasite and deep tissues of the host. Limb development in tadpoles exhibits

"regenerative decline" (Muneoka *et al.* 1986) whereby early limb buds can respond to perturbation by regenerating new limbs or duplicate limbs via intercalation. As the tadpole approaches metamorphosis, however, this ability gradually declines and is completely lost at metamorphosis. Thus, regenerative decline creates a critical "window of opportunity" for the parasites during early limb-bud stages, after which handicapped frogs will not be produced. The existence of these windows of opportunity/vulnerability involving the developmental response of limb buds and immunological competence of tadpoles suggests that co-evolutionary dynamics between *Ribeiroia* and their second intermediate amphibian hosts might feature adaptive changes in defence mechanisms in both host and parasite.

IV. AMPHIBIAN IMMUNITY AGAINST HELMINTHS

Host immune systems play an integral role in the life-cycles of parasites and thus must also be important in regulating trematode interactions with their hosts. Research on amphibian immune responses against trematodes has been limited. Nevertheless, amphibian immune systems are largely similar to those of other vertebrates (Rollins-Smith and Cohen 2005), so comparison with mammalian immunity can provide useful insights into amphibian responses (Charlemagne and Tournefier 1998). In mammals, the primary response to larval helminths is granuloma formation (Anthony *et al.* 2007), similar to the encapsulation response of invertebrates (Oksov 1991). Shortly after a trematode cercaria invades host tissue, neutrophils, macrophages and eosinophils surround the parasite, effectively separating the developing metacercaria from the surrounding tissue and sometimes killing the parasite using a cocktail of chemical weapons, including hydrogen peroxide (Anthony *et al.* 2007). This process, involving antibody-dependent cell-mediated cytotoxicity (ADCC) (Roitt *et al.* 1996), is facilitated by the action of CD4+ Th2 cells, which act to recruit more leukocytes to the site of infection, particularly during acquired immune responses to repeated infection by the same type of parasite (Anthony *et al.*, 2007). Increases in basophils and mast cells are also associated with infection by parasitic helminthes (Anthony *et al.* 2007). Recent evidence suggests that basophils act to recruit other effector cells, such as eosinophils and neutrophils, to sites of helminth infection (Min and Paul 2008).

Like mammals, amphibians possess all these leukocyte types; these probably play similar roles in combating trematode infections (Charlemagne and Tournefier 1998). One potential difference from mammals is that amphibian tissue macrophages often contain melanin (Gallone *et al.* 2002). These melanomacrophages have been associated with trematode infections in fish (Dezfuli *et al.* 2007) and probably play a role in amphibian granulomatous responses to larval trematode infections. Melanized granulomas are frequently observed surrounding trematode metacercariae, for example in the muscles and connective tissues of adult frogs (Johnson *et al.* 2004b) and in the livers of adult newts (T. R. Raffel, personal observation). Pesticide-induced reductions in eosinophils and melanomacrophages have been associated with increased susceptibility to trematode infection in tadpoles (Kiesecker 2002; Rohr *et al.* 2008b), suggesting a protective role for these cell types against trematode infections. Abundances of both eosinophils and melanomacrophages have been shown to be negatively associated with trematode load in amphibians (Rohr *et al.* 2008b). Furthermore, resistance to larval trematode infection is often higher in older tadpoles (Schotthoefer *et al.* 2003b; Dare *et al.* 2006; Holland *et al.* 2007; Raffel *et al.* in preparation a,b), perhaps corresponding to improved immune responses as tadpoles develop (Flajnik *et al.* 1987; Rollins-Smith 1998). *Ribeiroia* cysts do disappear soon after metamorphosis in some species of frogs (Stopper *et al.* 2002; Rajakaruna *et al.* 2008), but it is not known whether or not this is due to immunological attack. There is also evidence that cysts in surviving, peri-metamorphic frogs can excyst in their frog host (Sessions, unpublished) but the fate of the excysted flatworm is not known (Sessions *et al.*, unpublished observation).

Amphibians possess all the fundamental components for mounting an acquired immune memory response to parasites (Maniero *et al.* 2006), but little is known about their capacity to mount memory responses to helminths. Improved resistance to the monogenean *Protopolystoma xenopodis* has been demonstrated in *Xenopus laevis* following multiple exposures, indicating a significant immune memory response to this gastrointestinal parasite (Jackson and Tinsley 2001). There were, however, no apparent population-level effects of immune memory on any of four trematode taxa in a study of parasite age-intensity curves in red-spotted newts, despite evidence for memory responses to bacteria and a protist parasite (Raffel *et al.* 2009). Tadpoles exhibit immune memory responses to antigens, but acquired immune responses to actual parasites have not been documented (Flajnik *et al.* 1987; Rollins-Smith 1998). Such responses might be limited in tadpoles, because antibody-mediated eosoinophil activation requires the action of mature helper T-cells, which in turn requires a mature thymus. In the frog *Xenopus laevis*, however, the thymus does not complete development until near metamorphosis, so tadpoles are unable to express appropriate T-cell helper function (Roitt *et al.* 1998). Up to that point, the tadpoles might have a much more limited immunological defence. It is not known whether thymus development in *X. laevis* accurately reflects thymus development in other amphibians, although in *Rana pipiens*, the thymus does not complete its development until soon after tail resorption (stage XXV; Rugh 1951). Clearly more research is needed to determine the importance of acquired immunity to trematode infections in amphibians.

V. TREMATODIASIS AS AN EMERGING DISEASE OF AMPHIBIANS

Johnson and colleagues (Johnson *et al.* 2003; Johnson and Sutherland 2003; Johnson *et al.* 2004a), Beasley *et al.* (2004), and Skelly *et al.* (2006) have argued that certain amphibian trematodes are more common now than they have been historically. Although the arguments for the emergence of these trematodes are circumstantial, they are often quite convincing. Considering that historical data on trematode abundance and prevalence is scant, convincing circumstantial evidence of emergence might now be as good as it will ever be.

Johnson *et al.* (2002, 2003) surveyed numerous ponds and amphibians for malformations and *R. ondatrae* and compared these data to historical accounts and museum specimens to provide qualitative evidence that malformations caused by *R. ondatrae* have increased in prevalence. Johnson *et al.* (2003) could only identify seven historical (pre-1990) records of mass malformations in amphibians associated with *R. ondatrae*. In contrast, there are presently over 25 sites in the northwest and dozens of sites in Minnesota, Wisconsin, and Illinois that are presumably associated with mass malformations and *R. ondatrae*. Furthermore, it seems unlikely that most of these recent discoveries of sites where malformations occur are due to heightened surveillance, given that over 12,000 Pacific treefrogs were examined between 1950 and 1980 and few malformations relative to present levels were found (Johnson *et al.* 2003).

Beasley *et al.*(2004) argued that *Echinostoma trivolvis* infections of cricket frogs may be increasing in midwestern United States and might be associated with declines of these frogs. Frogs at sites with detectable levels of herbicides had higher *E. trivolvis* loads than at sites without detectable levels of herbicides. Beasley *et al.* (2004) proposed that agrochemical use and habitat modification might be promoting the emergence of *E. trivolvis* infections of amphibians.

Skelly *et al.* (2006) argued that urbanization was partly driving the increase in amphibian *E. trivolvis* infections. They showed a positive relationship between human densities surrounding ponds and both snail densities and the abundance of *E. trivolvis* infections in *Rana clamitans*. Skelly *et al.* (2006) proposed that humans might be inadvertently elevating snails' resources and densities by increasing inputs of nitrogen, phosphorus, and calcium into wetlands.

VI. VARIATION IN TREMATODE VIRULENCE AND AMPHIBIAN SUSCEPTIBILITY

In this section two questions are asked: (1) Which amphibian species are most susceptible to trematodes? and (2) Which species or morphotypes of trematodes are most deadly to amphibians? These questions are important because their answers assist in targeting conservation and mitigation efforts for populations and species of amphibians that are at the greatest risk from trematode infections. Most of this section, however, is hypothetical because little is presently known about variation in susceptibility among amphibians or variation in virulence among trematodes.

A. What Makes Certain Amphibian Species Particularly Susceptible to Trematodes?

It is proposed here that the frequency and duration of exposure to a given trematode species, or perhaps to trematodes in general, will affect the strength of selection for resistance to trematode infection. More specifically, amphibian species with long larval periods, such as many ranid species that overwinter in ponds and thus have two years of exposure to trematode cercariae, have greater exposure to cercariae and should have evolved stronger resistance to infection than have species with short larval periods. This hypothesis was supported by a recent comparison of two species of frogs. Tadpoles of the American toad, *Bufo americanus* (which has a larval period of about four to five weeks), were significantly more susceptible (as measured by mortality) to three species of trematodes than were green frog (*Rana clamitans*) tadpoles, which over-winter in ponds (Hall *et al.*, in preparation). Although the duration of the larval period will undoubtedly influence net exposure to trematodes, other factors will also affect exposure. For instance, species of frogs vary in the amount of time adults spend in the water where they are exposed to cercariae. The phenology of snails, trematodes, and amphibians could also be an important determinant of exposure levels. For example, in northern North America the abundance of snails and trematodes seems to peak in mid summer (Lemly and Esch 1984), so amphibians that breed early (e.g. *Rana sylvatica, Ambystoma maculatum*) probably have low exposure to trematodes and might be more susceptible if exposed. Finally, different species of amphibians, snails, and trematodes utilize different habitats, for example lotic versus lentic systems. Amphibian species found predominantly in lentic systems should have less exposure and be more susceptible to trematodes found predominantly in lotic systems, and vice versa.

Species-level variation in susceptibility to trematodes might also be a function of phylogenetic constraints. For instance, salamanders appear to be less capable of mounting an acquired immune response to parasites than are anurans (but see Raffel *et al.* 2009) and might be more susceptible to trematodes. It is not clear, however, whether this apparent difference between salamanders and anurans is generally true or merely due to unique characteristics of those few species of amphibians and parasites that have been examined so far (Raffel *et al.* 2009).

The age and size of a given amphibian are also likely to affect its susceptibility to trematodes. Recent evidence suggests that older tadpoles had lower proportions of cercariae that successfully encysted and were less likely to die, than was true for younger tadpoles, even when controlling for tadpole size (Schotthoefer *et al.* 2003b; Holland *et al.* 2007; Raffel *et al.*,in preparation a,b). This suggests that tadpole immunity improves with development. Amphibian size, independent of age, should also affect susceptibility to trematodes. Smaller amphibians have less space for trematodes and thus are more likely to succumb to lower trematode loads. In recent work, larger tadpoles exposed to a set number of cercariae had fewer trematodes and were less likely to die than was the case for smaller tadpoles, even after controlling for tadpole developmental stage (Hall *et al.*, in preparation).

B. What Makes Trematode Species Particularly Deadly to A Amphibians?

The mode of entry and the type and extent of migration through host tissues (if any) should affect trematode virulence to amphibian hosts. Certain trematode taxa enter through

amphibian orifices such as the cloaca (e.g. *Echinostoma* spp.), or are obtained by consuming infected intermediate or paratenic hosts followed by innocuous migrations through the host digestive tract (*Halipegus* spp.). In contrast, some trematode taxa cause substantial tissue damage by using stylets (*plagiorchid* and *telorchid* cercariae) or enzymes (*Ribeiroia ondatrae*) to burrow into the skin and muscle tissue. Others undergo damaging migrations within the host following infection. Trematodes that enter through the skin can cause substantial injury, with risk of subsequent bacterial infections.

The size of the trematode should also affect its virulence. Larger cercariae probably cause more tissue damage and elicit stronger immune responses than do smaller ones. Larger adult trematodes are likely to consume more host resources than are smaller trematode species. Trematode size is also likely to interact with mode of entry. For instance, *R. ondatrae*, with large cercariae that cause substantial tissue damage upon entry into the host, was more deadly to tadpoles of the American toad and green frog than were plagiorichid and *Echinostoma trivolvis* cercariae (Hall *et al.*, in preparation). Plagiorchid cercariae cause tissue damage but are smaller than *R. ondatrae*. *E. trivolvis* cercariae are similar in size to those of *R. ondatrae* but enter tadpoles through the cloaca, rather than through the skin. Indeed, many small tadpoles exposed to *R. ondatrae* cercariae seemed to consist more of "holes" than of tadpole tissue (Rohr, personal observation).

Infracommunity dynamics might also affect trematode virulence. Poor trematode competitors will be kept in check by other parasite species and thus might reach lethal levels less frequently (Kuris and Lafferty 1994; Sousa 1994). Certain combinations of parasites might also have synergistic positive effects on one another by adversely affecting different components of the host immune system. Thus, trematode virulence is likely to be dependent on co-infections. As discussed above, trematodes might also be indirectly lethal by making the host more susceptible to predation. Consequently, how deadly a trematode is will also depend on the strength of trematode-induced behavioural, physiological, and morphological modifications of its amphibian host (Dobson 1988; Moore and Gotelli 1996). Finally, all the factors mentioned above are unlikely to be independent. It is therefore likely that these factors interact in interesting synergistic ways that have yet to be revealed.

VII. NATURAL FACTORS AFFECTING AMPHIBIAN TREMATODE INFECTIONS

Despite the complexity of trematode life cycles, most seem to have one thing in common: snails are the kingpin. Nearly all trematode species utilize gastropods as first intermediate hosts, and they tend to specialize on a particular snail species more so than they do on any particular definitive or second intermediate host species. Furthermore, snails are particularly important for parasite dynamics due to embryonic amplification of trematodes within snails. Because each miracidium infecting a snail can potentially generate hundreds, thousands, or even hundreds of thousands of infectious cercariae, small fluctuations in snail population sizes might generate large fluctuations in cercarial production in a given pond. As a consequence, gastropod abundance and richness are often positive predictors of amphibian trematode infections (Johnson *et al.* 2002; Skelly *et al.* 2006; Rohr *et al.* 2008b). For example, ponds with the greatest snail densities had amphibians with the highest incidence of *R. ondatrae* and *E. trivolvis* infections (Johnson *et al.* 2002; Skelly *et al.* 2006), and gastropod richness was a significant positive predictor of total larval trematode loads (all trematode species combined) in *Rana pipiens* (Rohr *et al.* 2008b).

Except for the abundance and diversity of first intermediate hosts, amphibian age and the seasonality of cercarial shedding from snails might be the most important natural factors affecting larval trematode infections of amphibians. As mentioned above, susceptibility to larval trematode infections has been shown repeatedly to be a function of tadpole development (Schotthoefer *et al.* 2003b; Dare *et al.* 2006; Holland *et al.* 2007; Raffel *et al.* in preparation a,b). Fine-scale partitioning of tadpole ages revealed that the relationship between tadpole developmental stage and susceptibility to *E. trivovlis* cercariae is non-linear with the most susceptible tadpoles being of intermediate Gosner stages (Holland *et al.* 2007;

Raffel *et al.* in preparation a,b). Raffel *et al.* (in preparation b) suggested that the non-linearity could be explained by a general improvement in immunity with tadpole age, coupled with the smallest tadpoles having limited kidney space, making them less susceptible to heavy infections. Despite the general reduction in tadpole susceptibility with age, field-caught *Rana clamitans* tadpoles exhibited a curvilinear, asymptotic increase in *E. trivolvis* infections with age (Raffel *et al.* in preparation b). Because different mechanisms can generate similar age-parasite-intensity relationships, field data, experiments and model fitting were used to test among plausible drivers of this curvilinear age-intensity relationship for *R. clamitans*; these drivers included seasonal exposure, age-dependent susceptibility, density-dependent establishment, parasite-induced host mortality, acquired immunity, heterogeneity in susceptibility, and heterogeneity in exposure. The parsimonious explanation for the age-intensity relationship was seasonal exposure to trematodes (Raffel *et al.* in preparation b). That is, *R. clamitans* individuals that had the greatest overlap with the peak of seasonal shedding of *E. trivolvis* cercariae had the greatest *E. trivolvis* loads.

Amphibian behaviour also seems to affect the risk of trematode infection (Fig. 14). Gray treefrogs (*Hyla versicolor*) oviposited less often in pools containing snails shedding cercariae than in pools with uninfected snails or no snails, presumably thereby reducing infection risk for their offspring (Kiesecker and Skelly 2000). American toad (*Bufo americanus*) tadpoles avoided *E. trivolvis* cercariae and the strength of this avoidance was qualitatively similar to the strength of tadpole avoidance of predation-related cues (Rohr *et al.* 2009). Certain types of activity can also reduce the risk of infection (Taylor *et al.* 2004; Koprivnikar *et al.* 2006c). Thrashing of the body can remove cercariae from the body surface, and activity in general

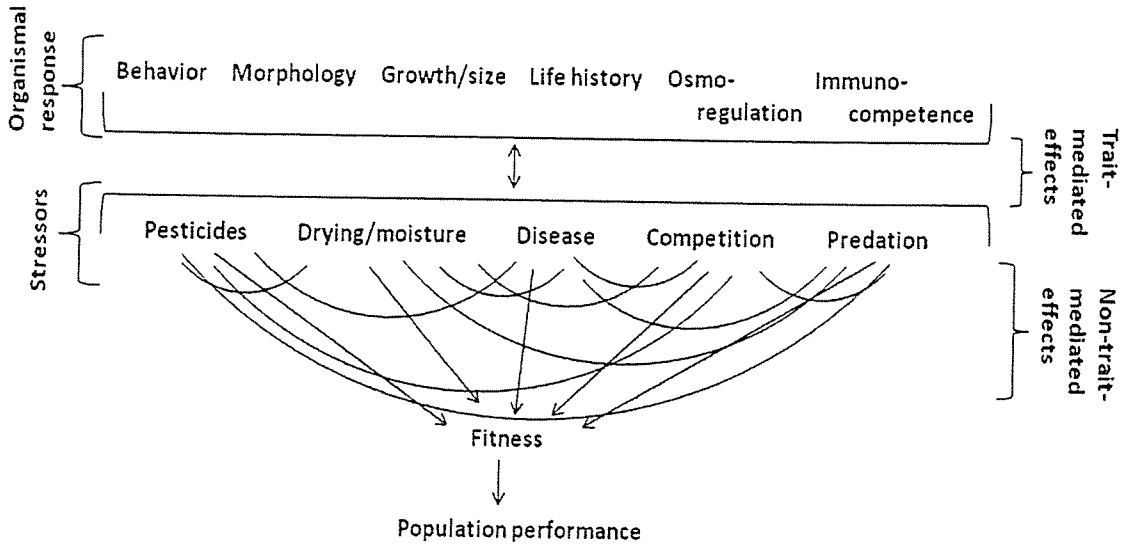


Fig. 14. Primary relationships among natural and anthropogenic stressors, organismic responses, and population performance. Arrows and lines represent connections among direct and indirect effects, respectively.

creates a moving target for cercariae that infect specific regions of the body (e.g. *E. trivolvis* must enter through the cloaca), thereby reducing trematode infections (Taylor *et al.* 2004; Koprivnikar *et al.* 2006c). Indeed, the mere smell of *E. trivolvis* cercariae appears to elevate *B. americanus* activity (Rohr *et al.* 2009).

Changes in community structure can also influence the spread of disease by changing the abundance of hosts or pathogens (density-mediated effects) or by altering host behaviour, host susceptibility, or parasite infectivity (trait-mediated effects) (Lafferty and Holt 2003). Changes in host or parasite abundance have the most obvious effects on parasite dynamics, but transmission can also be affected by factors influencing other trophic levels that then

have top-down or bottom-up effects on the parasite and/or host (Hudson *et al.* 2002). Predation, for instance, was recently discovered to be an important factor affecting amphibian trematode infections. In long-toed salamanders (*Ambystoma macrodactylum*), individuals that were missing limbs due to simulated cannibalism (amputation) were more likely to be infected by *R. ondatrae* and to exhibit limb deformities than were individuals that experienced *R. ondatrae* alone (i.e. without limb amputation) (Johnson *et al.* 2006). Caged predatory fish reduced the activity of *Rana clamitans* tadpoles and increased their *E. trivolvis* metacercarial loads (Thiemann and Wassersug 2000). Given the importance of activity as a defence against cercarial infections, it is likely that predator-induced reductions in activity made *R. clamitans* more susceptible to *E. trivovlis*.

In contrast, Raffel *et al.* (in preparation a) crossed caged predators (eastern red-spotted newts), tadpole density, and *E. trivolvis* exposure in a study on *Bufo americanus* tadpoles and found no effect of caged predators on *E. trivolvis* infections, despite *B. americanus* significantly reducing their activity in response to the caged predator. Raffel *et al.* (in preparation a), however, did detect strong effects of density. They revealed that tadpoles held at higher density were more susceptible to trematode infections when *per capita* trematode exposure was constant, a result comparable to that shown for a different trematode and amphibian species (Dare *et al.* 2006). Neither the change in tadpole activity nor the cellular immunity could account for this elevated susceptibility, suggesting an alternative mechanism. When exposure was kept constant at the level of the tank, rather than at the level of the individual, doubling density halved *per capita* exposure for the tadpoles, but resulted in no change in the number of metacercariae per tadpole. This was because the increased susceptibility associated with exposure to high tadpole density was offset by the reduced exposure to cercariae (Raffel *et al.* in preparation a).

Koprivnikar *et al.* (2008) examined the impact of *E. trivolvis* infections on the competitive ability of *Rana pipiens* tadpoles. *E. trivolvis* infection had no effect on the competitive ability of *R. pipiens* but it did reduce the growth rates of the tadpoles (Koprivnikar *et al.* 2008). Similarly, *E. trivolvis* infections slowed the growth of *B. americanus* (Raffel *et al.* in preparation a) and *R. clamitans* (Fried *et al.* 1997), but did not seem to affect the growth of *Rana sylvatica* (Belden 2006).

The hydroperiod of the pond also appears to influence trematode infections in amphibians. In ponds that were drying, both tadpoles and trematode-infected snails had decreased survivorship and the tadpoles had higher incidences of trematode infections, possibly because drying concentrated the amphibians and cercariae, thereby facilitating successful transmission (Kiesecker and Skelly 2001). With widespread climatic change, drying of ponds might be considered both a natural and an anthropogenic stressor (Rohr and Madison 2003).

Stress, in general, seems capable of elevating the incidence of trematode infections in larval amphibians. Stressors stimulate the release of glucocorticosteroid hormones that can be immunosuppressive (Belden and Kiesecker 2005). Gray treefrogs (*Hyla versicolor*) exposed to exogenous corticosterone had fewer circulating eosinophils and more *Alaria* sp. mesocercariae than did treefrogs receiving sham injections (Belden and Kiesecker 2005). These findings provide a mechanism by which both natural and anthropogenic stressors can elevate risk of disease.

VIII. ANTHROPOGENIC FACTORS AFFECTING AMPHIBIAN TREMATODE INFECTIONS

Anthropogenic changes to the environment can modify interactions between amphibians and their parasites. For instance, elevated trematode loads have been linked to nutrient and pesticide inputs into wetlands. The evidence for and against these relationships are reviewed and other potentially important anthropogenic factors influencing amphibian trematodes are discussed in the following sections.

A. Nutrient Inputs and Trematode Infections

Numerous studies have identified links between agriculture, amphibian helminth infections, and deformities (Johnson *et al.* 2002; Christin *et al.* 2003; Gendron *et al.* 2003; Beasley *et al.* 2004; Christin *et al.* 2004; Taylor *et al.* 2005; King *et al.* 2007; Koprivnikar *et al.* 2007a; McKenzie 2007), but only recently have the mechanisms underpinning this association been revealed. Johnson *et al.* (2003) suggested that an increase in artificial impoundments associated with agriculture partly drove increases in amphibian trematodes. These impoundments often receive heavy inputs of fertilizer and cattle waste, and these nutrient inputs were proposed to support a higher biomass of snails as intermediate hosts than in unaffected ponds (Johnson *et al.* 2003; Johnson and Chase 2004). Indeed, 44 of 59 wetlands associated with *R. ondatrae* infections were also artificial impoundments or human-altered wetlands (Johnson *et al.* 2002). "Created" wetlands also represented malformation hotspots in the midwestern United States (Lannoo *et al.* 2003) and higher helminth loads in frogs from agricultural wetlands (relative to frogs from forested wetlands) were attributed to elevated nutrient inputs (McKenzie 2007). To test the hypothesis that nutrients derived from agriculture could increase the level of *R. ondatrae* infections in frogs, Johnson *et al.* (2007) established mesocosms containing snail and amphibian hosts and conducted an experiment crossing fertilizer and additions of *R. ondatrae* eggs to these mesocosms. Fertilizer additions increased snail abundance, cercarial shedding rates, number of *R. ondatrae* infections in amphibians, and limb deformities, thereby verifying the postulated causal relationship (Johnson *et al.* 2007). Belden (2006) showed that nutrient inputs do not increase the pathogenicity or virulence of *E. trivolvis*, suggesting that the primary effect of eutrophication is to elevate exposure to trematodes. Skelly *et al.* (2006) suggested that the positive relationship between larval trematode infections of *R. clamitans* and human population density was due to humans releasing nutrients into wetlands, thereby causing increases in snail populations.

B. Pesticides and Trematode Infections

Pesticides common in agricultural landscapes have also been implicated as causes of elevated infections in amphibians. Various pesticides and pesticide mixtures have been shown to be immunosuppressive to both amphibians and snails (Taylor *et al.* 1999; Kiesecker 2002; Christin *et al.* 2003; Gilbertson *et al.* 2003; Christin *et al.* 2004; Russo and Lagadic 2004; Forson and Storfer 2006a,b; Hayes *et al.* 2006; Sandland and Carmosini 2006; Brodkin *et al.* 2007; Davidson *et al.* 2007; Rohr *et al.* 2008a,b) and this immune suppression has been linked to elevated amphibian infections (Taylor *et al.* 1999; Kiesecker 2002; Christin *et al.* 2003; Forson and Storfer 2006b; Hayes *et al.* 2006; Davidson *et al.* 2007; Rohr *et al.* 2008a,b). For example, Kiesecker (2002) demonstrated that three common pesticides, atrazine, malathion, and esfenvalerate, all suppressed immunity in *Rana sylvatica* and increased its larval trematode loads. Likewise, relatively high levels of atrazine negatively impacted the immune system of adult *Rana pipiens* (Houck and Sessions 2006). In a series of studies, Rohr, Raffel, and colleagues examined the lethal and sublethal effects of expected environmental concentrations of atrazine, glyphosate (herbicides), carbaryl, and malathion (insecticides) on *E. trivolvis* free-living stages and on its first and second intermediate hosts. These chemicals had no detectable effects on the survival of *E. trivolvis* miricidia, on the infectivity of *E. trivolvis* cercaria, on the growth, fecundity, or survival of snail first intermediate hosts, or on the survival of tadpole second intermediate hosts (Rohr *et al.* 2008a, Raffel *et al.* in press). The pesticides did, however, reduce cercarial survival and increase tadpole susceptibility to *E. trivolvis* infections (Rohr *et al.* 2008a). Most importantly, the reduction in exposure to trematodes due to pesticide-induced cercarial mortality (density-mediated effect) was 2.5 times smaller than the pesticide-induced increase in amphibian susceptibility (a trait-mediated effect), suggesting that the net effect of exposure to environmentally realistic levels of pesticides is to elevate the incidence of amphibian trematode infections (Rohr *et al.* 2008a).

In a follow-up study, Rohr *et al.* (2008b) surveyed wetlands in Minnesota where they quantified over 240 plausible predictors of larval trematode abundance in the declining northern leopard frog, *Rana pipiens*. They discovered that the widely used herbicide, atrazine, was the best predictor of larval trematode infections in *R. pipiens* and that this positive effect was consistent across different taxa of trematodes. Furthermore, they showed that phosphate, a primary ingredient in fertilizers, plays a complementary role, lending further support for the link between pond eutrophication and amphibian trematode infections. The combination of atrazine and phosphates accounted for 74% of the variation in larval trematode abundance (Rohr *et al.* 2008b). Path and regression analyses suggested that these agrochemicals caused an increase in snail abundance and diversity as well as immuno-suppression in *R. pipiens*.

To test whether the detected correlation between atrazine and amphibian trematode loads was causal, Rohr *et al.* (2008b) conducted a community-level mesocosm experiment in which tanks were dosed with atrazine. Tanks with atrazine had significantly less phytoplankton, greater water clarity, and more attached algae and snails than did control tanks (without atrazine). Tanks with atrazine also had immuno-suppressed tadpoles and tadpoles with elevated trematode loads when controlling for eosinophil abundance, further supporting a causal relationship between atrazine and elevated trematode infections in amphibians (Rohr *et al.* 2008b). An increased shedding rate of snails in tanks with atrazine (Johnson *et al.* 2007) was suggested as the potential mechanism by which atrazine increased trematode loads beyond its affect on immunity. The study of Rohr *et al.* (2008b) linked the work on pesticides and nutrient inputs by revealing that atrazine and phosphate, two common components of worldwide corn and sorghum production, seem to elevate larval trematode loads in a similar manner; both appear to increase exposure and susceptibility to trematodes by augmenting snail intermediate hosts and suppressing amphibian immune responses. Likewise, in an extensive study of *Bufo marinus* in Bermuda, Linzey *et al.* (2003) concluded that agrochemicals and environmental pollutants appear to cause immune suppression, increased susceptibility to trematode infections, limb malformations and possibly declines of amphibian populations.

C. Contrariwise Views on Amphibian Trematode Infections and Deformities

Not all studies have found a positive relationship between agricultural activities and larval trematode loads of amphibians or between *R. ondatrae* infections and deformities. Koprivnikar *et al.* (2006b) showed that 200 μg/L of atrazine reduced *E. trivolvis* cercarial survival and infectivity and that exposure of *R. sylvatica* only (cercarie were not exposed) to 30 μg/L of atrazine increased their *E. trivolvis* infections (2007b), similar to the findings of Kiesecker (2002) and Rohr *et al.* (2008a, 2008b). Furthermore, the increase in susceptibility of *R. sylvatica* could not be accounted for by an atrazine-induced change in activity (Koprivnikar *et al.* 2007b). Similarly, a mixture of metolachlor and atrazine also reduced *E. trivolvis* cercarial survival (Griggs and Belden 2008). Simultaneous exposure of *R. sylvatica* tadpoles and *E. trivolvis* cercariae to 30 μg/L of atrazine, however, caused no increase in tadpole cercarial infections relative to the control treatment (Koprivnikar *et al.* 2007b).

These results suggest that the primary mechanism by which atrazine elevates infection loads is by increasing periphyton, snails, and amphibian exposure to trematodes. Elevated susceptibility as a contributor cannot be ruled out, however, especially considering that it is not known how long after exposure to pollutants amphibians remain more susceptible to infections. For instance, atrazine exposure early in amphibian development (embryo and larval stages) had seemingly permanent effects on amphibian activity and water conservation, and had delayed effects on their survival (Rohr and Palmer 2005; Rohr *et al.* 2006b). In addition, Budischak *et al.* (2008) made a truly remarkable discovery. Just four days of exposure of ranid embryos to an environmentally realistic concentration of the insecticide malathion increased tadpole susceptibility to *E. trivolvis* infections seven weeks later, indicating that agrochemical exposure can have long-term effects on susceptibility.

Three recent field surveys examining the effects of agricultural activities and atrazine on parasite loads in frogs also cast doubt on the effects of pesticides on amphibian trematode infections (Johnson *et al.* 2002; Koprivnikar *et al.* 2006a; King *et al.*, 2007). An observational field study that examined over 100 sites and over 60 pesticides revealed that the frequency of frog deformities and *R. ondatrae* infections was not correlated with any of the measured pesticides; however, only three sites had detectable pesticide concentrations (Johnson *et al.* 2002). Koprivnikar *et al.* (2006a) stated that "we found no associations between combined trematode infection and . . . the presence of the herbicide atrazine". Only one out of 12 of their sampled wetlands, however, had concentrations of atrazine above the limit of detection; thus, there was insufficient power to test for a relationship. King *et al.* (2007) revealed that both the amount of agricultural and urban area around wetlands was negatively related to the abundance and diversity of helminth parasites in *R. pipiens*. Wetlands within each of their treatments (low, medium and high pesticide levels) were clustered spatially, thus making it impossible to ascertain whether the observed patterns were due to pesticide levels or spatial autocorrelation with other important factors (King *et al.* 2007).

King *et al.* (2007) suggested that agriculture and urbanization reduce parasite transmission to frogs by reducing the abundance of definitive vertebrate hosts, a suggestion similar to the findings of Rohr *et al.* (2008b), who showed that the effect of atazine on risk of trematode infection was dependent upon the number of habitat patches "suitable" for definitive hosts (forest, wetlands, and open water) around each wetland, a proxy for visitations by, and the abundance of, definitive hosts. The important message here is that chemical inputs into wetlands cannot increase trematode infections unless there is an ample supply of trematodes initially, a context-dependency that likely explains many conflicting results regarding amphibian trematode infections and anthropogenic factors. Hence, one cannot fully understand the effects of pollution or other anthropogenic factors without knowing something about proxies for the input of trematode eggs into wetlands (a natural factor), which depends upon wetland visitations by infected definitive hosts. For instance, agricultural or urban wetlands surrounded by nothing but unsuitable habitat for definitive hosts could have copious inputs of nutrients and agrochemicals, but are unlikely to have frogs with high levels of trematode infections because these wetlands are unlikely to have consistent inputs of trematode eggs.

It was proposed recently that a 'healthy' environment is one rich in parasite species (Marcogliese 2005; Hudson *et al.* 2006) because the abundance and diversity of parasites often reflects the abundance and diversity of host species on which parasites depend (Hechinger and Lafferty 2005). Although there is certainly merit to this hypothesis, the results presented here have revealed that the effects of anthropogenic factors on risk of disease are extremely complicated. Because of context-dependencies and non-linearities, one cannot categorically assume that high diversity of parasites means a healthy ecosystem. For instance, the effects of pollution are dependent upon the composition of the community (Relyea 2003; Rohr and Crumrine 2005), which can vary substantially across the landscape. While the free-living stages of parasites can be very sensitive to pollution (Lafferty 1997; Lafferty and Kuris 1999; Morley *et al.* 2003), the net effects of disease risk will depend on the concentration and duration of the exposure, the timing of exposure, delayed and long-term effects of anthropogenic factors, and impacts of environmental stressors on non-host species that compete with, or prey upon, the hosts (Rohr *et al.* 2006a; Rohr *et al.* 2008a). Likewise, the effects of other anthropogenic changes, such as modification of land use or change in climate, might also depend on the type and degree of change, the duration of the change, and the impact of any change on the traits and densities of parasites, hosts, and species that interact strongly with hosts.

The precise role of trematodes in amphibian limb deformities is also controversial. *R. ondatrae* appears to be common in the western United States and is responsible for many amphibian deformities there (Johnson *et al.* 2002). Substantial evidence, however, is building that, in other parts of the United States, many amphibian deformities cannot be explained

by *R. ondatrae* or by limb predation (Skelly *et al.* 2007). In fact, *R. ondatrae* is only rarely found in the northeast (Raffel, Rohr, Sessions personal observations) and therefore it does not account for the amphibian deformities found in a Vermont study (Taylor *et al.* 2005); it was also the least prevalent larval trematode infecting *R. pipiens* in Minnesota (found in only 5 of 18 ponds) (Rohr *et al.*, 2008b). A survey of 5,264 hylid and ranid metamorphs in 42 Vermont wetlands revealed that proximity to agriculture was the best predictor of nontraumatic limb malformations, providing support for the role of chemical toxicants, rather than of trematodes, as the cause of amphibian limb malformations (Taylor *et al.* 2005). Chemicals were also concluded as a likely cause of amphibian limb deformities in a recently released book on the topic (Lannoo 2008) (but see Sessions, in press). The identification of these missing limb malformations as "nontraumatic" has, however, recently come into question with evidence that limb trauma from selective predation can induce this type of deformity. (Sessions 2009; Ballengée and Sessions 2009).

Ultimately, there is much to learn about the effects of environmental change on amphibian deformities and trematode infections, as well as about parasite dynamics in general. The impacts of land use, pollution, invasive species, and climatic change on amphibian development and risk of disease will be important avenues of research for years to come.

IX. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Like many parasites, digenetic trematodes are important components of amphibian ecology. Although their role in amphibian population declines has yet to be elucidated, they have the potential to contribute to declines because they can cause substantial mortality in amphibians. As for many parasitic taxa of amphibians, there is much to learn about trematodes. Future research should focus on improving the understanding of amphibian immunity, the population-level effects of helminths, the impacts of reservoir hosts on the severity of amphibian trematode infections, which trematode species are highly virulent to particular amphibian species (and why), which natural and anthropogenic factors affect trematode abundance and virulence, the role of trematodes as drivers of host community dynamics, alternative explanations for amphibian deformities, and the impacts of trematode manipulation of amphibian hosts. Advances on these fronts will reveal the impact that trematodes have on the persistence of amphibian populations and on community dynamics generally.

Much progress has been made in understanding the effects of trematodes on amphibians. There are, however, undoubtedly more questions than answers. Following are suggested directions for research into the ecology of trematodes that parasitize amphibians.

Immunity of amphibians: Very little is known about the immunity of amphibians, such as the particular defences used against specific parasites, and amphibians' responses to trematodes are no exception. There is still much to learn about the contributions of cellular and hummoral immunity, and physical, biochemical, and behavioural responses to trematodes.

Population-level effects: It seems likely that trematodes can regulate amphibian populations given that many trematodes cause mortality in amphibians (Johnson *et al.* 1999; Johnson *et al.* 2001a; Schotthoefer *et al.* 2003a,b; Rohr *et al.* 2008a). Also, density-mediated increases in survival could compensate for trematode-induced mortality (Vonesh and De la Cruz 2002; Rohr *et al.* 2006b). Consequently, to assess the potential role, if any, that trematodes play in amphibian declines, one must determine whether they can regulate amphibian populations. Trematode removal studies seem to be the best approach for answering this question (Hudson *et al.* 1998). Furthermore, they might provide insight into potential anti-helmintics that could be effective in remedying infections contributing to population declines.

Reservoir hosts: Reservoir hosts that allow highly virulent parasites to persist after they reduce the populations of their primary hosts, are often important factors in declines of a host (de Castro and Bolker 2005). Are there reservoir hosts for the trematodes that are parasitic on amphibians, and if so, which species are they?

Natural factors: Despite a long history of ecological research on competition, predation, and parasitism, there is a paucity of knowledge on the effects of competition or risk of predation on the susceptibility and exposure of amphibians to parasites (Raffel *et al.* 2008). Are competition and predation stressors that can be immunosuppressive? How much of an impact does predation upon free-living stages of trematodes have on trematode infections of amphibians and snails (Schotthoefer *et al.* 2007)? Can predation on snails reduce the risk of infection of amphibians by trematodes?

Anthropogenic factors: There is now strong evidence that various agrochemicals can increase trematode infections of amphibians (Kiesecker 2002; Johnson and Chase 2004; Johnson *et al.* 2007; Rohr *et al.* 2008a; Rohr *et al.* 2008b), and evidence that climatic change might also increase risk of infection (Raffel *et al.* 2006). There is only a rudimentary understanding of the role of anthropogenic factors in amphibian declines and risk of disease, and little is known about the mechanisms by which anthropogenic factors might affect immunity and subsequent risk of infection.

Facilitation: Although the convention in ecology has been to focus on negative interactions among organisms (competition, predation, and parasitism), appreciation for positive interactions is growing (Raffel *et al.* 2008). Can trematodes have positive indirect effects on amphibians? Other amphibian species appear to be sinks for trematodes (dead end hosts), having positive effects on susceptible amphibian species (Johnson *et al.* 2008; Johnson and Hartson 2009). How common is parasite-mediated facilitation (Ostfeld and Keesing 2000; Dobson *et al.* 2006; Raffel *et al.* 2008b)? Is there selection for susceptible species to mimic species that are resistant to infections (Raffel *et al.* 2008)?

Alternative drivers of deformities: While many hot spots for malformed frogs in western United States and perhaps in midwestern United States are driven by exposure to *Ribeiroia ondatrae*, many hot spots cannot be explained by trematodes, especially on the eastern coast (Taylor *et al.* 2005; Skelly *et al.* 2007; Lannoo 2008). Furthermore, a monostome trematode has been discovered recently that also causes amphibian deformities (Rajakaruna *et al.* 2008), suggesting that *R. ondatrae* might not be the only culprit. Non-trematode drivers of deformities and alternative trematode species should be more thoroughly considered as factors contributing to amphibian malformations.

Trematodes' rhythms: Trematodes exhibit diurnal and circannual rhythms that remain understudied. What drives these rhythms, first intermediate, second intermediate, definitive hosts, or something else? What cues are used to entrain these rhythms?

Trematodes' manipulation of hosts: Helminths are well known for manipulating their hosts in ways that facilitate transmission of the parasite (Dobson 1988; Moore and Gotelli 1996). Limb deformities induced by *R. ondatrae* and oedema induced by echinostomes likely increase the incidence of predation by amphibians and thus transmission of the trematode (Sessions and Ruth 1990; Stopper *et al.* 2002; Johnson and Sutherland 2003; Johnson *et al.* 2004a; Holland *et al.* 2007), but this has yet to be demonstrated. Manipulations induced by other trematodes should also be explored.

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