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**An Investigation of Prevalence of *Aeromonas hydrophila* and of Measures
of Immune Function for Frogs Used in the Ontario Baitshop Industry:
Implications of Past and Present Practices**

by

David L. McRuer

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of
Master of Science**

Department of Biology

Carleton University

Ottawa, Ontario

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Chair, Department of Biology
Thesis Supervisor

Carleton University

ABSTRACT

I investigated the prevalence of *Aeromonas hydrophila* and measures of immune function for frogs used in baitshops to evaluate their potential to vector diseases to wild organisms. I tested whether prevalence of *A. hydrophila* was variable among *Rana pipiens* (leopard frogs), *R. clamitans* (green frogs), and *R. catesbeiana* (bullfrogs), sexes and age classes, and whether natural history with regard to habitat was predictive of this variation. Adult *R. pipiens* accounted for heterogeneity among species prevalence, which was not predicted. Variable prevalence could affect potential for transfer of parasites and pathogens, among captives or to the wild.

Currently, only leopard frogs are regulated for use in Ontario baitshops. Estimates of leukocyte density did not differ between baitshop and wild leopard frogs. Baitshop frogs show immunoredistribution suggesting that they are not overtly unhealthy, but are exposed to antigenic challenges. These conditions also could aid in transfer of disease to wild frogs. Further work on or near unregulated baitshops is warranted.

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GENERAL INTRODUCTION

Animal husbandry¹ practices (AHP) have been hypothesized to increase the risk of disease transfer to wild organisms via several means (Figure 1.0). For example, AHP can introduce new parasites and pathogens to susceptible populations (Davidson and Nettles 1992; Lyles and Dobson 1993; Cunningham 1996; Bergersen and Anderson 1997; Brown *et al.* 1999). Such risk of disease transfer can be associated with translocations including introductions and re-introductions. Further, disease agents also can be transferred from domestic animals to wild populations or vice versa.

There are a few well documented cases of such transfers. Whirling disease due to the metazoan parasite *Mxyobolus cerebralis* (native to Europe) has decreased the abundance of certain salmonids in many North American watersheds. This disease appears to have transferred to wild species after captive fish infected with *M. cerebralis* were released into streams (Bergersen and Anderson 1997). Disease transmission between wild and domestic animals also has been demonstrated with occurrence of foot-and-mouth disease in Chilean livestock, when farming practices encroached on national parks and reserves. This encroachment increased exposure of the Andean deer, *Hippocamelus bisulcus*, which is now a known foot-and-mouth disease reservoir (Simonetti 1995).

Conditions associated with housing/rearing during AHP are also known or hypothesized to increase the potential for parasite transmission at least among

¹ Terms that are operationally defined in the glossary (Appendix 1) appear bolded at first mention in this thesis.

Figure 1.0. Flow chart of risks associated with husbandry practices in terms of increased disease transmission from domestic or captive animals to wild animals of the same or of a different species. Flow chart does not include the problem of disease transfer from wild animals to domestics, although this can occur (see text). Those words bolded indicate factors seen in past or present frog baitshop practices. For exposure and immune status metrics, "+" indicates that practice is hypothesized to increase either exposure or help augment immune ability. A "-" indicates practice is hypothesized to depress or alter immune status. For probability of transfer, a "+" indicates that practice results in a potentially increased likelihood of transfer of parasitic or pathogenic organisms from captive animals to wild populations. Other problems with animal husbandry practices such as changes in the gene pool of wild populations with accidental releases of game or farmed animals, or changes in nutrient input from non-contained agriculture or aquaculture operations can occur, but are not considered in this thesis.

captive animals. These conditions include increased stress, hormone treatments, increased density, changed diet, and mixed species assemblages. However, less is known about the transfer of parasitic organisms from captive animals to wild populations.

Increased stress levels are acknowledged to influence immune status (Braude *et al.* 1999; Lochmiller & Deerenberg 2000) which, in the case of captive desert tortoises (*Gopherus agassizii*), is believed to have resulted in upper respiratory tract disease (due to *Mycoplasma agassizii*) and the subsequent spread to uninfected wild tortoises during reintroduction programs (Brown *et al.* 1994). In AHP geared toward protein production (e.g. aquaculture industry), hormone treatments have resulted in immunosuppression (Isayeva and Kozinenko 1992). Further, high densities of captive organisms associated with such AHP may increase exposure to disease agents and enhance transmission of pathogens that otherwise may not be encountered. This problem has been demonstrated in cattle farming where the prevalence of brucellosis (a disease caused by *Brucella abortus*) is only maintained when herd size is greater than 200 animals (Peterson *et al.* 1991). Increased density also may increase the risk of being physically wounded thereby creating portals for pathogen infection.

Other nutritional factors also may influence the risk of transfer of parasitic organisms. If food quantity or quality is reduced for extended periods of time, immune status should be negatively affected. Facilities holding mixed species assemblages are thought to increase the risk of disease by exposing the domestic or captive animals to multiple pathogen species and/or strains (CCAC

1984). These factors may not be universal to all animal husbandry practices. However, they must be considered when assessing risk of transfer of parasitic organisms.

This thesis deals specifically with the Ontario frog baitshop industry, an animal husbandry practice where disease is not uncommon and the potential for transmission of parasites and pathogens remains largely unexplored. Baitshop practices have many of the above listed risk factors and thereby could promote transmission of parasitic organisms. In particular, frogs are exposed to a variety of stresses that may increase the likelihood of disease. For example, frogs can suffer wounds during translocation (capture, transportation and housing) that can lead to infection. Frogs from different populations are often housed in high densities and occasionally in mixed species assemblages. High density can increase the rate of pathogen transmission and wounds while mixed assemblages potentially allow pathogens, which are benign on one host but pathogenic on another, to be transferred (CCAC 1984). Frogs also are rarely fed while in captivity. Chronic undernourishment may result in decreased ability to respond to antigenic challenges. Finally, the above factors may act in combination to affect the ability of the immune system to resist infection (Braude *et al.* 1999). If the immune system is suppressed, the likelihood of infection within baitshops increases, thus eventually augmenting the risk of transferring disease agents to wild populations.

I studied the bacterium *Aeromonas hydrophila* (the etiological agent of red-leg disease) as a model parasitic organism in this thesis (primarily in Chapter

1). *Aeromonas hydrophila* is known to infect amphibians in both the wild and captivity (Hunsaker and Potter 1960; Hird *et al.* 1981). In captivity, red-leg disease outbreaks are proposed to be associated with stressful conditions such as high density, lack or reduction of food, and physical wounds (CCAC 1984). In the wild, red-leg disease has been associated with other stressful conditions such as changing weather conditions (Hunsaker and Potter 1960; Nyman 1986), mating activities (Dusi 1949), and hibernation (Hunsaker and Potter 1960; Gibbs 1963; Glorioso *et al.* 1974; Nyman 1986; Cooper *et al.* 1992; Carey 1993). Finally, *A. hydrophila* has been implicated as one contributing factor to localized amphibian declines suggesting a greater need to assess "potential" disease transmission from baitshop to wild frogs (Alford and Richards 1999) .

The goal of my thesis research was to assess the "potential" for baitshop practices in Ontario to vector disease (such as red-leg) to wild hosts. Susceptibility of captives to disease depends on exposure and the ability to resist infection. In past practices, mixed- species assemblages occurred as did mixed assemblages of males and females of varying ages and red-leg disease was not uncommon (Forbes MR, pers. comm.). In 1999, the Ontario Ministry of Natural Resources changed regulations for the baitshop industry, by banning mixed species assemblages and by only allowing *Rana pipiens* (Schreber) to be housed and marketed. Reducing the number of species housed in baitshops should decrease exposure to a wider array of parasitic organisms, thereby lessening the risk of novel disease transfer among captives. As a preliminary investigation relating to thesis objectives outlined below, I first conducted a

survey of Ontario baitshops to determine whether outbreaks of red-leg disease still exist after the policy change and also if baitshops continue to house mixed species assemblages (Appendix 2).

Specific Objectives

In Chapter 1, I examine variation in prevalence of *A. hydrophila* on three wild frog species (*Rana pipiens*, *R. clamitans* (Rafinesque), and *R. catesbeiana* (Shaw)) known to have been used in Ontario baitshops. Higher prevalence of the pathogen in any species would suggest higher exposure of the pathogen(s) to heterospecifics, if housed in mixed species assemblages. Whether different strains of *A. hydrophila* differing in virulence exist on different species was not considered in this study. However, there is evidence that some strains of pathogens with low virulence can evolve in immuno-deficient hosts to highly virulent strains capable of inducing disease in immunocompetent hosts (Beck *et al.* 1994; Beck *et al.* 1995). Highly virulent pathogens may quickly kill the host, whereas strains with lower virulence can spread to a greater number of hosts, while still possessing the potential for disease outbreak (McCallum and Dobson 1995). In terms of my thesis, higher prevalence of *A. hydrophila* should indicate higher potential for transmission among captives regardless of the virulence found in baitshops.

More specifically, I tested the null hypotheses that there was no significant variation in *A. hydrophila* prevalence among species, sexes or age classes of frogs. Variation in background prevalence of *A. hydrophila* among frog species,

sexes or age classes would indicate variation in risk of disease transmission among captives or outbreaks within baitshops and variable transmission probability to wild animals. As an ancillary objective, I examined whether the natural history with regard to habitat of different categories of frogs was predictive of *A. hydrophila* prevalence. Different species, sexes or ages of frogs used previously and currently in baitshops may be expected to differ in their exposure to *A. hydrophila* before capture, such that some groups pose greater infection risks than others.

As mentioned, susceptibility to disease depends on exposure but also on the host's ability to resist infection. Under stressful conditions, the host may have reduced resources to allocate to immune responses (Lochmiller & Deerenberg 2000) resulting in an overall decrease in size, abundance, or functioning of immune elements (immunosuppression) (Svensson *et al.* 1998). Alternatively, hosts may reallocate certain components of the immune system to areas where they are most likely to be useful in resisting infection (immunoredistribution) (Dhabhar and McEwen 1996; Dhabhar 1998). Breeding male animals for example may show reductions in circulatory lymphocytes suggestive of androgen-mediated immunosuppression (Braude *et al.* 1999). However, other immune components may actually increase, such as neutrophils, which are a first line of defence following injuries sustained during displays or combat (Braude *et al.* 1999). If only one or a few immune components are measured, immunoredistribution may not be seen and researchers may falsely conclude that immunosuppression has resulted. If stresses associated with baitshop housing

cause either of these processes to occur, captive frogs may have a reduced ability to prevent infection and therefore may serve as vectors of disease if released. It is useful to know whether wholesale immunosuppression is occurring versus immunoredistribution. In the latter case, antigenically challenged frogs are still relatively healthy and may serve as better vectors if released.

Thus, I compared leukocyte profiles and relative mass of immunological organs of juvenile *R. pipiens* captured in the wild versus those housed in baitshops, to ascertain whether baitshop practices have any impact on the ability of captive frogs to respond to immunogenic challenges (Chapter 2). I compared juvenile leopard frogs because my survey indicated these were most often used in baitshops, but occasionally assemblages with different species of frogs and assemblages of different ages were seen. I tested the null hypothesis that baitshop and control wild caught frogs did not differ in relative weights of immunological organs or in density and proportion of leukocyte elements found in the blood. Again, my central argument was that altered immune ability in baitshop frogs could result in a greater potential of infection, and thus a larger risk of die-offs in baitshops or in potentially vectoring the pathogen. Importantly, baitshop frogs are often released following fishing at sites distant to where they were captured and often after being housed for some time (Appendix 2).

CHAPTER 1

Prevalence of *Aeromonas hydrophila* in wild-caught frogs of three ranid species: implications for the Ontario baitshop industry.

Introduction

Based on over 20 years of research, investigators have concluded that there is a global decline in amphibian populations and have argued that there is no one cause for this decline (e.g., Houlihan *et al.* 2000). The main factors hypothesized to cause declines include ultraviolet radiation, habitat and environmental modification (e.g., changes in acidity and toxicants), species introductions, changes in climate or weather patterns, and emerging infectious diseases (Beebee *et al.* 1990; Carey 1993; Kiesecker and Blaustein 1995; Long *et al.* 1995; Blaustein *et al.* 1998; Alford and Richards 1999; Pounds *et al.* 1999). These factors may act singly or synergistically. In cases of disease outbreaks, the importance of anthropogenic factors in hastening transmission or virulence remains largely unknown, but habitat fragmentation and transfer via humans have been implicated (Lyles and Dobson 1993; Cunningham 1996).

Despite global declines in frog abundance, humans continue to collect large numbers of frogs to supply baitshops and also high schools and universities using dissection as a teaching tool (Hird *et al.* 1981; Rosenberger 1998). In North America, leopard frogs (*Rana pipiens*) and bullfrogs (*R. catesbeiana*) are most

commonly collected (Hird *et al.* 1981; Rosenberger 1998); both are threatened in parts of their range (Wagner 1997). Market demand for amphibians may pose further threats to species, such as transfers of pathogens from distant sites to housing operations and then to the wild.

Suppliers of frogs for baitshops (or for education or research purposes) typically collect frogs from wild populations and house them in overcrowded containers while in transit to and from commercial supply houses. Such crowding is suspected to increase the likelihood of diseases (Oppliger *et al.* 1998). For example, red-leg is a bacterial disease caused by an infection by *A. hydrophila* (Russell 1898) and has been often recorded in captive and wild amphibian populations (Russell 1898; Dusi 1949; Hunsaker and Potter 1960; Shotts and Gaines 1972; Hird *et al.* 1981; Frye 1985; Nyman 1986; Carey 1993). This disease has been associated with overcrowding in animal husbandry operations, in research laboratories, and in wild mating congregations (Dusi 1949; Hunsaker and Potter 1960; CCAC 1984; Nyman 1986). Additionally, red-leg disease has been associated with other stresses such as hibernation (Cooper *et al.* 1992; Maniero and Carey 1997), or secondary infections (Carey 1993).

It is important to ascertain whether different frog species differ in natural levels of infestation by bacteria such as *A. hydrophila*. Different species of frogs are differentially used in baitshops or for educational purposes. In 1999, the Ontario Ministry of Natural Resources regulated that only leopard frogs could be used in baitshops in Ontario. Furthermore, mixed species housing is not permitted under the Canadian Council of Animal Care guidelines for Use of

Experimental Animals in Research because of the possibility of disease transmission. This policy change by OMNR would likely reduce transmission between species, even if it were not passed for this purpose. It is also relevant that *A. hydrophila* has been associated with cholera-like symptoms in humans (Janda and Abbott 1998). For this reason, educational studies involving live experimental frogs may potentially be hazardous with the risk increasing with the prevalence of infection seen in wild frogs used for such purposes. Although tetracycline can stop disease outbreaks in the facilities associated with research (CCAC 1984), it is unlikely to be used in many instances, particularly baitshops. The risk of undesirable antibiotic resistance may also result from tetracycline treatments. Taken together, Ontario baitshops in the past or present fulfill several criteria deemed important in spread of disease within baitshops and potentially to the wild. These criteria include mixed species housing, handling stress, high density housing, food limitation, and obtaining frogs from non-local areas.

There are established tests for among-species variation in prevalence of *A. hydrophila*. Prevalence is the proportion of hosts in a sample that score positively for a parasite or pathogen (Bush *et al.* 1997). In the context of this study, presence or absence of *A. hydrophila* was based on microbial tests of swab and other samples taken from wild-caught frogs. My working hypothesis is that natural variation in prevalence among ranid frogs should exist because frogs differ in aquatic habitat utilization and *A. hydrophila* is most common to aquatic environments. These differences in exposure among frog species include habitat preference, starting date and duration of breeding season, and maturation time

from tadpole to metamorph (Gilhen 1984; McAlpine and Dilworth 1989; Given 1990; Gilbert *et al.* 1994).

As mentioned, I assumed that presence of *A. hydrophila* indicates increased risk of disease occurrence to individual frogs and that high prevalence indicates increased risk of transmission of *A. hydrophila* or red-leg in captivity and possibly to wild populations. My specific interest is whether this surrogate of risk varies depending on the species being tested. As mentioned previously, other studies have recorded pathogens sweeping through commercial operations and indicated possible transfers to wild populations (Bergersen and Anderson 1997; Brown *et al.* 1999). As yet, no study has addressed the potential for disease spread within and from baitshop practices. I address this potential by examining prevalence of *A. hydrophila* in frogs used previously or currently in baitshops.

This first study assesses variation in prevalence of *A. hydrophila* among wild-caught leopard frogs, bullfrogs, and green frogs. I tested the null hypothesis of no significant variation in background levels of *A. hydrophila* among species, sexes and age classes. These host species were selected based on their previous use in baitshops, their differences in natural history with regard to habitat (see above) and their overall abundance at the study site relating to ease of capture (see below). By using standard microbiological techniques, I was able to determine prevalence of *A. hydrophila* and thus differential risk of red-leg disease transfer by juveniles and adult males and females of all three species.

Furthermore, by evaluating variation in *A. hydrophila* prevalence and assessing disease potential associated with the various species, sexes and age classes being tested, recommendations can be made regarding the use of frogs in baitshops. A survey this past year showed green frogs were used in 1 of 10 baitshops (Appendix 2) demonstrating that mixed species assemblages still exist. Keeping different species of frogs in common assemblages may be problematic.

Methods

Description of study sites and natural history of study species

Frogs were collected from small ponds and lake edges from April-June, 2000 at the Queens University Biology Station (QUBS) located in eastern Ontario, Canada (45° 34' N and 76° 13' W). QUBS was selected because of the abundance of frogs locally and to represent a typical area in which frogs would be collected for baitshops and used in fishing. In fact, collections have occurred within and around this area for at least 40 years (F. Phelan, pers. comm.).

In Ontario, all three species emerge from benthic hibernation in mid April. However, breeding seasons differ (leopard frogs: April-June, green frogs: late May-early August, and bullfrogs: June-August, (Wright 1914)). This difference in breeding seasons was confirmed for the three study species at my study sites by Schalk (1997). For all anuran species, males defend territories within the water and wait for females to approach and then mate (Wells 1977). During this time males are predicted to have higher exposure to *A. hydrophila* as wounds created by physical contact with other males during territory defence, increase risk of

infection by *A. hydrophila*. (Dusi 1949). After the breeding season, leopard frogs (explosive breeders) leave the water and live in terrestrial habitats whereas green frogs and bullfrogs (prolonged breeders) remain in the aquatic habitat (Wells 1977; McAlpine and Dilworth 1989). I predicted based on this information that leopard frogs might have lower exposure to *A. hydrophila* as compared to the other two species.

Aeromonas hydrophila is a gram-negative bacillus that is ubiquitous in fresh water and, as mentioned, is known to be the pathogenic agent responsible for red-leg disease in amphibians (Kulp and Borden 1942). Some strains of *A. hydrophila* are facultative psychrophiles and can maintain a selective advantage over other bacteria through the winter months (Carr *et al.* 1976). Hird *et al.* (1981) showed that frogs that breed in the spring soon after emergence from hibernation (in this case leopard frogs) have high prevalence of *A. hydrophila*. *Aeromonas hydrophila* produces both endotoxin passively (released from its cell walls after cell death) and the hemolytic toxin, aerolysin, actively (Rigney *et al.* 1978). Aerolysin produces holes approximately 3 nm in diameter in the cell membrane of erythrocytes by binding to a specific glycoprotein receptor on the surface of erythrocytes, and inserting into the lipid bilayer. This binding and insertion destroys the membrane permeability barrier and eventually results in cell death (Howard and Buckley 1985). As mentioned, red-leg is a highly transmissible and often virulent disease in captive amphibians (Glorioso *et al.* 1974; Kinney *et al.* 1999).

Field Protocol

I selected sites based on gross metrics of abundance of the three frog species. A field assistant and I did this by listening for frog choruses during the evenings and nights and then conducted quick surveys to see if enough frogs were available for a high catch per unit effort. I wanted *ca.* 100 frogs per species within about 4 weeks of sampling. Such considerations would certainly relate to collections made by suppliers to commercial operations or baitshops.

The field assistant or I captured frogs by hand while wearing latex gloves. We used a new pair of gloves for each frog to prevent the transfer of pathogens between frogs. Approximately 30 s after capture, the specimen was brushed with a sterile swab on the lower abdomen and the ventral side of its thighs. The swab was then stored in a phosphate buffered saline (PBS) solution for transport and storage. Immediately after the epidermal swab was stored, a cotton pad, pre-doused with 70% methanol, was gently rubbed externally over the ventral cardio-thoracic area of the frog to eliminate bacteria. Then by cardiac puncture, 0.02-0.04 ml of ventricular blood was obtained from the specimen using a 28 gauge (0.5 CC) needle, applied onto a second sterile swab and stored in a PBS solution.

The specimen was then placed upside-down on a 12cm X 20cm cardboard card and the length between the snout and vent (SVL) was recorded. The measurement was repeated on the other side of the card allowing for a mean SVL to be calculated and thus reducing measurement error. The specimen was then toe-clipped using a pair of human nail clippers stored in 70% methanol.

The first (outer) digit on the hind left foot was clipped to indicate that the animal had been sampled. The frog was then released on site.

In total, 142 green frogs, 130 bullfrogs, and 120 leopard frogs were completely processed in this way. Although not recorded specifically, < 10 frogs escaped during processing and partial data on these frogs were excluded from analysis. Frogs were placed into the following categories: breeding males, breeding females, and unsexed juveniles (non-breeding male or non-breeding females) based on SVL and secondary sexual characteristics. In all species, breeding males could be identified by enlarged thumbs, enlarged tympanums and by gender-specific colouration. Juvenile frogs could not be sexed without dissection.

Baitshop Survey

Eleven bait shops were chosen based on their close proximity to QUBS where the control frogs were captured. Collection dates ranged between July 30 and August 16, 2000 (Appendix 2). Upon arriving at each baitshop, I conducted a survey investigating the health status that evaluated 1) the number of dead frogs, 2) the number of frogs showing signs of disease and 3) the species and age classes housed in each baitshop. These records and others are reported in Appendix 2. The purpose of this survey was to assess whether red-leg disease or mixed species/age housing is still common in a sample of Ontario baitshops.

Laboratory Protocols

All tubes containing epidermal and blood swabs were returned to the laboratory and plated within 24 hours. Epidermal and blood swabs were initially plated onto slants containing 5mL of Ryan's Selective *Aeromonas* Media (Ryan 2000).

Swabs were removed from the PBS solution and transferred to the slant where they were rolled and spread over the media. Once all swabs had been applied onto separate media slants, they were incubated at $27^{\circ} \pm 1^{\circ}\text{C}$ for 48 hours. After incubation, the *Aeromonas* slants were scored for *A. hydrophila* colonies, characterized as being opaque green colonies with dark green centers.

As *Pseudomonas* colonies are often visually similar to *Aeromonas* colonies, all colonies that were thought to be *A. hydrophila* were aseptically re-plated for isolated colonies into petri dishes containing 12 mL of Ryan's Selective *Aeromonas* Media. These plates were incubated at $27^{\circ} \pm 1^{\circ}\text{C}$ for 48 hours.

Isolated colonies resembling an *Aeromonas* species were observed under a Leitzwetzlar 10X dissecting microscope for cloudy borders and then held up to a light to determine clarity of the colony. *Aeromonas* colonies do not have cloudy borders and are translucent when held to a light whereas *Pseudomonas* colonies have a distinctive cloudy border and are opaque when viewed in lighted conditions. For final confirmation of *A. hydrophila*, several suspected colonies were analyzed using a standard Biolog resource; this tests for patterns of carbon source utilization and compares results to a carbon utilization library.

Statistical analyses

Most analyses were conducted using SAS software (SAS Institute Inc. 2000). As indicated, prevalence of *A. hydrophila* was simply number of frogs scoring positively for *A. hydrophila* divided by the total number of frogs of a given category or species sampled. Confidence limits around prevalence were calculated following Zar (1996). This time-consuming method has advantages over normal approximation (Snedecor and Cochran 1980) including asymmetrical limits, and the fact that confidence estimates cannot go below 0 or above 1.0 (proportions), which are biologically meaningless. Additionally, one can calculate confidence limits using this method even when no frogs in the sample were infected, which cannot be done with the normal approximation method. Differences in prevalence between males and females, age classes (defined as adults and juveniles) and then among species, were assessed using χ^2 goodness of fit. Separate analyses were carried out for breeding adults and juveniles.

RESULTS

Overall prevalence of A. hydrophila and red-leg disease in wild frogs

Aeromonas hydrophila was isolated from epidermal samples on 30 separate occasions from presumably healthy leopard frogs, green frogs and bullfrogs. Red-leg diseased frogs were found only twice (both adult leopard frogs in the early spring) and *A. hydrophila* was cultured in both instances. The prevalence of *A. hydrophila* in the blood was very low (0.8%) considering all frogs (Table 1.1).

found only twice (both adult leopard frogs in the early spring) and *A. hydrophila* was cultured in both instances. The prevalence of *A. hydrophila* in the blood was very low (0.8%) considering all frogs (Table 1.1). This indicated a lack of systemic *A. hydrophila* infection in wild-caught frogs at my study location. Statistical comparisons of systemic infections were not done for this reason and all subsequent tests are based on epidermal samples.

Prevalence in relation to gender

Multiway contingency analysis showed no sex effect after controlling hierarchically for variation due to species (Mantel-Haensel $X^2 = 0.85$, d.f. = 3, $P = 0.35$, Fig. 1.1). Each species was then tested separately to confirm this result. There were no differences in *A. hydrophila* prevalence between adult sexes of leopard frogs ($X^2 = 0.002$, d.f. = 1, $P = 0.96$, Fig 1.1) or bullfrogs ($X^2 = 0.001$, d.f. = 1, $P = 0.98$, Fig 1.1). A significant difference in prevalence was found between male and female green frogs ($X^2 = 4.2$, d.f. = 1, $P = 0.04$, Fig 1.1), although the asymmetric confidence limits overlapped. Comparing adult males, leopard frogs had the highest prevalence of epidermal *A. hydrophila*, followed by bullfrogs and then green frogs (Fig. 1.1). The same trend was true for adult female frogs (Fig. 1.1) although not compared statistically.

Variation in prevalence among age classes

Age classes were compared for differences in prevalence of *A. hydrophila* within each species. Adult male and female frogs were grouped into a common adult

$P = 0.003$, Fig. 1.2), but not for green frogs ($X^2 = 1.93$, d.f. = 1, $P = 0.16$, Fig. 1.2) or bullfrogs ($X^2 = 0.95$, d.f. = 1, $P = 0.33$, Fig. 1.2).

Prevalence in relation to species

To determine whether *A. hydrophila* prevalence differs among species, frogs were first grouped into age classes because of the significant age class effect in leopard frogs. *Aeromonas hydrophila* prevalence data for juvenile frogs presented in Fig. 1.3 but remain unanalysed due to low prevalence counts found in *R. pipiens* and *R. clamitans* that produce associated statistical errors. Juvenile bullfrogs were the only juveniles with marked *A. hydrophila* prevalence. Adult frogs also were found to differ in *A. hydrophila* prevalence ($X^2 = 10.48$, d.f. = 2, $P = 0.005$, Fig. 1.3). Here post-hoc analyses were done by removing each species separately from the full model and repeating the chi-square analysis for the remaining two species following recommendations of Zar (1996). Leopard frogs had a significantly higher prevalence than green frogs ($X^2 = 10.04$, d.f. = 2, $P = 0.002$) and bullfrogs ($X^2 = 4.05$, d.f. = 2, $P = 0.04$), but no difference was observed between green frogs and bullfrogs ($X^2 = 1.3$, d.f. = 2, $P = 0.25$, Fig. 1.3).

Baitshop survey

I also present ancillary data concerning the commercial use and health status of wild frogs housed in bait shops (Appendix 2). As mentioned, leopard frogs were typically the only species found in captivity and therefore mixed assemblages

were rare. Although the majority of bait shops stocked juvenile leopard frogs, two shops had a mixed collection of both adults and juveniles (bait shops 1 and 9). In addition, 70 dead frogs were observed in baitshops: 20 in baitshop 6 and 50 in baitshop 9, two of six frogs purchased in baitshop 6 were symptomatic for red-leg disease.

Table 1.1. Summary of *Aeromonas hydrophila* prevalence in the blood of three wild frog species in relation to age and gender. Asymmetrical confidence limits (Zar, 1996) are reported for prevalence estimates.

Species	Age	Gender	N	Blood		
				Incidence	Prevalence	CL
Rana pipiens	Adult	Male	67	1	1.5	0.04-8.0
		Female	25	0	0	0-13.7
	Juvenile	Unknown	28	0	0	0-12.3
Rana clamitans	Adult	Male	67	0	0	0-5.4
		Female	45	0	0	0-7.9
	Juvenile	Unknown	30	0	0	0-11.6
Rana catesbeiana	Adult	Male	51	1	2.0	0.05-10.5
		Female	48	1	2.0	0.05-11.1
	Juvenile	Unknown	31	0	0	0-11.2

Figure 1.1. Prevalence ($\pm$ asymmetric confidence limits) of *Aeromonas hydrophila* swabbed from the epidermis of adult *Rana pipiens*, *R. clamitans*, and *R. catesbeiana* in relation to gender of adults. *Aeromonas hydrophila* was isolated from 11 and 4 *R. pipiens* males and females, respectively, and from 4 *R. clamitans* males and from 4 and 3 *R. catesbeiana* males and females respectively. No *A. hydrophila* was isolated from adult female *R. clamitans*. Sample sizes are reported above upper confidence limits. Multiway contingency analysis showed no effect after controlling hierarchally for variation due to species (Mantell-Haensel $X^2 = 0.85$, $P = 0.35$, d.f. = 3)

Figure 1.2. Prevalence ($\pm$ asymmetric confidence limits) of *Aeromonas hydrophila* swabbed from the epidermis of juvenile and adult *Rana pipiens*, *R. clamitans*, and *R. catesbeiana* in relation to age class. *Aeromonas hydrophila* was isolated from 15 adult *R. pipiens*, 4 adult *R. clamitans*, 7 adult *R. catesbeiana*, and 4 juvenile *R. catesbeiana*. No *A. hydrophila* was isolated from juvenile *R. pipiens* or *Rana clamitans*. Sample sizes are reported above upper confidence limits. Asterisk indicates $p < 0.05$.

Figure 1.3. Prevalence ($\pm$ asymmetric confidence limits) of *Aeromonas hydrophila* swabbed from the epidermis of *Rana pipiens*, *R. clamitans*, and *R. catesbeiana*. Juvenile and adult age classes are considered separately as juveniles cannot be sexed without dissection. *Aeromonas hydrophila* was isolated from 15, 4, and 7 adult *R. pipiens*, *R. clamitans*, and *R. catesbeiana* respectively and from 4 juvenile *R. catesbeiana*. *Aeromonas hydrophila* prevalence is reported for juveniles but remains unanalysed due to statistical errors resulting from low prevalence counts. Sample sizes are reported above upper confidence limits.

DISCUSSION

The purpose of this study was to assess whether there was significant variation in prevalence of *A. hydrophila* among species, sexes or age classes of ranid frogs and what, if any, implications such variation has for past or current baitshop practices. Further to this objective, I was interested in whether differences in natural history among frog species (or sexes or age classes) was predictive of variation in *A. hydrophila* prevalence. In brief, I am mainly interested in accounting for the causes and consequences of variation in prevalence of *A. hydrophila* from epidermal samples, because frogs were found to have a relatively low prevalence of systemic infections of *A. hydrophila* in the wild. I deal first with the nature of this variation, its potential consequences, and then whether differences in natural history were predictive of variation in susceptibility to *A. hydrophila* for the three frog species.

The results of this study suggest that adult leopard frogs were most likely to be carriers of *A. hydrophila* compared to the other ranid species and age classes surveyed. This study assumes that a high prevalence of *A. hydrophila* on wild frogs indicates a greater potential for red-leg transfer at least among captives. The risk of becoming infected should increase further when frogs are physically, socially or nutritionally stressed (CCAC 1984); conditions that are associated with husbandry practices such as baitshops (Oppliger *et al.* 1998). But whether frogs released from baitshops will have a higher prevalence of *A. hydrophila* and a greater likelihood of transmitting the bacterial pathogen to wild

frog populations (and other vectors or susceptible hosts such as leeches, fish and snakes, Appendix 3), will depend on the degree to which baitshop practices immunosuppress hosts.

In the past, baitshops have sold all three ranid species identified in this survey, a practice that continues in many provinces and in areas of the United States. The risk of novel parasite transfer among captives should be intensified when baitshops keep mixed species assemblages, depending on the extent to which species show variation in prevalence. My study shows significant variation in prevalence suggesting mixed species housing could bring individuals with little exposure in the wild (green frogs) into captive situations with other groups known to experience high exposure (adult leopard frogs). As of 1999, the Ontario Ministry of Natural Resources (OMNR) regulated commercial and private frog harvest in Ontario, now only allowing leopard frogs to be captured and sold. This practice should reduce the extent to which green frogs, for example, are exposed to harmful *A. hydrophila*. My survey of baitshops shows that with three exceptions (baitshops 1, 9 and 11), all frogs surveyed were juvenile leopard frogs (mean SVL = 5.06 cm). Wild caught juvenile leopard frogs have a relatively low prevalence of *A. hydrophila* compared to adult leopard frogs. Although capturing juvenile leopard frogs partly relates to their abundance (juvenile leopard frogs are found in high densities during July and August in terrestrial environments), baitshops are further selecting a group with low *A. hydrophila* prevalence thus reducing disease risk. The decision to restrict the sale of bait frogs to just leopard frogs was based on population abundance of various ranid species. However,

OMNR seems to have moved in the right direction, by also reducing the potential of red-leg disease in captive frogs by selecting a single species while practitioners select an age class with low *A. hydrophila* prevalence.

It therefore appears that the current risk of disease transmission from released baitshop frogs to susceptible hosts in the wild is not as bad as it could be. These results suggest that the likelihood of red-leg outbreak in or near baitshops was higher in the past or is higher in areas where mixed species assemblages are common. Frogs housed in mixed species (sex and/or age class) assemblages are not only more likely to become exposed to various parasites due to the presence of infected individuals, but variation in prevalence among groups also suggests the potential of multiple strains of *A. hydrophila*. Frogs resistant to one strain may not be resistant to others thus increasing the risk of disease. There are additional factors that may affect the exposure of frogs to *A. hydrophila* in the wild (such as population abundance and density). However, this study just deals with the "potential" for disease transmission among captives or from captive to wild animals.

Further to exploring consequences of variation in *A. hydrophila* prevalence, I found that although frogs differed in their natural prevalence of *A. hydrophila* depending on the age, gender or species being compared, differences in natural history with regard to habitat selection do not predict infection status. Leopard frogs, green frogs and bullfrogs differ remarkably in time spent in semi-aquatic habitats. Leopard frogs were expected to be less exposed to *A. hydrophila* than fully aquatic species such as green frogs or bullfrogs and thus

have a lower prevalence of *A. hydrophila*. In fact, adult leopard frogs had a significantly higher prevalence than adult green frogs and bullfrogs suggesting that perhaps those species living in contact most often with the etiological agent are best defended against it and show low prevalence as adults. In a recent study, Schalk (1997) found that green frog abundance was inversely correlated with abundance of *Macrobdella decora* leeches which are known vectors of *A. hydrophila* (see Appendix 3). This may account for the low prevalence of *A. hydrophila* in green frogs. Arguments based on exposure to the pathogen also cannot explain the prevalence trend in juvenile frogs; this one aspect of natural history predicts that all three should not differ in prevalence.

Other researchers have provided some information on prevalence of *A. hydrophila* in frogs that has bearing on this study. One study (Hird et. al 1981) showed a high prevalence of *A. hydrophila* in *R. pipiens* tadpoles, but not in juvenile *R. pipiens* suggesting that the bacteria did not persist through metamorphosis and must therefore be reacquired later in life with exposure. With this in mind, juvenile frogs were predicted to have lower prevalence than adults of the same species based on less exposure to the pathogen. This prediction was supported in leopard frogs. However, no difference between age classes was observed in either green frogs or bullfrogs. This result may reflect the inability to distinguish non-breeding juvenile green frogs and bullfrogs that had undergone metamorphosis during the sampling season from those that had undergone metamorphosis the previous year. Unlike juvenile leopard frogs in which time from metamorphosis and therefore exposure to *A. hydrophila* was

known, juvenile green frogs and bullfrogs may have been exposed to the pathogen for up to one year, thereby reducing the predicted effect between age classes. If this were the case, then the confidence limits on prevalence estimates are expected to be higher for juveniles than for adults in these latter two species. It appears that confidence limits are larger for juveniles than adults in all three species, although this difference is magnified for green frogs and bullfrogs as compared to leopard frogs.

Adult sexes might be expected to differ in *A. hydrophila* prevalence based on exposure. As mentioned, males compete with males for females throughout their respective breeding seasons. During this time, males have an increased risk of becoming physically wounded as a result of aggression with other males. The potential for females to become wounded during the breeding season is less than males as their contact with other frogs (usually males during amplexus) decreases after egg deposition. Wounds suffered during the breeding season were thought to contribute to the outbreak of red-leg disease observed in a population of wild American toads (*Bufo americanus*) (Dusi 1949). Increased wounding potential found in baitshops due to high rearing densities somewhat emulates wounding in male frogs during breeding aggregations. Contrary to predictions, my results indicate that males did not have higher *A. hydrophila* prevalence than females.

As indicated, red-leg diseased leopard frogs were found and *A. hydrophila* was cultured in both instances. This indicates that high prevalence on adult leopard frogs may progress to red-leg disease under natural conditions. Both

cases were discovered in April in Lake Opinicon, presumably after frogs emerged from hibernation. As previously mentioned, the immune system is suppressed during hibernation and the potential for pathogenic infection is high (Cooper *et al.* 1992; Carey 1993; Maniero and Carey 1997). In addition, during a brief survey of shorelines surrounding marshy bays in Lake Opinicon, numerous dead leopard frogs and bullfrogs were found lying on the bottom. Although no attempt to isolate *A. hydrophila* from these carcasses was made, the fact remains that winter mortality exists, and stress is likely associated. I further suggest that since leopard frogs breed not long after emerging from hibernation compared to green frogs and bullfrogs (Wright 1914; Schalk 1997), high prevalence of *A. hydrophila* on males may be due to incomplete immune recovery following hibernation coupled with breeding activity.

Finally, it is important to note that despite the relatively high prevalence in some groups, not all frogs are carriers of the bacteria and, as demonstrated in both the wild and in the survey of baitshops (Appendix 2), most frogs tested were not symptomatic for red-leg disease. However, the potential to become infected with microbial pathogens is not only based on exposure but depends also on the immune status of the individual. Frogs have a capable antibacterial defense system, that includes physical barriers such as mucous membranes, cellular defenses such as defensive cells, **phagocytes** and **natural killer cells**, and molecular defenses such as the **complement system**, **interferons**, and **magainins** (Horton and Ratcliffe 1998), and at least magainins appear to be induced seasonally (Matutte *et al.* 2000). Although these are formidable

defenses, there is much evidence that suggests that organisms in AHP have reduced immune responses that make them more susceptible to disease (Holt 1993; Cunningham 1996). Therefore, in order to understand the risk of transmission to wild hosts, the immune system of baitshop frogs relative to wild control frogs must be assessed.

Chapter 2

Baitshop practices and their effects on the immune profile of captive juvenile leopard frogs (*Rana pipiens*): implications for disease transmission.

Introduction

Past baitshop practices in Ontario housed assemblages of different frog species, sexes, and ages which may affect exposure to a wide diversity of pathogens of varying strains and degrees of virulence (CCAC 1984). A survey of Ontario baitshops during the summer of 2000 revealed that juvenile *R. pipiens* were most commonly encountered; however, mixed-species assemblages still existed (despite restrictions imposed by the Ontario Ministry of Natural Resources). In addition to pathogen exposure, susceptibility to disease depends on the host's ability to resist parasitic organisms. Baitshop practices may compromise this ability by exposing captive frogs to an array of stresses found less frequently or not at all in wild conditions. High housing/rearing density has been shown experimentally to increase the likelihood of infection and disease outbreaks by suppressing the immune response (Oppliger *et al.* 1998). In addition, high density increases contact rates among animals.

Multiple forms of stress such as physical handling (Gobbetti & Zerani 1996), dense housing conditions (Peterson *et al.* 1991), and associated toxicity from waste products, and nutritional deprivation (Beck *et al.* 1994) have been shown to decrease the ability of the host to resist infection (Maule *et al.* 1988; Cooper *et al.* 1992; Gobbetti and Zerani 1996; Maniero and Carey 1997; Oppliger *et al.* 1998; Hopster *et al.* 1998; Braude *et al.* 1999).

As a consequence of such stressful conditions, energy allocated to the immune system by the host may decrease resulting in immunosuppression (Ellsaesser and Clem 1986; Svensson *et al.* 1998). Immunosuppression indicates the inability of the host to produce a full complement of immune cells or a reduction in the total number of immune cells, usually leukocytes (Norris and Evans 2000). This is generally observed as a reduction in total circulating leukocyte density in the blood (Cooper *et al.* 1992) and reduced lymphocyte density in the lymphomyeloid organs (spleen, thymus, jugular bodies, and bone marrow) (Maule *et al.* 1988; Cooper *et al.* 1992; Maniero and Carey 1997; Hopster *et al.* 1998). The consequence of immunosuppression is the increased likelihood of disease among captives, but whether this means increased probability of transmission to wild animals is largely unknown, although certain well documented cases suggest immunosuppression leads to increased transmission.

Reduced leukocyte density in the blood has also been attributed to an immunoredistribution process. In response to certain stresses, leukocytes can migrate from the blood to the skin, lymph nodes and immunological organs

where they are more likely to encounter antigens (Dhabhar 1998). If immune status is only assessed using one type of cell, tissue or organ, results may indicate immunosuppression when in fact, immunoredistribution (or both) is occurring (Braude *et al.* 1999). Unlike immunosuppression, immunoredistribution may not contribute to an increased likelihood of disease, but it does mean that carriers (potential vectors) are present even if asymptotic. In other cases, certain components of the immune system could increase the individual's ability to resist infection, in response to stimuli from disease agents or conditions indicative of transfer or parasitic or disease organisms.

In this chapter, I compare metrics of immune response between captive and wild frogs, asking the question: do frogs from baitshops differ in their immune profile from wild caught frogs, and if so, are baitshop frogs potentially more susceptible to disease (immunosuppressed) or showing increased antigenic challenge as indirectly assessed by immunoredistribution? I deal with several immune metrics to avoid the pitfall of falsely concluding immunosuppression of baitshop frogs, including relative weights of immune organs, total leukocyte estimates and differential leukocyte counts between baitshop and wild frogs.

Study species and selection of immune response indicators

Juvenile leopard frogs were used in this study because of their abundance in both baitshops and the wild. As mentioned, the majority of baitshops surveyed housed juvenile leopard frogs, although frogs of different ages and species were

found within and between baitshops (Appendix 2). Wild juvenile leopard frogs were abundant during the sampling period, allowing for a large sample size in a short period of time. In addition, sampling juvenile frogs should be more appropriate for conservation purposes; collecting breeding adults could impact upon local leopard frog populations more so than collections of juveniles. However, I could not find evidence of this differential impact of collections of adults versus juveniles on frog populations from the published literature. Juvenile male and female *R. pipiens* do not differ in life history traits and therefore I predicted no differences in organ or leukocyte characteristics between genders.

Specific metrics of immune response were quantified. For each frog, the spleen was harvested (see methods) as it is one of the primary sites of interaction between leukocytes and antigens in the circulatory system (Horton and Ratcliffe 1998). Relatively larger spleens in baitshop frogs should indicate the presence of a recent or current immune response whereas smaller spleens may indicate immunosuppression. Although the liver and kidney also have some immunological properties in amphibians (Horton and Ratcliffe 1998), they are not a site of leukocyte/antigen interaction and are therefore considered unaffected by the immunoredistribution response. Leukocytes were also assessed. Amphibian leukocytes are similar to those of higher vertebrates, consisting of eosinophils, basophils, monocytes, heterophils and lymphocytes. Heterophils are synonymous to neutrophils in mammals and serve as the first line of defense against internalized microbes (Black 1996; Horton and Ratcliffe 1998). For

specific functions of amphibian immunological organs and leukocyte types, refer to Tables 2.1 and 2.2.

Specific objectives

As mentioned, I addressed the question of whether juvenile leopard frogs used in baitshops were potentially more susceptible to disease based on a profile of their immune system compared to wild caught frogs. Frogs more susceptible to disease in baitshops could indicate a greater potential to vector pathogens if released. Alternatively, otherwise "healthy frogs" that are antigenically challenged in baitshops may pick up several parasitic organisms that have direct life cycles (such as *A. hydrophila*) and/or may act as vectors for novel pathogens upon release or following their use in fishing. I tested two contrasting predictions. The first was that baitshop frogs may be immunosuppressed compared to wild caught control frogs. For this prediction to be met, spleen weights and total leukocyte density should both be lower in baitshop frogs compared to wild caught frogs.

Secondly, I predicted that baitshop frogs could be antigenically challenged in response to rearing conditions. In this scenario, frogs should redistribute the densities and proportions of leukocyte elements within the blood so that cells most important to general immune responses (heterophils and monocytes) exist in greater numbers and cells involved in specific host immune defences (lymphocytes) exist in lower numbers. In addition, leukocytes responding to challenges should be reallocated to sites of antigen interaction. Therefore the spleen is predicted to be enlarged in baitshop frogs compared to control frogs, if

immunoredistribution is occurring. It is important to recognize that immunoredistribution may precede full-blown immunosuppression and that residency times in baitshops should affect which response is observed.

Methods

Baitshop Frogs

Eleven baitshops were chosen based on their close proximity to the Queens University Biological Station (QUBS) where the control frogs were captured. Collection dates ranged between 30 July and 16 August, 2000. Upon arriving at each shop, I conducted a survey investigating the health status of captive frogs also noting the species and age classes housed in each shop (Appendix 2).

In each baitshop, six frogs were selected by the baitshop owners, placed in a plastic bait bucket and then brought immediately back to QUBS. The maximum time any frog spent in transit was 2 hours and this travel emulated the movement of baitshop frogs from the site of purchase to the fishing location.

Control Frogs

Control frogs were collected in 2 fields that were situated next to ponds approximately 10 kilometers west of QUBS. Frogs were located by walking throughout the fields looking for the juvenile leopard frogs in the short grass (field had been hayed 1 week prior to first collection date). As indicated, only juvenile frogs were collected and compared to juvenile baitshop frogs. Collections were timed with baitshop visits to control for possible seasonal effects (two collection

dates: July 22 and August 13, 2000). Each frog was caught by researchers wearing new latex gloves so as not to contaminate the frog with foreign bacteria. Next, a blood smear was made for later leukocyte analysis. In this procedure, 0.03 mL of blood was aseptically extracted via cardiac puncture, and spread onto a new microscope slide using another new slide (Maniero & Carey 1997). The blood was then air dried and placed into a slide storage container. After this procedure, frogs were placed in separate containers, set in an ice bath, and transported back to QUBS.

Laboratory protocols

The same lab procedures were conducted on wild frogs as baitshop frogs. Upon reaching QUBS, blood smears were prepared from baitshop frogs and sent with blood slides from control frogs to Vita-tech (Veterinary Laboratory Services) for differential leukocyte analysis and leukocyte estimates (see amphibian blood protocols below). Frogs were then placed separately in plastic containers and set in an ice bath for approximately 1 hour (or until their body temperature was lowered to 4°C). Frogs were then removed from the ice bath, measured (for SVL) and double pithed. Two SVL measurements were recorded on a 12cm X 20cm piece of cardboard and the two measures were later averaged to reduce measurement error. All frogs were dissected to allow for sex determination and for organ harvest (kidney, liver, and spleen). Wet weights were taken for each organ and measured with a Mettler AE 100 electronic balance scale (accurate to 0.1 mg).

Estimation of Leukocyte Numbers and Differential Leukocyte Counts from Peripheral Blood Smears (performed by Vita-tech)

Blood smears were first viewed on low power using a Nikon microscope to assess the quality of the preparation. For low quality smears, a visual sub-selection of areas was performed after viewing the slide at 40X to ensure that all areas were proportionately represented. For example, if 25 % of the smear was thick and 75 % was thin, 5 fields would be viewed in the thick area and 15 fields would be viewed in the thin area for a total of 20 fields on each slide. Fields from high quality slides and from within sub-selections of low quality slides were always randomly selected. Leukocytes in each of the 20 fields were counted and averaged then multiplied by a correction factor ($1.0 \times 10^9/L$) that was previously determined using known blood cell densities. This gives a reasonable estimate of leukocyte numbers per litre (Dr. Tanya Stirtzinger, pers. comm.).

Cells that were subject to drying artifact making the cytoplasmic and nuclear details unrecognisable were not included in the differential leukocyte count. In addition, to reduce the impact of these cells, fields of view were considered only if all leukocytes could be identified. For standard differential counts, 100 leukocytes were evaluated per smear and classified into the various sub-classes. Where individuals were leukopenic, fewer than 100 cells per smear were counted as many were not adequately preserved to allow for sub-classification. Although counting fewer than 100 cells does reduce the accuracy of the differential count somewhat, a comparison between leukopenic and non-leukopenic smears revealed that the proportions of sub-classified cells in relation

to the total leukocyte count on each did not differ. The percentage of each leukocyte type was then multiplied by the total leukocyte count to give the absolute differential count. In diagnostic hematology only the absolute count is accepted as being truly representative of an individual's condition (Dr. Tanya Stirtzinger, pers. comm.).

Statistical analysis

Baitshops were initially compared using a one-way ANOVA to control for differences in snout-to-vent length between shops. Shops with significantly higher snout-to-vent lengths were excluded from the following analyses. As a first analysis, baitshop and wild-caught leopard frogs were compared with respect to their immunological profile (size of immunological versus control organs and comparison of leukocyte densities and proportions of particular cell types). All analyses were conducted using SAS software (SAS Institute Inc. 2000). Organ weights were compared using a multiple analysis of covariance (multiple ANCOVA), testing the linear relationship between organ mass and corrected body weight (body weight – organ weight) for different levels of site location and gender. Separate multiple ANCOVA's were used for the spleen, liver and kidney.

A two-way analysis of variance (ANOVA) was used to test for immunosuppression of baitshop frogs relative to wild frogs while examining any effects due to gender. The two-way ANOVA was performed using the natural logarithm transformed total leukocyte density to test for overall immunosuppression between treatments. This was followed by separate two-way

ANOVA using the density of each of the five leukocyte components (heterophils, lymphocytes, monocytes, eosinophils, and basophils, all transformed using natural logarithms) as dependant variables in order to test for immunoredistribution between baitshop and control frogs. In addition to density, the proportions of leukocyte elements were used to test immunoredistribution by performing a two-way ANOVA, using the proportion of each of the five leukocyte components to total leukocytes (normalized data) as the dependent variable and baitshop versus control, and gender as independent variables.

Results

Certain data were excluded from the main comparisons for a variety of reasons. From the organ weight data set, 12 frogs were dropped due to high SVL (a correlate of age). All frogs sampled from baitshop number one were breeding adults and had significantly larger mean SVL than the other baitshops ($F_{10,65} = 8.78$, $P < 0.001$, Fig. 2.1). They were therefore inappropriate to compare to juvenile frogs. Likewise, baitshop number seven was dropped from the analysis because the juvenile frogs had significantly larger snout-to-vent lengths than the rest of the baitshops ($F_{9,50} = 4.53$, $P = 0.0002$). There was no difference in SVL in the remaining nine baitshops ($F_{8,53} = 0.90$, $P = 0.52$). In addition, three frogs were rejected due to the presence of microfilarial and protozoan parasites, and five frogs were rejected due to extremely poor quality blood smears which did not yield reliable data.

To compare organ weights between the baitshop and wild caught frogs, analyses of co-variance (ANCOVA) were performed in order to detect whether the relationship between organ weight and corrected body weight (body weight – organ weight) was affected by locations (baitshop versus wild) or gender. For the spleen analysis, baitshop frogs had significantly heavier spleens with regard to corrected body weight than wild frogs ($F = 12.88$, d.f. = 1, $P = 0.0005$; Fig. 2.2) but no difference existed between sexes ($F = 0.015$, d.f. = 1, $P = 0.9$). Comparing the kidney weights (with regard to corrected body weight), no significant difference was found between baitshop and control frogs ($F = 2.19$, d.f. = 1, $P = 0.14$; Fig. 2.3) or between males and females ($F = 0.008$, d.f. = 1, $P = 0.93$). Finally, liver weights were found to be significantly larger (with regard to corrected body weight) in wild frogs as compared to baitshop frogs ($F = 13.53$, d.f. = 1, $P = 0.0004$; Fig. 2.4) and no difference existed between sexes ($F = 0.01$, d.f. = 1, $P = 0.92$).

The hypothesis that baitshop frogs should be immunosuppressed compared to wild caught frogs was also investigated using the total circulating leukocyte density. The two-way ANOVA revealed no significant difference between the two locations ($F_{1,73} = 0.66$, $P = 0.42$; Fig. 2.5). No differences in gender were expected or observed ($F_{1,73} = 0.08$, $P = 0.78$).

I next tested for differences in immunoredistribution using the five leukocyte elements, comparing both the density and proportion (number of each cell type to the total number of circulating leukocytes) between baitshop and control frogs. No difference was observed between the densities of heterophils

between baitshop and control frogs ($F_{1,73} = 0.77$, $P = 0.38$; Fig. 2.6) but heterophils represented a significantly larger proportion of the leukocyte composition in baitshop compared to wild frogs ($F_{1,73} = 4.63$, $P = 0.03$; Fig. 2.7). Comparison of lymphocytes showed that both density ($F_{1,73} = 7.43$, $P = 0.008$; Fig. 2.6) and proportion ($F_{1,73} = 11.42$, $P = 0.001$; Fig. 2.7) were significantly lower in baitshop frogs compared to wild frogs. I also found significantly higher monocyte densities ($F_{1,73} = 3.98$, $P = 0.05$; Fig. 2.6) and monocyte proportions ($F_{1,73} = 4.61$, $P = 0.04$; Fig. 2.7) in baitshop frogs compared to wild frogs. Baitshop and control frogs did not differ in either eosinophils or basophils based on cell density ($F_{1,73} = 0.73$, $P = 0.39$, Fig. 2.6; $F_{1,73} = 0.55$, $P = 0.46$, Fig. 2.7), or cell proportions ($F_{1,73} = 0.39$, $P = 0.53$, Fig. 2.6; $F_{1,73} = 0.16$, $P = 0.69$, Fig. 2.7).

Thus baitshop and control frogs did not differ in 3 of 5 blood cell elements and 1 of 3 organs. In the cases where they did differ baitshop had heavier spleens, lighter livers, lower lymphocyte density, higher monocyte density, higher proportions of heterophils and monocytes, and lower proportions of lymphocytes.

Table 2.1. Summary of *Rana* immune tissue and their functions¹.

Organ	Organ status	Immunological Function	Notes
Thymus ²	Primary	<ul style="list-style-type: none"> -Production of lymphocytes with T-cell function -Produces dendritic cells, macrophages, cysts, granular cells, myeloid cells -houses B cells 	-This organ is alternately known as the jugular bodies
Spleen ³	Secondary	<ul style="list-style-type: none"> -traps antigen, houses proliferating lymphocytes after their stimulation by antigen, provides the release of these cells and their products. -B cell development 	
Lymphomyeloid nodes ²	Secondary	<ul style="list-style-type: none"> -mainly blood filtering organs -antibody producing cells 	<ul style="list-style-type: none"> -Superficial functional resemblance to mammalian lymph nodes -In the small intestine
Gut-associated lymphoid tissue ²	Secondary	<ul style="list-style-type: none"> -IgM and IgX – secreting cells -houses intra-epithelial T cells 	
Kidney and Liver ³	Secondary	<ul style="list-style-type: none"> -B cell development -early differentiation of erythroid, lymphoid, and myeloid cells 	-Immunological function of these organs wanes in reptiles, birds and mammals
Bone marrow ²	Primary	-antibody production	-Lymphomyeloid tissue

¹ after Roitt, Brostoff, Male (1998)

² not assessed in this study

³ assessed in this study

Table 2.1 cont'd

B-cell - Lymphocytes that give rise to antibody-producing plasma cells.

Dendritic cells - A set of cells present in tissue, which capture antigens and migrate to lymph nodes and spleen, where they are particularly active in presenting the processed cells to T-cells.

Granulocyte - a leukocyte (basophils, mast cell, eosinophils, or neutrophils (known also as heterophils in amphibians and birds)) with granular cytoplasm and irregularly shaped, lobed nuclei.

IgM - The first class of antibody secreted into the blood during the early stages of a primary immune response or found on the surface of B-cells.

IgX- The possible precursor of IgA; an antigen found in blood and secretions in higher vertebrates.

Lymphomyeloid system - produces and stores lymphocytes, granulocytes, and other blood cells and allows appropriate immunological/antigen interaction.

Myeloid cells - The lineage of bone-marrow-derived phagocytes, including neutrophils, eosinophils, and monocytes.

Primary lymphoid organ - Tissues that are major sites of lymphocyte development.

Secondary lymphoid organ - Tissues that provide an environment in which lymphocytes can interact with antigens.

T-cell - Lymphocytes that are central to the control and development of immune response.

Table 2.2. Summary of amphibian leukocyte form and function¹

Leukocytes	Element	Function
Granulocytes	Basophil²	Release histamine and other chemicals during inflammation.
	Eosinophil²	Release defensive chemicals to damage parasites; phagocytic
	Heterophil²	Phagocytic; contain oxidative chemicals to kill internalized microbes
Agranulocytes	Monocyte²	In tissue and blood, develop into macrophages, which are phagocytic
	B Lymphocyte³	Specific host immune defences. Produced and matured in bursal-equivalent tissue, gives rise to antibody-producing plasma cells.
	T Lymphocyte³	Specific host immune defences. Thymus-derived cell used in cellular response
	Natural killer cell⁴	Destroys virus-infected cells, malignant tumors, and cells of transplanted tissue

¹ after Roitt *et al.* (1998)² assessed separately during this study³ combined and assessed during this study⁴ not assessed during this study

Figure 2.1. Mean snout-to-vent lengths (SVL) and 95% confidence intervals for *Rana pipiens* found in eleven Ontario baitshops. Baitshops with letter designations above confidence interval have frogs with significantly larger SVL than the remaining baitshops.

Figure 2.2. Mean ($\pm$ one standard error) spleen mass of juvenile *Rana pipiens* as found in baitshop and wild control frogs.

Spleen

$P < 0.05$

Figure 2.3. Mean ($\pm$ one standard error) kidney mass of juvenile *Rana pipiens* as found in baitshop and wild control frogs.

Kidney

Figure 2.4. Mean ($\pm$ one standard error) liver mass of juvenile *Rana pipiens* as found in baitshop and wild control frogs.

Liver

Figure 2.5. Mean ($\pm$ one standard error) density of leukocytes counted in juvenile *Rana pipiens* as found in baitshop and wild treatments.

Figure 2.6. Mean ($\pm$ one standard error) density of leukocyte elements counted in juvenile *Rana pipiens* as found in baitshop and wild control frogs. Baitshop frogs have lower lymphocyte density and higher monocyte density than wild control frogs.

Figure 2.7. Mean ($\pm$ one standard error) proportion of leukocyte elements counted in juvenile *Rana pipiens* as found in baitshop and wild control frogs. Baitshop frogs have higher proportions of heterophils and monocytes than wild control frogs. Wild control frogs have a higher proportion lymphocytes than baitshop frogs.

Discussion

It appears that frogs housed in baitshop practices are not immunosuppressed compared to wild caught frogs. No significant change in total leukocyte density was found between frogs housed in baitshops versus those collected in the wild suggesting that baitshop frogs are capable of mounting immune responses. Current housing practices in Ontario baitshops may not stress frogs beyond what they can tolerate.

Differences in leukocyte density or proportions between baitshop and control frogs have to be evaluated relative to organ weights. The spleen acts immunologically as the primary site of interaction between the leukocytes and invading pathogens (Horton and Ratcliffe 1998). If immunosuppression occurred, I should expect spleens from baitshop frogs to be relatively smaller than spleens from wild caught frogs. Instead, my results indicate the opposite: baitshop frogs had larger spleens than wild frogs. Amphibian livers and kidneys do have some immunological abilities; however their role in immune responses is secondary compared to other physiological functions (Horton and Ratcliffe 1998). Therefore little weight change was predicted and these organs were designated as controls for the immunologically active spleen. No weight change was observed in the kidney but liver weights were significantly larger in wild frogs. Perhaps baitshop practices suppress immunological functions of the liver however, this result may be misleading since the liver has many other functions that may be affected by baitshop captivity and associated stresses. For example, one of its primary roles

is to store and process fats and proteins (Martini 1998). Since frogs are not fed in baitshops and can be housed for long periods of time (Appendix 2), the natural metabolism of the liver may account for its loss in weight. Thus, the liver is a good choice for an organ indicating nutritional status. More importantly, another explanation is required for the relationship between circulating leukocytes and the gross measure of immune response found in the spleen.

In response to stress, elevated blood levels of **corticosteroids** or oestradiol-17 β in amphibians (Gobbetti and Zerani 1996), are known to affect the vertebrate immune system by shunting leukocytes out of peripheral blood circulation and into areas such as the lymph nodes, skin and immunological organs where they are most likely to interact with antigens (Dhabhar and McEwen 1996; Dhabhar 1998). I suggest that this process, coined immunoredistribution, explains why baitshop frogs have enlarged spleens compared to wild caught frogs. Baitshop frogs are likely exposed to higher concentrations and diversity of pathogens (especially if housed in mixed-species and/or mixed-age assemblages) and physical injury (resulting from dense housing conditions) requiring that the immune system be especially responsive. An enlarged spleen could be the result of either a heightened immune response, a current infection, or a combination of both factors. Reallocating more leukocytes to the spleen must be accomplished while maintaining the density of circulating leukocytes in the blood since no immunosuppression was observed between baitshop and wild control frogs. This may be accomplished by increasing the production rate or decreasing the

degradation rate of leukocytes, however, this rate was not measured and so cannot be confirmed.

Additionally, the significantly larger proportion of heterophils, higher proportion and density of monocytes, and the lower proportion and density of lymphocytes found in the blood of baitshop frogs compared to wild frogs can be explained by immunoredistribution. Under certain conditions, the immune system could alter both the types of leukocytes produced at high rates and the locations to which they migrate (Dhabhar and McEwen 1996; Dhabhar 1998). Monocytes, which are capable of producing phagocytic cells responsible for internalizing microbes (Black 1996), should exist in higher proportions and densities under conditions of heightened antigenic challenge. Since lymphocytes are less active and less important in general immune responses, it stands to reason that their proportions and densities should lessen if a fixed allocation to immunity is maintained. Heterophils are the first response of the immune system to invading microbes such as *A. hydrophila* (Horton and Ratcliffe 1998) and should comprise a greater proportion of the total leukocytes when exposed to stressful conditions.

Thus, it appears that immunoredistribution is occurring in baitshop frogs. However, caution should be exercised when regarding these results which likely depend on the length of time frogs spend in captivity. Immunoredistribution is thought to be a temporary response to stress and the immune system is known to revert back to its original state following the stressful event (Braude *et al.* 1999). If nutritional stress becomes chronic and the demands to maintain the process of immunoredistribution or the entire immune system are not met,

immunosuppression may then occur. The addition of other baitshop practices such as mixed-species housing could then further increase the risk of disease. In this study, baitshop frogs were housed for up to 165 hours with no food (Appendix 2) and showed no signs of immunosuppression. These results can not be extrapolated to frogs housed in baitshops for longer periods of time.

In this chapter, I set out to discover whether juvenile leopard frogs used in Ontario baitshops are potentially more susceptible to disease or become potential vectors based on a profile of their immune system compared to wild caught frogs. Ethically, I could not infect frogs and determine their immunocompetence defined as “the ability of a host to prevent or control infection by pathogens and parasites” (Norris and Evans 2000). Results from this study indicate that baitshop frogs are not wholly immunosuppressed compared to wild frogs. In response to baitshop associated stresses, it appears that frogs redistribute elements of their leukocytes (in some cases density and/or proportion) thereby increasing their ability to resist general antigenic challenges by pathogenic microbes such as *A. hydrophila*. This suggests that based on immune metrics, current Ontario baitshop practices may not greatly increase the risk of disease, however outbreaks in baitshops are still seen, although possibly less frequent than elsewhere or following changes in guidelines.

Summary and Conclusions

Overall, it appears that Ontario baitshops have a lower than predicted potential of transmitting *Aeromonas hydrophila*/red-leg disease from captive frogs to susceptible wild hosts but cannot be regarded yet as completely harmless practices. Furthermore, this is not to say that baitshop practices have no other effects on captive or wild frogs. Conditions associated with baitshops such as malnutrition and physical injury may kill frogs while in captivity or after release, the translocation of frogs from one area to another may have unforeseen consequences other than parasite and pathogen transmission, and harvesting wild frogs may affect local species abundance which is already a matter of global concern.

As reported in Chapter 1, the prevalence of *A. hydrophila* appears to be heterogeneous among species, sexes, and age classes. This implies that the choice of frogs used in baitshops is important in terms of reducing both the risk of infection and the transmission of *A. hydrophila* within and from baitshops. Adult leopard frogs have the highest prevalence of *A. hydrophila* and therefore are the most risky to house in captivity with regard to red-leg outbreak. The current risk of infection should be less than past practices and less than unregulated baitshops outside Ontario since mixed-assemblages are less common. However, despite current conditions, red-leg symptoms and die-offs within baitshops still occur, indicating that disease may be transferred from baitshop frogs into the wild. This will depend most on whether pathogen strain or virulence differs between baitshop and control frogs: a point not assessed in this study.

My research has also determined that natural history (with regard to habitat preference) relating to exposure is not predictive of *A. hydrophila* prevalence. Other factors that may explain observed prevalence differences among groups include: the persistence of the infection through frog metamorphosis, the inability of the researcher to distinguish the exact age of juvenile green frogs and bullfrogs, the emergence time from hibernation for each species compared to their breeding period, and the possibility that those frogs most exposed to the pathogen are also most resistant.

Infection status is also important in determining the susceptibility of the host. Stresses associated with baitshop practices may influence the ability of the captive frog to resist parasites and pathogens such as *A. hydrophila*. In Chapter 2, I tested two competing hypotheses of immune response during baitshop captivity (immunosuppression and immunoredistribution) by measuring immune metrics (circulating leukocytes and immunological organs) and comparing them to the same metrics in wild control frogs. Baitshop and wild frogs were found to differ in their immune profiles but this was indicative of differences in immunoredistribution. Immunoredistribution may indicate that baitshop frogs are antigenically challenged and may otherwise be healthy individuals with previous or current infections. Contrary to prediction, baitshop frogs were not immunosuppressed compared to wild control frogs however, immunoredistribution may be a precursor to immunosuppression if residency times in baitshops were longer. Small liver weights in baitshop frogs suggest nutritional deprivation, a state that will eventually lead to immunosuppression.

Additionally, more research should be conducted on the various strains of *A. hydrophila*. Some strains may be locally adapted to specific host species. One could assess this by experiments cross infecting hosts with various *A. hydrophila* strains to determine their influence on infection status and disease transmission. Different locally adapted strains could prove problematic if released elsewhere. This research applies not only to red-leg disease but also to other pathogens such as *Rana* iridioviruses and chytrid fungi that have also been implicated in frog die-offs in captivity and the wild.

One recommendation of this thesis is that residency times for frogs in baitshops should be as short as possible to decrease the risk of immunosuppression. Although it is not known whether releasing moribund immunosuppressed frogs is better or worse than releasing antigenically challenged frogs that have redistributed their immune elements accordingly, reducing the residency time will at least decrease exposure to pathogens. Further, more emphasis should be placed on reducing release of frogs that are purchased but not used in fishing although its not clear how this could be done. More work should be conducted regarding parasite and pathogen transmission from unregulated baitshops to surrounding areas and the costs of baitshops other than risk of disease transmission relative to the benefits to the baitshop owner and society in general.

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Appendix 1. Glossary of operational definitions used in this thesis.

animal husbandry – Broadly defined as the maintenance of domestic or wild animals as captives for the purpose of their future sale or sale of their products for consumption (e.g. cattle) or for recreational activities (game farms) and not excluding maintenance of rare, threatened, or endangered animals for future re-introductions or augmentation programs.

complement system - A group of serum proteins involved in the control of inflammation, the activation of phagocytes, and the lytic attack on cell membranes. The system can be activated by interaction with the antibodies of the immune system (Black 1996).

corticosteroid – A steroid hormone known to influence immune response.

differential leukocyte count – The proportion of each leukocyte element (heterophils, monocytes, basophils, lymphocytes, eosinophils) found in haematological samples.

endotoxin – Liposaccharide produced by Gram-negative bacteria, which activates B cells and macrophages.

etiological agent - Any living or nonliving thing, power, or substance capable of causing a disease.

facultative psychrophile – An organism that grows best at temperatures below 20°C but can grow at temperatures above 20°C.

hemolytic toxin – A poisonous substance that lyses red blood cells.

immune profile – An inventory of magnitude of investment in different components involved in immune response.

immunocompetence - The ability of a host to prevent or control infection by pathogens and/or parasites.

immunoredistribution – The re-allotment of immune cells and their elements, independent of each other, in response to stress.

immunosuppression – The reduction in density of circulating immune cells and in size and functioning of immune organs, usually in response to intrinsic or extrinsic stresses. Reduction in one or more components of the immune system followed by increased allocation to another is better described as **immunoredistribution**.

interferon – A group of molecules involved in signaling between cells of the immune system, and in protection against viral infection.

species introduction – The accidental or intentional introduction of animals to previously unoccupied areas.

leukocyte estimate – The density of total leukocytes and leukocyte elements (heterophils, monocytes, basophils, lymphocytes, eosinophils) found in the circulatory system.

leukopenic – Decreased complement of circulating leukocytes.

magainin – A family of anti-microbial peptides that are secreted from glands in the skin and gut of amphibians.

metamorph – A juvenile frog that has recently undergone metamorphosis.

microfilial parasite – An immature microscopic roundworm larva.

natural killer cells – A group of lymphocytes which have the intrinsic ability to recognize and destroy some virally infected cells and some tumour cells.

phagocyte – A cell that ingests and digests foreign particles.

prevalence – The proportion of sampled hosts of a particular species, age, or gender infected by a particular parasite.

protozoan parasite – Single celled, microscopic, animal-like protists in the kingdom Protista.

re-introduction – The release of wild-caught or captive-bred animals into areas where the species has declined or disappeared.

translocation – The transfer of animals from one part of its historic geographical range to another.

virulence – The degree of intensity of the disease produced by a pathogen usually assessed as mortality rates of infected individuals (Ewald 1994) .

Appendix 2: Health status survey for baitshop captive frogs. All baitshops were located in Lanark and Frontenac counties Ontario.

Baitshop	Species	Age	Rearing Conditions ¹	Rearing Density ² (m ²)	Number of Dead Frogs	Diseased frogs
1	R. pipiens	Juvenile and Adult	D	13.3	0	0
2	R. pipiens	Juvenile	B	1061	0	0
3	R. pipiens	Juvenile	A	88.9	0	0
4	R. pipiens	Juvenile	D	75.0	0	0
5	R. pipiens	Juvenile	B ²	2222.2	0	0
6	R. pipiens	Juvenile	B	763.9	20	2
7	R. pipiens	Juvenile	C	358.3	0	0
8	R. pipiens	Juvenile	D	150.0	0	0
9	R. pipiens	Juvenile and Adult	C	191.0	50	0
10	R. pipiens	Juvenile	D	416.7	0	0
11	R. pipiens and R. clamitans	Juvenile	D	333.3	0	0

Appendix 2 (cont'd)

Baitshop	Time in Captivity (hrs) ⁴	Percent water cover ³	Water Depth (cm)	Source of frogs ⁵
1	24	20	3	Local
2	165	100	2	Local
3	48	100	4.5	Commercial
4	72	10	2	Local
5	120	100	6	Commercial
6	72	100	12	Commercial
7	24	20	2	Commercial
8	120	10	3	Local
9	165	25	4	Local
10	72	15	2	Local
11	20	15	2	Local

Appendix 2 (cont'd.)

¹ A = Unfiltered Flow-through tank

B = Still water tank

C = Dry steel tank

D = Wire cage

² Frogs kept in refrigerator at 3°C

³ Standardized density measurements of the number of frogs per meter² based on frog rearing densities observed in Ontario baitshops.

⁴ Time in captivity was determined from baitshop owner

⁵ Any data < 100% indicates that water was contained in a vessel, usually a low plastic container. 100% indicates frogs were kept in water at variable depths.

⁶ Local refers to within the same or surrounding communities as the bait shop. Commercial frogs are potentially gathered from areas distant from where they are being sold.

Appendix 3. Examples of other species that tested positive for *Aeromonas hydrophila* surrounding the Queen's University Biological Station.

Species	Comments
Black Rat Snake (<i>Elaphe obsoleta</i>)	<p>A mature black rat snake was captured at the Queens University Biological Station on April 30, 2000 with symptoms of mouth-rot; a bacterial infection in the oral region caused by <i>Aeromonas hydrophila</i>.</p> <p>Ulcers on the periphery of the mouth were swabbed according to the protocols outlined in Chapter 1 for the isolation of <i>A. hydrophila</i> from amphibians. The sample showed the presence of the <i>A. hydrophila</i> colonies on Ryan's selective media. Although this does not indicate the prevalence of the bacteria in the black rat snake population nor the frequency of which <i>A. hydrophila</i> infection results in disease, it does show that black rat snakes (and potentially other snake species) are susceptible to mouth-rot. <i>Aeromonas hydrophila</i> may also directly infect snakes (and other susceptible hosts such as frogs) from its free life stage in the water column.</p>

Appendix 3 cont'd

Leech

Leeches may use hemolytic *A. hydrophila* to digest blood meal from their prey, which often

(*Macrobrdella decora*) includes amphibians. Seventeen actively feeding leeches were separated on May 5, 2000 from breeding American toads (*Bufo americanus*). Leeches were individually packed in plastic bags and taken back to the laboratory at QUBS where their crops were dissected. Using the same protocols outlined in Chapter 1 to isolate *A. hydrophila*, I swabbed each crop and plated the blood on Ryan's selective media. Using this method, I determined that the prevalence of *A. hydrophila* in the sampled leeches was: $P(0.28 \leq 0.53 \leq 0.75)$. Confidence limits were calculated by hand for population proportions according to Zar (1996) as previously demonstrated in Chapter 1. It was also noted that 4 of 17 leeches had regurgitated in the bag during transfer. Although not tested, it is also probable that leeches may regurgitate while feeding thus transmitting *A. hydrophila* from their crop into their prey. This increases both the exposure of frogs to the pathogen and the potential for infection. Leech/amphibian interaction may be especially high during the amphibian breeding season due to the high density of breeding adult frogs that will increase the rate of transmission.