Characterizing the Impact of Multiple Potential Enemies (Predators and Parasites) on the Behaviour of Ranid Tadpoles

by

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Thesis Abstract

In order to fully understand an organism's behaviours the interactions between multiple enemies or selective pressures need to be considered, as these interactions are usually far more complex than the simple addition of their effects in isolation. In this thesis, I consider the impact of multiple enemies (fish predators and parasites) on the behaviour of three larval anurans (*Lithobates sylvaticus*, *L. clamitans* and *L. catesbeianus*). I also determine whether species that differ in life-histories and habitat preferences possess different antipredator mechanisms and how this affects species responses to multiple enemies. I show that the three Ranid larvae respond differently to the trade-off imposed by the presence of both fish predators and trematode parasites within the environment. The two more permanent pond breeders (*L. clamitans* and *L. catesbeianus*) increased activity when in the combined presence of predators and parasites. In contrast, the temporary pond breeder (*L. sylvaticus*) decreased activity in the combined presence of predator and parasites, in the same manner as they responded to fish alone. Further, the presence of fish along with parasites increased the susceptibility of both *L. sylvaticus* and *L. clamitans* to trematode infection, whereas parasite infection in *L. catesbeianus* was unaffected by the presence of fish. A second experiment to assess palatability of the three anuran species to fish, revealed a range of palatabilities, with *L. catesbeianus* being least palatable, *L. clamitans* being somewhat unpalatable, and *L. sylvaticus* being highly palatable. This result helps to explain the species differences in the observed behaviour to the combined presence of fish and parasites. In conclusion, the

results from this study highlight the importance of considering multiple selective pressures faced by organisms and how this shapes their behaviour.

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Chapter 1

General Introduction

Over the last century, it was generally thought that parasites did not play an important role in structuring animal communities (Poulin 1999). This view, unfortunately, has been a major oversight on our part, as parasites in some ecosystems can account for a large percentage of total biomass (Kuris et al. 2008), and it is probable that there are as many or more species of parasites in a community as non-parasitic species (Poulin 1999). Thus, the general view of parasites being unimportant determinants of animal community structure has been slowly changing over the last few decades and now parasitology and ecology have become linked, which is important as these two disciplines were previously viewed as being mutually exclusive from each other (Poulin 1999, Lefevre et al. 2009). In fact, most ecologists are now aware that the introduction or extinction of a single parasite species in an ecosystem can alter the interactions among multiple trophic levels within the community, which can have an impact on overall biodiversity (Thomas et al. 2005).

There are three major ways in which parasites alter community structure of free-living animals, or their hosts (Poulin 1999). First, parasites can have differential effects on multiple host species and thereby change their relative abundances. For example, Park (1948) showed that when two species of flour beetles (*Tribolium confusum* and *Tribolium castaneum*) were kept together, *T. castaneum* would drive *T. confusum* to extinction, as *T. castaneum* was a better competitor. However, some of the containers housing both beetle species contained a sporozoan parasite (*Adelina tribolii*, which lives in the beetles'

haemocoel) thereby parasitizing the beetles. The importance of this parasite became clear, as *T. castaneum* instead of *T. confusum* went extinct when the parasite was present. Because *T. castaneum* was more susceptible to the parasite than *T. confusum*, the parasite reversed the outcome of the interspecific competition and completely changed species' abundance.

Second, parasites can debilitate keystone species, which by definition affects other species within the community. For example, Wood et al. (2007) showed that herbivorous snails (*Littorina littorea*) parasitized by a trematode (*Cryptocotyle lingua*), consume 40% less macroalgae compared to uninfected snails. In the field, weaker grazing by infected snails resulted in significantly more ephemeral macroalgae cover relative to areas grazed by the same density of uninfected snails, as these snails are the dominant herbivores within this intertidal community. This change in the macroalgal community indirectly caused by parasitized snails, in turn affects the abundance of other species that rely on the macroalgae for resources or habitat structure.

Third, parasites can alter the phenotype of their host (whether it is morphology, behaviour or physiology), which in turn can change the importance of the host species for the community. For example, when the foot of cockles (*Austrovenus stutchburyi*) becomes heavily parasitized by a trematode (*Curtuteria australis*), the cockle cannot burrow under the mud as it naturally would and instead lies on the sediment surface. This behavioural modification facilitates faster transmission to the definitive host because cockles that do not burrow under the mud are more susceptible to avian predators (Mouritsen 2002, Babirat et al. 2004). While this behavioural modification increases transmission success of the parasite, it also changes the community by creating a habitat

for limpets (*Notoacmea helmsi*) allowing them to coexist with the otherwise out-competing sea anemone (*Anthopleura aureoradiata*) (Thomas et al. 1998). Further, a long-term field study that manipulated the infection intensity of buried cockles and the density of uninfected experimental cockles on the sediment surface, showed that both high parasite loads in buried cockles and those placed on the surface increased species richness and the density of major systematic and functional groups of benthic macroinvertebrates (Mouritsen and Poulin 2005). Parasitized animals are thus complex organisms which retain characteristics that are similar to uninfected individuals but also display new characteristics involving them in novel interactions with other trophic levels, which inevitably re-configures the community (Lefevre et al. 2009).

In natural settings, species face numerous diverse selective pressures from herbivores and predators as well as parasites. Most studies done to date on trophic interactions consider only one species pairing (e.g. one predator/one prey item or one host/one parasite species) (Hochberg 1996, Poitrineau et al. 2003). However, in order to truly understand behaviour and other phenotypic traits, the interactions between multiple potential enemies needs to be considered, as these interactions are usually far more complex than the simple addition of their effects in isolation (Hochberg 1996, Poitrineau et al. 2003). For example, depending on the specifics of the system, the addition of one enemy may enhance or reduce the risk of attack by another enemy. In extreme cases, the addition of a second natural enemy can cause the population to destabilize and lead all three species to run the risk of extinction (Begon et al. 1996, Hochberg 1996, Poitrineau et al. 2003).

The combined effect of predatory mites and fish predators on larval damselflies (*Ischnura verticalis*) illustrates the need to consider multiple enemies in natural systems. Larval damselflies increase their activity (crawling and vigorously grooming) in an attempt to remove attacking mites (Forbes and Baker 1990, Baker and Smith 1997, Rutherford et al. 2007). However, larval damselflies are also vulnerable to many fish predators and, as such, generally decrease their activity in the presence of fish (Dixon and Baker 1988). Thus, when a larval damselfly is confronted with both natural enemies at the same time, a conflict between antipredator and antiparasite behaviour is generated. Two studies examined this conflict in detail using two different species of larval damselflies: *Ishnura verticalis* (Baker and Smith 1997) and *Enallagma ebrium* (Rutherford et al. 2007). Both studies showed that in the presence of mites alone antiparasite behaviours (grooming, crawling, swimming and turning) increased. In the presence of fish alone, *E. ebrium* decreased grooming behaviour; presumably this is a conspicuous behaviour. However, in both studies when larval damselflies were exposed to mites and fish simultaneously, larval damselflies increased their grooming and antiparasite behaviour similarly to when mites were present alone. In addition, Baker and Smith (1997) showed that larval damselflies exposed to both mites and fish were also more likely to be attacked and killed by fish than those exposed only to fish. Thus, it would appear that there is interference between defences against two enemies for larval damselflies and if encounter rates with one enemy is sufficiently high, then optimal fitness may be achieved by the abandonment of defence against the other enemy (Poitrineau et al. 2003; Rutherford et al. 2007). These types of interactions that lead to conflicting behaviours need to be further explored in a variety of systems in order to

elucidate the impact of multiple selection pressures on organisms within their communities.

Larval anurans make an ideal system in which to investigate the impact of multiple trophic interactions and the subsequent impact on behaviour. First, anurans have a complex life cycle (an aquatic larval stage and a terrestrial adult stage) that requires individuals of a single genotype to persist in two very different selective environments. Second, anuran species have diversified into different environments exposing them to a diverse range of stressors (such as different predator types). There are generally three classifications of habitat types used for breeding into which anuran species can be grouped (Wellborn et al. 1996). (1) Ephemeral pond breeders - these species use ponds that have relatively short hydroperiods, meaning that they frequently dry out, and include flooded fields, woodland pools, ditches, or even puddles (Wellborn et al. 1996, Skelly 1997, Harding 2006). As such, ephemeral ponds generally have only a few smaller invertebrate predators or none at all and tadpoles that inhabit these environments are very active and develop rapidly in order to avoid desiccation (e.g. *Lithobates sylvaticus, Spea hammondii,* also sometimes, *Hyla versicolor* and *H. chrysoscelis*) (Skelly 1997). (2) Intermediate pond breeders - these species use ponds that are characterized by longer hydroperiods, drying infrequently (e.g. once every 5 – 10 years), and include lakes, swamps and bogs (Wellborn et al. 1996, Harding 2006). The major predators found within these habitats are invertebrates, salamander larvae and sometimes fish (Skelly 1997). Tadpoles that are moderately active and develop in a moderate time frame (e.g. a few weeks to a year) flourish in these habitats (e.g. *L. clamitans* and *L. pipiens*) (Wellborn et al. 1996, Skelly 1997, Harding 2006). (3) Permanent pond breeders – these

species breed in ponds that rarely or never dry out (e.g. river back waters, lakes, shallow Great Lakes bays; Harding 2006). As such, these water bodies can support larger predators that always require water for survival such as fish (Skelly 1997). As there is no constraint imposed by desiccation, tadpoles that inhabit these types of water bodies are relatively inactive and develop slowly, spending as long as two years in the larval form before transforming (e.g. *L. catesbeianus* and *L. septentrionalis*) (Wellborn et al. 1996, Skelly 1997, Harding 2006).

Species segregating along this permanency gradient have acquired different strategies to cope with the unique constraints/stressors imposed by each habitat (Wellborn et al. 1996). In addition, the different predators that inhabit each system select for different morphological and behavioural adaptations. For example, some ephemeral pond breeders show morphological plasticity when in the presence of invertebrate predators (e.g. *L. sylvaticus, Hyla versicolor* and *H. chrysoscelis*). *Hyla chrysoscelis*, *H. versicolor* and *H. fermoralis* tadpoles in the presence of dragonfly nymphs develop large and brightly coloured tail fins, which appear to function by diverting lethal strikes from the body to the less vulnerable tail (McCollum and Van Buskirk 1996, Van Buskirk et al. 2004). Toxicity is another morphological adaptation that has been observed to occur in larval anurans such as *Bufo* spp. This species contains a known toxin called bufotoxin (Flier et al. 1980) that makes tadpoles unpalatable to predators and thereby minimizes predation risk.

In addition to morphological changes, there are three major behavioural anti-predator mechanisms used by a wide variety of larval anuran species: aggregation (as seen in larvae of *Bufo* spp., Watt et al. 1997), refuge use (e.g. Petranka et al. 1987), and

an overall decrease in activity (Benard 2004). A decrease in activity is the main behavioural antipredator mechanism used by tadpoles as it decreases the likelihood that a predator will visually detect the tadpole. This behaviour has consequences if the tadpole cannot accurately assess the predation risk. For example, a decrease in activity in the presence of a predator translates into an overall decrease in foraging opportunities as foraging success is determined by activity levels (e.g. Lawler 1989, Werner and Anholt 1993, Anholt et al. 2000, Relyea 2001, Richardson 2001). A decrease in activity can thus have severe consequences for reproductive success through size at metamorphosis for anuran species (Werner and Anholt 1993). In general, the effects of predators and the way in which predators shape anuran communities have been extensively researched and characterized over the last few decades.

On the other hand, parasites, which are also important determinants of animal community structure as discussed above, have received relatively little attention particularly within larval anuran communities. This, however, has been slowly changing and a number of parasite species have now been identified that use tadpoles as an intermediate host. Further, the presence of attacking parasites, (e.g. *Echinostoma trivolvis*, *Ribeiroia ondatrae*, *Alaria mustelae*) produces a change in tadpole behaviour as it attempts to dislodge the parasites (Taylor et al. 2004). Some of these behavioural responses displayed by tadpoles in the presence of parasites include an overall increase in activity and multiple uncharacteristic explosive behaviours (Thiemann and Wassersug 2000, Taylor et al. 2004, Koprivnikar et al. 2006). Because a decrease in activity is the main antipredator behaviour employed by larval anurans and in the presence of parasites tadpoles increase their activity, this leads to a trade-off between antipredator and

antiparasite behaviour when faced with both potential enemies simultaneously. This trade-off between antipredator and antiparasite behaviours when both enemies are present has received little attention. Investigating how different larval anuran species that are adapted to life in different habitats respond to the trade-off when both enemies are simultaneously present can help us understand how anurans have successfully diversified into different habitat types while persisting alongside potential enemies.

In this research, I consider the impact of multiple enemies on the behaviour of three larval anurans (*Lithobates sylvaticus*, *Lithobates clamitans* and *Lithobates catesbeianus;* all were formerly in the genus *Rana*). To characterize patterns, I asked the following questions:

1. How do larval anurans respond to the trade-off between antipredator/antiparasite behaviours when both fish predators and trematode parasites are present within the environment?
2. How does the behavioural response to the trade-off differ among species? In particular, do species that possess additional antipredator mechanisms, such as unpalatability, respond differently from those species that do not?

The system

Amphibians

The three larval anurans used in this study were chosen because they all commonly occur in southern Ontario and are closely related (Hillis and Wilcox 2006) but have different life-histories and habitat preferences. For example, *L. sylvaticus* is found in ephemeral ponds that tend to lack parasites and have mostly invertebrate predators

(Skelly 1997). Because *L. sylvaticus* typically never encounter fish in the wild, this species lacks any additional antipredator mechanisms against fish (e.g. toxicity or bad taste) and as such, may respond more to the predation threat when both fish and parasites are present. *Lithobates catesbeianus,* on the other end of the spectrum, inhabit only permanent bodies of water and are exposed to both a number of parasite species and larger predators like fish, but less to invertebrate predators (as fish also consume macroinvertebrates). This species is likely to have additional antipredator mechanisms against fish predation and thus is expected to respond behaviourally more to attacking parasites than to fish predators. Finally, *L. clamitans* tends to breed in intermediate pond types and can be exposed to higher densities of invertebrate predators than *L. catesbeianus,* although larvae of this species can also encounter fish predators. Thus, it is unclear how this species will react to the simultaneous presence of fish and parasites.

Predators

Sunfish are native to North America and are widely distributed throughout a variety of streams, rivers, ponds, and lake habitats (Scott and Helfman 2001). In addition, sunfish are major predators of native anuran species (Werner and McPeek 1994, Hecnar and McLoskey 1997); thus, non-lethal visual and chemical cues of *Lepomis gibbosus* were used to produce the predatory threat to which the three anuran species were exposed.

Parasite

Echinostoma trivolvis is a digenetic trematode that occurs in lentic aquatic environments all across North America and infects the intestines of numerous vertebrate hosts, typically aquatic or semi-aquatic birds and mammals (e.g. muskrats and raccoons) (Beaver 1937, Huffman and Fried 1990). *Echinostoma trivolvis* has a complex life cycle requiring them to infect three different host species namely, planorbid snails, larval amphibians or fish, and semi-aquatic birds or mammals (Beaver 1937, Huffman and Fried 1990). The larval form of *E. trivolvis* (cercariae), actively seek out tadpoles and upon contact, attach to the epidermis and crawl along the body until the cloaca is reached (Huffman and Fried 1990). Cercariae that locate the cloaca enter, lose their tail, and migrate up the ureters where encystment occurs (Huffman and Fried 1990).

This parasite species was chosen for this study because previous research indicates that tadpoles react to attacking cercariae with an increase in activity and large explosive behaviours in attempt to dislodge cercariae (Thiemann and Wassersug 2000, Taylor et al. 2004, Koprivnikar et al. 2006). In addition, this parasite species appears to cause mortality only in the earlier developmental stages of tadpoles (e.g. below Gosner stage 25; Gosner 1960) and not in the later stages (Schotthoefer et al. 2003), unlike other parasites such as *R. ondatrae* which has been shown to cause significant mortality at later developmental stages (Gosner stage 27; Johnson et al. 2001).

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Chapter 2

On the Behavioural Response of *Lithobates* Tadpoles to the Combined Presence of Predators and Parasites

Animals are confronted with many trade-offs on a daily basis. However, none is as fundamental as the trade-off between the need to eat while simultaneously evading predators (Lima and Dill 1990). For example, almost all free-living animals must be active in order to acquire resources and obtain mates, but this activity also makes prey vulnerable to predators (Taylor 1984). This is particularly clear within the larval anuran system. Larval anurans are filter feeders, sometimes scraping attached algae and detritus into the water column to filter suspended particulates while swimming (Seale and Wassersug 1979, Seale and Beckvar 1980, Anholt and Werner 1995). The more active a tadpole is, the more conspicuous it is to predators and therefore, more vulnerable relative to an inactive conspecific (Anholt and Werner 1995). Thus, it is no surprise that larval anurans in general reduce their activity in the presence of a predator in order to minimize predation risk (e.g. Lawler 1989, Werner and Anholt 1993, Anholt et al. 2000, Relyea 2001b, Richardson 2001).

In addition to reduced activity, larval anurans have developed other defence mechanisms as protection from predators. For example, some anuran larvae have noxious or toxic skin secretions, which originate from granular glands of the skin (Liem 1961). Others exhibit morphological phenotypic plasticity, altering their shape (e.g. tail fin depth, tail length and shorter bodies) in response to predators (e.g. Van Buskirk and Relyea 1998, Relyea 2001a, b, 2003, 2004). This can minimize encounter rates or

decrease the likelihood that the predator will capture the tadpole upon encounter (Van Buskirk and Relyea 1998, Van Buskirk et al. 2003).

While the importance of predators in aquatic systems is clear, parasites that are commonly present have been overlooked until recently. A large body of research has focused on determining why amphibian populations are in worldwide decline (Stuart et al. 2004). The results of these studies have shown that in conjunction with human interference, parasitism is increasingly linked to limb deformities and population declines (Johnson et al. 1999, Johnson and Sutherland 2003, Holland et al. 2007). Echinostomes are a group of trematode parasites that are now known to be an important disease agent in amphibian populations (Holland et al. 2007).

Echinostomes have a complex life cycle involving three different hosts (Figure 2.1). Adult echinostomes reside within the intestinal tract of aquatic birds and mammals (Beaver 1937, Huffman and Fried 1990). More specifically, *Echinostoma trivolvis* uses a snail (*Planorabella trivolvis*) as the first intermediate host, which then produces free-swimming cercariae that can infect a wide range of secondary intermediate hosts, including tadpoles (Huffman and Fried 1990, Martin and Conn 1990, Fried et al. 1997, Taylor et al. 2004, Koprivnikar et al. 2006b). Once cercariae contact a tadpole, the cercariae crawl along the epidermis much like an inch worm until the cloaca is contacted (Beaver 1937). Once the cercariae enter the cloaca, their tails drops off and they migrate

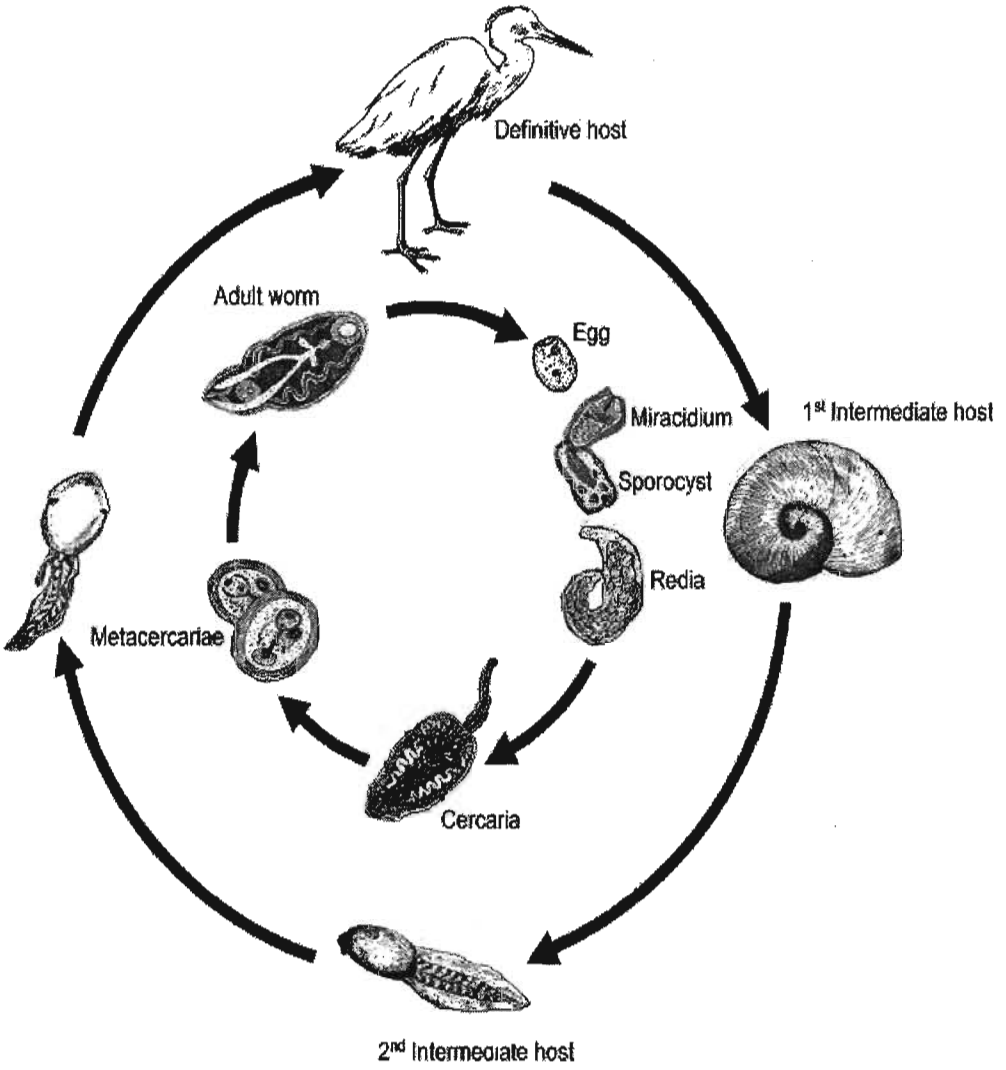

Figure 2.1: Generalized life cycle of the trematode parasite *Echinostoma trivolvis*. Clockwise from the top (outer circle), is the definitive host (avian or semi–aquatic mammal), followed by the first intermediate host (aquatic snail) and finally the second intermediate host (Ranid tadpoles), where *E. trivolvis* preferentially encysts in the developing kidney system. The inner circle depicts the various life stages of the parasite as it is transferred from host to host. Modified from Figure 1 in Szuroczki & Richardson (2009).

up the ureters (ducts that carry urine from the kidney) where encystment occurs (Beaver 1937, Prudhoe and Bray 1982, Huffman and Fried 1990, Martin and Conn 1990, Fried et al. 1997, Thiemann and Wassersug 2000a, Holland et al. 2007). In response to attacking parasites, tadpoles increase their activity and exhibit numerous peculiar behaviours or evasive maneuvers (such as explosive swimming with high angular accelerations) as a means to effectively dislodge cercariae (Taylor et al. 2004).

If an increase in activity is used to lower parasitism rates, this is directly counter to the best response if a fish predator is present. For example, Relyea (2001b) showed that the presence of a predatory fish reduced tadpole activity. However, this reduction in activity can reduce the tadpole's ability to effectively detach cercariae leading to a trade-off between antipredator and antiparasite behaviours.

Thiemann & Wassersug (2000) investigated this trade-off between antipredator and antiparasite behaviours in two larval anuran species, *Lithobates sylvaticus* and *Lithobates clamitans*. Larvae of both species were exposed to parasites and caged fish predators and subsequent activity, parasite loads and the effect of predators and parasites on the shape, growth and development of larval *L. clamitans* were measured. Both species unexpectedly reduced activity in the presence of parasites. This was unexpected because preliminary observations of tadpoles exposed to cercariae showed that tadpoles responded with explosive outbursts of movement. The experiments, however, ran for multiple days (7 days for *L. sylvaticus* and 28 days *L. clamitans*) and activity was scored over multiple brief periods throughout the experiment. In fact, activity was not scored upon parasite introduction; thus, the unexpected result of a decrease in activity in response to parasites over an extended period of time was speculated to be an adaptation

for minimizing parasite detection. The experimental design, however, would have inevitably missed any short-term responses to cercariae. These short term responses, however, are important to consider, especially when looking at the combined presence of predators and parasites, because these short-term bursts of activity are more likely to attract visually oriented predators.

In light of this potential trade-off, I investigate the behavioural responses of larvae from three anuran species, *Lithobates sylvaticus* (formerly *Rana sylvatica*), *L. clamitans* (formerly *R. clamitans*) and *L. catesbeianus* (formerly *R. catesbeiana*), to the combined presence of fish predators and parasites in the short-term (15 min exposure period to both fish and parasites). I measure overall tadpole activity, and quantify behaviours displayed by tadpoles in response to attacking cercariae, as previously noted in the literature (Taylor et al. 2004; Koprivnikar et al. 2006b). In addition, tadpoles exposed to parasites were dissected to determine the number of parasites that successfully encysted within the developing nephric system. I predict that predator presence may increase infection risk for those species that never encounter fish predators (i.e. *L. sylvaticus* larvae) or encounter them less frequently (i.e. *L. clamitans* larvae), as the presence of an unknown predator has been shown to induce a general antipredator response (namely a decrease in activity) in tadpoles and larval newts (Manteifel 1995, Mathis and Vincent 2000). If tadpoles decrease their activity in response to a potential predator, this increases the chances of successful encystment of parasites, as tadpoles will be unable to freely engage in any antiparasite behaviours in an attempt to shed attacking cercariae. I also predict that *L. catesbeianus* larvae, which inhabit permanent ponds with fish and have been hypothesized to possess the additional antipredator mechanism of unpalatability (Kruse

and Francis 1977, Kats et al. 1988, Werner and McPeek 1994, Eklov and Werner 2000), will be unaffected by fish predators, responding freely to attacking cercariae and thereby decreasing infection intensity by successfully dislodging cercariae.

Methods

Collection of Animals and Husbandry

Amphibians

Nine *L. sylvaticus* egg masses were collected in May 2008 from Bat Lake (45° 35' N, 78° 31' W) at the Wildlife Research Station, Algonquin Park, Ontario, Canada (45° 35' N, 78° 30' W). Nine egg masses were collected to ensure sufficient numbers of hatchlings were available, as a single *L. sylvaticus* egg mass typically only has 500 eggs (compared to a single *L. catesbeianus* egg mass, which has upwards of several thousand eggs; Harding 2006). Individual *L. sylvaticus* egg masses were placed into either 1.5 L glass bowls or, for smaller egg masses, 300 mL glass bowls which were all placed into two growth chambers and cooled to 5°C prior to hatching (cooling egg masses is required as *L. sylvaticus* egg masses have very low hatching success at warmer temperatures; it also slows growth, necessary because this species develops much faster than *L. clamitans* and *L. catesbeianus*).

Lithobates clamitans hatchlings (Gosner stage 20; Gosner 1960) from the same egg mass, were collected in July 2008 from a pond near Rock Lake in Algonquin Park, Ontario, Canada (45° 31' N, 78° 24' W). Hatchlings instead of an egg mass were collected, as no fully intact egg mass was found. Hatchlings of both species were housed in 38 L

(61 x 40.6 x 22.2 cm) Rubbermaid® tubs filled with a combination of filtered pond water and animal-ready water (carbon-filtered and aged tap water with pH adjusted to 7.0). Finally, a single *L. catesbeianus* egg mass was collected July 2008 from Lake Sasajewun (45° 36' N, 78° 31' W) at the Wildlife Research Station, Algonquin Park, Ontario, Canada and placed into a 121 L (84 x 51 x 61 cm) Rubbermaid garbage canister filled approximately half way with Lake Sasajewun water and allowed to hatch. The larger Rubbermaid® container was used as previous unsuccessful attempts (in unrelated studies) to hatch out *L. catesbeianus* egg masses were attributed to use of a shallow container. Hatchings were then removed and placed into 11.4 L (30 x 25 x 15 cm) white plastic tubs. In all species, as tadpoles developed and grew larger, they were housed in more tubs so that at testing size there were no more than 20 tadpoles per tub. All tadpoles were maintained on ground Spirulina Algae Discs (Wardley®, Secaucus, New Jersey) and approximately 0.5 L of suspended unicellular green algae (from a lab culture) placed into tubs once a week. Tubs were cleaned of feces every second day and a complete water change was performed weekly.

Experiments began for *L. sylvaticus* and *L. clamitans* when they reached Gosner stage 26 (meaning that the hind limb buds had started to develop and were visible under a dissecting microscope; Gosner 1960) and ended using individuals that were at stage 28 for *L. sylvaticus* (as *L. sylvaticus* develop quickly) and stage 26 for *L. clamitans*. Larval *L. catesbeianus* develop more slowly compared to larval *L. sylvaticus* and *L. clamitans*, so late Gosner stage 25 and 26 individuals were used. Tadpoles in early Gosner stage 25 were not used in any experiments because the mortality rate due to Echinostome infection that early in development has been shown to be high (Schotthoefer et al. 2003).

Fish predators

Four *Lepomis gibbosus* (approximately 8-9 cm in length) were collected in May 2008 from a pond located within St. John's conservation area, Pelham, Ontario (43° 3' N, 79° 17' W). Fish were housed in transparent 10 L (30.5 x 22.9 x 17.8 cm) aquaria (Tom Pla-House Clear Vue) filled with approximately 8.5 L of animal ready water and fitted with a sponge filter (Dirt Magnet® Aquarium Filter, Junior Model). All tanks received five drops of Stress Coat® and five drops of Kent Freshwater Essential™ (mineral supplement for aquaria), as well as one piece of rounded pvc tubing as cover for fish. Fish were maintained on approximately 2 mL of wet blood worms 3 times a week.

Snails/Parasites

Throughout May to September 2008, *Planorbella trivolvis* snails were collected by handpicking snails from vegetation (they are often found clinging to cattails, *Typha spp.* and other submerged or floating vegetation) from the Glenridge Naturalization Site in Niagara region, Ontario, Canada (43° 7' N, 79° 14' W). They were also collected using dip net sweeps through the debris on the pond floor. Snails were housed communally in small aquaria and fed lettuce *ad libitum*. To obtain cercariae, snails were placed into small plastic dishes (100 mL Petri dishes) filled with animal-ready water and placed approximately 20 cm away from a 100 W incandescent light bulb (the combination of light and heat from the bulb stimulates cercarial emergence). *Echinostoma trivolvis* cercariae were identified by the anterior collar of spines, distinct swimming, and size (for a more detailed description on how to identify cercariae refer to Schell (1970) and Szuroczki & Richardson (2009)). Cercariae were pipetted and placed into containers

filled with either 150 mL of animal-ready water or 150 mL of fish cue water (see below) in preparation for subsequent introduction into the experiments.

All animals were kept at room temperature within the laboratory (e.g. between 23 – 25°C) and on a 14:10 light:dark cycle.

Experimental Design

Experiments with *L. sylvaticus*, *L. clamitans* and *L. catesbeianus* followed identical designs and protocols. I used a 3 x 4 factorial design, replicated 20 times (Figure 2. 2). This design resulted in four separate treatments (1) predator- and parasite-free control, (2) parasite only, (3) fish only, presence of fish visual/chemical cues, and (4) combination, presence of fish visual/chemical cues and parasites (I will refer to this treatment as "combo" from here on in).

Experimental tanks were transparent 10 L (30.5 x 22.9 x 17.8 cm) aquaria (Tom Pla-House Clear Vue) filled with approximately 8.5 L of animal ready water for the fish and combo tanks or tap water for the control and parasite only treatments. In order to expose tadpoles to non-lethal fish cues and to ensure the use of a fixed density of cercariae, all experimental tanks had an inner 1 L transparent cylindrical container (13 cm in height with a diameter of 13 cm) mounted on top of a 8 x 5 cm ABS bushing (Figure 2.3). For the fish only and combo treatments, one fish was placed into each tank; an air stone and a piece of pvc tubing for cover were added to the tanks when trials had finished

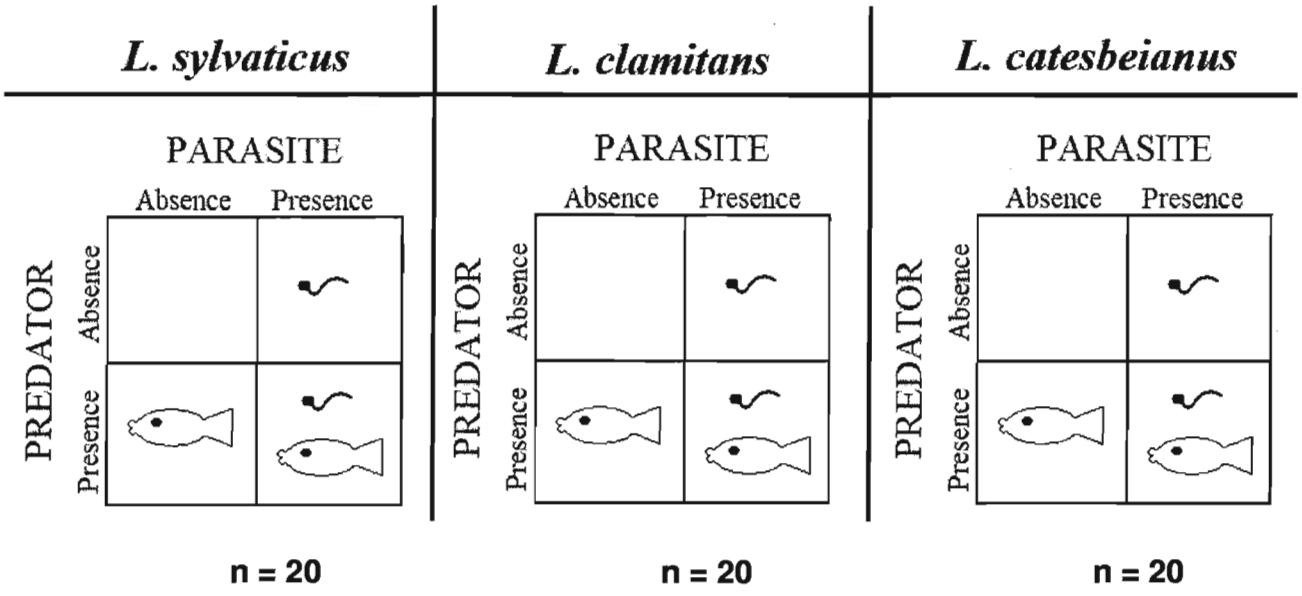

Figure 2.2: Cartoon overview of the 3 x 4 factorial experimental design used, where the presence/absence of a fish predator was crossed by the presence/absence of parasites. This resulted in 4 separate treatment conditions (control, parasite only, fish only and combo) requiring different individual tadpoles for each of the four treatments. This was replicated 20 times for all 3 species.

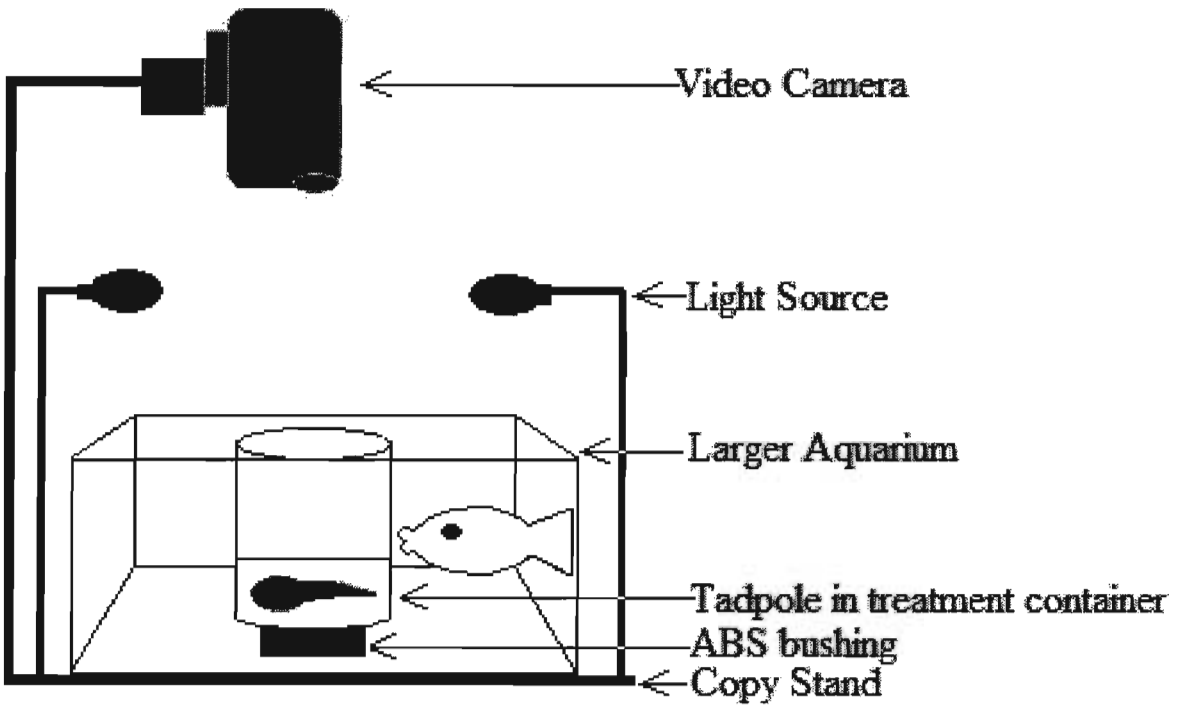

Figure 2.3: The experimental tank setup. Each experimental tank received an inner tadpole treatment container (depicted in the middle of the tank) mounted on a 8 x 5 cm ABS bushing which exposed the tadpole to the non-lethal predator cues and ensured parasites were contained. The experimental tanks sat on a copy stand which had a video camera positioned approximately 25.4 cm above the tadpole treatment container. In addition, there were 2 light sources positioned on either side of the copy stand. Drawing is not to scale.

for the day. Fish in the experimental tanks were fed 2 mL of wet bloodworms three times a week and partial water changes were completed weekly.

All experimental treatment conditions were conducted in the lab using the set-up shown in Figure 2.3. A Canon (HV30) camcorder was attached to a copy stand and positioned approximately 25.4 cm above the tadpole treatment container. The camcorder was equipped with a polarizing lens to minimize glare from the water and the copy stand had a set of lights attached to provide proper lighting conditions for filming. The order in which the treatments were recorded was randomized within each replicate. The appropriate experimental tank was placed on the copy stand and an individual tadpole in 150 mL of animal ready water was placed into the treatment container, which was covered using a larger piece of silver duct piping (24 cm in height with a diameter of 15 cm). The duct piping fit completely over the tadpole treatment container and blocked out external cues from the larger experimental aquarium (e.g. fish visual cues). The tadpole was given five minutes to acclimate to the treatment container before the trial began. Each trial lasted a total of 40 minutes and was broken down into three segments (Figure 2.4). The first 15 minutes of the trial (denoted as "baseline"), the tadpole was filmed devoid of any cues/treatment to get an estimate of the individual's activity. This was done to allow individual variation in activity to be statistically removed from the analysis. Once the 15 minute period had elapsed, 150 mL of one of the four treatments was added to the tadpole treatment container (bringing the total volume within the container to 300 mL): (1) predator- and parasite- free control (animal-ready water was added), (2) parasite only (150 mL of water inoculated with 36 *E. trivolvis* cercariae was added to give a final density of 36 cercariae per 300 mL), (3) fish only, presence of fish visual/chemical cues

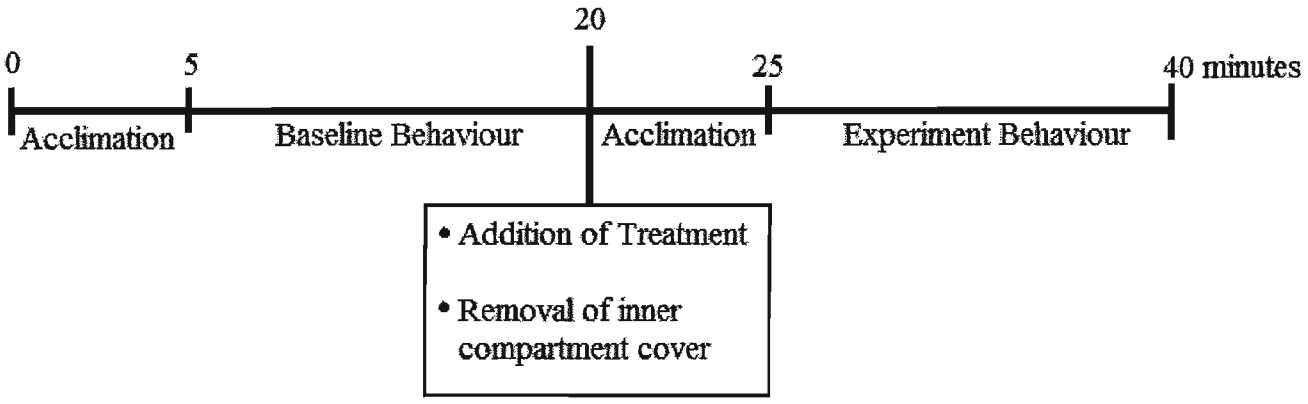

Figure 2.4: Timeline of one 40 minute experimental trial with a 5 minute acclimation period prior to the start of the experimental trial. Tadpoles were placed into the treatment container (which was covered using a larger piece of silver duct piping, to block external cues) for five minutes. The tadpole was then filmed for 15 minutes to assess baseline activity. After 20 minutes, the treatment was added and the duct piping was removed. The tadpole was allowed an additional five minutes to acclimate to the disturbance while filming. Finally, the tadpole was filmed for an additional 15 min to assess the response to the added treatment.

(150 mL of water from sunfish's home tank was added), (4) combo, presence of fish visual/chemical cues and parasites (150 mL of water from the sunfish's home tank was inoculated with 36 cercariae and then added). The density of cercariae used was selected based on published literature to reflect a moderate level of infection realistic of what a tadpole might encounter in the wild (Fried and Bradford 1997, Schotthoefer et al. 2003, Koprivnikar et al. 2006b, Skelly et al. 2006).

As the treatment was being added to the tadpole treatment container, the silver duct piping was removed, exposing the tadpole to all external cues. The tadpole was given an additional 5 minutes (while filming) to acclimate to the disturbance. After the acclimation period, the tadpole's behavioural response to the treatment was recorded for an additional 15 minutes (Figure 2.4). Two copy stands, two camcorders and a second set of tanks specific to each treatment (for a total of eight tanks) allowed me to film up to eight replicates per day.

Response Variables

Tadpole Activity

Prior to the start of watching videos for data collection, five videos were randomly selected and the baseline activity for these individuals was estimated three times to assess repeatability of the measure. On average, my activity estimates for the same tadpole were within ± 8 seconds (average time active for the five tadpoles was 400 seconds, out of a potential total of 15 minutes, or 900 seconds). In addition, to minimize observer bias, treatment labels were removed from the videos and the video order

randomized so that I was blind to the treatment of the replicate I was scoring. All videos were watched and scored by me.

For all videos in all treatments and species, total time active in both the 15 minute baseline and 15 minute treatment period was quantified using the free software JWatcher 0.9 (Blumstein et al. 2000). **Activity** was defined as any movement of the tadpole through the water. The mean time active in the baseline was subtracted from the mean time active in the treatment to give change in activity and this response variable was used in all subsequent analyses.

Additional Behaviours

In addition to total time active, three behaviours identified in preliminary trials as associated with cercariae presence were quantified using JWatcher 0.9 (Blumstein et al. 2000). **Extreme Swimming** (number and duration of each bout was quantified): tadpole initiated swimming with a fast start and high angular acceleration from a resting state. This behaviour was typically of brief duration (typically 10-20 seconds) and led to little displacement in space. This differs from the burst swimming observed in response to a stimulus, which tends to be linear and leads to a large displacement away from the stimulus (JML Richardson, *personal communication*). **Body Twisting** (number was quantified): the tadpole turned its entire body sharply in any direction, bending at the body-tail junction, immediately twisted its body around the dorsal axis and then rolled its body 180° around either its longitudinal axis or lateral axis. This was a very fast movement taking only a few milliseconds to complete. **Tail flicking** (number was quantified): tadpole was in a resting state with its tail fully extended, then abruptly bent

its tail approximately halfway along its length, and quickly swept the distal portion of the tail forward to one side of the body, and then extended it again. A single tail flick took less than one second to complete.

These three behaviours are similar to those observed in other studies that parasitized tadpoles using *E. trivolvis* (e.g. fast swimming and extremely rapid twisting, turning and tumbling; Thiemann and Wassersug 2000b, Taylor et al. 2004). Note that total time active was recorded continuously even while these specific behaviours occurred.

Parasite Load Determination

For all tadpoles exposed to parasites (in either of the parasite only or combo treatments), once the final 15 minute filming period had lapsed, tadpoles were carefully removed from the tadpole treatment container using a plastic spoon and placed individually into 745 mL plastic containers filled with 300 mL of animal-ready water (i.e. the tadpoles were kept in the same volume of water used during the experiment). The tadpoles were housed in these containers with a small piece of an algae disc (food) for 24 hours to allow sufficient time for any cercariae that had attached to the epidermis or successfully located the cloaca during the treatment period to encyst within the tissues of the nephric system (encystment within the nephric system has been shown to take as little as 8.5 hours; Fried et al. 1997).

The following day, tadpoles were euthanized with an overdose of the anesthetic MS-222 and preserved in 10% neutral buffered formalin for subsequent dissections.

Dissections took place after all filming had been completed for all three species. Tadpoles from each treatment-species combination were preserved in a single container. Containers were labeled with a code by another individual, so that I was blind to treatment while dissecting the tadpoles. The dissection procedure followed that outlined by Thiemann & Wassersug (2000a). Six places within the developing nephric system (the right and left pronephroi, right and left Wolffian ducts, and right and left mesonephroi) were examined for metacercariae (Figure 2.5). If any metacercariae were found, the tissue was carefully teased apart and the number of metacercarial cysts was counted for each tadpole. Cysts were clearly visible under a dissecting microscope.

Infection Intensity from Wild Populations

In addition to those tadpoles that were exposed to parasites in the lab, multiple larger (Gosner stages 27-28) tadpoles were collected from the field. *Lithobates clamitans* were collected from a pond near Rock Lake (where the hatchlings used in the experiment were collected) and *L. catesbeianus* were collected from the Glenridge naturalization site, which houses a large population of *L. catesbeianus* and is where snails containing parasites were collected for the experiment). These field-caught tadpoles were used to estimate the rates of *E. trivolvis* parasitism in the wild. Immediately upon collection, these tadpoles were euthanized and preserved in 10% buffered formalin, and subsequently dissected in the same way as experimentally parasitized tadpoles above.

All methods presented were approved by the Brock University Research Committee on Animal Care Use (AUPP 08-01-01).

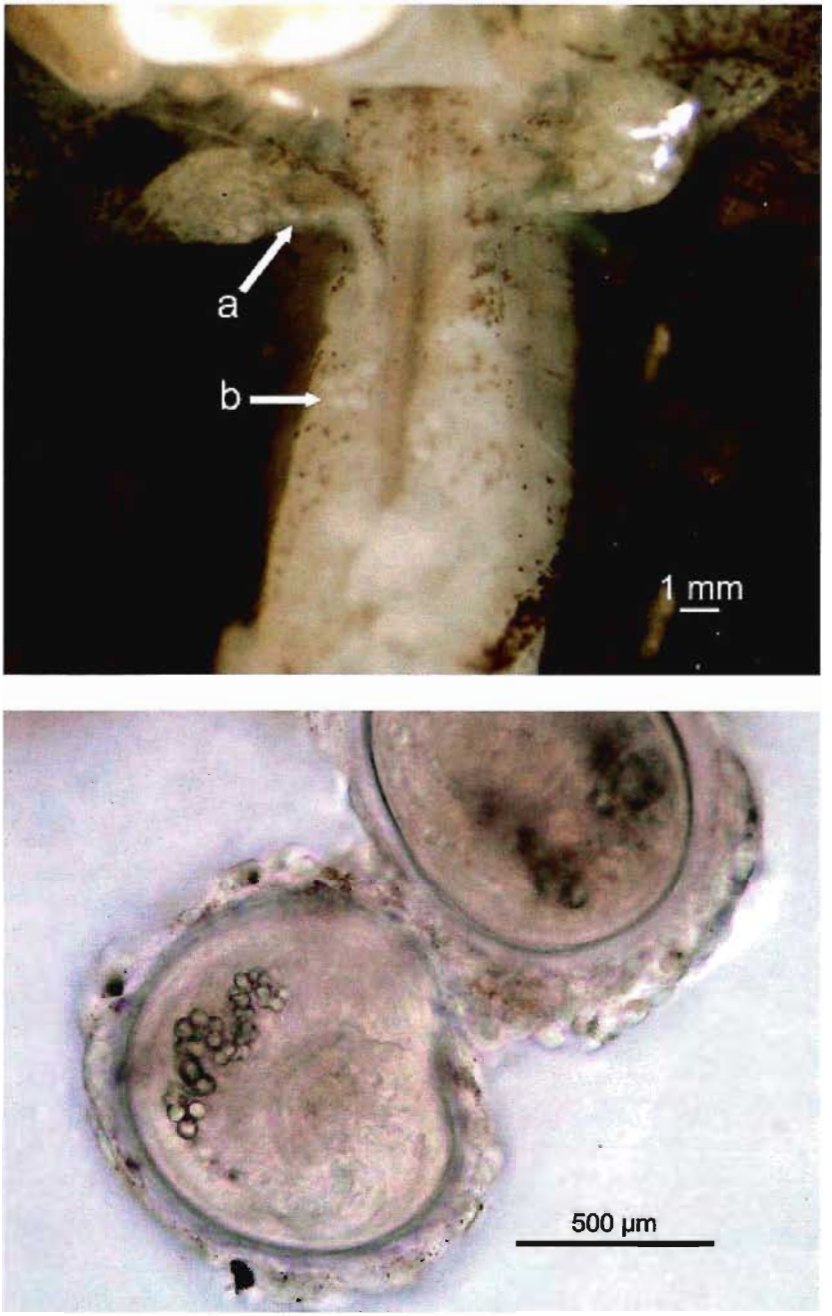

Figure 2.5: (Top) Dissected *L. sylvaticus* tadpole's nephric system. Arrow (a) points to encysted metacercariae within the right pronephroi. Arrow (b) points to encysted metacercariae within the right mesonephroi. (Bottom) depicts two metacercariae excised from an infected tadpole and fixed on a microscope slide.

Statistical Analyses

All data were analyzed using SAS 9.1 (SAS Institute Inc., 2003). In order to determine if the different treatments (control, parasite only, fish only, and combo) had an effect on the change in tadpole activity for the three different species (*L. sylvaticus*, *L. clamitans,* and *L. catesbeianus*), a two-way ANOVA was used. Independent variables were species (*L. sylvaticus, L. clamitans,* and *L. catesbeianus*) and treatments (control, parasite, fish, and combo), and the dependent variable was change in time active. Tukey post-hoc comparisons were completed to determine which species by treatment pairs differed significantly in activity.

When analyzing the three additional behaviours namely, extreme swimming, body twisting, and tail flicking, the average number of occurrences for each behaviour could not distinguish whether it was a global response expressed by numerous tadpoles to the treatment or one individual performing the behaviour numerous times (for graphs of the average number of occurrences for each behaviour, please see Appendix A). Therefore, the number of individual tadpoles that performed each behaviour at least once (instead of the mean number of occurrences) was analyzed, as I considered this a better metric for assessing whether a particular behaviour was related to treatment type. The number of tadpoles engaged in each behaviour in only the treatment period was compared using a log-odds ratio logistic regression, with the number of tadpoles engaged in the specific behaviour as the response variable, and the four treatments and species as independent class variables. Contrast statements were used to make pairwise comparisons between specific treatment combinations.

Finally, in order to test differences in the mean number of metacercariae that encysted in the nephric systems of each species in the parasite only and combo treatments, a stratified analysis of contingency tables was performed (using proc "freq" with the "cmh" option in SAS).

Results

Tadpole Activity

A two-way ANOVA done to examine the interaction between the different treatments and species, revealed a strong interaction between treatment and species ($F_{6, 228} = 20.95$, $P < 0.0001$; Table 2.1). For all species, mean change in activity in the control treatment was not significantly different from zero. All species increased activity significantly when in the presence of parasites only, compared to the control (Figure 2.6). Differences in change in activity between the species occurred in the presence of fish only and the combo treatments. For example, in the fish only treatment, *L. sylvaticus* larvae decreased activity significantly from the control and the other two species (Figure 2.6). *Lithobates clamitans* larvae decreased their activity significantly relative to *L. catesbeianus* larvae, but did not decrease activity to the same extent as *L. sylvaticus* larvae as this decrease was not significantly different from the control (Figure 2.6). On the other hand, *L. catesbeianus* larvae did not decrease their activity in the presence of fish and change in activity was similar to what was observed in the control (Figure 2.6). Finally, in the combo treatment, *L. sylvaticus* larvae also decreased their activity significantly relative to the other two species and the control; however, they did not

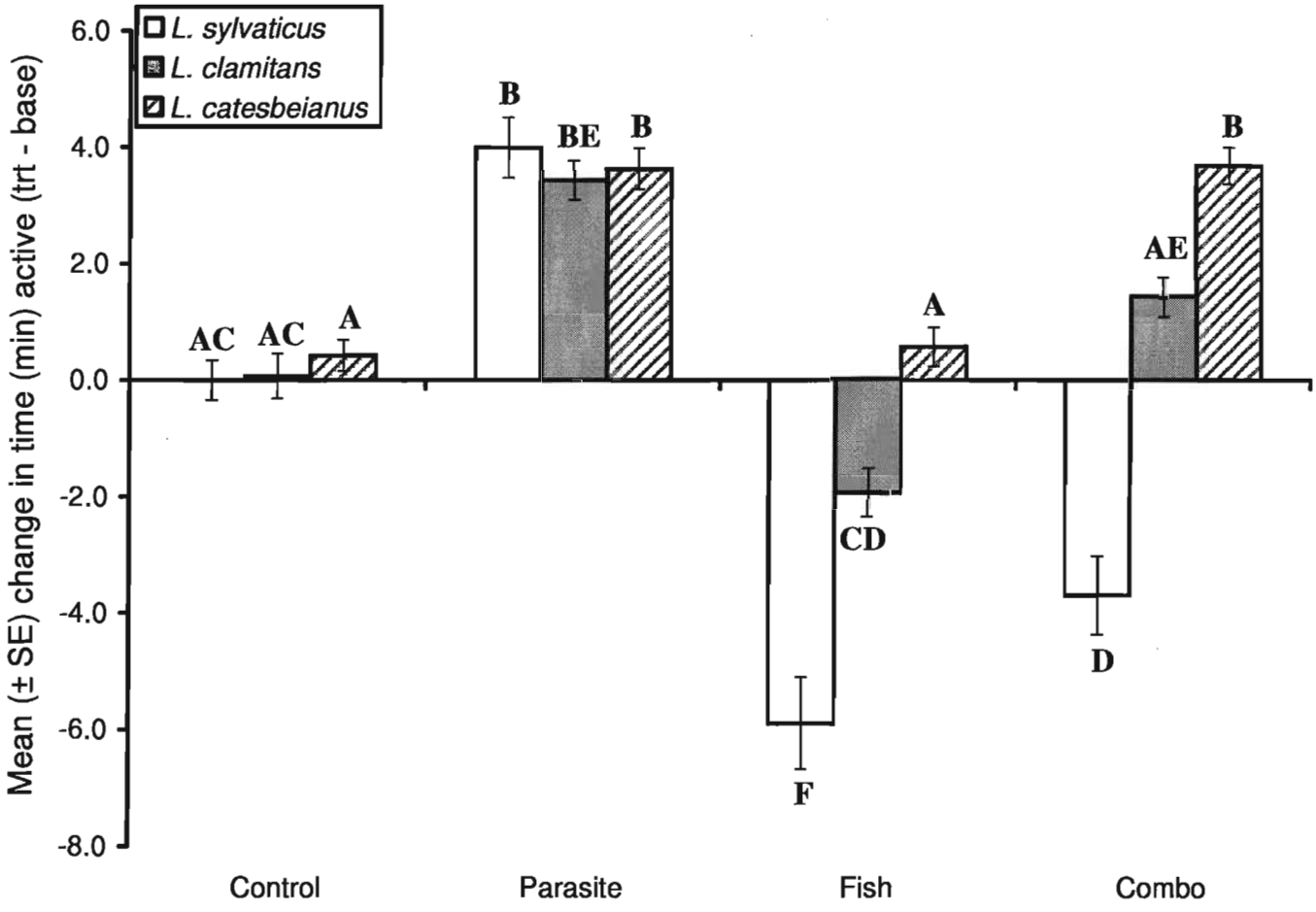

Figure 2.6: Mean (± SE) change in time active (time active in treatment condition – time active in baseline measure, in minutes) for three ranid species (*L. sylvaticus*, *L. clamitans* and *L. catesbeianus*) under four different treatments (control, parasite present, fish present, and combo, both fish and parasite present). A significant species*treatment interaction was present (ANOVA, $F_{6, 228} = 20.95$, $P < 0.0001$). Bars with different letters are significantly different (based on Tukey's post-hoc analysis).

Table 2.1: Results of a Two-Way ANOVA examining the impact of four treatments (control, parasite only, fish only and combo) on the mean change in time active (treatment-base) for three species of larval anurans (*L. sylvaticus*, *L. clamitans* and *L. catesbeianus*).

Source	**DF**	**Type III SS**	**MS**	**F value**	**P value**
Species	2	491.87	245.94	60.72	<0.0001
Treatment	3	1124.53	374.84	92.55	<0.0001
Species*Treatment	6	509.17	84.86	20.95	<0.0001
Error	228	923.43	4.05		

reduce activity to the same extent as in the fish only treatment (Figure 2.6). *Lithobates catesbeianus* larvae however, increased their activity to the same extent as when parasites only were present within the environment (Figure 2.6). In the combo treatment, *L. clamitans* larvae increased their activity significantly when compared to their behaviour in the fish only treatment and their response did not differ significantly from the parasites only treatment. However, their response was significantly less than that of *L. catesbeianus* in the combo treatment (Figure 2.6).

Additional Behaviours

Extreme Swimming

One *L. sylvaticus* tadpole engaged in extreme swimming behaviour in each of the fish and combo treatments prior to the addition of the treatment (baseline measure) (Figure 2.7a). The behaviour was not observed during baseline observations in any individuals of *L. clamitans* or *L. catesbeianus*.

A significant interaction effect for the number of tadpoles engaged in extreme swimming during treatment conditions (Logistic regression, species*treatment Wald X^2_6 = 14.71, P = 0.02; Table 2.2). The number of tadpoles (for all three species combined) displaying extreme swimming increased significantly in parasite only (Contrast between control and parasite treatments, Wald X^2_1 = 7.27, P = 0.007) and combo (Contrast between control and combo treatments, Wald X^2_1 = 10.83, P = 0.001) treatments relative to the control (Figure 2.7b). In the parasite only treatment, significantly more *L. sylvaticus* and *L. clamitans* tadpoles engaged in extreme swimming than did

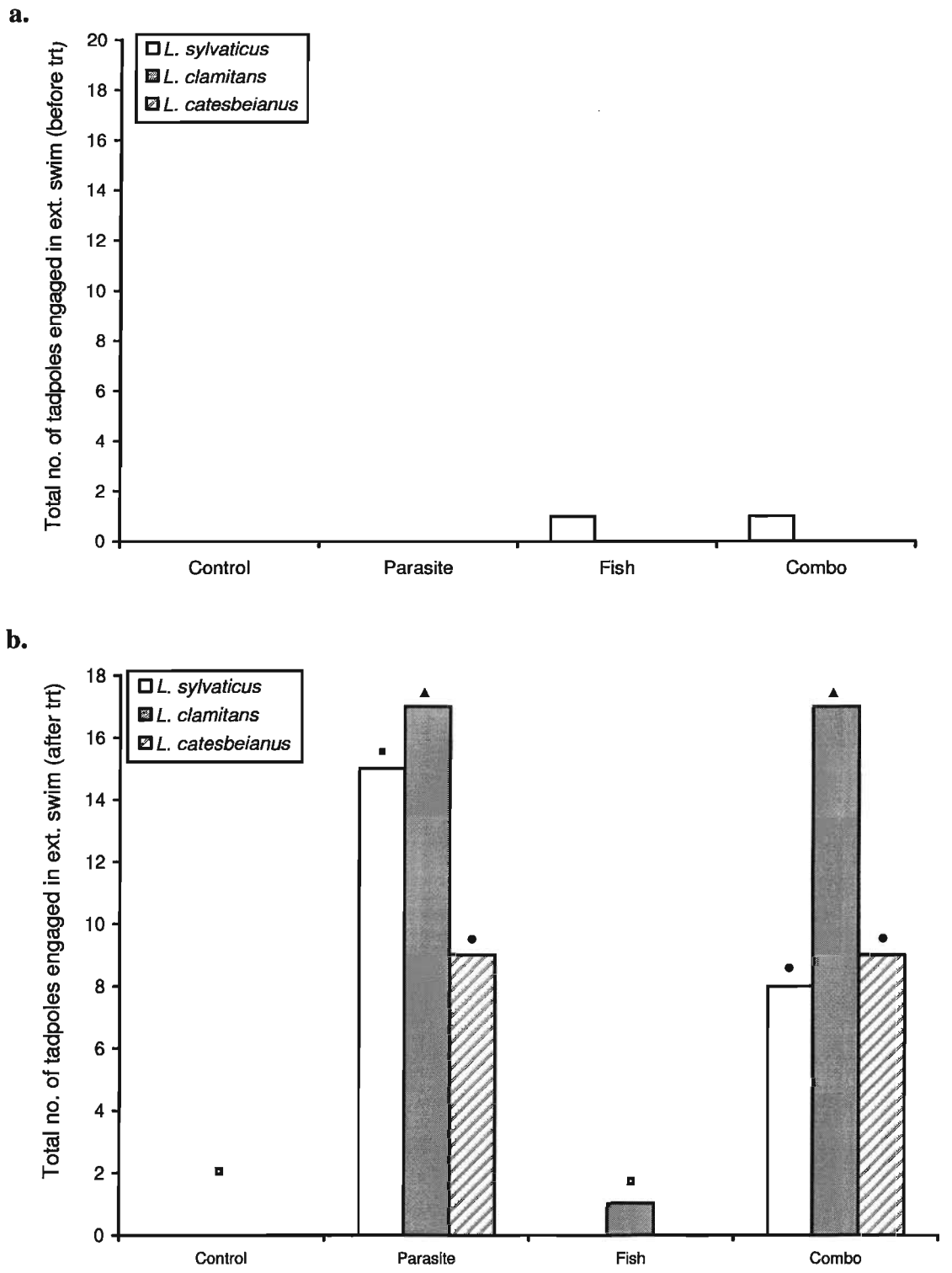

Figure 2.7: (a) Number of tadpoles engaged in extreme swimming before the treatment was added. (b) Number of tadpoles engaged in extreme swimming after the treatment was added. There was a significant species*treatment interaction (Logistic regression, Wald $X^2_6 = 14.71$, P = 0.02). Different symbols above bars are significantly different (based on contrast statements).

Table 2.2: Logistic regression summary table for the number of tadpoles engaged in extreme swimming only after the treatment had been added. The logistic regression compares the three species (*L. sylvaticus*, *L. clamitans* and *L. catesbeianus*) and the four treatments (control, parasite, fish and combo).

Source	**DF**	**Wald X^2**	**P value**
Species	2	12.58	0.0019
Treatment	3	26.84	<0.0001
Species*Treatment	6	14.71	0.0227

L. catesbeianus larvae (Contrast between *L. sylvaticus* vs. *L. catesbeianus* in parasite treatment, Wald $X^2_1 = 8.84$, P = 0.003 and contrast between *L. clamitans* and *L. catesbeianus* in parasite treatment, Wald $X^2_1 = 13.82$, P = 0.0002; Figure 2.7b); in addition, significantly more *L. clamitans* larvae engaged in extreme swimming than did *L. sylvaticus* larvae (Contrast between *L. sylvaticus* and *L. clamitans* in the parasite treatment, Wald $X^2_1 = 8.02$, P = 0.005). *Lithobates sylvaticus* tadpoles engaged in significantly less extreme swimming in the combo treatment compared to the parasite treatment (Contrast between *L. sylvaticus* in combo treatment vs. *L. sylvaticus* in parasite treatment, Wald $X^2_1 = 7.30$, P = 0.007). For both *L. clamitans* and *L. catesbeianus,* there was no difference between the combo and parasite treatments (Figure 2.7b).

Body Twisting

During baseline observations, one *L. clamitans* tadpole and one *L. sylvaticus* tadpole engaged in body twisting in the control and parasite treatments, respectively (Figure 2.8a). With the addition of treatments, a significant main effect of treatment was observed (Logistic regression, treatment Wald $X^2_3 = 27.20$, P < 0.0001; Table 2.3). Significantly more tadpoles engaged in body twisting in the parasite treatment compared to the control (Contrast between parasite and control treatments, Wald $X^2_1 = 4.35$, P = 0.04) and in the combo compared to the control (Contrast between combo and control treatments, Wald $X^2_1 = 4.10$, P = 0.04) (Figure 2.8b).

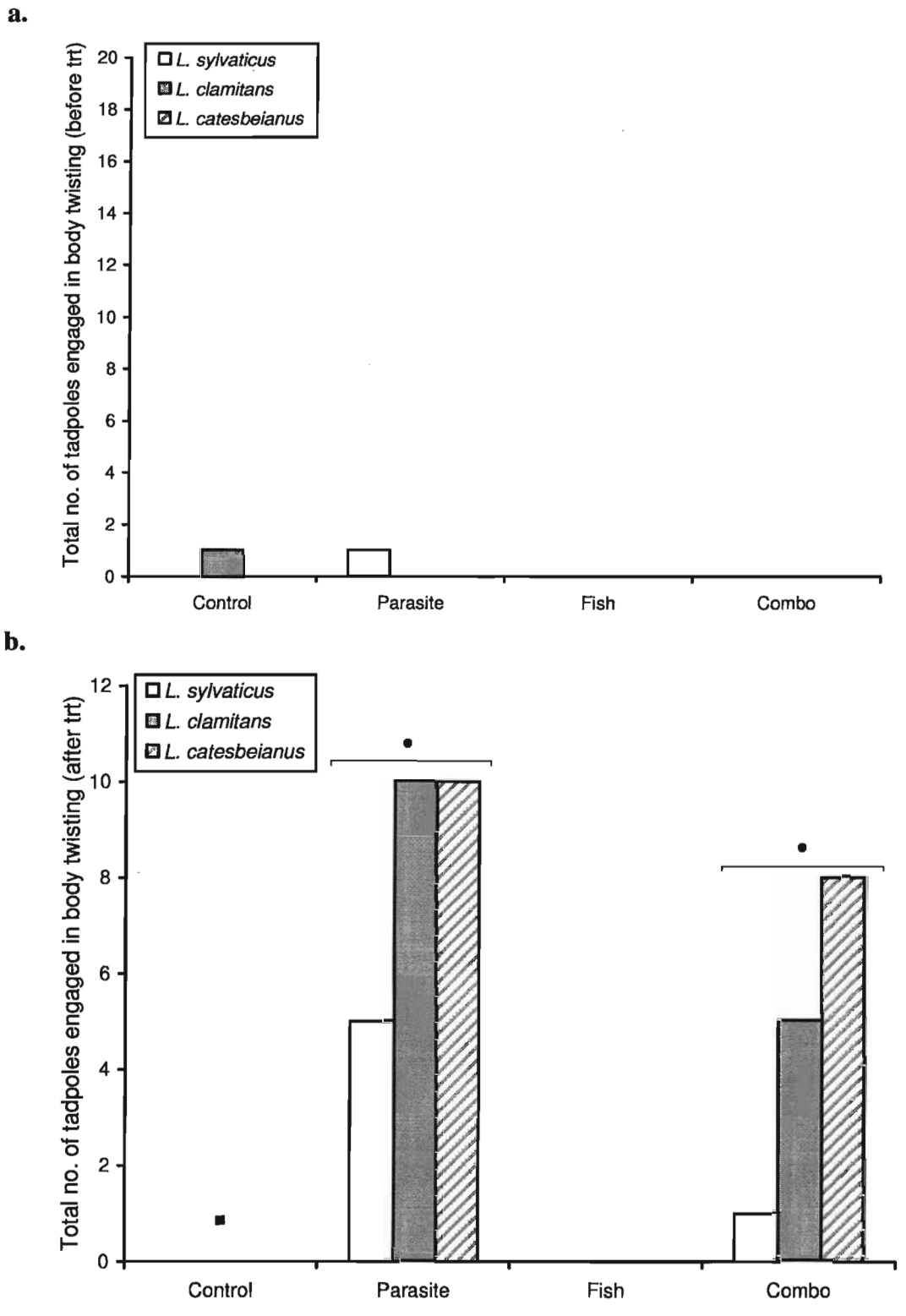

Figure 2.8: (a) Number of tadpoles engaged in body twisting before the treatment was added. (b) Number of tadpoles engaged in body twisting after the treatment was added. The main effect of treatment was significant (Logistic regression, Wald $X^2_3 = 27.20$, $P < 0.0001$). Different symbols are significantly different (based on contrast statements).

Table 2.3: Logistic regression summary table for the number of tadpoles engaged in body twisting only after the treatment had been added. The logistic regression compares the three species (*L. sylvaticus*, *L. clamitans* and *L. catesbeianus*) and the four treatments (control, parasite, fish and combo).

Source	**DF**	**Wald X^2**	**P value**
Species	2	2.41	0.3002
Treatment	3	27.20	<0.0001
Species*Treatment	6	3.19	0.7846

Tail flicking

All three species in all four treatments in the baseline portion engaged in tail flicks (Table 2.4). However, the addition of the treatments significantly affected the number of tadpoles performing tail flicks and a species by treatment interaction was present (Logistic regression, species*treatment Wald $X^2_6 = 17.74$, P = 0.007; Table 2.5). This significant interaction occurs because while both *L. catesbeianus* and *L. clamitans* increase their tail flicking in both the parasite and combo treatments relative to the control, *L. sylvaticus* decreases its tail flicking in the combo treatment relative to the control (Table 2.4). In addition, *L. sylvaticus* also decreases its tail flicking in the fish treatment relative to the control, while there is no change in tail flicking for both *L. clamitans* and *L. catesbeianus* relative to the control. The contrast statements revealed that within the parasite treatment, more *L. sylvaticus* individuals engaged in tail flick when compared to *L. clamitans* (Contrast between *L. sylvaticus* and *L. clamitans* in the parasite treatment, Wald $X^2_1 = 4.80$, P = 0.03) and *L. catesbeianus* (Contrast between *L. sylvaticus* and *L. catesbeianus* in the parasite treatment, Wald $X^2_1 = 18.02$, P < 0.0001; Table 2.4). Within the combo treatment, *L. clamitans* larvae engaged in significantly more tail flick than both *L. catesbeianus* and *L. sylvaticus* (Contrast between *L. clamitans* vs. *L. catesbeianus* in the combo treatment, Wald $X^2_1 = 4.20$, P = 0.04 and contrast between *L. clamitans* and *L. sylvaticus* in the combo treatment, Wald $X^2_1 = 3.99$, P = 0.04).

Table 2.4: Summary table of the number of tadpoles for all species (*L. sylvaticus*, *L. clamitans* and *L. catesbeianus*) engaged in tail flick before and after the treatment had been added. There was a significant species*treatment interaction (Logistic regression, species*treatment Wald $X^2_6 = 17.74$, P = 0.007) in the after treatment data. Arrows represent the direction of change (e.g. increase or decrease) after the treatment had been added. A "no change (↔)" was based on an increase/decrease of four tadpoles or less.

		Number of tadpoles engaged in tail flicking in each treatment			
Species		**Control**	**Parasite**	**Fish**	**Combo**
L. sylvaticus	Before	16	13	15	13
	After	19 ↔	20 ↑	8 ↓	8 ↓
L. clamitans	Before	6	7	5	3
	After	10 ↔	18 ↑	6 ↔	15 ↑
L. catesbeianus	Before	2	0	0	0
	After	0 ↔	8 ↑	0 ↔	6 ↑

Table 2.5: Logistic regression summary table for the number of tadpoles engaged in tail flicking after the treatment had been added. The logistic regression compares the three species (*L. sylvaticus*, *L. clamitans* and *L. catesbeianus*) and the four treatments (control, fish, parasite and combo).

Source	**DF**	**Wald X^2**	**P value**
Species	2	34.12	<0.0001
Treatment	3	24.08	<0.0001
Species*Treatment	6	17.74	0.0069

Parasite Load

Mean number of metacercariae found in the nephric system of individuals exposed to parasites revealed a significant species by treatment interaction (stratified analysis of contingency tables, species*treatment $X^2_2 = 84.95$, $P < 0.0001$; Figure 2.9). Overall, *L. sylvaticus* incurred more metacercariae than each of the other species. In addition, significantly more cercariae successfully encysted in those *L. sylvaticus* tadpoles that were exposed to the combo treatment compared to the parasite only treatment ($X^2_1 = 213.64$, $P < 0.0001$; Figure 2.9). *Lithobates catesbeianus* larvae had the smallest parasite load and parasite only and combo treatments did not differ (stratified analysis of contingency tables, treatment $X^2_1 = 0.0059$, $P = 0.9387$; Figure 2.9). Finally, the average number of metacercariae encysted within larval *L. clamitans* was intermediate (particularly within the parasite only treatment) to the number encysted within larval *L. sylvaticus* and *L. catesbeianus*. For example, there were significantly fewer metacercariae found in *L. clamitans* when compared to *L. sylvaticus* however, significantly more metacercariae when compared to *L. catesbeianus* (Figure 2.9). *Lithobates clamitans* that spent time in the combo treatment had significantly more metacercariae than those in the parasite only treatment (stratified analysis of contingency tables, $X^2_1 = 5.27$, $P = 0.022$; Figure 2.9).

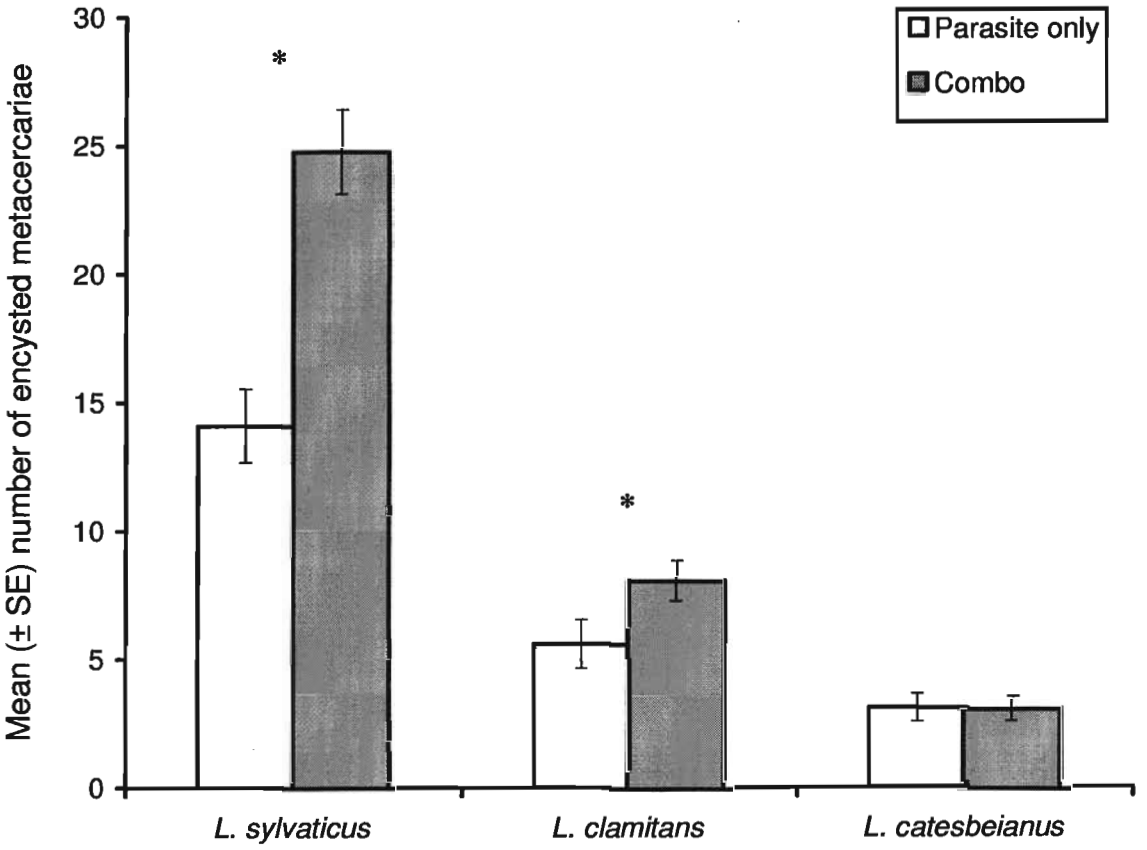

Figure 2.9: Mean (± SE) number of encysted metacercariae (out of potential 36 cercariae) for all three species in both the parasite only and combo treatments. A significant species*treatment interaction was present (stratified analysis of contingency tables, species*treatment= $X^2_2 = 84.95$, $P < 0.0001$). An asterisk denotes a significant difference between the two treatments for that particular species.

Infection Intensity from Wild Populations

Of a total of 15 *L. clamitans* tadpoles caught from the pond near Rock Lake, three individuals were infected by Echinostome, as evidenced by cysts found in the nephric system. Therefore, a crude estimate of Echinostome infection intensity within that pond is 20% (Table 2.6). Infection intensity of tadpoles within the Glenridge Naturalization site is much higher (Table 2.6). Of 15 *L. catesbeianus* tadpoles collected, eight individuals harboured Echinostome metacercariae, giving an infection intensity of 53%.

Table 2.6: Natural infection intensities for both *L. clamitans* and *L. catesbeianus* tadpoles collected from Rock Lake (where *L. clamitans* hatchlings used in the experiment were collected from) and the Glenridge Naturalization Site (where snails harbouring *E. trivolvis* cercariae were collected from).

Species	**n**	**% Infection Intensity**
L. clamitans (Pond near Rock Lake)	15	20
L. catesbeianus (Glenridge)	15	53

Discussion

Both *L. sylvaticus* and *L. clamitans* larvae decreased their activity in the presence of a fish predator, while *L. catesbeianus* larvae were completely unaffected by the presence of a fish predator. When both parasites and predators are present within the environment, different responses are observed in each of the species, which match my hypothesis that those species that either never encounter fish (*L. sylvaticus*) or rarely encounter fish (*L. clamitans*) will decrease activity resulting in higher infection intensities compared to a species that encounters fish all the time (*L. catesbeianus*). Here, I discuss the effects of both predators and parasites on the activity and behaviour of all three species. I will also discuss the potential mechanism behind the differential response and subsequent infection intensity (parasite load) in the three different species to the combined presence of parasites and predators observed.

Tadpole Activity and Behaviour

It is clear that the response to the fish is much stronger than the response to the parasite in *L. sylvaticus*. For example, the greatest decrease in activity was observed in the fish only treatment followed by the combo treatment. In the absence of the predator, *L. sylvaticus* tadpoles responded freely to the parasite and activity increased significantly from the control (Figure 2.6). In addition, the number of individual *L. sylvaticus* tadpoles engaged in any conspicuous behaviours that could potentially aid in dislodging cercariae that have attached to the epidermis, such as extreme swimming, decreased in the combo treatment when compared to the parasite only treatment (Figure 2.7). Also, the number

of individuals tail flicking decreased significantly in the presence of fish relative to the control and parasite only treatments (Table 2.4).

It is somewhat surprising that *L. sylvaticus* tadpoles respond so strongly to the fish predator given that they do not encounter fish in the wild. This is not uncommon, as larvae of various species have been shown to increase antipredator behaviour, even if the individual has not had prior experience with the predator because an unknown predator represents a high-risk situation (e.g. Kiesecker et al. 1996, Mathis and Vincent 2000). In addition, Chivers and Mirza (2001) demonstrated that *L. sylvaticus* tadpoles decreased activity when exposed to dietary chemical cues from fish. Furthermore, fish avoidance has been shown to be strong in this species; Hopey and Petranka (1994) were able to show that adult *L. sylvaticus* can assess the presence of predatory fish in ponds prior to oviposition. Therefore, the results of the current study for *L. sylvaticus*, support my hypothesis that species that never encounter fish predators, and thus never evolve additional antipredator mechanisms effective against fish, respond more strongly (decreasing activity) to the fish than to the parasites in the combo treatment, presumably because the unknown potential predator poses a greater risk and as such, elicits a greater antipredator response. The potential cost of predation (zero fitness) outweighs the cost of parasitism in this case, as *E. trivolvis* does not kill the tadpole host unless they are very small (less than Gosner larval stage 25; Schotthoefer et al. 2003).

The response of *L. sylvaticus* to the combined presence of both predators and parasites is in agreement with other published literature (Thiemann and Wassersug 2000b, Koprivnikar et al. 2006b). However, the response to parasites alone has produced a conflicting result in comparison to the results obtained by Thiemann and Wassersug

(2000b). Thiemann and Wassersug (2000b) noted that the proportion of tadpoles active decreased significantly in the presence of parasites relative to the control. This was an unanticipated result as they hypothesized that in the presence of parasites alone, activity would increase, as preliminary observations of cercariae contacting tadpoles resulted in explosive burst of activity. The authors postulate that the response exhibited by *L. sylvaticus* to parasites is adaptive and is parallel with the response to predators (decreasing activity), which is aimed at reducing tadpoles' risk of detection.

Methodological differences between Thiemann and Wassersug (2000b) and the current study may explain conflicting results. In Thiemann and Wassersug (2000b), experiments went on for a longer period of time (7 days) and activity was scored over brief periods throughout the experiment instead of scoring activity upon parasite introduction. In the current study, activity was measured once parasites were added and experiments ran for a much shorter time frame (40 minutes total). The outbursts of activity tadpoles display in response to attacking parasites are usually interspersed with periods of inactivity; thus, numerous scores of brief activity throughout the day and not immediately after parasite introduction, may miss short term responses to attacking parasites. For example, in the current study, tadpoles would start to display bursts of antiparasite behaviours sometimes in as little as 60 seconds after the introduction of parasites. Therefore, it is likely that Thiemann and Wassersug (2000b) captured the response to only long term parasite exposure. While the current study would miss such long term effects to chronic exposure, I designed the study to capture the initial response of tadpoles to attacking parasites. I suggest that characterizing these short term responses is essential for understanding natural tadpole communities that are in the presence of high

predator and parasite densities, because these short term conspicuous responses will clearly attract visually oriented predators and increase the likelihood of a tadpole being successfully captured by a predator.

In *L. clamitans*, the presence of fish reduced activity, although not to the same extent as in the *L. sylvaticus* tadpoles. The decrease in activity in the fish only treatment suggests that *L. clamitans* perceives fish as a potential threat but that this perceived threat is lower than that perceived by *L. sylvaticus* larvae (as activity decreased significantly for this species). This is not unexpected as *L. clamitans* can encounter fish predators in the wild (Werner and McPeek 1994). Antipredator behaviour is a costly investment (Lima 1998). For example, tadpoles are filter feeders, scraping attached algae and detritus into the water column to filter suspended particles while swimming (Seale and Wassersug 1979, Seale and Beckvar 1980, Anholt and Werner 1995). A decrease in activity equates to a loss of foraging opportunities, which may negatively affect long term survivorship, but a decrease in activity also has the benefit of decreasing the encounter rate with a potential predator (Lima 1998). Therefore, it is crucial for larval anurans to be able to assess the level of danger posed by a potential predator and act accordingly to balance the trade-off between eating and evading predators. As *L. clamitans* have been found to coexist occasionally with fish predators and have also been hypothesized to be unpalatable to fish (Werner and McPeek 1994), perhaps, *L. clamitans* perceive the potential risk of fish to be less than do *L. sylvaticus*, allowing *L. clamitans* tadpoles to better balance the trade-off between foraging and predation.

When parasites are present in combination with a predator, *L. clamitans* responds more to the attacking parasites than the predator (Figure 2.6). For example, in addition to

an increase in activity in the combo treatment relative to the fish treatment, the number of tadpoles engaged in extreme swimming and body twisting also increased significantly relative to the control and was similar to the number of tadpoles engaged in both behaviours in the parasite only treatment (cf. Figures 2.7b and 2.8b). If *L. clamitans* possess additional antipredator defence mechanisms against fish (such as unpalatability), then I would expect individuals to be relatively free (compared to *L. sylvaticus*) to respond to attacking parasites, as observed in this study (Figure 2.6). However, it is directly counter to the response observed by Thiemann and Wassersug (2000b), who observed a decrease in activity in response to parasites alone. Again, the results obtained by Thiemann and Wassersug (2000b) can be explained by an antiparasite mechanism, whereby tadpoles exposed to parasites chronically decrease activity to avoid further detection.

Lithobates catesbeianus, on the other hand, appears to be completely unaffected by fish. In fact, in the presence of fish alone, activity is not significantly different from the control. In addition, activity increases significantly in the combo treatment (to the same extent as in the parasite only treatment) when compared to the response in the control and fish treatments (Figure 2.6); thus, the response to the parasite appears to be much stronger than the response to the fish predator in *L. catesbeianus*. This is not surprising, as *L. catesbeianus* has coevolved with fish and is extremely successful in these permanent ponds. In fact, Smith et al. (1999) have shown that predatory fish (*L. macrochirus*) can indirectly facilitate increased larval *L. catesbeianus* abundance by consuming predacious invertebrates or by removing competitors. One of the most common co-evolved defence mechanisms that has been postulated to aid *L. catesbeianus*

larvae in escaping fish predation is unpalatability. Thus, if fish learn to avoid *L. catesbeianus* and feed on invertebrates that *L. catesbeianus* larvae may also be vulnerable to, this will clearly increase the success of *L. catesbeianus* tadpoles in fish ponds. Further work is needed to confirm the existence and effectiveness of this particular antipredator mechanism (see Chapter 3).

Additional Antiparasite Behaviours

Extreme swimming and body twisting predominately occur in the presence of parasites (for all three species), suggesting that these behaviours might aid in dislodging attached cercariae or prevent cercariae from attaching. These additional "explosive" behaviours in response to *E. trivolvis* exposure have been documented repeatedly in the literature, further suggesting that tadpoles use these behaviours in an attempt to reduce parasitism (e.g. Taylor et al. 2004, Koprivnikar et al. 2006b). Tail flicking, on the other hand, appears to occur even when no *E. trivolvis* are present thus, this is a more general behaviour not necessarily associated with the presence of parasites.

These behaviours are generally conspicuous and if tadpoles employ these additional behaviours to shed attacking cercariae, *L. catesbeianus* larvae might be expected to perform more of these behaviours in the combo treatment (as the predator poses no threat) than either *L. clamitans* or *L. sylvaticus*. This however was not the case, for example, the number of *L. catesbeianus* tadpoles engaged in both extreme swimming and tail flicking behaviours was consistently lower in the combo treatment relative to *L. clamitans*. However, *L. catesbeianus* engaged in more body twisting (which is the most conspicuous behaviour out of the three; pers. obs.) than the other two species in the

combo treatment, although this difference was not statistically significant. One potential explanation for this trend (*L. catesbeianus* engage in fewer antiparasite behaviours), is that perhaps *L. catesbeianus* maybe much better at effectively removing cercariae using fewer of these "explosive" behaviours than either *L. clamitans* or *L. sylvaticus*, thus minimizing energy expenditure; presumably maintaining these behaviours over an extended period of time requires a great deal of energy.

This fine-tuning of antiparasite behaviour by *L. catesbeianus* may be a direct reflection of overlapping habitat types between *L. catesbeianus* and *E. trivolvis* and frequency of exposure. *Echinostoma trivolvis* requires the aquatic snail *P. trivolvis* to serve as its first intermediate host. *Planorbella trivolvis* is typically found in permanent ponds as are *L. catesbeianus* (namely, well vegetated lentic or still waters and farm ponds, dams, lakes; Johnson et al. 2004). In addition, *L. catesbeianus* typically grow slowly and overwinter as larvae at least once and often two or three times in northern latitudes (Harding 2006). As such, it is likely that *L. catesbeianus* encounter *E. trivolvis* cercariae frequently and are repeatedly exposed as larvae, making *L. catesbeianus* more likely to have evolved effective antiparasite behaviours than either *L. clamitans* or *L. sylvaticus*. For example, the prevalence of infection by *E. trivolvis* in *L. catesbeianus* tadpoles collected from a wild population was high (53%) and much higher than that observed in wild caught *L. clamitans* tadpoles (20%) (this is, however, based on a small sample size from only two localities; more sampling from multiple localities should be conducted). However, Koprivnikar et al. (2006a) sampled 11 ponds across southern Ontario, and found natural *E. trivolvis* prevalence in *Hyla versicolor* (which breed in both

temporary and permanent ponds, Kiesecker and Skelly 2000, Harding 2006) to range from 8 – 94%.

In addition to *E. trivolvis*, there are numerous other species of parasites that require freshwater snails as a first intermediate host and tadpoles as the second intermediate host (e.g. *Ribeiroia* sp, *Alaria* sp., *Echinoparyphium* sp.; Prudhoe and Bray 1982, Kostadinova and Gibson 2000, Johnson et al. 2004, Koprivnikar et al. 2006a, Szuroczki and Richardson 2009). As *P. trivolvis* is typically found in permanent ponds like *L. catesbeianus* (Johnson et al. 2004), this can increase the number and species of parasites that *L. catesbeianus* tadpoles can potentially encounter. As some species of parasites are more detrimental to overall fitness than *E. trivolvis* (e.g. *R. ondatrae* causes limb malformations; Johnson et al. 1999, Johnson et al. 2001, Johnson et al. 2002), it makes sense that there would be strong selection pressure for more efficient and effective antiparasite behaviours within this species. For example, Orlofske et al. (2009) demonstrated that there is no real consequence on the physiology and fitness-related traits of tadpoles exposed to moderate levels of *E. trivolvis* infection. The study measured the effects of infection on survival, growth, metabolism and intestine size (as intestine size has been shown to exhibit plastic responses to predation). There was no change in any of these parameters in the absence of any other additional environmental stressors (e.g. pesticides). Therefore, in nature, the level of infection by *E. trivolvis* to which tadpoles are most likely exposed does not seem to have any negative consequence. However, if *L. catesbeianus* tadpoles cannot distinguish among cercariae of different parasite species, there may be selection for tadpoles to respond behaviourally to *E. trivolvis* as it attaches

and crawls along the epidermis in the same manner as cercariae from other, more detrimental, species do.

Lithobates clamitans, while sharing similar habitat types as *P. trivolvis* and other freshwater snails, generally only over winter once as larvae in northern latitudes (Harding 2006), reducing the exposure time for larvae of this species to *E. trivolvis* cercariae and other species of parasites. This, in turn, will lessen the selection pressure for antiparasite behaviours. In contrast, *L. sylvaticus* grows quickly, typically transforming in 6 to 15 weeks in northern latitudes (Harding 2006). This faster growth rate is an adaptation to life in temporary ponds that often dry in late summer. As such, *L. sylvaticus* are not exposed to cercariae for more than one season. Further, these temporary ponds are less likely to sustain freshwater snails such as *P. trivolvis* and so *L. sylvaticus* are also less likely to encounter *E. trivolvis*. Hence, selection for effective antiparasite responses to *E. trivolvis* may be relatively weak in *L. sylvaticus*. *Lithobates sylvaticus* may however, encounter other parasites such as *Telorchis sp.,* which use the freshwater lymnaeid snail (*Pseudosuccinea columella*) as a first intermediate host, because this snail can survive in both temporary and permanent ponds (Kiesecker and Skelly 2000). *Telorchis* sp. require tadpoles as an intermediate host and the general antiparasite response observed in *L. sylvaticus* to *E. trivolvis* suggests that *L. sylvaticus* does in fact encounter other species of parasites.

Parasite Load

The number of cercariae that encysted within the developing nephric system in the combo treatment is a direct reflection of how each species responded behaviourally to the trade-off imposed by both predator and parasites and the expected susceptibility of

each species to fish predators. *Lithobates sylvaticus* had the greatest parasite loads of the three species. In addition, *L. sylvaticus* individuals in the combo treatment had 43% more metacercariae than those in the parasite only treatment (Figure 2.9).

The number of encysted cercariae in *L. clamitans* was moderate relative to the other two species. Individuals that spent time in the combo treatment however, incurred 30% more metacercariae than those that were in the parasite only treatment (Figure 2.9). This suggests that *L. clamitans* tadpoles perceive fish to be a moderate threat, but that when both parasite and fish are present, *L. clamitans* will respond to the parasites to a greater degree than *L. sylvaticus* do in the same treatment. These results again are counter to Thiemann and Wassersug (2000b) who found that in the combined presence of fish and parasites, *L. clamitans* incurred a greater number of metacercariae (16% more than the parasite only treatment), although this significance is arguably borderline at P = 0.0494; Thiemann and Wassersug 2000b). The authors claim that the predator presence eliminated crucial parasite-avoidance behaviours. However, if this were the case, one would expect that the elimination of parasite behaviours would be stronger in *L. sylvaticus,* resulting in even higher parasite loads, particularly in the combination treatment (Thiemann and Wassersug 2000b). This was not the case, as there was no significant difference between parasite loads in the parasite only and combination treatment in *L. sylvaticus*. The sample sizes however, were only based on 10 individuals per treatment (Thiemann and Wassersug 2000b). Perhaps, Thiemann and Wassersug (2000) did not have enough power to detect significant differences in parasite loads and as such, an increase in the sample size might have produced different results.

Lithobates catesbeianus had the fewest number of metacercariae among the three species, and there was no difference between the number of metacercariae in the parasite and combo treatments (Figure 2.9). *Lithobates catesbeianus* was also the most active species in the combo treatment; there was no difference in activity between the parasite only and combo treatment. This presumably reflects the fact that *L. catesbeianus* are completely unaffected by fish predators; *L. catesbeianus* tadpoles have also been hypothesized to be protected from fish predation by unpalatability (e.g. Kats et al. 1988, Werner and McPeek 1994). If this is the case and *L. catesbeianus* tadpoles are protected from fish predation by some chemical repellent, perhaps this repellent also makes it much more difficult for cercariae to attach or to creep along the epidermis making the species much less susceptible. Work aimed at determining whether or not additional antipredator mechanisms such as unpalatability may function as an effective antiparasite mechanism as well, warrants further investigation.

In conclusion, the three closely related Ranid larvae tested have very different life histories and habitat preferences and respond differently to the trade-off imposed by the presence of both predators and parasites within the environment. All three species increased their activity in the presence of parasites. The two permanent pond breeders (*L. clamitans* and *L. catesbeianus*) increased their activity in the combined threat of predation and parasitism where as the temporary pond breeder (*L. sylvaticus*) decreased activity in the combo treatment as response to fish predation was much stronger than the response to parasites. Further, the presence of fish increased the susceptibility of *L. sylvaticus* and *L. clamitans* to trematode infection, but *L. catesbeianus* was unaffected by the presence of fish. This interaction between predator threat and parasite infection is

important, as it adds to a growing body of evidence showing the need to consider the impact of multiple trophic levels, including parasites, when looking at community level dynamics (Hatcher et al. 2006).

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Chapter 3

Assessing palatability in larvae of three Ranid species

North American anuran larvae exhibit a variety of defence mechanisms against an array of aquatic predators. Potential prey can reduce predation risk by reducing the likelihood of encountering a predator through mechanisms that minimize the chances a predator will detect their presence: cryptic colouration, sensitivity to water-borne indicators of predator presence (chemical cues), decreased activity behaviour, etc. (Wisenden 2000). Potential prey can also reduce predation risk by decreasing the probability of consumption once detected by a predator. This tactic is used in adult salamanders with toxic skin secretions that act as a predator deterrent (Brodie et al. 1979). Furthermore, Brodie et al. (1978) found that toxins accumulate and become more potent during metamorphosis in the toad *Bufo americanus*, suggesting toxins may also be present in larvae. While toxicity refers to the presence of a known chemical compound that causes some type of physical harm, such as loss of muscle coordination (Liem 1961), decreased consumption risk may also be achieved more simply from unpalatability, which refers to a general defence mechanism whereby prey apparently have a taste that a predator perceives as disagreeable (Gunzburger and Travis 2005). The term unpalatability has been invoked extensively in anuran larvae literature (as reviewed in Gunzburger and Travis 2005). In practice, it is difficult to distinguish between unpalatability and toxicity, as toxicity is nearly always associated with unpalatability; throughout this paper, I use the term unpalatability to include that occurring with or without toxicity.

Fish predators can dramatically affect anuran populations through the consumption of eggs or early larval stages (e.g. Bronmark and Edenhamn 1994, Skelly 1996). For anuran larvae that inhabit permanent ponds, such as *L. catesbeianus*, selection to minimize fish predation is likely the driving force behind evolved antipredator defence mechanisms. Unpalatability has been proposed frequently as the mechanism by which *L. catesbeianus* larvae minimize predation risk (Kruse and Francis 1977, Kats et al. 1988, Werner and McPeek 1994, Eklov and Werner 2000), yet no study has definitively demonstrated it (Gunzburger and Travis 2005). The majority of these claims are based on the peculiar behaviour exhibited by sunfish when offered a *L. catesbeianus* tadpole. Some of these behaviours include expectoration, avoidance and the tendency to take the alternative "more palatable" tadpole when offered a choice between larvae of two species (Werner and McPeek 1994, Smith et al. 1999, Eklov and Werner 2000). Alternatively, some species reported as unpalatable (e.g. *Bufo americanus, Hyla cinerea)* are readily consumed by hungry individuals of two sunfish species (*Lepomis gibbosus and L. punctatus*) (Richardson, unpublished data). Thus, a rather large controversy exists as to whether unpalatability functions as an antipredator mechanism in larval anurans and more specifically, *L. catesbeianus* larvae.

Three general problems are associated with most palatability studies done to date: failure to incorporate the existence of a range of relative preferences or palatability, failure to effectively control for predator hunger levels and failure to consider confounding variables such as prey behaviour and prey appearance. The first problem, as noted by Gunzburger and Travis (2005), arises because preference or choice experiments provide little evidence of palatability per se. Rather, these types of experiments provide

information on predation rates and profitability. As a result, this methodology can only provide information on relative palatability; a predator may prefer a *L. clamitans* tadpole over a *L. catesbeianus* tadpole when both are present (as shown by Werner and McPeek, 1994), but this does not mean the fish will not readily eat *L. catesbeianus* tadpoles when no *L. clamitans* tadpoles are present. Or that *L. clamitans* will always be consumed upon every presentation when offered with *L. catesbeianus*. The existence of a range of relative preferences or palatability may also influence the ability of a predator to learn with experience.

The second problem associated with most studies of palatability is that hunger levels of fish predators are insufficiently controlled. Motivation to forage may vary greatly in predators and we might expect a fish that refuses a slightly unpalatable prey species when it has energy reserves remaining, will rapidly take the same prey when energy reserves are depleted. The difficulty comes in determining what qualifies as being "sufficiently hungry" for the potential predator. For example, Kats et al. (1988) reported both *L. catesbeianus* and *L. clamitans* tadpoles to be unpalatable, but fail to indicate how much alternative food the fish received.

The third problem deals with an inability to control for confounding variables such as prey behaviour or prey appearance which can also alter consumption or predation rates (Gunzburger and Travis 2005). This could be especially true in choice experiments, where two species of prey are offered to a predator. If the predator is visually oriented, like fish, then subtle differences in activity levels or slight differences in body colouration might attract the predator toward one of the particular prey items. This in turn can skew the results of any palatability study. For example, if the predator is drawn to one species

due to behaviour or appearance and does not attempt to consume the other species, this leads the observer to conclude that the predator selected the more palatable prey item (when selection was based not on taste but rather on prey appearance) and that the other prey species was unpalatable, which can be incorrect.

Here, I test the vulnerability of three species of larval anurans hypothesized to range in palatability to fish predators by attempting to feed *Lepomis gibbosus* with a common food staple (bloodworms, Chironomid spp.) spiked with skin samples of each larval anuran. This method allowed me to isolate consumption rates based solely on the taste of skin alone while controlling for the three problems outlined above. The three anuran species were chosen based on the habitats in which the larvae are found, as variable levels of palatability has been hypothesized to be an indirect function of habitat. For example, *L. sylvaticus* larvae inhabit temporary pond settings (such as vernal ponds, flooded areas, wooded swamps and quiet stream backwaters; Harding 2006) that do not contain fish predators but do contain invertebrate predators (Skelly 1997). As a direct result of inhabiting fishless ponds, *L. sylvaticus* larvae lack any additional evolved defence mechanisms against fish such as unpalatability (Walters 1975, Kats et al. 1988).

On the other end of the spectrum, *L. catesbeianus* larvae are found in permanent water bodies that contain fish (e.g. *Lepomis* spp.). In light of this, it has been suggested that *L. catesbeianus* larvae have evolved unpalatability as a means to co-exist with fish predators (Kruse & Francis 1977; Kats et al. 1988; Werner and McPeek 1994). Finally, *L. clamitans* larvae were chosen to serve as the intermediate species, because they inhabit both permanent and temporary pond settings where they can encounter predatory fish (Werner and McPeek 1994), and are more closely related to *L. catesbeianus* than to *L.*

sylvaticus (e.g. sister species, Hillis and Wilcox 2006, Wiens et al. 2009). In addition, it has been suggested that *L. clamitans* larvae are also somewhat unpalatable to fish predators (Werner and McPeek 1994).

Methods

Collection of Animals and Husbandry

Nine *L. sylvaticus* egg masses were collected in May 2008 from Bat Lake (45° 35' N, 78° 31' W) at the Wildlife Research Station, Algonquin Park, Ontario, Canada (45° 35' N, 78° 30' W). Nine egg masses were collected to ensure sufficient numbers of hatchlings were available because a single *L. sylvaticus* egg mass typically has only 500 eggs (compared to a single *L. catesbeianus* egg mass, which has upwards of a several thousand eggs; Harding 2006). Individual *L. sylvaticus* egg masses were placed into either 1.5 L glass bowls or, for smaller egg masses, 300 mL glass bowls, which were then placed into two growth chambers at 5°C prior to hatching (cooling egg masses is required as *L. sylvaticus* egg masses have very low hatching success at warmer temperatures; it also slows growth, necessary because this species develops much faster than *L. clamitans* and *L. catesbeianus*).

Lithobates clamitans hatchlings (Gosner stage 20; Gosner 1960) from the same egg mass, were collected in July 2008 from a pond near Rock lake in Algonquin Park, Ontario, Canada (45° 31' N, 78° 24' W). Hatchlings, instead of an egg mass, were collected because no fully intact egg mass was found. Hatchlings of both species were

housed in 38 L (61 x 40.6 x 22.2 cm) Rubbermaid® tubs filled with a combination of filtered pond water and animal-ready water (carbon-filtered and aged tap water with pH adjusted to 7.0). In order to minimize animal usage, larger (Gosner stages 30-34; Gosner 1960) *L. catesbeianus* tadpoles (instead of eggs or hatchlings; this species grows slowly) were collected using dip nets in September 2008 from the Glenridge Naturalization Site in Niagara region, Ontario, Canada (43° 7' N, 79° 14' W). *Lithobates catesbeianus* tadpoles were housed in 11.4 L (30 x 25 x 15 cm) Sterilite® tubs filled approximately 3 L of animal ready water with up to 15 tadpoles/ tub. All tadpoles were maintained on ground Spirulina Algae Discs (Wardley®, Secaucus, New Jersey) and approximately 0.5 L of suspended algae (from a lab culture of mixed unicellular spp.) placed into tubs once a week. Tubs were cleaned of feces every second day and a complete water change was performed weekly. All animals were maintained on a 14:10 light:dark cycle.

Six *Lepomis gibbosus* (approximately 8-10 cm in length) were collected in May 2008 from a pond located within St. John's Conservation Area, Pelham, Ontario (43° 3' N, 79° 17' W). Fish were housed in transparent 10 L (30.5 x 22.9 x 17.8 cm) aquaria (Tom Pla-House Clear Vue) filled with approximately 8.5 L of animal ready water and fitted with a sponge filter (Dirt Magnet® Aquarium Filter, Junior Model). All tanks received five droplets of Stress Coat® and five droplets of Kent Freshwater Essential™ (mineral supplement for aquaria), as well as one piece of rounded pvc tubing as cover for fish. Fish were maintained on approximately 2 mL of wet bloodworms 3 times a week.

Experimental Feeding Regime

Feeding experiments with *L. sylvaticus*, *L. clamitans* and *L. catesbeianus* followed identical designs and protocols. The six fish were first fed five bloodworm-only pellets (control) three times in the first week. The following week, the same fish were fed five *L. sylvaticus* skin containing pellets three times (Figure 3.1). The above procedure was replicated for both *L. clamitans* and *L. catesbeianus*, such that all fish received bloodworm-only control pellets for a week prior to the pellets containing anuran skin (Figure 3.1). A one month period elapsed between the last *L. sylvaticus* skin feeding and the next bloodworm-only feeding to allow *L. clamitans* tadpoles to grow to a large enough size for use in the experiments. Fish were maintained on bloodworms during this interval. The control bloodworm-only week of feeding for *L. catesbeianus* followed immediately after the week testing *L. clamitans*. Finally, to determine if fish would consume bloodworm-only pellets after all the anuran skin feeding experiments were completed, the six fish were fed bloodworm-only pellets for an additional final week (Figure 3.1).

Approximately three hours prior to feedings (to allow fish sufficient recovery time after disturbance in the tank), all waste/debris and aquaria accessories were removed from each of the fishes' home aquaria. During feedings, fish were given 5 minutes between each pellet to eat any "leftovers" before the next pellet was offered. After the last pellet was introduced and the 5 minute period had lapsed, all remaining food was reclaimed using a fine mesh fish net and a pipette. All unconsumed food was dried for 24 hours in a drying oven at 50 °C after which it was combusted in a muffle furnace.

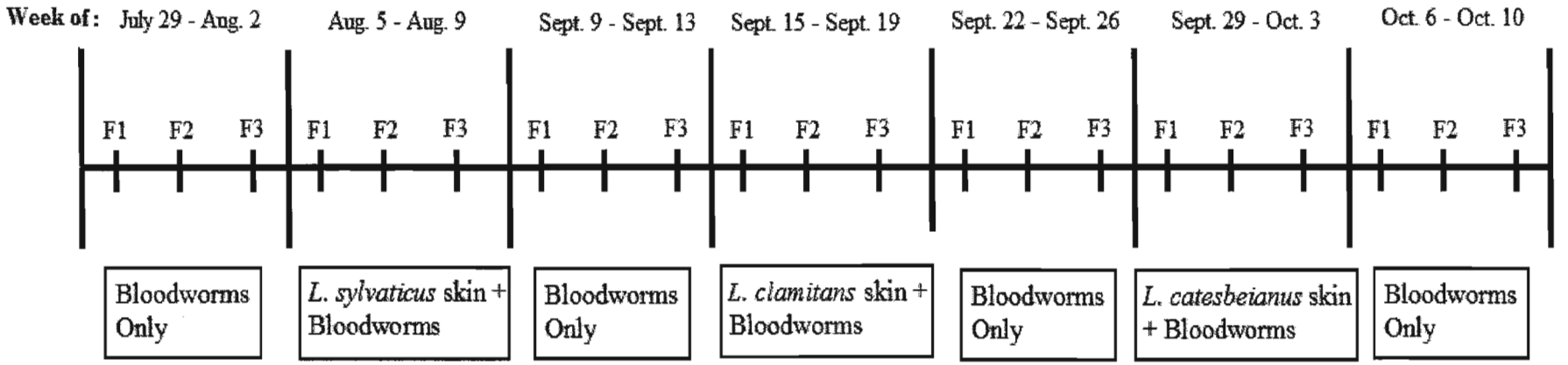

Figure 3.1: Schematic of experimental feeding regime. Six fish were first fed five bloodworm-only pellets for three days starting in week 1 (July 29 – Aug. 2). The same fish were then fed five *L. sylvaticus* skin containing pellets three times the following week (Aug. 5 – Aug. 9). A month passed between the *L. sylvaticus* skin feedings and the next bloodworm-only feedings to allow *L. clamitans* tadpoles to grow to a sufficient size. The procedure was then repeated in the exact same manner for both the *L. clamitans* and *L. catesbeianus* species (e.g. one week of bloodworm-only pellets fed three times, then skin containing pellets the following week fed three times). Finally, bloodworm-only pellets were fed to fish for a final week after all the anuran skin feedings were completed.

Pellet Construction

Control pellets

Wet bloodworms (*Chironomidae* spp.) were divided into 2 g samples and dried for approximately 2 hours or until the bloodworm samples were leather hard. The dried bloodworms were packed into a "pellet mold" (a piece of plexiglass approximately 6.4 mm in thickness with a hole that had a 6.4 mm diameter drilled into it). The dried bloodworms were packed tightly together to form uniform pellets that held together once removed from the mold (Figure 3.2a). Each pellet was weighed to insure they were all within 0.01 g of each other. A total of six pellets were made for each of the six fish. Five of the pellets for each fish were given to the fish during feeding trials and the sixth pellet (Figure 3.2b) was placed into the drying oven for 24 hours to obtain an estimate of pellet final dry weight and then into the muffle furnace to obtain an estimate of ash-free dry weight per pellet. For each fish and feeding, the ash-free dry weight of this sixth pellet was multiplied by five to give the total amount of food presented to each fish.

Pellets with skin

To obtain skin samples, tadpoles were first euthanized by immersion into liquid nitrogen for approximately 30 – 45 seconds. Liquid nitrogen was chosen over chemical agents such as MS-222 as I was concerned that the chemical would either change or mask the true "taste" of the skin. Once the tadpoles had thawed, a small incision in the

a.

b.

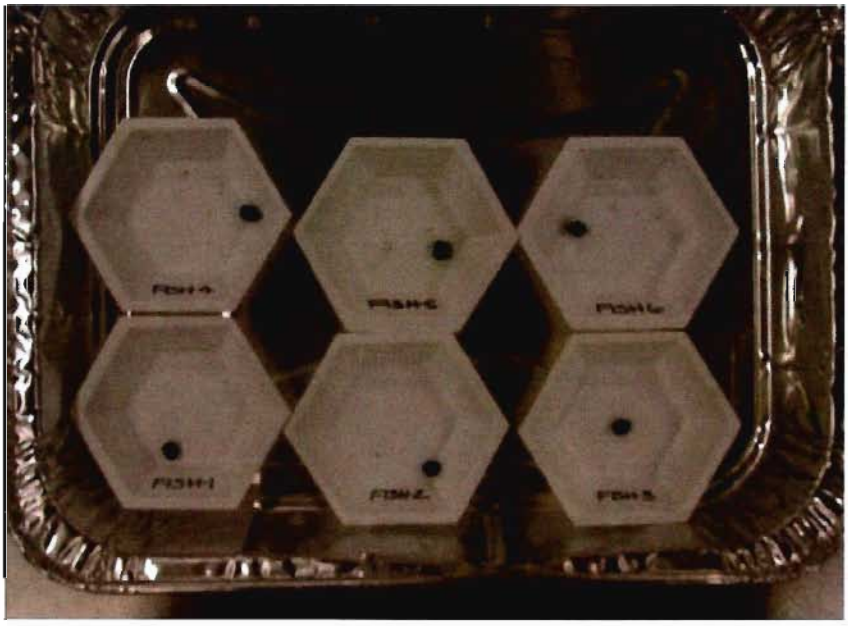

Figure 3.2: Food pellets used in feeding experiments. (a) Example of 5 pellets presented to each fish. (b) Control pellet constructed for each fish, and each feeding used to measure amount offered.

epidermis was made all around the base of the tail. With a pair of forceps the skin covering the body was gently pulled forward until completely removed. Skin was weighed to insure that samples being placed into the pellets were within ± 0.002 g of each other. *Lithobates sylvaticus* and *L. clamitans* tadpoles used were between Gosner stages 27-30 (Gosner, 1960), and *L. catesbeianus* tadpoles used were Gosner stages 30-34 (Gosner 1960). To construct pellets containing the skin samples, dried bloodworms were packed into the bottom of the "pellet mold". The skin sample was placed on top of the dried bloodworms within the pellet mold. Finally, more dried bloodworms were placed on top to cap off the pellet. The dried bloodworms/skin samples were packed tightly together to form uniform pellets that stayed together. Each pellet was weighed to insure they were all within 0.01 g of each other. As with the control pellets, a total of six pellets were made for each of the six fish, five of which were used in subsequent feeding trials. The sixth pellet was placed into the drying oven for 24 hours and then into the muffle furnace to obtain the ash-free dry weight.

This protocol was approved by the Brock University Research Committee on Animal Care Use (AUPP 07-08-01, 07-04-01, and 07-09-06).

Statistical Analyses

To calculate consumption rates, the ash-free food remains for each fish was subtracted from the total amount of ash-free food offered to each fish to give the amount of organic material consumed.

Data were analyzed using SAS 9.1 (SAS Institute Inc., 2003). To determine whether the addition of skin samples from larval anurans changed the amount consumed a repeated measures MANOVA was performed on the change in consumption (i.e. consumption of bloodworms only was subtracted from the bloodworms + skin). Change in consumption was calculated separately for each fish at each feeding. The "proc glm" statement in SAS was used, with the nine repeated measures (each fish individual had nine values: 3 feedings x 3 anuran species) as the response variables in the model statement.

To test the willingness of fish to consume bloodworms at the end of the experiment (after all tadpole skin feedings were completed), a paired t-test was used to compare the amount of bloodworm-only pellets consumed before the addition of any skin to the pellets to the amount consumed after all the larval anuran skin feedings were completed. Subsequently, a power test was conducted in order to validate the results of the paired t-test. The "proc power" statement in SAS was used, using a within individual correlation of 0.5 between the two treatments and the observed standard deviation of the difference between the treatments.

Results

The amount of food consumed by fish depended on both the anuran species and the feeding number (anuran species by feeding number interaction, Repeated measures MANOVA, Wilks' lambda $F_{2,4} = 23.03$, $P = 0.0421$; Figure 3.3). The greatest decrease in

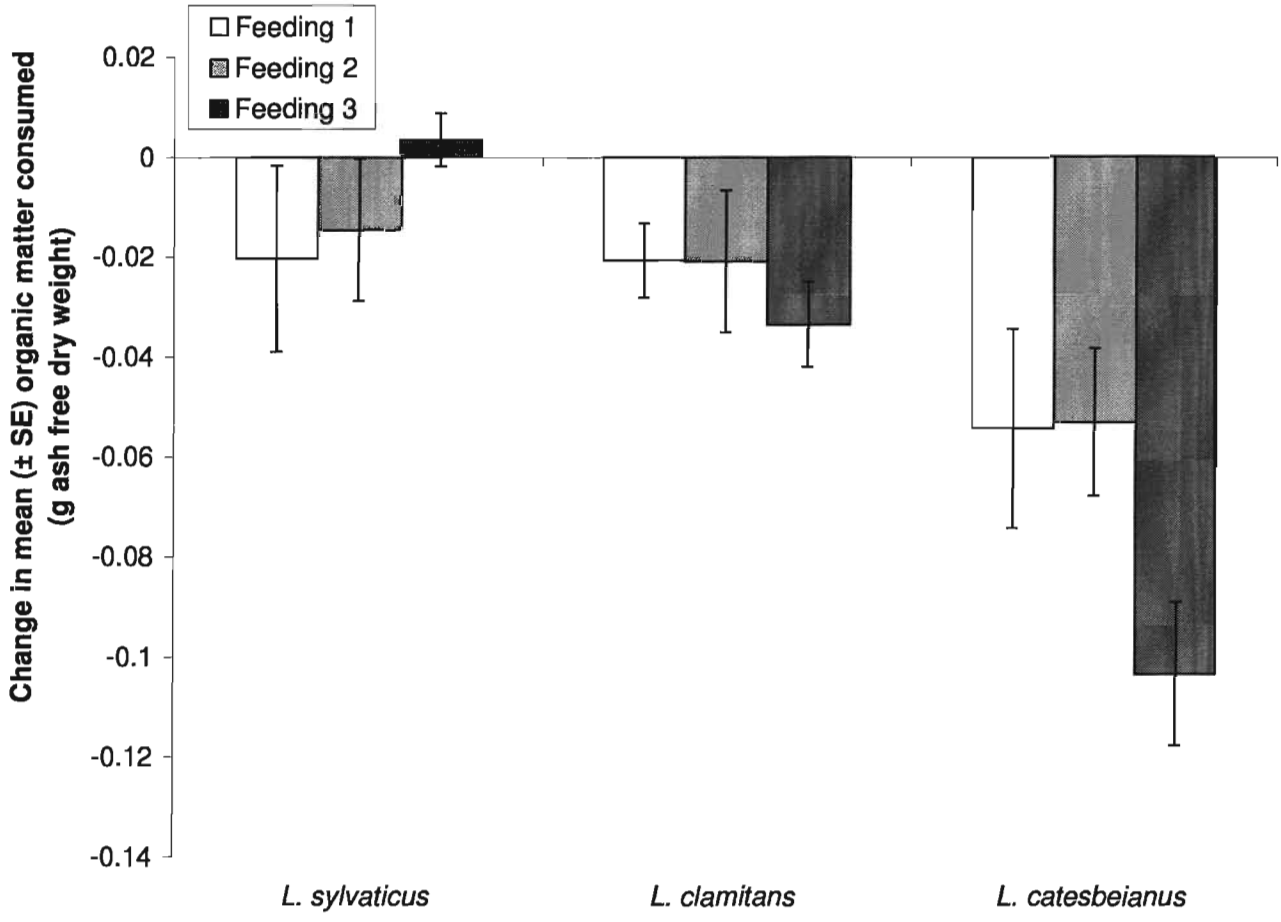

Figure 3.3: Change in mean organic matter consumed (skin-containing pellets – bloodworm-only pellets) collapsed (i.e. averaged) across the six fish. Feedings refers to each of the three feeding days in which fish were given pellets containing tadpole skin. There is a significant species by feeding effect (Repeated measures MANOVA, Wilks lambda $F_{2,4} = 23.03$, $P = 0.04$).

consumption occurred with the addition of *L. catesbeianus* skin, which is especially evident by the third feeding where fish consumed very little (Figure 3.3). The addition of *L. sylvaticus* skin had little impact on consumption; in fact, by the third feeding, consumption rate was equal to that of the prior week when no skin was in the pellet (Figure 3.3). Finally, the addition of *L. clamitans* skin elicited an intermediate response; consumption rate decreased relative to the addition of *L. sylvaticus* skin but was greater than the consumption rate of pellets containing *L. catesbeianus* skin (Figure 3.3). Thus, the large decrease in consumption as feeding number increased for *L. catesbeianus* is what generates the significant interaction term.

There was no difference between the amount of bloodworm-only pellets consumed before the addition of any anuran skin compared to the amount consumed after the experimental trials (mean difference in consumption = - 0.06; Paired *t*-test: t_5= 1.54, P = 0.1847). The power of this test to detect a difference in consumption rate of 0.10 g (the decrease in consumption observed when fish were fed *L. catesbeianus* skin for the third time; Figure 3.3) was 45%. However, one fish consumed a lot less after the experiment compared to the other fish (-0.26 g compared to an average of -0.03 g for the other five fish). This one value likely inflated my estimate of variance used in the power analysis, so I re-ran the power analysis using standard deviation calculated with this one fish removed. That power analysis gave a power of 92% for a difference of 0.10 g.

Discussion

The current study is unique in that I was able to clearly demonstrate the existence of unpalatability in *L. catesbeianus* solely based on the taste of skin. I achieved this by first training six fish to consume standardized food rations (pellets) for a week and then feeding those same fish with the same standardized food ration containing the amphibian skin. I was able to directly compare the impact of the skin on predator feeding rate by comparing consumption of skin containing pellets to the control pellets. In addition, the measure of predator feeding rate was streamlined by considering specifically the total amount of organic matter available for digestion in each pellet (all samples were placed into muffle furnace to obtain ash-free dry weights). This methodology has allowed me to show that predator feeding rate decreases significantly when *L. catesbeianus* skin is added to the pellets devoid of any confounding variable associated with predator-prey interactions (e.g. prey behaviour, foraging effectiveness and prey appearance).

In addition, I was also able to show that the closely related *L. clamitans* species are also relatively unpalatable, as consumption of pellets containing *L. clamitans* skin decreased significantly by the third feeding when compared to the consumption of pellets containing *L. sylvaticus* (the most palatable species; Figure 3.3). This is not a novel result, as unpalatability has been hypothesized to exist within this species (Werner and McPeek, 1994). However, what is novel is that I was able to show that there is a clear range in palatability that seems to vary substantially in three anuran species. This is important, as it may aid in resolving some of the apparent contradictions in previous literature regarding palatability (see Gunzburger and Travis 2005), as the existence of a range of palatability may influence the ability of a predator to learn with experience. As

such, a predator might need to experience more than a few moderately unpalatable organism before it learns to avoid subsequent individuals. Thus, future researchers need to start thinking about palatability as a continuous trait rather than a simple presence or absence.

Showing that *L. clamitans* is somewhat unpalatable raises another interesting question as to why it is maintained in this species that typically prefer ponds lacking fish (Werner and McPeek, 1994). It is unclear whether unpalatability or distastefulness is simply maintained because there is no negative selection acting on the trait (meaning that there is no real cost associated with producing a chemical repellent or toxin) or is maintained (even if there is a cost associated with unpalatability) as it allows *L. clamitans* larvae to exist in permanent ponds with fish if required.

Whether it is unpalatability, low levels of an unidentified toxin, or some other type of chemical repellent, my results also suggest that unpalatability as an antipredator mechanism requires experiential learning for individual sunfish. For example, fish consumed more pellets containing *L. catesbeianus* skin on feeding days 1 and 2 compared to the final feeding day (Figure 3.3). Perhaps, the fish associated the pellets with a post-ingestion negative effect. While I never observed regurgitation of *L. catesbeianus* skin-containing pellets, a previous study conducted by Werner and McPeek (1994) found remains of partially digested bullfrog tadpoles that had been regurgitated in some of the aquaria, suggesting that fish consumed a few *L. catesbeianus* tadpoles but that tadpoles were noxious in some way, leading to regurgitation. In fact, it has been suggested that consumption of *L. catesbeianus* tadpoles causes a digestive dysfunction in largemouth bass (*Micropterus salmoides*) (Kruse and Francis 1977), which belong to the

same family as sunfish (Centrarchidae) (Neff et al. 1999). It would appear that predatory fish (such as sunfish) can associate either a bad taste or some kind of negative post-ingestion effect with a *L. catesbeianus* tadpole and learn to avoid it in the future (Garcia and Koelling 1966, Ralphs and Provenza 1999).

Experiential learning that occurs in sunfish when encountering a *L. catesbeianus* or *L. clamitans* tadpole, in addition to a range of palatability, may also help to resolve some of the contradictions in the literature regarding the palatability of anuran species. Failure to account for fish experience in palatability studies can lead to different results and thereby generate controversy regarding the existence of unpalatability within the species. For example, if fish are naïve to *L. catesbeianus* tadpoles, then fish might consume a few tadpoles once or twice, leading researchers to conclude that *L. catesbeianus* tadpoles are palatable. J.M.L Richardson (unpublished data) found that all predators used in her study (*L. gibbosus, L. punctatus, Notophthalmus viridescens* and *Anax junius*) would consume *L. catesbeianus* tadpoles when hungry enough. However, this was based on a single feeding trial per individual and, as such, would have inevitably missed the learned aversion to subsequent *L. catesbeianus* tadpoles similar to what was observed in the current and previous studies (Szuroczki and Richardson, unpublished data).

On the other hand, if sunfish had prior experience with *L. catesbeianus* tadpoles, then upon experimental presentation with a subsequent *L. catesbeianus* tadpole, fish would most likely refuse to consume the tadpole. This would lead the investigator to conclude that *L. catesbeianus* tadpoles are unpalatable. In fact, Szuroczki and Richardson (unpublished data) observed a very similar trend which suggested that there was a

combination of both inexperienced and experienced sunfish (as their natal pond also housed breeding *L. catesbeianus*) used in their palatability study. For example, five fish which were later hypothesized to have had prior experience, refused to consume any *L. catesbeianus* tadpoles even after nine days of food deprivation. In contrast, two sunfish that were later hypothesized to be naïve consumed *L. catesbeianus* tadpoles on two separate presentations and then refused any subsequent tadpoles, showing that fish appear able to learn to avoid larval *L. catesbeianus* after only one or two experiences and with tadpoles that are at a very early stage of development. Thus, incorporating both fish experience and the existence of various levels of palatability in future palatability or predation studies using larval anurans is essential for obtaining accurate results.

Finally, another one of the major problems of most palatability studies done to date is that predator hunger levels are insufficiently controlled. Both extremes of hunger level (satiated or starved) could skew the results of a palatability study because motivation to forage is likely to play an important role in an individual's willingness to consume food (Gunzburger and Travis 2005). The current study maintained a constant feeding schedule (feeding either of the two types of pellets three times a week) that ensured that fish would be hungry enough to be sufficiently motivated to forage, while at the same time not starved to the point that fish could not readily discriminate by food palatability. I also tried to ensure that no residual preference based on the anuran skin in the previous feedings affected consumption of the pellets containing skin of the next species. This was achieved by alternating skin-containing pellets with bloodworm-only pellets for a week and by offering the fish the hypothetically most palatable species (*L. sylvaticus*) first, followed by *L. clamitans* and finally *L. catesbeianus*. This also ensured

that fish were not forming an aversion to the bloodworm pellets themselves. In fact, there was no significant difference between the control pellets offered before the addition of larval anuran skin compared to the consumption of bloodworm-only pellets after the addition of all anuran skin. One fish out of the six however, appeared somewhat averse to bloodworms post skin feedings, which further showcases unpalatability as an effective defence mechanism in larval anurans.

The results of the current study also suggest that fish can discriminate between pellets that look virtually identical but taste differently depending on the addition of tadpole skin (Figures 3.3). Fish are generally regarded as visually oriented predators (Guthrie and Muntz 1993), but because all pellets looked virtually identical (regardless of the addition of skin), fish presumably could not distinguish them visually. Therefore, fish must have either tasted each of the five pellets offered separately upon each of the three feedings, for all of the conditions (control, and then the three anuran species) or fish learned to associate a chemical cue from the skin detectable prior to ingestion with some kind of bad taste or post-ingestion consequence, and subsequently ignored pellets with the same chemical signature. It is likely that fish were using chemical cues to detect skin in the pellets, as fish have also been shown to possess sensitive chemosensory organs (Toshiaki 1993). Either way, this result suggests that fish possess a fine-tuned ability to detect food items that are offensive and then modify foraging to reflect a learned aversion.

In conclusion, this study is the first to show that unpalatability conclusively exists in both *L. catesbeianus* and to a lesser extent *L. clamitans,* devoid of any confounding variables associated with predator-prey interactions. Further work however, should be

aimed at determining whether or not *L. catesbeianus* and *L. clamitans* larvae contain a chemical toxin or what skin compound causes them to be unpalatable, and whether sunfish can learn to avoid larvae of both anuran species through observational learning.

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Chapter 4

General Conclusions

The importance of considering how organisms behave in the context of their community is essential, as behaviour cannot be fully understood when considering individuals in isolation or in the presence of only one component of the community (e.g. predators). While predator-prey interactions can be complex, the addition of parasites adds a whole other facet to the complexity and, in some cases, parasites can alter the host in such a way that it responds to a potential predator in a completely different manner than when unparasitized. This is especially clear when considering manipulative parasites (those parasites which induce phenotypic changes in their hosts in an attempt to increase the probability it is transmitted to the next host), which have been shown to completely modify host population ecology, competition processes, food web structures, and even habitat creation (Lefevre et al. 2009). Thus, not only is it important to consider the effects of parasites within the community, but also how multiple trophic levels, such as predators and parasites, influence a particular organism. Larval anurans are an ideal group of animals in which to investigate these types of questions, as anurans have invaded a multitude of habitats (e.g. see Chapter 1). By characterizing the behavioural and morphological adaptations to multiple enemies (namely, parasites and predators) present simultaneously in larval anurans, we can begin to better understand the adaptations that have helped anurans become one of the most successful vertebrate groups on the planet (Harding 2006).

The goal of this thesis was to answer two very specific questions aimed at investigating how three different species of larval anurans (*L. sylvaticus, L. clamitans* and

L. catesbeianus) that inhabit different environments and, as such, are exposed to very different predator and parasite guilds, respond to the threat of both potential enemies. In addition, I was interested in determining if this response to the combined presence of parasites and predators differed among those species that possess additional antipredator mechanisms against fish predators compared to those species that do not.

In this thesis I demonstrate that species often found to co-occur in natural systems with fish predators, such as *L. catesbeianus* and *L. clamitans* (to a lesser extent), have tadpoles that increase their activity and antiparasite behaviour when both predator and parasites are present, suggesting the threat of parasitism outweighed the cost of predation (Chapter 2). In *L. sylvaticus,* which is never found coexisting with fish, activity and antiparasite behaviours decreased when both fish and parasites were present, suggesting that for this species, the response to the fish was much stronger than the response to the parasites. I predicted that the difference observed in the behavioural response of these three species to the combined presence of fish and parasites was a function of additional antipredator mechanisms that have coevolved in those species that co-occur with fish in nature. In support of this, I was able to demonstrate that *L. catesbeianus,* and to a lesser extent *L. clamitans,* do possess an additional antipredator mechanism against fish predators, namely unpalatability (Chapter 3). Unpalatability apparently protects these species from fish predation, as fish learn to associate a bad taste or a negative post-ingestion consequence with that species and avoid it in the future.

The result that unpalatability functions as an antipredator mechanism in both *L. catesbeianus* and *L. clamitans* helps to explain how these two species of larval anuran are able to increase activity and conspicuous antiparasite behaviours in the presence of fish

and parasites. It is of no surprise that larval anurans alter behaviour in a manner that is reflective of predation risk; it has been shown repeatedly in the literature (e.g. Lawler 1989, Skelly 1994, Anholt and Werner 1998, Van Buskirk and Yurewicz 1998, Anholt et al. 2000, Richardson 2001). If a larval anuran is not vulnerable to a specific predator, then it would not make sense to decrease activity, as decreasing activity has serious consequences for growth (as feeding opportunities decrease) and subsequent size at metamorphosis, which can impact overall fitness of the individual (Werner 1986, Smith 1987, Werner 1991).

The presence of unpalatability or an additional antipredator mechanism while useful in decreasing the frequency of predation may also indirectly serve to minimize parasite infection. Permanent ponds are home to numerous parasites that require tadpoles as an intermediate host. Thus, by being less susceptible to fish predators (the major predator guild in permanent ponds), tadpoles can be more active and this may increase the frequency with which they successfully dislodge attacking parasites, which, in turn, may reduce the number of cercariae that successfully encyst. This could also be true for other antipredator mechanisms. For example, if a predator induces particular morphological changes within an organism, better equipping it to escape predation, it is likely that some of these changes would also aid in decreasing parasite infection. For example, Wilson et al. (2005) showed that *R. lessonae* larvae raised in the presence of predatory sunfish had 2% shallower tails and tail musculature was 2.5% higher than non-predator-exposed tadpoles. In addition, they showed that these morphological changes significantly influenced swimming performance (they swam 9.5 – 15% faster than non-predator exposed tadpoles). Thus, if swimming speed increases in response to being

raised with fish, then perhaps faster swimming speeds might also aid in reducing the number of attached cercariae.

In conclusion, when taking chapters two and three together, the importance of considering multiple trophic interactions and the combined effects on behaviour, especially in anuran communities, is clear. This type of work is extremely important as parasite-host interactions among ecologists have received far less attention than any other interaction (e.g. competition or predation; Price 1980, Baker and Smith 1997). Further, the trade-off between antiparasite and antipredator behaviour when encountering a parasite and predator simultaneously within the environment has received even less attention. Thus, by understanding the dynamic interactions that exist between multiple trophic levels within a community, it will enable ecologists to make informed decisions regarding conservation strategies for those species that are in danger of extinction in an attempt to maintain global biodiversity.

Future studies, in addition to characterizing the impact of multiple enemies on the behaviour, physiology and morphology of a wide array of organisms, should also focus on determining how foreign stressors (both biotic and abiotic) added to the system change the dynamics among multiple trophic levels. Specifically within the anuran community, it would be interesting to investigate how pesticides or other anthropogenic substances stress infected individuals, thereby changing the host-parasite dynamic and the predator-prey dynamic. This would be particularly interesting to test using *E. trivolvis,* as infection from this parasite has been shown to have no physiological or fitness related traits when individuals that become infected are not experiencing any other type of stressor. Investigating the synergistic effects between a multitude of abiotic and biotic factors (e.g.

pesticides, increased UV-B radiation, parasites and predators) and the impact on larval anuran communities as a whole seems to be the next logical step.

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Appendix A: Average Frequency within Individuals of Three Parasite Related Behaviours

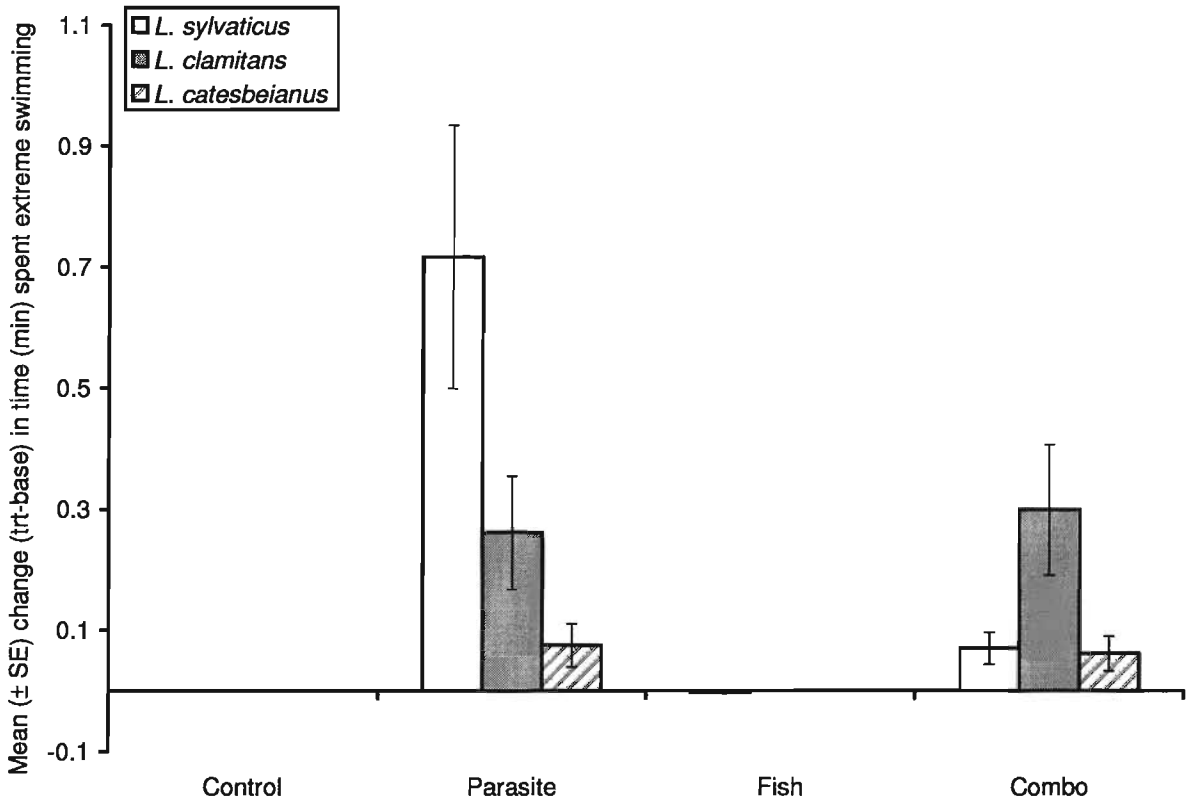

Figure A1: Mean (± SE) time (min) spent extreme swimming for all 20 tadpoles and all three anuran species in each of the four treatments. See chapter 2 for more details.

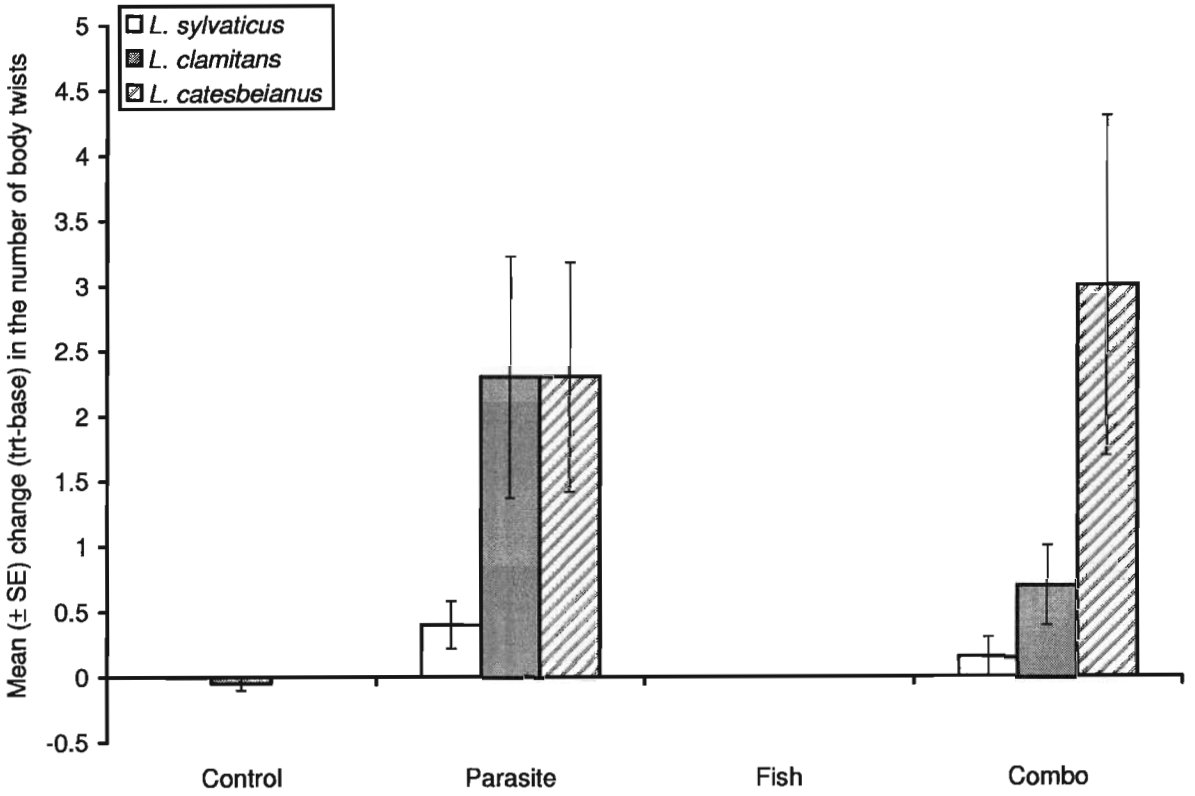

Figure A2: Mean (± SE) number of body twists for all 20 tadpoles and all three anuran species in each of the four treatments.

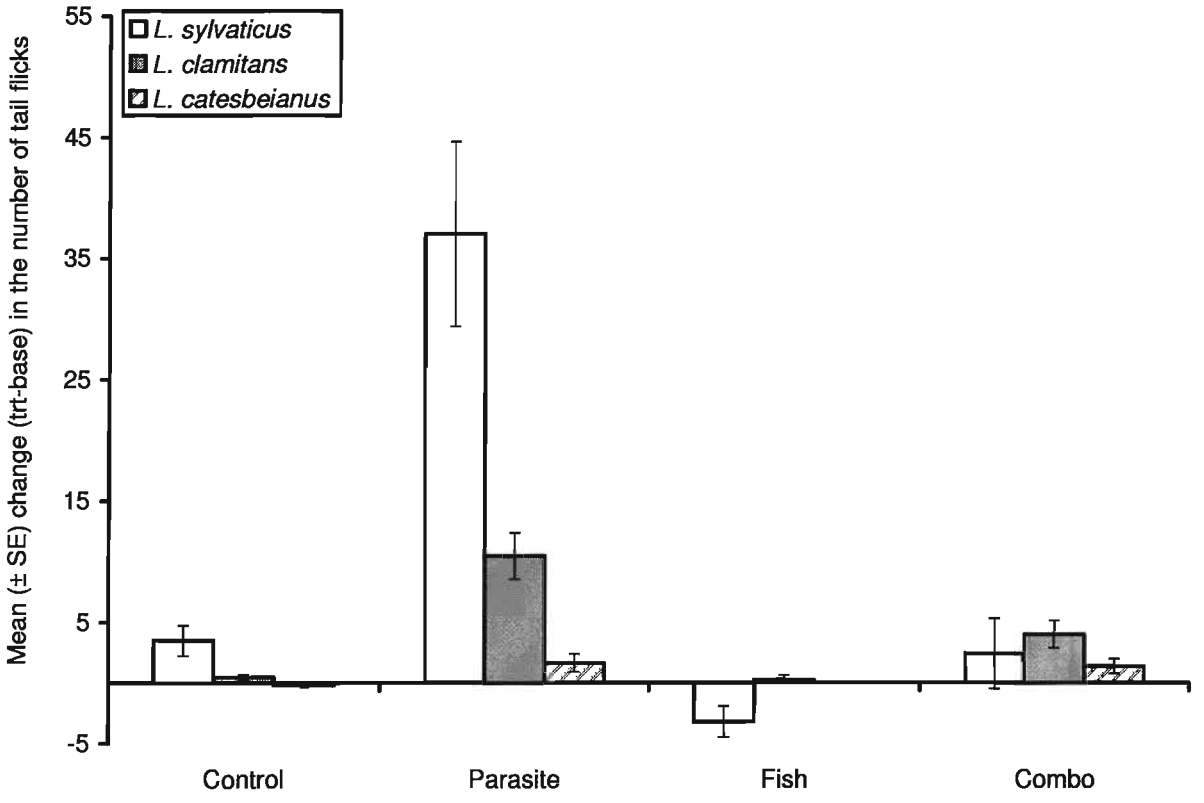

Figure A3: Mean (± SE) number of tail flicks for all 20 tadpoles and all three anuran species in each of the four treatments.