Detection of the parasite *Ribeiroia ondatrae* in water bodies and possible impacts of malformations in a frog host.

by

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Abstract

This study devised a method to detect *Ribeiroia ondatrae* (class Trematoda) in water-bodies using environmental DNA (eDNA) collected from filtered water samples from selected ponds in the USA and Canada. Species-specific PCR primers were designed to target the *Internal Transcribed Spacer-2* (*ITS*-2) region of the parasite's genome. The qualitative PCR method was 70% (n=10) accurate in detecting *R. ondatrae* in ponds previously found to contain the parasite, while the qPCR method was 88.9% (n=9). To examine how the retinoic acid (RA) pathway gene expression may be perturbed during *R. ondatrae* infections, leading to limb development abnormalities in the wood frog (*Lithobates sylvaticus*). Multiple sequence alignments were used to design degenerate PCR primers to eight RA biosynthesis genes, but only two gene fragments were identified using this approach. Without effective primer sets it was not possible to measure changes in gene expression in infected frogs.

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1 GENERAL INTRODUCTION

1.0 THE EMERGENCE OF FROG MALFORMATIONS

Malformed frogs have been documented occasionally in the literature for centuries (Sessions and Ruth, 1990). Most amphibian populations show a normal deformity rate of approximately 5% (Roberts and Dickinson, 2012). However, a noticeable increase in the distribution, frequency, and severity of amphibian malformations in North America has occurred since 1994 (Johnson *et al.*, 2003). Sessions and Ruth (1990) first observed malformed frogs in 1986 during field studies in Aptos, California (Reinitz, 2007; Stopper *et al.*, 2002). In Minnesota, for example, there was a six-fold increase in frog deformities between 1958 and 1998 (Blaustein and Johnson, 2003). To date, 60 amphibian species including anurans and urodeles have been observed with deformities in parts of Canada, 46 US states, Europe and Japan, with the mid-western United States being the most affected (Blaustein and Johnson, 2003). Malformed frogs collected in field surveys show varied limb deformities such as: extra complete limbs; missing limbs; missing digits; extra digits; mirror image duplicates; gross distortions; ectopic structures; skin fusions; truncated limbs; duplicated pelvic girdles; dorsal-ventral duplications; reversed dorsal-ventral axis; missing elements; multiple feet; bifurcations; and trifurcations (Schotthoefer *et al.*, 2003; Sessions and Ruth, 1990). A variety of possible causes for the sudden increase in limb deformities have been suggested, including increased predation, changes in ultraviolet radiation, exposure to chemicals such as retinoic acid, infection by parasites, and mechanical disruption (Blaustein and Johnson, 2003). Each of these will be considered briefly below.

1.0.1 PREDATION AND ULTRA VIOLET RADIATION (UV)

Missing and truncated limbs can in part be explained by non-lethal predatory attacks on the tadpole by fish, invertebrates and other amphibians. Recent studies have shown that dragonfly nymphs selectively target limb buds of developing tadpoles and rarely kill the tadpole (Bowerman *et al.,* 2010; Ballengee and Sessions, 2009). These frogs develop into adults with either missing or partially missing limbs but rarely, if ever, develop extra limbs. Very little scar tissue or signs of predation appear on the amputated limbs due to the regenerative strength of the developing tadpole (Bellengée and Sessions, 2009; Bowerman *et al.,* 2010). Therefore, missing limbs and limb elements in frogs in the absence of parasites, such as most of those seen in Vermont (Skelly *et al.,* 2007), could be considered the result of predation (Ballengée and Sessions, 2009). Field surveys in 41 Vermont wetland sites were conducted on *Rana pipiens* (Leopard frog) and *Rana clamitans* (Green frog) between 2002 and 2003. These sites showed average population deformities of 3.4%, with the most severe population containing 20% deformed animals. No parasite infections, such as *Ribeiroia ondatrae* infections were found in any of these cases (to be further discussed in section 1.0.3) (Skelly *et al.,* 2007).

In laboratory studies, UV radiation has been shown to produce extra limbs, skin webbing, and twisted limbs, but these malformations do not resemble what is commonly seen in the wild (Blaustein and Johnson, 2003). UV radiation is unlikely to explain high frequencies of deformities in wild populations because most frogs will exhibit behavioral responses to find shelter from the UV radiation before this level of damage is acquired (Blaustein and Johnson, 2003).

1.0.2 CHEMICALS: RETINOIDS

Of all the chemicals examined thus far, disruption of retinoid levels in developing frogs is considered one of the most likely causes of the deformities seen in wild frog populations. Retinoic acid (RA) is an important chemical signal in chordates that mediates anteroposterior spatial patterning during time-sensitive stages of development and has been implicated in proximal limb, eye, hind brain, and heart development (Marletaz *et al.*, 2006). RA is derived from Vitamin A (retinol) (Marletaz et al., 2006) in two biosynthetic steps. First, retinol is enzymatically oxidized into retinal by alcohol dehydrogenases. Retinal is then oxidized into retinoic acid via retinaldehyde dehydrogenases (*Raldh1-3* enzymes, also known as ALDH1A1-3 enzymes) (McEwan et al., 2011; Marletaz et al., 2006).

The combined interaction of Raldh enzymes, the ensuing RA accumulation, and the induction of genes relevant to limb formation are required to produce normal limbs in the developing frog. Not surprisingly, excess retinoids in frogs can then create perturbations in limb development, and experimentally altered retinoid concentrations in frogs have been shown to cause extra complete limbs, bony triangles (BT), missing digits, fused digits, truncated limbs, bifurcations, proximodistal (PD) serial duplications, duplicated pelvic girdles, and missing limbs (Alsop *et al.*, 2004; Gardiner *et al.*, 2003; Maden and Corcoran, 1996). Maden and Corcoran (1996) showed that tadpoles exposed to excess retinoids in conjunction with an amputated tail during the time of limb development would produce limbs growing out of the wound site. Measurements of gene expression in the normal tails and mutant “limb –tails” revealed that RARs and RXRs were up-regulated in the limb-sprouting tails.

A likely source of environmental retinoids are cyanobacteria blooms, which have been shown to greatly increase aquatic retinoic acid (RA) and 4-oxo RA in affected water systems (Wu *et al*, 2012). Cyanobacteria, such as *Microcystis flos-aquae* and *Microcystis aeruginosa*, produce about 1.4 x 10^3 and 3.7 x 10^2 ng/g dry weight of RA and 4-oxo RA respectively. Depending on the magnitude of the algal bloom, it could be possible for environmental levels of RA to reach concentrations of $8x10^{-9}$M to $8x10^{-7}$M. Similar concentrations of TTNPB, a synthetic RAR-specific activator that has a higher affinity for the receptors than all trans RA, were found to be necessary to induce limb deformities in developing frogs without killing the animal during neural tube and heart development (Maden and Hind, 2003). While many of these same deformities appear in wild-caught amphibians, naturally occurring high levels of retinoids have not been found in a water system, and hence, the environmental retinoids theory for limb deformities is not well-supported as a sole cause to explain the bulk of the deformities being observed in nature. The main point of skepticism is that PD serial duplications typical of those induced by exogenously applied RA have not been observed in nature (Sessions *et al.,* 1999). However, localized retinoids caused by a parasite coupled with mechanical disruption caused by the clusters of cysts around limb-buds may help explain how some of the deformities arise. High-pressure liquid chromatography (HPLC) analyses of RA levels in *R. ondatrae*-infected *Lithobates sylvaticus* frogs found that infected tissue contained 70% more RA than non-infected controls . Interestingly, the free-swimming and recently-infected parasites contained about 56% more RA than the encysted organism infecting the host. It is possible that the parasite may serve as a localized source of RA within this species of tadpole (Szuroczki *et al.*, 2011), and that this added source of RA could disrupt

normal RA concentration during the critical period of limb development.

1.0.3 PARASITE INDUCED MECHANICAL DISRUPTION

While environmental sources of retinoids have been ruled out as the primary cause of the high incidences of frog limb deformities, several research groups have since been exploring the possibility that retinoid pathways could be disrupted in developing tadpoles by parasites. Sessions and Ruth (1990) showed that during *Ambystoma macrodactylum* (Long-toed salamander) and *Hyla regilla* (Pacific tree frog) tadpole limb development, deformities are often observed in conjunction with parasite infections. This parasite was later identified as *Ribeiroia ondatrae* by Sessions *et al.* (1999). *Ribeiroia ondatrae* possesses a complex lifecycle that utilizes amphibians or fish as a second intermediate host to later infect piscivorous birds or mammals as a final host through trophic transmission (Sessions *et al.,* 1999; Figure 1.1). For a full parasite life history, see Section 1.1. Sessions and Ruth (1990) suggested that the malformations in the amphibian host may result from simple mechanical disturbance caused by *R. ondatrae* metacercariae cysts located in developing host limb buds. Similar deformities (including extra limbs) were induced by physically implanting small beads (200μm) in the tissues of the limb bud (Sessions and Ruth, 1990). This research suggests that the presence of a physical obstruction within the host limb bud may rearrange the dividing cells and cause the cells to reset or lose their spatial organization, resulting in gross morphological deformities such as supernumerary limbs, skin webbing, and limb bifurcations. However, certain species of frogs, such as the Gray treefrog (*Hyla versicolor*) and Cope's tree frog (*Hyla chrysoscelis*) appear to be “resistant” to the development of such malformations even if

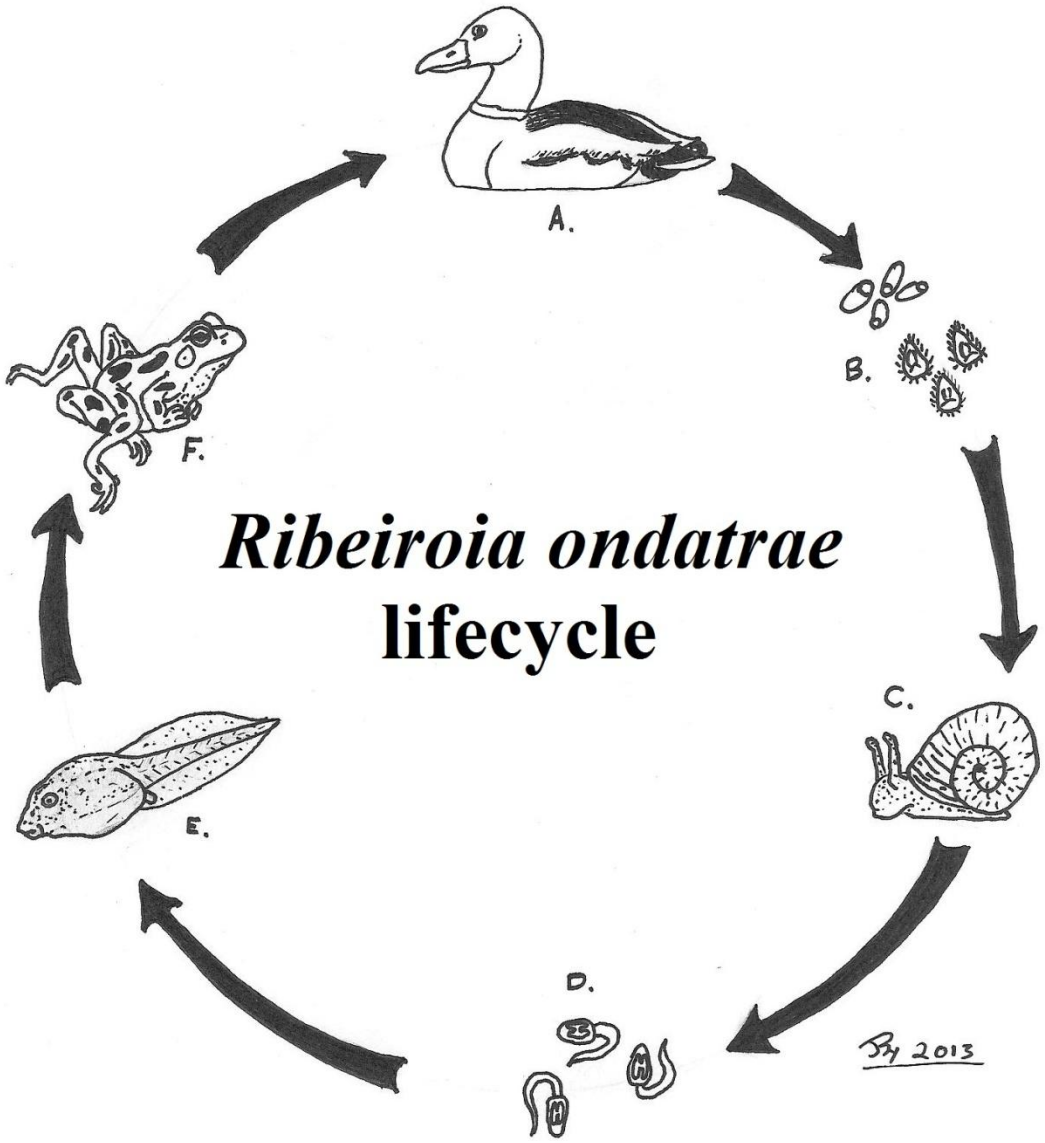

Figure 1.1 *Ribeiroia ondatrae* lifecycle. Adult worms live in the digestive tract of a final host bird or mammal (A). Eggs are shed via host feces into the water column which hatch into miracidia (B). A miracidium will locate and infect a first intermediate host planorbid snail (C). The parasite will undergo life stage changes and multiply within the snail host and release cercariae into the water-column (D). Cercariae will then locate a fish or larval amphibian second intermediate host, encyst, and transform into a metacercaria. In the case of amphibians, the cercariae will primarily encyst in the developing limb-bud region (E). The metacercaria will remain in the host. In the case of amphibians, malformations will usually occur in the limb region (F) which is thought to increase the chances of trophic transmission to the final host (A) completing the lifecycle.

infected with hundreds of *R.ondatrae* cysts, while other species such as the Wood frog (*Lithobates sylvaticus*) and the Cascades frog (*Rana cascadae*) will exhibit a 60%

malformation rate with as little as 20 parasites (Johnson *et al.* 2012;Johnson and Hartson, 2009). Hence, mechanical disruption may not fully explain why limb deformities occur following a *R. ondatrae* infection. In some cases, it is possible that the parasite is altering the host's limb development by both mechanical disruption and chemical interference of developmental signaling pathways (Goodman and Johnson, 2011).

Manipulation of a host's gross morphology during parasite infection is not unique to *R. ondatrae.* Many parasites have been documented to alter their intermediate hosts to behave and/or develop in a way that enhances the transmission of the larval parasite to the final host such as a bird or mammal. For example, the trematode *Diplostomum spathaceum* has been shown to increase aggressiveness in rainbow trout (*Oncorhynchus mykiss*), resulting in the loss of territory and shelter, and hence increasing the chance of predation by the definitive hosts, piscivorous birds (Mikheev *et al.,* 2010). Another example of gross morphology manipulation that results in trophic transmission to the final host involves echinostomes (*Curtuteria australis* and *Acanthoparyphium spp.*), which infect cockles (*Austrovenus stutchburyi*). The parasites will displace the muscle in the foot, preventing the organism from burrowing effectively, resulting in easy predation by the definitive host, the oystercatcher (Keeler and Huffman, 2009). Goodman and Johnson (2011) have shown it is possible that *R. ondatrae* also alters its intermediate host by inducing limb deformities in order to increase the chances of trophic transmission. *Ribeiroia ondatrae* -infected frogs were less competent in basic predator evasion skills, exhibiting a 37% reduced swimming speed, 66% reduced endurance, and a 41% reduced jumping distance compared to their non-infected kin, thus potentially increasing the

chances of transmission of the parasite to the final host (Goodman and Johnson, 2011). It is still currently unclear why some species of frogs are unaffected by this parasite, whereas others show numerous limb deformities.

1.1 *RIBEIROIA ONDATRAE* BACKGROUND

1.1.1 THE PARASITE'S LIFE HISTORY

Ribeiroia ondatrae is a parasitic flatworm from the family Psilostomatidae found in still-water environments within North and South America. Like most trematodes, *Ribeiroia ondatrae* has a complex life cycle involving many hosts (Figure 1.1). Planorbid snails serve as first intermediate hosts, fish and amphibians as second intermediate hosts, with various birds and mammals serving as final hosts (Johnson *et al.*, 2004). *Ribeiroia ondatrae's* complex life cycle begins with the adult worm residing in the digestive tract of a bird or mammal. After about a week of infection of the final host, eggs are released by the adult worm into the water body via host feces. Temperature plays a large role in the gestation period of egg development. In moderate water temperatures (25-28°C), the egg will hatch into a miracidium within 2 weeks (Johnson *et al.*, 2004). Experiments performed by Paull and Johnson (2011) have shown that eggs in 17°C water will take up to 58 days to hatch and temperatures lower than 12ºC will result in no development of the parasite embryo even after temperature increase to 26 ºC. Water-bodies which have temperatures lower than 17 ºC year round or during final host occupation therefore are unlikely to host a full lifecycle because eggs are unable to develop. Once hatched, the free-swimming miracidium will find a planorbid snail and embed into the host by

digesting the epidermis via apical glands. The parasite will locate itself within the pulmonary and renal veins. The parasite will then transform into a sporocyst within this host. Little is known about the transformation and sporocyst stage except that it will produce about a dozen mother rediae and about a dozen germ balls (Johnson *et al.*, 2004). These mother rediae can produce both daughter rediae and cercariae. The germ balls produce more mother rediae. Mother rediae will continue to produce daughter rediae until the entirety of the snail's gonad is destroyed and replaced with rediae resulting in full reproductive castration (Johnson *et al.* 2004; Paull and Johnson, 2012). During infection, the host snail will undergo parasitic gigantism causing infected snails to grow larger than non-infected snails. This phenomenon is more pronounced in higher temperature water-bodies resulting in a doubled daily growth rate at 26 ºC when compared to non-infected snails (Paull and Johnson, 2012). The development and release of cercariae by mature rediae is dependent on temperature with the average release beginning after 4-6 weeks of infection (Johnson *et al.*, 2004; Paull and Johnson, 2012). Paull and Johnson (2012) have shown that infected snails maintained at 26 ºC and 20 ºC will begin to release cercariae after 28 and 50 days respectively. Infected snails maintained in waters 13 ºC and below did not shed cercariae until the water temperature reached 20 ºC. Therefore, cercarial development does occur at these lower temperatures but they will only emerge at warmer temperatures (Paull and Johnson, 2012). Cercariae emerge from the snail late spring until fall, when water temperatures promote this behavior, which allows them to infect developing tadpoles during sensitive limb development stages. Cercariae will most commonly exit the snail after dark and actively swim towards the next intermediate host (a fish or frog) (Johnson *et al.*, 2004). When the cercaria locates a suitable host, the

parasite will latch on to the host with its ventral and oral suckers. The cercaria then loses its tail and crawls to a suitable location on its host. The cercaria will then encyst itself and transform into the metacercaria stage. Cyst clusters are usually formed around the hind limb bud region, eyes, and around the tail absorption area. The cyst will then be enveloped in host- and parasite- derived tissue. The metacercaria will remain in the host until it is consumed by a suitable final host. Once consumed by a bird or mammal, the metacercaria will transform into the adult form in the host's digestive tract, where it will release eggs to continue the life cycle (Johnson *et al.*, 2004).

An increasing number of malformed frogs have been documented in the United States and Canada over the last decade, which is of growing concern due to worldwide amphibian declines (Johnson *et al.*, 2004). It is important to note that the overall issue of amphibian deformities cannot be attributed to a single mutagen or parasite but is likely a range of factors that often work in in synergy to result in the increased deformities found in nature. *Riberorira ondatrae* however, appears to be a key player in many of the sites of amphibian malformations and will be the focus of this paper. This parasite has been occasionally documented in final hosts in Manitoba (Johnson *et al.*, 2004). Amphibians infected by *R. ondatrae* have been documented in Ontario (Szuroczki *et al.*, 2011), and British Columbia (Johnson *et al.*, 2004; Roberts et al., 2012) but there is little known about the extent of *R. ondatrae* distribution within Canada (Johnson *et al.*, 2004; Roberts et al., 2012).

Environmental alterations such as climate change are likely to affect parasite distributions (Lafferty, 2009) and the distribution of *R. ondatrae* may also be affected. Other environmental factors are also known to affect *R. ondatrae* presence and dynamics.

For example, eutrophication and increased water temperature could result in a larger population of snail hosts due to an increased food supply and metabolism as well as an increased cercarial output from infected snails (Lafferty, 2009). Single and interactive effects may have significant influences on parasite-induced frog malformations under certain scenarios. For example, the reduced immunity of the amphibian host and increased presence of cercariae, coupled with increased levels of environmental RA posed by cyanobacterial blooms could result in an increased abundance of malformed amphibians and have population-level effects (Figure 1.2). The increased occurrence in amphibian malformations and population declines are serious events which need to be understood and hopefully reversed to help maintain a healthy ecosystem for future generations.

For the reasons outline above, it is critical to understand the distribution of *R. ondatrae* to better predict how environmental changes may alter future infection and malformation patterns. *Ribeiroia ondatrae* may still occur in sites currently lacking deformed frogs and could become problematic under certain conditions as explained later. The current methods of *Ribeiroia ondatrae* detection are very inefficient, in that they require collection and sacrifice of living snails, frogs, fish, birds, and mammals, to visually inspect the affected tissues for parasites. This process can be expensive and time consuming, and similar parasitic species can also be confused with *R. ondatrae*. In addition, permits are often required to collect and transport specimens from the site which require early preparation to ensure that collections can be made during peak infection seasons. While I have a modest understanding of the lifecycle and pathology induced by *Ribeiroia ondatrae*, I currently know very little regarding the actual mechanisms

involved in the development of amphibian limb deformities due to this parasite and its distribution and prevalence within Canada. Such information will allow us to better identify which amphibian populations and species may be most vulnerable.

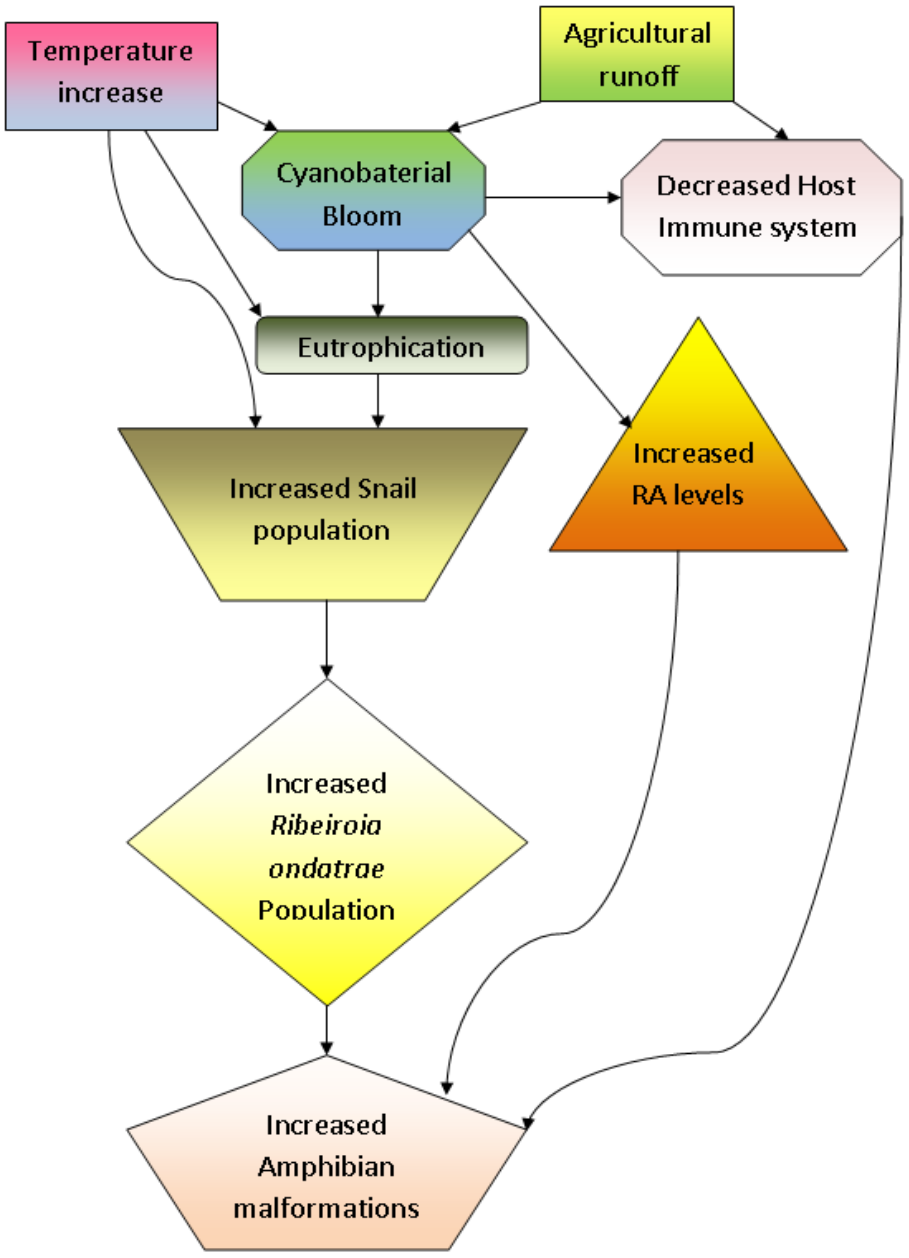

Figure 1.2 *Sample factors which may lead to increased amphibian malformations.* This diagram assumes that all hosts and the parasite are present in the water-body. Increased RA concentrations may cause malformation on their own or they could increase the chances of malformations when coupled with *R. ondatrae* infections.

1.2 RESEARCH OBJECTIVES

1.2.1 Protocol to detect *Ribeiroia ondatrae* in water bodies using environmental DNA (eDNA)

One objective of my MSc research was to assess the prevalence of *R. ondatrae* in water bodies throughout parts of Manitoba, Ontario, and California. In this study, I demonstrate the utility of using PCR-based methods to detect environmental DNA (eDNA) for *R. ondatrae.* The goal was to develop a suitable method of collecting water samples from a variety of *R. ondatrae*-infected and non-infected ponds, and to assess the efficacy of the end-point PCR and quantitative real-time PCR screening method to accurately detect *R. ondatrae* -infected waters. This information will provide tools to better understand the distribution and density of *R. ondatrae* which can help predict threat levels posed to amphibians living in these water-bodies.

1.2.1 Effect of parasite infection on retinoic acid pathways during host limb development

The other aspect of my research was to determine how these parasites induce limb malformations in frogs. Studies with *Xenopus laevis* have shown that the presence of a small, parasite-sized glass bead can induce a supernumerary limb (Sessions & Ruth, 1990), suggesting that the parasite is somehow physically obstructing normal limb formation. However, it is yet to be determined which chemical signals are being disrupted, if any. In contrast, in *Lithobates sylvaticus* (wood frog, formally *Rana sylvatica*), it is suggested that *R. ondatrae* may release a large amount of RA upon encystment near the developing limb bud (Szuroczki et al., 2011). As discussed earlier, it

was found that infected tissue contained 70% more tissue RA than controls and the free-swimming parasite contained about 56% more RA than the encysted organism. My goal was to build upon these observations by examining whether changes in RA levels and the enzymes that synthesize RA (e.g. retinaldehyde dehydrogenases *Raldh1, Raldh2, and Raldh3*) are correlated with parasite infections. Given that different frog species appear to respond differently to the *R. ondatrae* parasite (Johnson *et al*, 2012), *Lithobates sylvaticus* (a vulnerable species) was examined in this study. With the recent release of the *X. tropicalis* genome, it was possible to use a bioinformatic approach to attempt to identify candidate genes involved in RA biosynthesis in this species, and attempt to use this information to assist in identifying related genes in other species. Frogs were subjected to a variety of treatments including *R. ondatrae* infections and glass bead implants to assess whether the RA biosynthesis pathway were altered. Using a quantitative reverse transcriptase PCR approach, I aimed to examine whether the various treatments caused perturbations in the expression of genes involved in the RNA biosynthesis or reception pathways. The goal of this project was to obtain a better understanding of which mechanisms are involved in the development of these malformations, as well as compare aspects of the developmental pathology exhibited by different frog species when exposed to the *R. ondatrae* parasite. With this information, I may be better placed to identify and protect vulnerable amphibian populations by understanding the vulnerability of different species.

2. ENVIRONMENTAL DNA (eDNA)

2.0 Environmental DNA Background

DNA can be found in water, ice, soil, the atmosphere and even other organisms. The DNA is released into the environment by cell lysates of sloughed cells, spores, feces, decomposition of dead organisms, and external fertilization. This environmental DNA (eDNA) leaves a molecular imprint in the medium which can be used to identify organisms present via various PCR techniques. PCR methods have been used previously to identify cryptic, endangered, pathogenic, and invasive organisms such as *Leishmania sp.* (Aviles *et al*., 1999) , *Acanthaoeba sp.*(Chang *et al.,* 2010), *Lithobates catesbeianus* (Ficetola *et al.*, 2008; Goldberg *et al.,* 2011), *Dicamtodon aterrimus* (Goldberg *et al.,* 2011) and *Batrachochytrium dendrobatidis* (Kirshtein *et al.,* 2007).The sensitivity of these techniques depends on various factors such as the primer sensitivity, inhibitors, and degradation rate of the eDNA.

The stringency of a PCR technique depends on the uniqueness of the primer set and target sequence amplified. The more unique the primer target sequence, the less likely the primer will bind to a non-specific target gene (such as a gene from a non-target organism) which will result in a false positive. If the primer set is too stringent, and the target sequence contains a variation in the DNA code, the primer will not bind and a false negative will occur. In order to prevent non-specific target binding, primers should be tested *in silico* to determine if they will amplify non-target genes or target genes of other organisms (Dejean *et al.,* 2011).

When designing an eDNA protocol, it is important to consider the optimal temporal window for collection. DNA will degrade over time in an aquatic environment. Shorter

target sequences are more resistant to degradation; while DNA sequences of 300-400bp tend to be detectible for up to a week, shorter sequences, such as the 98bp and 79bp have been shown to remain intact in the water column for 14 and 25 days with a detection level of greater than 5% (Dejean *et al.*, 2011).

2.0.1 CURRENT *RIBEIROIA ONDATRAE* DETECTION TECHNIQUES

The most common method to detect *R. ondatrae* in water-bodies is by host sampling of frogs and planorbid snails. Tadpoles and emerging frogs are collected from the site and unless malformations are obvious, they are examined for cysts under a dissection microscope (often involving sacrificing the animal). Cysts are most often found near the limb-bud region and cloaca (Szuroczki and Richardson, 2009). Infection of snails can be determined by either cracking the snail open and looking for rediae and sporocysts or by placing the snail in a small amount of water and allowing the cercariae to emerge after sunset. However, presence of infection within snails is not enough. For example, to the untrained eye, echinostomes can easily be mistaken as *R. ondatrae. Riberoria ondatrae* cercariae can be identified and distinguished from echinostomes by their swimming pattern and the following 3 key anatomical features (Szuroczki and Richardson, 2009; Johnson *et al.*, 2004): 1. *R. ondatrae* does not possess collar spines); 2. the presence of esophageal diverticula which extend laterally from the mid-length of the esophagus in *R. ondatrae*; and 3. *R. ondatrae* cercariae possess a pink colored tissue located between the ventral and oral sucker that is unique but varies in intensity and presence among individuals and should not be used as a primary identification feature.

Examining and dissecting hosts requires considerable time and effort, particularly

because these organisms can be hard to find and catch in some field situations. Misidentification of the parasite is another factor which can occur using traditional methods. A PCR-based method to detect the presence of *R. ondatrae* infections in snails was previously developed (Reinitz *et al.*, 2007). Reinitz *et al.* (2007) developed PCR primers that would amplify a portion of the internal transcribed spacer 2 (*ITS-2)* sequence (GeneBank ID: AY761142.1) of *R. ondatrae.* The primers enabled the detection of as little as 1/50th of a cercariae or just 100fg of a 290bp DNA fragment derived from snail tissues. The primers were species-specific, detecting only *R. ondatrae* and not closely related snail-infecting trematodes such as *Fasciola hepatica* and *Echinostoma spp.* (2007). The shortfall of this technique is that it still requires researchers to collect often hard to find planorbid snails. A method to detect the parasite within the water, without the need to collect snails would be useful.

Ficetola *et al.* (2008) devised a method to screen water samples for the presence of environmental DNA that indicated the presence of invasive bullfrogs (*Lithobates catesbeianus*). A similar method, used to detect *R. ondatrae* directly from water samples, is described below. The technique outline below detects the presence of *R. ondatrae* in a water body but cannot determine which aquatic life cycle stage (egg, miracidium, or cercaria) is present since all contain the same genome and can thus be detected using the following PCR-based method.

2.1 METHODS AND MATERIALS eDNA

2.1.1 ANIMAL CARE AND TREATMENT

2.1.1.1 SNAIL COLLECTION AND CARE

Ribeiroia ondatrae- infected snails were obtained from a lab-maintained stock originating from California or wild caught from Glenridge pond located in Southern Ontario (a known *R. ondatrae* infected site). Planorbid snails were also collected from St. Ambrose (Manitoba) where other trematode species could be collected for primer specificity tests. To test for infection, snails were placed in small Petri-dishes overnight to allow the cercariae to emerge. Cercariae were identified using the criteria outlined by Szurocki and Richards (2009). Infected snails were sorted by trematode species and placed in separate 10 gallon aquaria containing dechlorinated tap water. Snails were fed boiled organic spinach *ad libitum.* Aquaria were kept under a 16:8 hour light:dark cycle to simulate natural light conditions.

2.1.1.2 CERCARIAE COLLECTION

When cercariae were needed for experimentation, infected snails were placed in small Petri-dishes of water. *Echinoparyphium spp.* and fasciolid -infected snails were placed under a bright light to encourage cercariae emergence. *R. ondatrae*-infected snails were placed in a dark place such as a cabinet or drawer. This was done no earlier than 2 hours before the onset of darkness for optimal shedding of cercariae. All Petri dishes were examined every 30 minutes for cercariae, which were removed with a 1ml disposable pipette and placed in a larger Petri-dish under a dissection microscope. Fresh

water was added to the snail dishes to ensure sufficient water and promote further shedding. Using a micro-pipette set at 2µl to prevent excessive water transfer, cercariae were then carefully selected and transferred to their next location depending on the experiment. After sufficient cercariae were collected, the snails were returned to their holding tank.

2.1.2 PCR DETECTION OF *RIBEIROIA ONDATRAE* eDNA.

2.1.2.1 OPTIMIZATION OF PCR DETECTION OF *RIBEIROIA ONDATRAE* DNA.

To detect *Ribeiroia ondatrae* DNA in water samples, a PCR-based method was developed using primers designed to amplify the internal transcribed spacer 2 (*ITS-2*; GeneBank ID: AY761142.1) of the trematode's ribosomal DNA sequence. To develop PCR primers that would be species-specific and sensitive enough to detect eDNA in minute concentrations, primers previously developed by Reinitz *et al.* (2007) and two other primers designed using Primer3 (Version 0.4.0) software were initially tested on serial dilutions of DNA purified from *R. ondatrae.* To acquire this DNA, 25 cercariae were collected from infected snails, and DNA was extracted from these pooled trematodes using the DNA Wizard Purification Kit (Promega) according the manufacturer's instructions. The concentration of the DNA was measured using a Nanodrop spectrophotometer (General Electric) and the DNA was then serially diluted (10X dilution) in water to assess the sensitivity of PCR detection using the different primer pairs (Table2.1). Negative controls contained nuclease-free water in lieu of DNA. Positive controls containing DNA derived from 10 *R. ondatrae* cercariae were included in every experiment. PCR products were amplified using a BioRad **My**Cycler^{tm}

thermocycler in 25µl reactions (12.5µl EconoTaq PLUS GREEN 2X Master Mix (Lucigen), 1µl fwd primer, 1µl rev primer, 2µl template DNA, and 8.5 nuclease-free water). A gradient PCR method was first used to select the optimal annealing temperature, using the following cycling conditions: 94°C for 5 minutes followed by 40 cycles of: [94°C for 30 seconds; a variable annealing temperature ranging from 40°C to 60°C (the optimal temperature was observed to be 46°C; see Results) for 30 seconds; and 72°C for 30 seconds], followed by 72°C for 5 minutes. Following PCR amplification, the PCR products were resolved on 1.5% agarose gels, either stained with ethidium bromide, or SYBR Gold (Invitrogen) and visualized with a UV transilluminator (Syngene Bio Imaging: GENE FLASH [for ethidum bromide-stained gels] and BioRad: Universal Hood II [For SYBR Gold-stained gels]).

Table 2.1. eDNA primer information

Primer Name	Sequence	Tm ºC	Product size (bp)
Ro-ITS 1 fwd	TCACGACGCTCAAATAGTCG	63.9	240
Ro-ITS 1 rev	GAGCATAGCTCCACCCGTAG	63.6	240
Ro-ITS 2 fwd*	AGTCATGGTGAGGTGCAGTGA	65.9	290
Ro-ITS 2 rev*	AGACCGCTTAGATAGCAG	54.5	290
Ro-ITS 3 fwd†	CGTGTTTGGCGATTTAGT	58.7	164
Ro-ITS 3 rev†	TCAAAAATGAAGCAACAGT	55.7	164

*Derived using Primer3 software but was identical to Reinitz *et al.* (2007) 21up and 18dn respectively.
†
Primer designed by Reinitz *et al.* (2007) referred to as 18up and 19 dn respectively in the original paper.

2.1.2.2 PRIMER STRINGENCY TEST

In addition to testing sensitivity of the primers to detect small amounts of *R. ondatrae* DNA, the primers were also assessed for species-specificity by testing their

ability to amplify 25 *Echinoparyphium spp.* and fasciolid cercariae. DNA from these organisms was extracted using the DNA Wizard Purification Kit (Promega) according the manufacturer's instructions. The primers listed above were used in the following PCR protocol to test their ability to detect these related trematodes' DNA: 25µl reactions (12.5µl EconoTaq PLUS GREEN 2X Master Mix, 1µl fwd primer, 1µl rev primer, 2µl template DNA, and 8.5 nuclease-free water), using the following cycling conditions: 94°C for 5 minutes followed by 40 cycles of [94°C for 30 seconds, 46°C for 30 seconds, and 72°C for 30 seconds], followed by 72°C for 5 minutes. Following PCR amplification, the PCR products were resolved and examined as described above.

2.1.2.3 eDNA DEGENERATION

The ability of the primers to detect degraded DNA was tested by placing 5 *R. ondatrae* cercariae each into 16 1L Mason jars containing 900ml of dechlorinated tap water and 100ml of uninfected aquarium water containing zebrafish (for bacterial culture). The 16 jars were divided among 4 experimental groups: 10 days stored at 20ºC or 25ºC, and 21 days stored at 20ºC or 25ºC. Samples were then filtered, and processed as described below to determine the detectability of the DNA.

2.1.3 COLLECTION OF eDNA WATER SAMPLES

Water was collected from 10 field sites in California and southern Ontario for which *R. ondatrae* infection presence/absence has been verified through traditional means, along with one unverified site in Manitoba. Environmental DNA (eDNA) was collected by pushing 500ml of pond water through a filtration unit equipped with filters

to catch suspended particulates, including *R. ondatrae*. I designed an inexpensive filter prototype called the "Cost-effective Hand-made eDNA And Particulate (C.H.E.A.P.) Filtration unit" that could be made for around CAD $20.00(Figure 2.1).

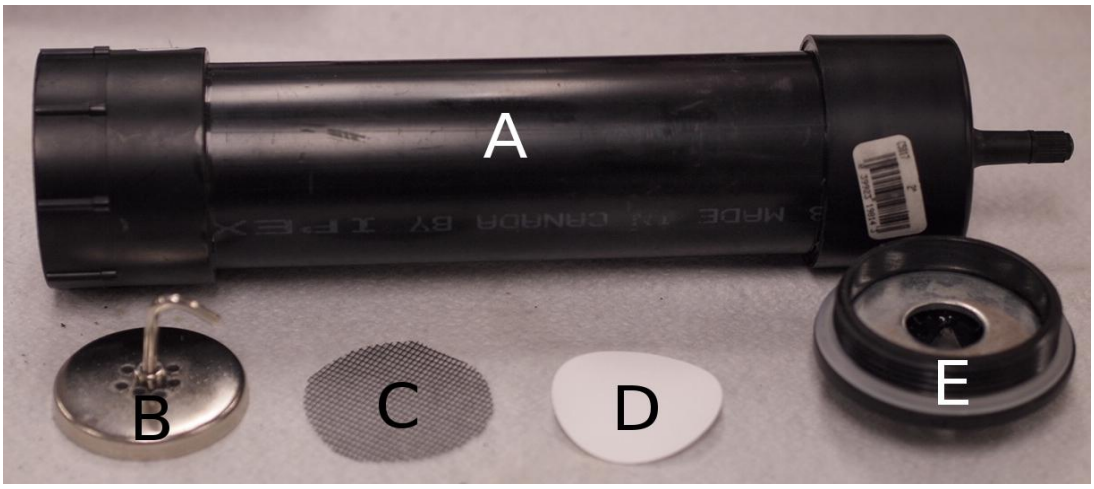

Figure 2.1. C.H.E.A.P filtration unit. Filter body (A), filter support retaining magnet (B), filter support screen (C), membrane filter (D), and the filter support unit (E).

For details on the construction of this device, please see Appendix I. Whatman cellulose nitrate membrane filters (Sigma-Aldrich) with a 3µm pore size were preloaded into the filter support units as seen in Figure2.2 prior to filtration of the water sample.

Figure 2.2 Filter support assembly. Screen was placed over the filter support unit (1) filter was then placed upon the filter support unit (2) the filter support retaining magnet was then pressed firmly into filter support system to ensure a proper seal (3).

Five water samples were filtered per site. Water was collected near the shorelines (15-45 cm water depth) 5 meters apart from one another at the sites listed in Table 2.2. Water was scooped up into the filter body and filled to the max line (500ml). The filter support unit was then screwed onto the filter body. The filter system was then inverted and attached to a bicycle pump to provide pressure to push the water through the filter. Air was pumped until all water was drained. Collectors were careful not to exceed 40 PSI to prevent rupture of the filter. The filter support unit was removed and the filter was placed in 10 ml of 70-95% ethanol until further processing. A new filtration unit was used for each pond to ensure no cross-contamination.

Table 2.2. eDNA collection site information. Sites are named with their location and GPS co-ordinates. Longevity refers to whether the site is a permanent pond site or dries up depending on precipitation levels. Origin refers to whether the site was man-made or natural. Assmt_Date refers to the date that the samples were collected. Rib History refers to whether there was a history of *R. ondatrae* in that site. The 2012 Rib Status corresponds to whether tadpoles and snails were observed with *R. ondatrae* during collection in that site.

Site Name	Location	Latitude	Longitude	Longevity	Origin	Assmt_Date	Rib History	2012 Rib status
Frog	California	37°08'56.14"N	121°73'95.61"W	Permanent	Artificial	07.25.2012	Present 2011	Present
PRPND007	California	37°62'22.90"N	121°88'66.38"W	Semi-permanent	Artificial	07.21.2012	Absent 2011	Absent
PRPND008	California	37°61'99.43"N	121°89'14.01"W	Semi-permanent	Artificial	07.21.2012	Absent 2011	Present
Murky Bullfrog	California	37°64'52.78"N	121°91'83.19"W	Permanent	Artificial	07.20.2012	Present 2011	Present
VPPND006	California	37°58'52.87"N	121°93'56.27"W	Permanent	Artificial	07.21.2012	Present 2011	Present
Pond 3	Ontario	43°28'21.43"N	80°14'24.76"W	Permanent	Natural	07.03.2012	Absent 2010	Absent
Pond A	Ontario	43°27'35.78"N	80°13'13.91"W	Permanent	Natural	07.03.2012	Absent 2010	Absent
Pond C	Ontario	43°29'53.50"N	80° 7'14.40"W	Permanent	Natural	07.03.2012	Absent 2010	Present
Pond D	Ontario	43°31'36.71"N	80° 5'16.20"W	Permanent	Natural	07.03.2012	Present 2010	Present
Glenridge	Ontario	43° 7'31.32"N	79°14'8.57"W	Permanent	Artificial	07.04.2012	Present 2010	Present
Alf Hole Goose Santuary	Manitoba	49° 50'96.8"N	95°32'98.6"W	Permanent	Natural	08.17.2012	unknown	unknown

2.1.4 EXTRACTION OF eDNA FROM WATER SAMPLE FILTERS.

eDNA was extracted from the filters using a modified TRIZOL reagent (Invitrogen) protocol. Water samples from different sites were processed separately from one another with a thorough bleaching of the bench and equipment between samples to ensure that no cross contamination between samples occurred. Filters were air-dried in 60mm Petri dishes until all of the ethanol was evaporated. Filters were then placed in a sterile 2ml tube using flame-sterilized tweezers. Filters were then chopped up using small flame sterilized scissors. TRIZOL reagent (1.5ml) was added to the filter, vortexed for 15 sec, and allowed to incubate for 5 minutes at room temperature to dissolve the DNA from the filter. The TRIZOL solution was then transferred to another 2ml tube, leaving the filter behind. Chloroform (300µl) was added to the TRIZOL mixture, vortexed for 15 seconds, and allowed to incubate at room temperature for 15 minutes. The tube was then centrifuged at 15,000 xg for 15 minutes at 4°C. The aqueous phase, containing the RNA, was removed carefully using a pipette and discarded. The DNA in the interphases and phenol phase was precipitated by adding 450 µl of 100% ethanol, followed by a gentle vortex and incubation at room temperature for 3 minutes. The DNA was pelleted by centrifuging at 15,000 xg for 5 minutes at room temperature. The supernatant was discarded and the pellet was washed twice in 1 ml of 0.1M sodium citrate for 30 minutes using a rocking table. The DNA was then washed with 1ml of 70% ethanol and pelleted by centrifugation. The pellet was then vacuum dried using a vacuum centrifuge and was then resuspended in 100µl of nuclease-free water. To enhance re-suspension, the DNA was warmed at 55°C for 10 minutes. To remove any particulate debris from the sample that had carried over from the initial extractions, the resuspended DNA was centrifuged at

15,000 x g for 15 minutes at 4°C and the supernatant was transferred to a clean vial for storage at -20°C until further processing.

2.1.5 QUALITATIVE-END POINT PCR DETECTION OF *RIBEIROIA ONDATRAE* DNA FROM FIELD-COLLECTED WATER SAMPLES.

Ro-ITS 3 primer sets were chosen due to their high sensitivity and stringency compared to the other primer sets (see Results). Each site sub-sample was replicated 3 times for a total of 15 PCR reactions per site (5 replicate sub-samples from each site). PCR reactions were prepared and conducted as described above, using the previously described PCR protocol, optimized to selectively amplify the Ro-ITS sequence using an annealing temperature of 46°C. PCR products were resolved on 1.5% agarose gels in TBE stained with ethidium bromide. Gels were examined for bands in the negative control or lack of bands in the positive control. Pond sample sites were considered positive if bright bands of 164 bp were observed or if multiple samples from one site consistently produced light intensity bands of the expected size (164 bp).

2.1.6 ESTABLISHING A CALIBRATION CURVE FOR QUANTITATIVE PCR OF *RIBEIROIA ONDATRAE* DNA

In order to quantify the amount of eDNA in a water sample, a series of dilutions of *R. ondatrae* DNA was used to create a calibration curve. Previously extracted DNA from 1 cercaria was used as template in a 100µl PCR reaction (50µl EconoTaq PLUS GREEN 2X Master Mix, 4µl Ro-ITS 3 fwd primer, 4µl Ro-ITS 3 rev primer, 8µl template DNA, and 34µl nuclease-free water) using the same thermocycler protocol to ensure that the

starting DNA concentrations would be high enough to be accurately estimated. The concentration of this stock DNA was then determined using a General Electric NanoVue spectrophotometer. The stock DNA was then subjected to multiple 10-fold serial dilutions and these diluted standards were used in quantitative real time PCR (q-PCR) using BioRad's iQ SYBR Green Supermix on a BioRad iQ5 Multicolor Real-Time Detection System as per the manufacturer's protocols. Samples were analyzed in 10µl reactions (5µl iQ SYBR Green Supermix, 0.5µl Ro-ITS 3 fwd primer, 0.5µl Ro-ITS 3 rev primer, 3.5µl sterile water, and 0.5µl site cDNA template) using the manufacturer's protocol. Real time data was compiled using iQ (BioRad) software. The cycle threshold (C_t) values for each DNA concentration were plotted to generate a standard curve. A linear regression formula was calculated using the linear and R^2 trend function in Open Office Calc (version 3.4.1) which allows interpolation of unknown concentrations of sample sites by their Ct value.

2.1.7 Q-PCR OF *RIBEIROIA ONDATRAE* DNA FROM FIELD-COLLECTED WATER SAMPLES

Site samples were analyzed with quantitative real time PCR (q-PCR) using BioRad's iQ SYBR Green Supermix on a BioRad iQ5 Multicolor Real-Time Detection System as per the manufacturer's protocols. Samples were analyzed in 10µl reactions using the protocol listed above. Concentrations of *R. ondatrae* in each pond sample were determined by converting the C_t values to µg *R. ondatrae* DNA/mL using the calibration curve described above. Each DNA sample was analyzed by q-PCR in duplicate, and the average DNA concentration was then calculated for each sampling point in every pond. The mean DNA concentration and standard error for each pond was compared to the

effective range of the calibration curve. If the range of the standard error dropped below the effective interpolation range of the calibration curve, the site was considered negative (void of *R. ondatrae*). If the values ranged higher than the cut off, the site could safely be considered positive. A Generalized Linear Mixed Model (GLMM with normal distribution and identity link function) statistical analysis was performed in SPSS 20.0. Ribeiroia presence was used as a categorical fixed factor (present =1, absent = 0 based on examination of tadpoles in 2012) while the pond identity for each sub-sample was used as a categorical random factor. To meet the assumptions of a normal distribution, the qPCR value was log-transformed before analysis.

2.1.8 CLONING *RIBEIROIA ONDATRAE ITS-2* GENE FOR SEQUENCING

California and Ontario *Ribeiroia ondatrae* DNA was extracted from gel bands of approximately 164bp and then purified using QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's instructions. These samples were then ligated into a vector using the Clone Jet Sticky end Protocol and were transformed into Sub cloning Efficiency™ DH5α Chemically Competent *E. coli* cells (Invitrogen) following the manufacturer's protocol. Cells were allowed to incubate overnight (18 hours) and 10 colonies were chosen for PCR screening to examine if the ligation of the insert had worked. Each colony was scraped with a pipette tip and swirled in a sterile PCR tube then rubbed onto an agar plate that had grids numbered 1-10. The PCR was run as described above using the Ro-ITS 3 primer set protocol. The agar plates were incubated at 37ºC overnight. PCR products were then resolved on a 1.5% agarose gel stained with ethidum bromide and examined for bands. Colonies that exhibited bands were then scraped off of the agar

plate with a pipette tip and were inoculated into 3ml of LB Broth + 3µl ampicillin and allowed to incubate for 12 hours. Samples were then processed using the Qiagen QIAprep miniprep kit as per manufacturer's instructions. The products were then analyzed for purity and concentration using a General Electric NanoVue Spectrophotometer. Samples were then diluted with nuclease-free water to ensure that a total of 200-300ng of DNA were loaded into a total volume of 7µl in a PCR tube. The samples were then shipped off to TCAG Facilities: DNA Sequencing/synthesis for DNA sequencing.

2.2 eDNA RESULTS

2.2.1 PCR detection of *Ribeiroia ondatrae* eDNA.

The PCR method detected minute traces of *Ribeiroia ondatrae* DNA. The PCR product, estimated to be 160bp long on the gels, correlated well with the predicted target length of 164bp. The Ro-its 3 primer set was able to detect as little as 1/2500000th of a single cercaria under optimal conditions using molecular grade water and directly extracting DNA without using a filter (Figure 2.3). The Ro-its 3 primer set was also found to be the most stringent primer set used as it did not amplify genes from *Echinostoma sp.* or fasciolid cercariae (Figure 2.3). Ro-its 3 was then used for the remaining experiments to ensure that related trematodes were not detected.

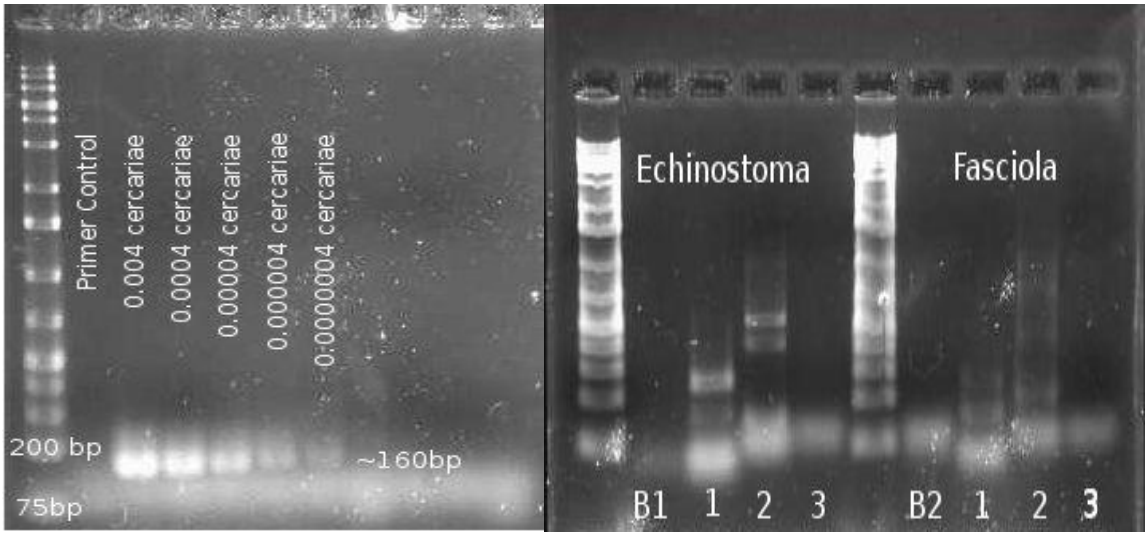

Figure 2.3.Sensitivity and stringency tests. (left) A pilot experiment using Ro-its 3 detects as little as 0.0000004 (1/2500000th) of a cercariae (Right) Stringency test of primer pairs Ro-its 1, Ro-its 2, and Ro-its 3. B1, B2, are DNA template blanks, containing Ro-its 1 and Ro-its 2 (respectively) primer pairs only, to test for primer dimerization. 1, 2, or 3 indicate the primer pair used in each lane. Each primer was tested on *Echinostoma* sp. and fasciolid cercariae. Primer pairs Ro-its 1 and Ro-its 2 both detected 2.4×10^{-2} of a *Echinostoma* sp.or fasciolid cercaria. Primer pair Ro-its 3 did not react with any of the other test parasites.

2.2.2 COLLECTION OF eDNA WATER SAMPLES

The C.H.E.A.P. water filtration system was effective in collecting suspended particulates from most pond water samples. One of the samples collected required two filters to process a 500ml volume of water due to clogging of the filters from high amounts of sediment and algae in the water body. Another minor issue with the system occurred when high pressure caused by a clogged filter made the cap difficult to release. This issue could be resolved by installing a quick release valve to future models of the water collecting device.

Some of the cellulose nitrate filters showed signs of degradation when stored and shipped in the ethanol-filled containers, perhaps due to prolonged storage in sun-warmed containers during the field collection process. The Glenridge pond site filters were degraded to the consistency of a semi-viscous gel. Nevertheless, they were subjected to the DNA extraction protocol, although there is some uncertainty that the maximal amount of DNA was recovered from these filters, as it was difficult to collect all of the gelatinous material from the shipping containers. The filters from ponds VPPND006, Frog, and Murkey Bullfrog had fully degraded in the ethanol. These samples were filtered again and processed with the standard protocol.

2.2.3 EXTRACTION OF eDNA FROM WATER SAMPLE FILTERS.

In pilot tests, DNA extractions from the cellulose nitrate filters were mostly successful in detecting *Ribeiroia ondatrae*. Filter extractions did not exhibit the same sensitivity as direct DNA extractions from the organism but were able to detect the following in test solutions: 1 out of 3 samples with a single cercaria, 3 out of 3 samples

with 10 cercariae and 1 out of one sample with 20 cercariae (Figure 2.4).

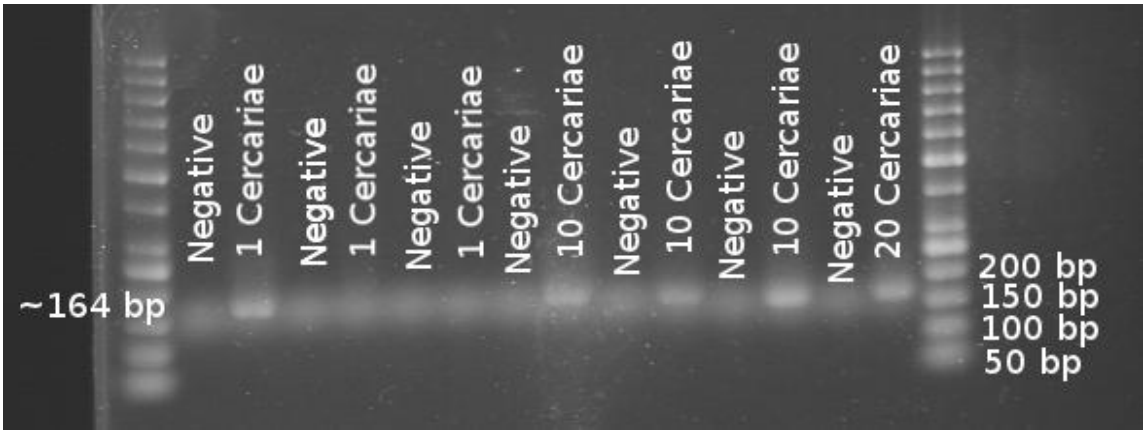

Figure 2.4. Filter Extraction test. Gel electrophoresis resolution of a sample eDNA extraction from 3µm cellulose membrane filters. Water samples (500ml) were spiked with the indicated number of *R. ondatrae* cercariae. The apparatus was washed and a negative control sample, containing no cercariae, was collected, DNA extracted and PCR-amplified between each spiked sample. The target product size was 164 bp long. 1.5 % agarose gel stained with Sybr Gold.

2.2.4 eDNA DEGRADATION

Neither time (10 or 21 days) nor temperature (20ºC or 25ºC) caused enough degeneration to affect the effectiveness of the primer set to amplify the target sequence (Figure 2.5).

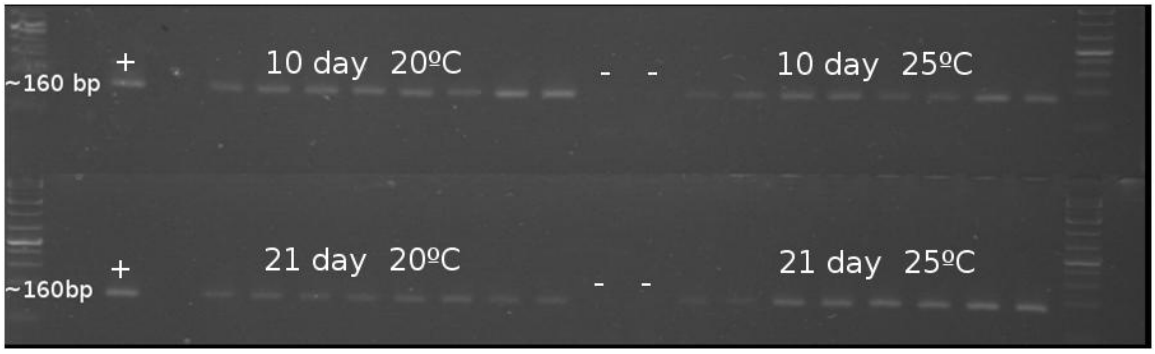

Figure 2.5. End-point PCR gel of time and temperature degeneration test on 5 cercariae in 1L of water. 1kb Ladder (Fermentas).

2.2.5 END-POINT PCR DETECTION OF *R. ONDATRAE*

Sites were scored as negative if fewer than three out of fifteen replicate water samples produced faintly visible PCR products after 40 rounds of PCR amplification. Sites were considered positive if 4 or more faint PCR products were observed or one or more intensely-staining PCR products were observed in the electrophoresis gels. The end-point PCR method was determined to be 70% accurate when compared to *R. ondatrae* infections in the water body that were detected via host examination (Table 2.3). The Alf Hole Goose sanctuary was excluded from this calculation because snails and frogs were not examined for infection in this site. The end-point PCR method did not show any false positives. The Frog, PRPND008, and Glenridge ponds appeared as false negatives using the above method. Notably, the Frog site filters were completely degraded in the ethanol, thePRPND008 site filters were caked with sediment and silt (even the purified DNA sample had a brown coloration), and the Glenridge pond filters were partially degraded in the ethanol as described earlier.

Table 2.3. End-point PCR results for water body eDNA. Gel positives indicate the number of positive bands that appeared in the 15 replicates on a single gel. The Gel verdict indicates whether the site was considered positive or negative according to the test. Rib History shows the presence or absence of the parasite in the frog or snail host and what year it was verified. 2012 Rib Status shows if the parasite was observed in the snail or frog host during the time of collection of the eDNA water samples.

Site Name	Location	Gel Positives	Gel Verdict	Rib History	**2012** Rib status
Frog†	California	0/15	False Negative	Present 2011	Present
PRPND007	California	0/15	Negative	Absent 2011	Absent
PRPND008*	California	0/15	False Negative	Absent 2011	Present
Murky Bullfrog†	California	4/15	Positive	Present 2011	Present
VPPND006†	California	5/15	Positive	Present 2011	Present
Pond 3	Ontario	1/15	Negative	Absent 2010	Absent
Pond A	Ontario	0/15	Negative	Absent 2010	Absent
Pond C	Ontario	8/15	Positive	Absent 2010	Present
Pond D	Ontario	7/15	Positive	Present 2010	Present
Glenridge Ponds††	Ontario	0/15	False Negative	Present 2010	Present
Alf Hole Goose Sanctuary	Manitoba	4/15	Positive	unknown	unknown

* These samples were of a brown coloration even after DNA purification.

† Filters were completely degraded in ethanol

††Filters were semi-viscous due to degradation in ethanol

2.2.6 QUANTITATIVE PCR DETECTION OF *RIBEIROIA ONDATRAE* DNA FROM FIELD-COLLECTED WATER SAMPLES.

A calibration curve was established for quantitative PCR (qPCR) of *Ribeiroia ondatrae* cercarial DNA. The effective range of the test was 10 to 5.54 x 10^{-11} cercariae (25-35 real time PCR Cycle threshold (Ct)) (Figure 2.6). When the cercarial concentrations dropped below 5.54 x 10^{-11}per filter sample, the Ct values began to plateau, making the test inaccurate beyond this level. To reduce the chances of a false positive, sites were considered negative if the mean Ct value of the site was 35 or higher, which correlated to 5.54 x 10^{-11} cercariae or less. The strength of the test with parasite loads higher than Ct = 10 was not tested, but Ct scores of less than 25 will indicate a positive site. Sample site data could be interpolated from the Ct by using the following formula:

$$\#\ Cercariae = e^{\frac{Ct-29.1951281697}{-0.2457886476}}$$

The line of best fit had a R^2 value of 0.81, which indicates that Ct values that were lower than 35 could provide reasonably strong estimations of cercariae abundance in the water samples (Figure 2.6).

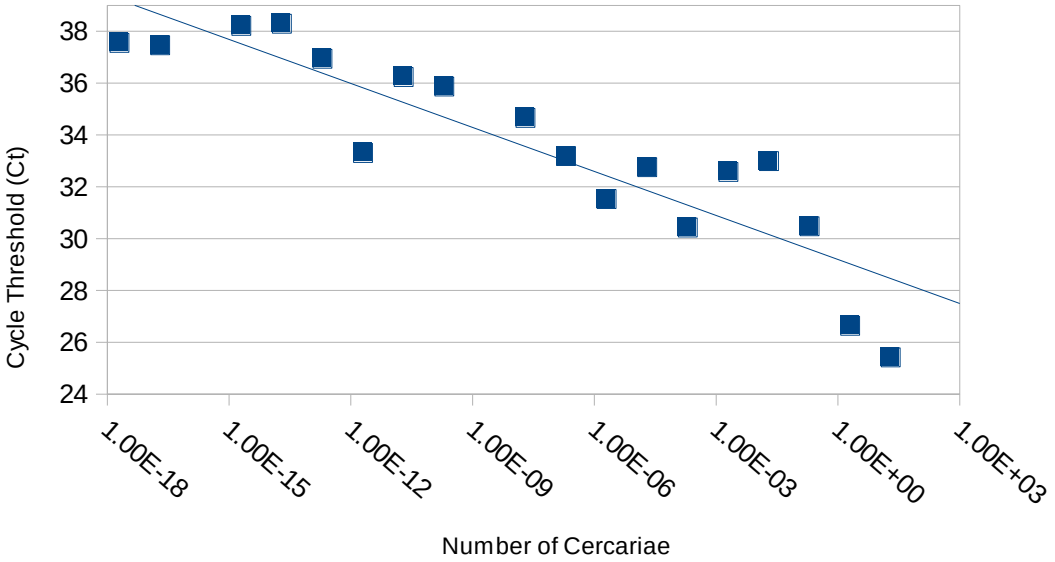

Figure 2.6. Calibration curve of 10X serial dilutions of cercariae DNA. The trend line shows a best line of fit (y = -0.2457886476ln(x) -29.1951281697, R^2 = 0.81).

The qPCR technique showed 88.9% accuracy when compared to the observed field collections (Table 2.4 and Figure 2.7). The PRPND008 samples, which were observed to be contaminated with dark pigments, failed to produce any detectable PCR products in the qPCR analyses, and were therefore excluded from the accuracy rating.

Table 2.4. Quantitative PCR results for water body *Ribeiroia ondatrae* eDNA. The n represents the number of samples (out of 10) that produced a detectable qPCR signal. The qPCR Verdict shows whether the test indicated that the site was positive or negative. The Rib history and 2012 status show if the parasite was observed in the frog or snail host at the dates indicated.

Site	Mean Cercariae	Std. Error Min	Std. Error Max	n	qPCR Verdict	Rib History	2012 Rib status
Frog†	5.54079E-06	3.08E-07	9.96E-05	2	Positive	Present 2011	Present
PRPND007	1.45826E-08	2.26E-11	9.4E-06	8	Negative	Absent 2011	Absent
PRPND008*	N/A	N/A	N/A	0	Unknown	Absent 2011	Present
Murky Bullfrog†	7.54692E-07	5.19E-10	0.001098	10	Positive	Present 2011	Present
VPPND006†	0.000701833	1.95E-10	2521.107	6	Positive	Present 2011	Present
Pond 3	9.0889E-13	2.33E-14	3.54E-11	3	Negative	Absent 2010	Absent
Pond A	2.47223E-13	4.33E-16	1.41E-10	8	Negative	Absent 2010	Absent
Pond C	6.05086E-13	3.18E-15	1.15E-10	7	False Negative	Absent 2010	Present
Pond D	8.94957E-09	1.66E-10	4.82E-07	9	Positive	Present 2010	Present
Glenridge Ponds††	1.11508E-07	3.16E-08	3.94E-07	4	Positive	Present 2010	Present
Alf Hole Goose Sanctuary	1.02003E-05	4.06E-10	0.256036	9	Positive	unknown	unknown

* None of the replicates of PRPND008 resulted in viable data.
† Filters were completely degraded in ethanol
††Filters were semi-viscous due to degradation in ethanol

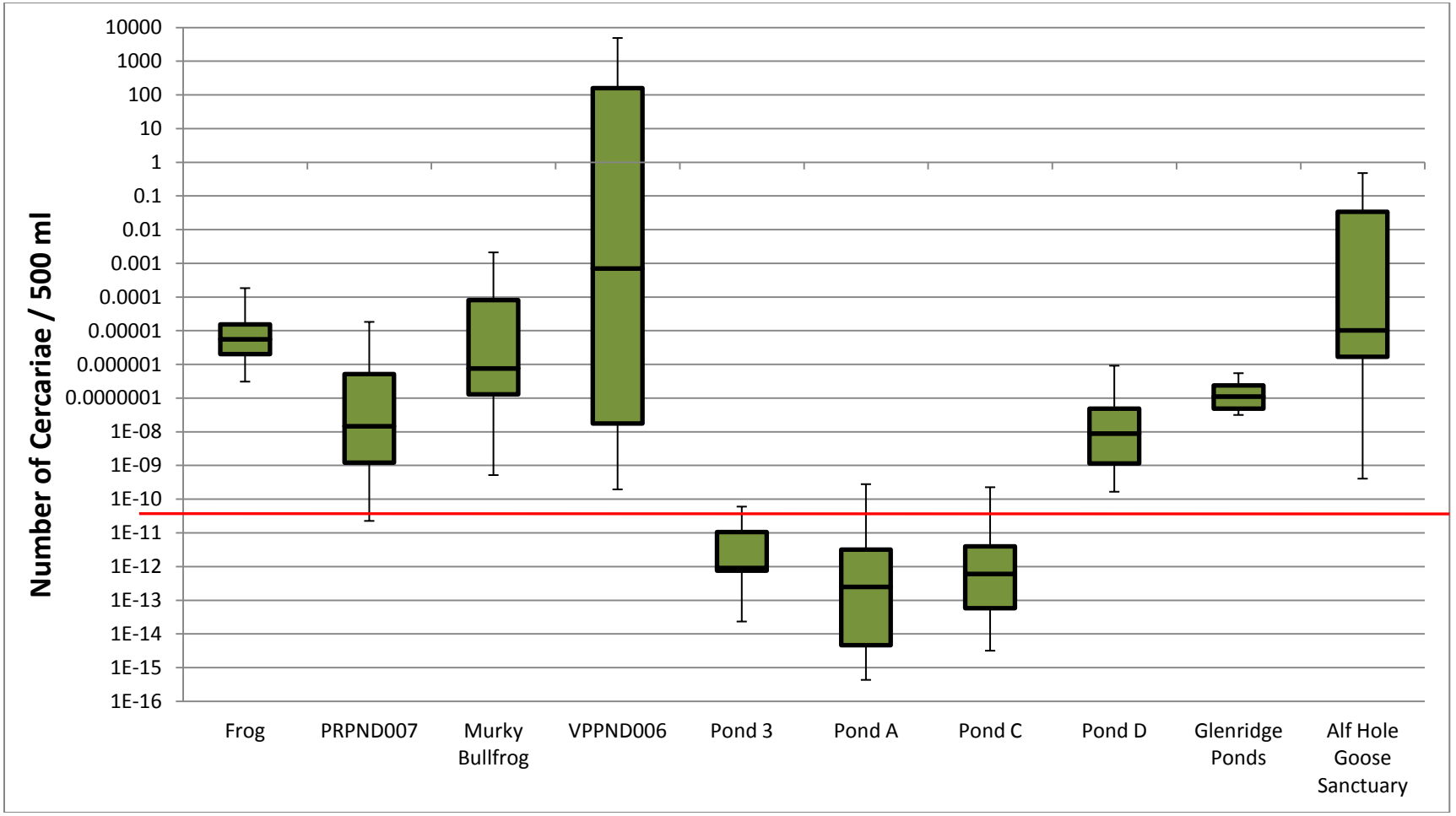

Figure 2.7. Quantitative real time PCR results of water body eDNA. Box plots contain the mean number of cercariae per filter, 25% and 75% confidence intervals as well as the standard error bars. The red line represents the cut off value of $5.54X10^{-11}$ cercariae. Any data range that crosses below this line indicates a negative site. None of the replicates of PRPND008 worked in the reactions so no data is available for this site

The results of the Generalized Linear Mixed Model statistical analysis showed that there was a significant difference in the qPCR value between the two pond types (Rib 1 versus Rib 0): $F_{1,63} = 4.634$, $P = 0.035$. Infected sites had a mean qPCR Ct value of 33.0063±0.34644 (n=46) and clean sites had a mean Ct of 35.1995±0.44923 (n=19) (Figure 2.8).

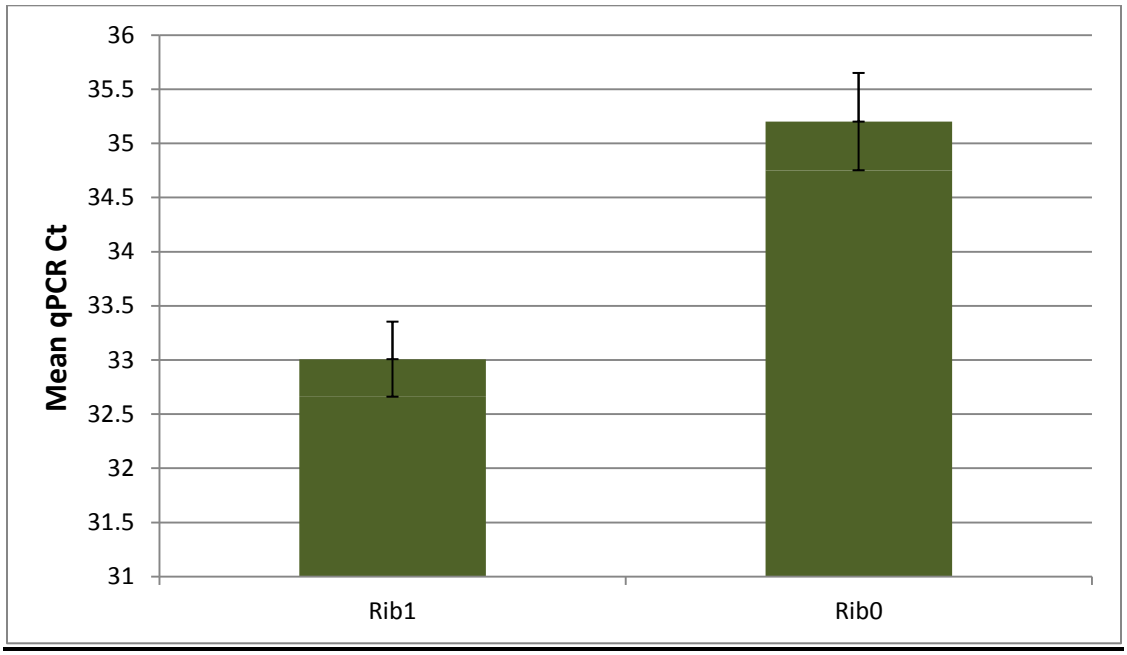

Figure 2.8. Generalized Linear Mixed Model statistical analysis. Statistically significant difference in mean (± S.E.) qPCR values between *Ribeiroia ondatrae* positive (Rib1) and negative (Rib0) sites.

2.2.7 CLONING *RIBEIROIA ONDATRAE ITS-2* GENE FOR SEQUENCING

The product sequence resulted in a 100% identity match with the desired target sequence (Fig 2.9).

A. Ro-ITS 3 primer set amplicon

AAAATGAAGCAACAGTAACGAAACGTGCATGGTATGGAATTACCTCAAAGATCATATGTGAGCCGACTAGAAGCGCCAAACTCACTGAT
AATCAAGTACCAACCCGAGCATAGCTCCACCCGTAGTCATATGCATGCCGACTAAATCGCCAAACACG

B.

```
Sequence       39     AAAATGAAGCAACAGTAACGAAACGTGCATGGTATGGAATTACCTCAAAGATCATATGTG  98
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R. ondatrae    354    AAAATGAAGCAACAGTAACGAAACGTGCATGGTATGGAATTACCTCAAAGATCATATGTG  295

Sequence       99     AGCCGACTAGAAGCGCCAAACTCACTGATAATCAAGTACCAACCCGAGCATAGCTCCACC  158
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R. ondatrae    294    AGCCGACTAGAAGCGCCAAACTCACTGATAATCAAGTACCAACCCGAGCATAGCTCCACC  235

Sequence       159    CGTAGTCATATGCATGCCGACTAAATCGCCAAACACG  195
                      |||||||||||||||||||||||||||||||||||||
R. ondatrae    234    CGTAGTCATATGCATGCCGACTAAATCGCCAAACACG  198
```

Figure 2.9. A. Ro-ITS 3 primer set amplicon. B. BLAST pair wise alignment of the Ro-ITS 3 primer set amplicon resulted in a 100% identity to ITS-2 gene in *R. ondatrae* Genbank ref: gb|AY761142.1|

2.3 eDNA DISCUSSION

2.3.0 eDNA DEGRADATION

As discussed earlier, DNA will degrade over time in aquatic environments, lasting less than one month in most cases (Dejean *et al.*, 2011). Surprisingly, the 164bp target used in this paper was detectable 100% of the time regardless of the experimental temperatures (20ºC and 25 ºC) and degradation time (10 days and 21days) to which the sample was exposed. The success of the detection method over the range of times and temperatures in this experiment could be a consequence of the sensitivity of the primers as well as the stability of the DNA in the water. However, it is worth noting that the DNA in those experiments was dissolved in clean 90% de-chlorinated tap water, rather than in pond water, which likely contains many compounds that can either facilitate DNA degradation or inhibit detection by PCR-based methods. Nevertheless, eDNA was readily detected within pond water, which indicates at least that it can persist in the natural environment. The ease of detecting the target sequence may also reflect an innate stability of the rDNA; being located within the nucleolus, rDNA is more densely packed and surrounded by many proteins (e.g. ribosomes). As the cells degrade in the water, the rDNA may be somewhat more protected than other portions of the genome. The other (more likely) explanation for the ease of detection of the target DNA is its relative abundance compared to other sequences within the genomic DNA. The ITS-2 sequence is tandemly repeated within the ribosomal DNA, often in thousands of copies, and therefore has a higher probability than most other sequences that at least some of the target sequence will be intact after prolonged exposure in an aquatic environment (Prokopowich *et al*, 2002).

2.3.1 PERFORMANCE OF THE END POINT PCR TECHNIQUE

The primer set designed by Reinitz *et al.* (2007) (designated as RoITS 3 in this paper) proved moderately effective (70% accuracy) for detecting DNA in an aquatic environment. The end point PCR technique was able to detect as little as 1/2 500 000th of a single cercaria under optimal conditions. Under natural pond water conditions, the technique was unable to detect a single cercaria two thirds of the time. These results compare well to other studies using eDNA to detect difficult-to-locate organisms. Ficetola *et al.* (2008) similarly encountered a high percentage of false negatives when trying to detect invasive frogs in low density areas. The high rate of false negatives (often caused by low densities of eDNA or inhibitors [see Section 2.3.3.1]) are why multi-tube PCR reactions are required when performing eDNA extractions.

2.3.2 PERFORMANCE OF THE QPCR TECHNIQUE

The cut off value of $5.54X10^{-11}$ cercariae ($1.14X10^{-11}$ ng/µl DNA) or less (Ct >35) resulted in a statistically significant (P = 0.035) representation of what was observed in the water body. This method worked well to quantify the amount of eDNA observed in a water sample. Ct levels above 35 were considered negative and were best explained by non-specific amplification or background fluorescence of the dyes which reflected background noise rather than the presence of target DNA (Kirshtein *et al,* 2007).

2.3.3 TROUBLE SHOOTING

2.3.3.1 INHIBITORS IN THE PCR

Many chemicals used during sample processing and collected alongside environmental samples will negatively interact with PCR reactions. Some of these environmental factors will persist during DNA purification causing PCR inhibition. There are three main modes of inhibition that can impede the PCR process, which can all operate together to impact PCR amplification (Wilson, 1997). Firstly, agents can bind directly to DNA (single- or double-stranded), preventing the DNA from being involved in the amplification. Secondly, agents can also interact with the DNA polymerase, which will prevent DNA amplification due to restriction of access of the template to the enzyme (Matheson *et al.* 2010, and Wilson, 1997). And thirdly, agents that reduce the Mg^{2+} ability to operate as a cofactor will prevent the binding of DNA to the DNA polymerase, thus reducing or preventing PCR amplification. Most contaminants related to sample processing such as phenol, ethanol and isopropanol (which will bind to, and condense DNA), excess salts such as KCl, NaCl, and ionic detergents such as sodium deoxycholate, sarkosyl and SDS, are usually removed during the normal DNA extraction and purification steps (Matheson *et al.*, 2010; Wilson, 1997). Some contaminants such as humic acids, which are found in soils and sediment, are very resistant to purification and inhibit PCR amplification by binding to both the DNA template and DNA polymerase (Matheson *et al.*, 2010; Wilson, 1997). The effect of humic acid inhibition can be reduced by adding 400ng/µl bovine serum albumin (BSA) or 150 ng/µl T4 gene 32 protein during DNA extraction or added to the PCR mix (Kreader, 1996). BSA added to the PCR reactions in my experiment did not seem to improve my ability to detect the parasite in

PRPND008 samples, which based on microscopic analyses, contained *R. ondatrae*, which suggests that factors other than humic acid may have interfered with these water samples.

2.3.3.2 CROSS CONTAMINATION

Cross contamination was a real challenge during these analyses, which resulted in a positive negative control in many of my early tests. To prevent cross-contamination in the field, separate C.H.E.A.P. filtration units were used for each site. The problem of cross-contamination became apparent once the samples were being manipulated in the lab. Negative controls (no template) are very important to ensure that contaminated samples are not read as false positives. One reason why cross-contamination is so rampant is that short PCR products can persist on dark dry surfaces (such as work benches, drawer handles, fridge doors, and sample boxes) almost indefinitely and can aerosolize quite easily (usually from opening sample tubes). For this reason, it is very important to ensure that positive PCR products are kept away from the pre-PCR preparation areas or that the area is sterilized between steps and samples. Once cross-contamination was observed to be an issue in this experiment, all manipulations were performed in a laminar flow fume hood and all equipment and water was UV irradiated for 15-30 minutes between steps and prior to use. This technique seemed to greatly reduce the chances of cross-contamination.

2.3.3.3 EXPLANATION OF POTENTIAL 'FALSE' POSITIVES

Based on the findings of the experiments performed in this project, there is very little chance for false positives to occur using this method (0% of performed tests). As

discussed in section 2.3.3.2, cross-contamination can result in the appearance of a false positive but the negative control will allow the researcher to identify the error quite readily. The appearance of 'false' positives may occur when a water sample shows a positive result but one or more of the host species are parasite-free or absent from the water-body altogether. The major weakness of the current method is that it does not indicate which parasite life cycle stage is present in the water-body - it merely indicates the presence or absence of the parasite target sequence. This implies that the test may be detecting one or more of the following: 1. eggs or miracidia shed into the water-body by the feces of an adult host; 2. cercariae shed by snails in a water-body; or 3. metacercariae in decomposing frog or fish hosts. Migratory birds leaving *R. ondatrae* infected water-bodies can easily shed eggs on their journey. If the hatching miracidia do not find a suitable host, the life cycle will not be completed. In a water body that has infected snails but no frogs, the lifecycle could still be maintained if there are suitable fish, and piscivorous mammal or bird hosts. Sites such as these may be overlooked due to the lack of frog deformities. Alternatively, they may have such high infections that malformed metamorphs are absent for most of the year and difficult to detect. One method to determine which stages of the lifecycle are present in an aquatic habitat would be to examine environmental RNA (eRNA). This method would have an even smaller temporal window of collection due to the rapid degeneration of RNA. If future researchers determined species specific genes that are up-regulated during specific life stages, the detection of specific parasite stages in the water-body should be possible (Juthikumar *et al.*, 2010).

2.3.4 PERFORMANCE OF THE C.H.E.AP. FILTRATION SYSTEM

The C.H.E.A.P. filtration system was able to provide efficient collection of eDNA from the water-column in the field. The filter extraction protocol above provided effective extraction of eDNA from the filter. The results of the eDNA experiments matched parasite infection observations in the field. The great benefit of this method was the reduction in transport costs by shipping small filters in small quantities of ethanol over shipping many liters of water. Another benefit of this method was the preservation provided by the alcohol which helped reduce further degradation of DNA that would have occurred if the samples were shipped as pure water samples.

The downside of the method outlined in Section 2.1.3 is that the ethanol appears to react with the cellulose nitrate filters on occasion. If the filter was fully dissolved, filtering remaining ethanol worked well for extraction (66% accurate for end-point PCR and 100% for qPCR). If the filters were only partially degraded (as observed in Glenridge pond samples) end-point PCR did not detect the target sequence. Conversely the qPCR technique was able to detect the target sequence accurately (Table 2.3 and 2.4). These filters likely degraded due to heat and direct sunlight during travel.

2.3.4.1 POTENTIAL MODIFICATIONS FOR FUTURE FILTRATION PROTOCOLS

After using the outlined method to collect and filter eDNA there are some modifications to future applications that should be considered to enhance DNA collection. The storage of filters can be adjusted in three ways to prevent damage to the filter: 1. Filters could be stored in a lower concentration of ethanol (70%) to reduce the chances of degradation; 2. Filters could be stored on dry ice in bags or containers until

further processed. This option may be awkward to conduct in the field and may not be practical; and 3. Filters can be air dried to desiccate the DNA in the filters until further processed. This method is the cheapest and would reduce shipping cost further. The drawback to air drying the filters is the greater chance of cross-contamination when handling by making the DNA particles airborne. The other option is to choose a different filter (Such as nylon) to collect the DNA which does not react with ethanol but still binds DNA passing through it. The filtration unit itself should be modified to include a quick release valve to relieve the air pressure to allow for easy removal of the filter retaining unit if the filter becomes clogged.

2.3.4.2 OTHER POTENTIAL USES FOR THE C.H.E.AP. FILTRATION SYSTEM

The C.H.E.A.P. filtration unit can be utilized for a variety of other purposes such as collecting proteins, RNA, and other particulates. Filter membranes should be chosen according to the binding properties of target particles to ensure optimal collection. This system can also be used for collecting multiple target eDNA sequences while conducting biodiversity research in various water-bodies for which multiple PCRs can be used to collect multi-organism information from the same sample. In the case of *R. ondatrae* research, multiple PCR target techniques could help determine the presence and biomass of potential hosts in the water-body along with parasite presence. This information can allow researchers to better estimate the threat level posed by the parasite in that water-body.

2.3.5 CONCLUDING REMARKS

Obtaining a better understanding of *R.* ondatrae's distribution within North America is imperative to help predict and prevent amphibian declines. While large-scale patterns of *Ribeiroia ondatrae* infections are not yet fully understood, they have been linked to local amphibian population declines (Koprivnikar *et al.*, 2012). *Ribeiroia ondatrae* is a serious pathogen that causes gross morphological pathology and high mortality in infected amphibian populations. The methods outlined in this paper can provide researchers with an efficient means to calculate the distribution and threat levels posed to water-bodies throughout North America. Compared to the traditional method of screening water-bodies for *R. ondatrae* infections, this technique offers six major advantages. 1. This technique does not require sampling permits to collect host organisms. 2. Filters are much easier to transport compared snails or amphibians (dead or alive). 3. This technique offers faster field collection compared to finding and collecting host organisms. 4. Performing PCR-based techniques for multiple sites is faster than examining hosts for parasites, especially when one considers that the host population may have a low prevalence of infection requiring many samples to be dissected and examined to ensure an accurate representation of parasite's hold on the water-body. 5. This technique allows for a representative estimate of parasite quantity within the water-body. 6. In theory, using the same filter samples, this method could be used to detect the presence (and biomass) of the host species in the water-body by designing host-specific primers. Knowing that a water body has been contaminated by *R. ondatrae* can help researchers determine threat levels to amphibian populations in the future even if the water body does not contain a full lifecycle yet. For example, if an infected bird delivers infected fecal

material into a water-body after frog metamorphosis, that cohort of amphibians will unlikely to acquire any cercariae that infected snails could produce that year. However, snails infected by the parasite can over winter and infect next year's cohort of amphibians. Even if the water-body produces no amphibians in the following year, infected snails can live for up to 2-3 years. The overwintering of the snail host means that a site could still maintain an incomplete lifecycle for years until the situation allows for a full *R. ondatrae* lifecycle to establish in the water-body. This information is important because as little as 10 cercariae can prove fatal to pre-limb bud tadpoles and some species of frog (including *L. sylvaticus*) exhibit high mortality rates when exposed to increasing levels of *R. ondatrae* infection (Johnson *et al.* 2012). Changes in *R. ondatrae* presence or intensity in these water-bodies could harm amphibian populations, which can result in a shift in the food web and a cascade of events that could disrupt the entire local ecosystem. The techniques described in this paper provide a time efficient and cost effective way to screen a large number of water bodies with a reasonable amount of certainty to determine threat levels posed to amphibian populations by this pathogenic parasite.

3. MALFORMATIONS AND RETINOIC ACID(RA)

3.0 RETINOIC ACID BACKGROUND

Retinoic acid is an important mediator of early limb development that controls anteroposterior spatial patterning of the tadpole limb bud. Retinoic acid is derived from retinol via enzymatic activity. The speed by which retinol can be transformed into RA is typically limited by the amount of *Raldh1-3* (a.k.a. *Aldh1a1-3*) enzymes available within each tissue during various stages of development (McEwan *et al.,* 2011). In frogs, *Raldh1* (*Aldh1a1*) has not been observed during hind-limb development (McEwan *et al.,* 2011). *Raldh2* is expressed in the proximal limb bud in anurans until Nieuwkoop and Faber stage 54 (when all digits can be seen in the autopod) and thereafter, expression ceases in that portion of the developing limb (McEwan *et al.,* 2011). *Raldh2* (*Aldh1a2*) is also expressed between the digits of the forming autopod from stage 52-55 in anurans (McEwan *et al.,* 2011). *Raldh3* (*Aldh1a3*) is expressed after stage 52, along the distal portion of the hind limb to where digit IV is formed and in patches surrounding the other digits as well throughout the autopod's development (McEwan *et al.,* 2011).

There are several proteins that help regulate RA production and transport. CYP26 (a cytochrome p450 hydroxylase) degrades RA to prevent RA from persisting beyond the necessary period for it to induce its target developmental genes (Marletaz *et al.,* 2006; McEwan et al. 2011). Knock outs of CYP26 have been shown to cause pathology similar to excess RA (Marletaz *et al.,* 2006; McEwan *et al.,* 2011). RA signals are also regulated by cellular retinol binding proteins (CRBPs) and retinoic acid binding proteins (CRABPs) (Marletaz *et al.,* 2006). Crabp1 binds to free RA, which helps prevent RA from interacting with non-target proteins (McEwan *et al.,* 2011). Crabp2 enhances the

ability of RA to bind or interact with specific transcription factors, which in turn bind to retinoic acid response elements (RAREs) within a variety of genes' regulatory sequences (McEwan *et al.,* 2011).

The above interactions help form RA gradients in developing and regenerating organisms (McEwan *et al.*, 2011). Retinoids themselves interact with retinoic acid receptors (RARs) and retinoid X receptors (RXRs). For both RARs and RXRs, there are three isoforms, known as alpha (α), beta (β), and gamma (γ) type receptors. Once a RA molecule binds to an RAR, the receptor-ligand complexwill heterodimerize with an RXR. The heterodimer will then bind with retinoic acid response elements (RAREs), which then regulate the expression of many genes such as, *Fgf-8* (Han and Kim, 2002), *shh, lmx-1,En-1, fgf-4, wnt-7a* (Stratford *et al.*, 1999), *Hox* genes, *HNF-3α, Cdx1*, and *CRABP1*and*2* (Marletaz *et al.*, 2006) during limb development and regeneration. Retinoids have been shown to affect regeneration ability in a variety of amphibians such as the axolotl (*Ambystoma mexicanum*)(Maden and Hind, 2003; Satoh *et al.*, 2010; Yakushiji *et al.,* 2009), *Xenopus laevis* (Lynch *et al.*, 2011; Yakushiji *et al.*, 2009), and common frog (*Rana temporaria*) (Maden and Corcoran, 1996; Maden and Hind, 2003), but also chickens (Reijntnes *et al.*, 2010), quail (Stratford *et al.*,1999), mice (Zeller *et al.*, 2009) and humans (Blomhoff and Blomhoff, 2005). In order to fully understand the effect of *Ribeiroia ondatrae* infections on the host's RA pathways during limb development, I must determine the up- and down-regulation of the *RAR* and *RXR* gene suite as well as *Aldh1a2* and *Aldh1a3*.

3.1 METHODS AND MATERIALS

3.1.1 ANIMAL CARE AND TREATMENTS

3.1.1.1 *LITHOBATES SYLVATICUS* COLLECTION AND CARE

Lithobates sylvaticus egg clutches were collected in May 2011 from Brandon Manitoba. Eggs were kept in 10 gallon tanks in aerated and dechlorinated tap water and allowed to develop. Tadpoles were fed Nutrifin flaked fish food *ad libitum*. Animals were kept in a 16:8 hour light:dark cycle to reflect natural summer light cycles.

3.1.1.2 *LITHOBATES SYLVATICUS* TREATMENTS AND MANIPULATION

Two rounds of larval manipulation were performed. In the first round of experiments, wood frog tadpoles were infected or manipulated at Gosner stage 26-27 (Gosner, 1960), which exhibits the beginnings of a limb bud and has been shown to be the optimal window to induce deformities with highest survivorship of individuals (Schotthoefer *et al.,* 2003). Three hundred tadpoles (60 per treatment) were randomly selected and placed into 5 manipulation groups: negative controls; jab controls; *R. ondatrae*-infected; 150-200µm glass bead implants (approximating the size of the parasite cyst; see Session *et al.*, 1990); and retinoic acid (RA) injections. All tadpoles were anaesthetised in buffered 0.1%MS-222(Sigma-Aldrich) for 2 minutes, which resulted in unresponsiveness for about 45 minutes. The negative controls did not receive any further treatment, while jab controls were poked in the developing left hind limb bud with a 29 gauge needle. Tadpoles in the parasite group were infected with 10 cercariae by

placing the cercariae and tadpole in 500ml of water overnight. Tadpoles in the glass bead injection group had 5-10 150-200µm glass beads (Sigma-Aldrich) injected into the left developing hind limb using glass capillary tubes. The retinoic acid group was injected with 150µg/g of body weight of RA (40 mg/mL RA in DMSO, Sigma-Aldrich) (Maden and Corcoran, 1996). Tadpoles were weighed individually to ensure that the proper dosage was administered. A 29 gauge syringe needle fixed onto a 10µl micro-pipette and tip was used to inject the desired amount of RA into the left developing hind limb bud. After treatment, all tadpoles were placed in individual cups containing 500ml dechlorinated water and allowed to develop. Fifteen tadpoles were sacrificed at days 7, 14, and 21 from each treatment group by immersion in buffered 1% MS-222 for 10 minutes. Ten frogs were to be used for molecular analysis and 5 for histological examinations. The molecular analysis entailed biopsies of developing limbs from the treated and untreated limb buds, as well as the 2mm surrounding region from each frog. The dissected tissues were preserved in individual tubes of RNAlater (Ambion) until they could be further analyzed. RNAlater tissues were stored at -80ºC until processed further. Histological samples were preserved in 10% buffered formalin and stored at room temperature until processed.

In a second experiment, sixty wood frog tadpoles (20 for each treatment group) were infected or bead-manipulated at Gosner stage 27-30 (Gosner, 1960). Tadpoles were randomly selected and placed into 3 manipulation groups: negative controls, *R. ondatrae*-infected, 150-200µm glass bead implants. All tadpoles were anaesthetized in buffered 0.1% MS-222 for two minutes. The negative controls did not receive any further treatment. Infected groups were infected with 20 cercariae by placing the cercariae and

tadpole in 500ml of water. Glass bead injection groups had 8-10 150-200µm glass beads injected as described above. After treatment, all tadpoles were placed in individual cups containing 500ml water and allowed to develop. Five tadpoles were sacrificed after 13 days from each treatment group and the remainder were kept until their hind limbs fully developed in order to determine the frequency of malformations and then sacrificed by immersion in buffered 1% MS-222 for 10 minutes. The first five frogs were to be used for molecular analysis and were processed in the same manner as the first experiment. The remainder were placed in 10% neutral buffered formalin to be examined for skin and bone malformations.

3.1.2 RA PATHWAY GENE PRIMER TARGET DESIGN

I planned to target the following retinoic acid pathway genes: *retinaldehyde dehydrogenases (Raldh2 and Raldh3);* and the retinoic receptors *RARα, RARβ, RARγ, RXRα, RXRβ, and RXRγ.* The genome and the aforementioned genes of *Lithobates sylvaticus* have not been sequenced, making primer design difficult. In order to acquire species-specific primers, degenerate primers had to be constructed using known sequences from other species. Degenerate primers were designed to target the genes listed above using the following methods (Table 3.1). Firstly, multiple known sequences of the target genes previously documented in other organisms were collected using the National Center for Biotechnology Information (NCBI) nucleotide database (http://www.ncbi.nlm.nih.gov/). Secondly, these sequences underwent a multiple sequence alignment using either ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) or Geneious Pro 5.5.6 software to examine similarity between the sequences. Lastly,

degenerate primers were designed using Geneious software or designed by eye if Geneious could not resolve a good primer set by choosing regions of conserved sequences, or if Geneious primers did not work when tested. Degenerate primers were then run through the Standard Nucleotide Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to see if the primers were likely to amplify non-specific genes.

Table 3.1. Primer data

Gene	Primer Name	Sequence	TmºC	Product Size
ALDH1A3	ALDH1A3 g1 fwd	TCCACAGAGGTTGGWMARCTGRT	63.5	176
	ALDH1A3 g1 rev	KGTRCAGSMYTGRCCYTGGT	59.6	
RARα	RARa MC fwd	AAGCACTGAAAGTCTACGTCCG	64.4	201
	RARa MC rev	TTAGAGTGTCCAAGCCCTCAG	63.2	
	RARa 2 fwd	AGGACCTGGAGCAGCCMG	65.8	162
	RARa 2 rev	CKGGCTGCTCCAGGTCCT	65.9	
RARβ	RARb1 fwd	GCWTGTGAGGGATGYAAGGG	63.3	153
	RARb 1 rev	GGACATKCCCACTTCAAAGCA	66.1	
Beta Actin	ACTB-1 fwd	TTCACCACCACAGCAGAAAG	63.9	298
	ACTB-2 rev	GCACAGTGTTGGCATACAGG	64.2	

3.1.3 EXTRACTION OF *LITHOBATES SYLVATICUS* RNA AND CONVERSION TO cDNA

RNA was extracted from biopsied wood frog limb bud regions using the Qiagen: Quantitect® Reverse Transcription protocol according to manufacturer's instructions. cDNA was stored at -80ºC until further processing.

3.1.4 CLONING OF *LITHOBATES SYLVATICUS* RA PATHWAY GENES FOR SEQUENCING

Gel bands of appropriate size (Table. 3.1) were extracted and then purified using

QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's instructions. The samples were then ligated into a vector using the Clone Jet Sticky end Protocol and were transformed into Sub cloning Efficiency™ DH5α Chemically Competent *E. coli* cells (Invitrogen) according to the manufacturer's protocol. Cells were incubated overnight (18 hours). The next morning, 10 colonies were chosen for PCR screening to examine if the ligation of the insert had been successful. Each colony was scraped with a pipette tip and swirled in a sterile PCR tube then rubbed onto an agar plate that had grids numbered 1-10. The PCR was run as follows: 25µl reactions (12.5µl EconoTaq PLUS GREEN 2X Master Mix, 1µl fwd primer, 1µl rev primer, 2µl template DNA, and 8.5 nuclease-free water), using the following cycling conditions: 94°C for 5 minutes followed by 40 cycles of [94°C for 30 seconds, 10°C less than Tm°C of specific primer (Table 3.1) for 30 seconds, and 72°C for 30 seconds], followed by 72°C for 5 minutes . The agar plates were incubated overnight at 37ºC. PCR products were run on a 1.5% agarose gel stained with ethidum bromide and examined for bands. Colonies that exhibited bands were then scraped off of the agar plate with a pipette tip and were inoculated into 3ml of LB Broth + 3µl ampicillin and allowed to incubate for 12 hours. Samples were then processed using the Qiagen QIAprep miniprep kit as per manufacturer's instructions. The products were then analyzed for purity and concentration using a General Electric NanoVue Spectrophotometer. Samples were diluted with nuclease-free water to ensure that a total of 200-300ng of DNA were loaded into a total volume of 7µl in a PCR tube. The samples were then shipped off to TCAG Facilities: DNA Sequencing/synthesis for DNA sequencing.

3.2 MALFORMATION RA RESULTS

3.2.1 TREATMENT MORTALITY

All treatment groups experienced varying ranges of mortality; their rates are as follows: 4.4% (n=45) mortality in control treatments, 6.6% (n=45) mortality in jab treatments, 8.3% (n=60) mortality in bead treatments, 43.3 % (n=60) mortality in RA treatments, and 16.7% (n=60) mortality in parasite treatments. A mortality analysis was performed on this data set using the Crosstabs procedure in SPSS 20.0. Each tadpole coded as 0 or 1 for early death (not due to sacrifice). Treatment codes were assigned as follows: 1 = control, 2 = RA, 3 = bead, 4 = jab, 5 = parasite. The Pearson Chi-square value was 40.393 (df=4) with a P-value of <0.0001 indicating a significant difference in mortality among treatments.

3.2.2 OBSERVED MALFORMATIONS

In experiment number two, three malformed frogs were found among the treatment groups. One parasite-treatment frog had three forearms, two trifurcating from one elbow. Another parasite-treatment subject was observed with a right leg deformity which appeared to be a rotation of the foot, however, the frog was far too desiccated before being placed in formaldehyde to confirm. One bead-treatment subject had a rotation of the left foot. Another bead-treatment frog appeared to have a misshapen body and eye on the right side with two beads observed on the pectoral girdle. This specimen however was quite desiccated before being placed in formaldehyde so a thorough examination was not possible.

3.2.3 PRIMER SET RESULTS

Degenerate primers were used to PCR-amplify *RARγ, Aldh1a3,* and beta-actin from tadpole cDNA (Figures, 3.1 - 3.3). In addition to amplifying the intended genes, other primer sets amplified some unintentional targets, including the chloride channel *clns1a*, and SRY-box 11 (SOX11) (Figures 3.4 and 3.5). Degenerate primers were unsuccessful in amplifying *RARα, RARβ, RXRα, RXRβ, RXRγ*, and *Aldh1a2*.

A. RARa MC primer set amplicon

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GGTGGCATCTCGTAGCTGTCTGATACCACCACCTCTTCTTTCACCTCTTTCTTCTTTTTGTTCCTGTCATTTCGCACCGCTATACCACT
CCCCCAGCTGCATGGACCTTCTTTTGACATGCCAACCTGGAAACATTTTTGTAGTCGGCAAAACTGGCAGCGATTCCGTGTCACCTTAT
TGATTTGGCAATTC
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B.

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L. sylvaticus  1      GGTGGCATCTCGTAGCTGTCTGATACCACCACCTCTTCTTTCACCTCTTTCTTCTTTTTG  60
                      || |||||||| |||||||||| |||||||||||| ||||| |  || |||||||| |||
X. tropicalis  659    GGGGGCATCTCATAGCTGTCTGGTACCACCACCTCCTCTTTTATTTCCTTCTTCTTCTTG  600

L. sylvaticus  61     TTCCTGTCATTTCGCACCGCTATACCACTCCCCCAGCTGCATGGACCTTCTTTTGACATG  120
                      || ||||| |||| ||||||                              ||||| |||||
X. tropicalis  599    TTTCTGTCGTTTCTCACCGCC----------------------------TCTTTAGACATT  567

L. sylvaticus  121    CCAACCTGGAAACATTTTTGTAGTCGGCAAAACTGGCAGCGATTCCGTGTCACCTTATTG  180
                      || |||||||| ||||| || |||||||||||||||| ||||||||||||||||||||
X. tropicalis  566    CCGACCTGGAAGCATTTCTGCAGTCGGCAAAACTGGCAACGATTCCGTGTCACCTTATTG  507

L. sylvaticus  181    ATTTGGCAATTC  192
                      |||||||| |||
X. tropicalis  506    ATTTGGCAGTTC  495
```

C.

```
L. sylvaticus  1      GGTGGCATCTCGTAGCTGTCTGATACCACCACCTCTTCTTTCACCTCTTTCTTCTTTTTG  60
                      || |||||||| |||||||||| |||||||||||| ||||| |  || |||||||| |||
X. tropicalis  563    GGGGGCATCTCATAGCTGTCTGGTACCACCACCTCCTCTTTTATTTCCTTCTTCTTCTTG  504

L. sylvaticus  61     TTCCTGTCATTTCGCACCGCTATACCACTCCCCCAGCTGCATGGACCTTCTTTTGACATG  120
                      || ||||| |||| ||||||                              ||||| |||||
X. tropicalis  503    TTTCTGTCGTTTCTCACCGCC----------------------------TCTTTAGACATT  471

L. sylvaticus  121    CCAACCTGGAAACATTTTTGTAGTCGGCAAAACTGGCAGCGATTCCGTGTCACCTTATTG  180
                      || |||||||| ||||| || |||||||||||||||| ||||||||||||||||||||
X. tropicalis  470    CCGACCTGGAAGCATTTCTGCAGTCGGCAAAACTGGCAACGATTCCGTGTCACCTTATTG  411

L. sylvaticus  181    ATTTGGCAATTC  192
                      |||||||| |||
X. tropicalis  410    ATTTGGCAGTTC  399
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D.

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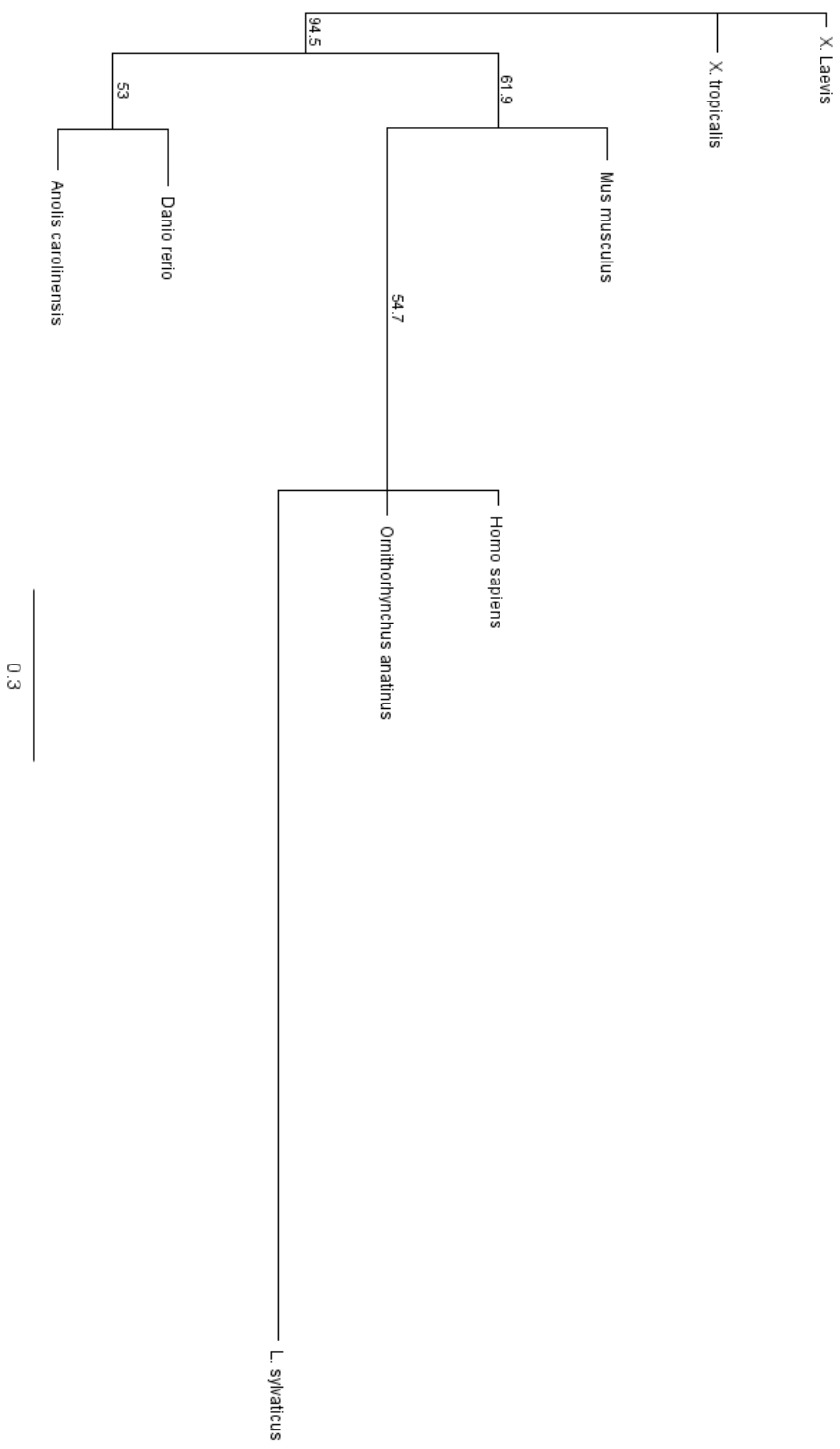
L. sylvaticus  1      GGTGGCATCTCGTAGCTGTCTGATACCACCACCTCTTCTTTCACCTCTTTCTTCTTTTTG  60
                      || |||||||| |||||||||| |||||||||||| ||||| |  || |||||||| |||
X. tropicalis  901    GGGGGCATCTCATAGCTGTCTGGTACCACCACCTCCTCTTTTATTTCCTTCTTCTTCTTG  842

L. sylvaticus  61     TTCCTGTCATTTCGCACCGCTATACCACTCCCCCAGCTGCATGGACCTTCTTTTGACATG  120
                      || ||||| |||| ||||||                              ||||| |||||
X. tropicalis  841    TTTCTGTCGTTTCTCACCGCC----------------------------TCTTTAGACATT  809

L. sylvaticus  121    CCAACCTGGAAACATTTTTGTAGTCGGCAAAACTGGCAGCGATTCCGTGTCACCTTATTG  180
                      || |||||||| ||||| || |||||||||||||||| ||||||||||||||||||||
X. tropicalis  808    CCGACCTGGAAGCATTTCTGCAGTCGGCAAAACTGGCAACGATTCCGTGTCACCTTATTG  749

L. sylvaticus  181    ATTTGGCAATTC  192
                      |||||||| |||
X. tropicalis  748    ATTTGGCAGTTC  737
```

Figure 3.1a. BLAST pair wise alignment of the RARa MC primer set amplicon(A) resulted in a 75% identity to RARγ-A-like receptor in the following three *X. tropicalis* sequences. B. Genbank ref|XM_002936635.1|24701 C. Genbank ref|XM_002936634.1|23741 D. Genbank ref|XM_002936633.1|27121



3.1b.Tamura-Nei Neighbor-Joining tree (1000 Bootstap;Geneious Pro 5.6.5.) of RARg genes against L. sylvaticus sequence. Sequences compared: X. laevis (NCBI Reference Sequence: NM_001088194.1); X. tropicalis (NCBI Reference Sequence: XM_002936635.1); Mus musculus (NCBI Reference Sequence: NM_001042727.1); Homo sapiens (NCBI Reference Sequence: NM_000966.5); Ornithorhynchus anatinus (NCBI Reference Sequence: XM_001518383.2); Danio rerio (NCBI Reference Sequence: NM_131339.1); Anolis carolinensis (NCBI Reference Sequence: XM_003216663.1).

A. ALDH1A3 primer set amplicon:

TGGTACAGCCCTGGCCCTGGTTGAAGAAAACTCCTTGATGGGCACATTCCACAGCCAGCTCCATGTCGGCG
TCTTTAAACACAATACATGGATTTTTCCCACCGAGCTCCAGGGTGACGCGCTTCAAATTGCTGGCAGAAGC
GGCTTCTTTAACCAGTTGACCAACCTCTGTGGAATC

B.

```
L. sylvaticus  3      GTACAGCCCTGGCCCTGGTTGAAGAAAACTCCTTGATGGGCACATTCCACAGCCAGCTCC  62
                      || ||||  ||| || |||||||||| |||||||||| ||||||| ||||||||||
X. tropicalis  944    GTGCAGCACTGACCTTGGTTGAAGAAGACTCCTTGATGAGCACATTCTACAGCCAGCTCT  885

L. sylvaticus  63     ATGTCGGCGTCTTTAAACACAATACATGGATTTTTCCCACCGAGCTCCAGGGTGACGCGC  122
                      | ||| | ||||  |||||| || || |||||||||| |||||||||  ||||| ||
X. tropicalis  884    AAGTCAGAGTCTGCAAACACGATGCAGGGATTTTTCCCTCCGAGCTCCAAAGTGACACGT  825

L. sylvaticus  123    TTCAAATTGCTGGCAGAAGCGGCTTCTTTAACCAGTTGACCAACCTCTGTGGA  175
                      |||| ||| ||   |||||||||||| |||| |||||  ||||||||||||||
X. tropicalis  824    TTCAGATTACTTCTAGAAGCGGCTTCCTTAATCAGTTTTCCAACCTCTGTGGA  772
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C.

```
L. sylvaticus  3      GTACAGCCCTGGCCCTGGTTGAAGAAAACTCCTTGATGGGCACATTCCACAGCCAGCTCC  62
                      || ||||  ||||| |||||||||| |||||||||| |||||||| ||||||||||
X. laevis      1016   GTGCAGCATTGGCCTTGGTTGAAGAAGACTCCTTGATGAGCACATTCTACAGCCAGCTCT  957

L. sylvaticus  63     ATGTCGGCGTCTTTAAACACAATACATGGATTTTTCCCACCGAGCTCCAGGGTGACGCGC  122
                      | ||| |  |||  ||||||||| |||||||||||||| |||||||||  ||||| ||
X. laevis      956    AAGTCAGAATCTGCAAACACAATGCATGGATTTTTCCCTCCGAGCTCCAAAGTGACACGT  897

L. sylvaticus  123    TTCAAATTGCTGGCAGAAGCGGCTTCTTTAACCAGTTGACCAACCTCTGTGGA  175
                      |||| ||| ||   |||||||||||| || | |||||  ||||||||||||||
X. laevis      896    TTCAGATTACTCTTAGAAGCGGCTTCCTTGATCAGTTTTCCAACCTCTGTGGA  844
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D.

```
L. sylvaticus  3      GTACAGCCCTGGCCCTGGTTGAAGAAAACTCCTTGATGGGCACATTCCACAGCCAGCTCC  62
                      || ||||  ||||| |||||||||| |||||||||| |||||||| ||||||||||
X. laevis      1016   GTGCAGCATTGGCCTTGGTTGAAGAAGACTCCTTGATGAGCACATTCTACAGCCAGCTCT  957

L. sylvaticus  63     ATGTCGGCGTCTTTAAACACAATACATGGATTTTTCCCACCGAGCTCCAGGGTGACGCGC  122
                      | ||| |  |||  ||||||||| |||||||||||||| |||||||||  ||||| ||
X. laevis      956    AAGTCAGAATCTGCAAACACAATGCATGGATTTTTCCCTCCGAGCTCCAAAGTGACACGT  897

L. sylvaticus  123    TTCAAATTGCTGGCAGAAGCGGCTTCTTTAACCAGTTGACCAACCTCTGTGGA  175
                      |||| ||| ||   |||||||||||| || | |||||  ||||||||||||||
X. laevis      896    TTCAGATTACTCTTAGAAGCGGCTTCCTTGATCAGTTTTCCAACCTCTGTGGA  844
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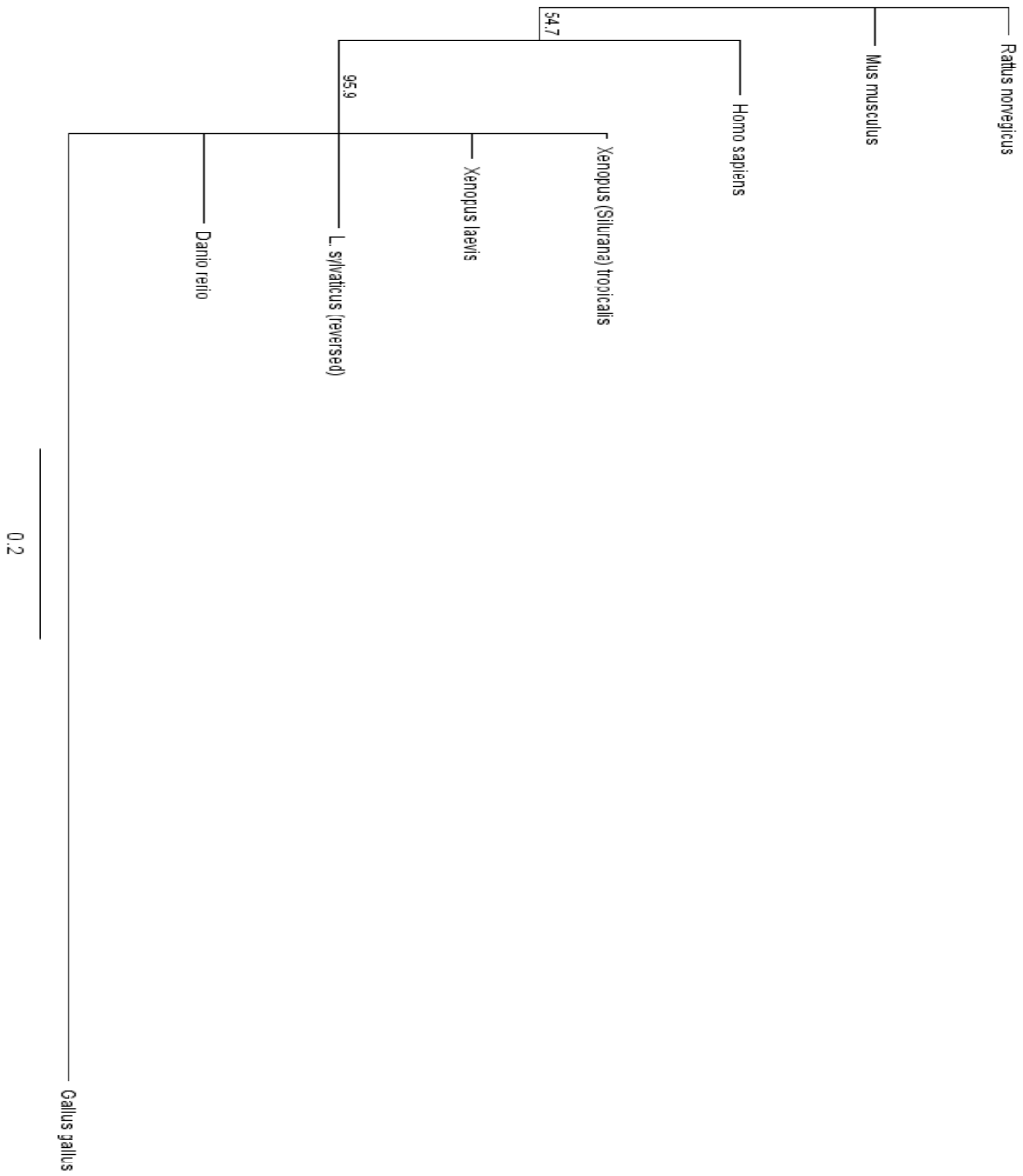
Figure 3.2a. BLAST pair wise alignment of the ALDH1A3 primer set amplicon(A) resulted in an 83% to the aldehyde dehydrogenase family 1 member A3 (ALDH1A3 or RALDH3) gene in the following sequences. B. *X. tropicalis* (ref|XM_002939264.1|20111) C. *X. laevis* (gb|BC169603.1|18971) D. *X. laevis* (gb|BC169605.1|18971).



3.2b. *Figure 3.2b. Tamura-Nei Neighbor-Joining tree (1000 Bootstap; Geneious Pro 5.6.5.) of Aldh1a3 genes against L. sylvaticus sequence. Sequences compared: Rattus norvegicus (GenBank: BC166415.1);Mus musculus (NCBI Reference Sequence: NM_053080.3); Homo sapiens (GenBank: AK302607.1), Xenopus (Silurana) tropicalis (NCBI Reference Sequence: XM_002939264.1); Xenopus laevis (GenBank: BC169603.1); Danio rerio (NCBI Reference Sequence: NM_001044745.1); Gallus gallus (NCBI Reference Sequence: NM_204669.1).*

A. Beta-actin primer set amplicon:

CAGNTTCACCACCACAGCAGAAAGAGAAATCGTGCGTGACATCAAGGAGAAGCTCTGCTACGTCGCCCTGG
ACTTCGAGCAGGAGATGGCCACCGCTGCCTCCTCCTCATCCCTGGAGAAGAGCTATGAGCTTCCCGACGGT
CAAGTCATCACCATCGGAAACGAGAGGTTCAGGTGTCCAGAGGCCCTCTTCCAGCCATCCTTCTTGGGTAT
GGAATCATGCGGNATTCACGAAACCACATTCAACTCAATCATGAAGTGCGACGTANATATCCGTAAGGACC
TGTATGCCAACACTGTGCTGTCTGGAGGCACCACCATGTACCCNGGA

B.

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L. sylvaticus  1      CAGNTTCACCACCACAGCAGAAAGAGAAATCGTGCGTGACATCAAGGAGAAGCTCTGCTA  60
                      ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R. catesbeiana 36     CAGCTTCACCACCACAGCAGAAAGAGAAATCGTGCGTGACATCAAGGAGAAGCTCTGCTA  95

L. sylvaticus  61     CGTCGCCCTGGACTTCGAGCAGGAGATGGCCACCGCTGCCTCCTCCTCATCCCTGGAGAA  120
                      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
R. catesbeiana 96     CGTCGCCCTGGACTTCGAGCAGGAGATGGCCACCGCTGCCTCCTCCTCATCCCTGGAGAA  155

L. sylvaticus  121    GAGCTATGAGCTTCCCGACGGTCAAGTCATCACCATCGGAAACGAGAGGTTCAGGTGTCC  180
                      |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
R. catesbeiana 156    GAGCTATGAGCTGCCCGACGGTCAAGTCATCACCATCGGAAACGAGAGGTTCAGGTGTCC  215

L. sylvaticus  181    AGAGGCCCTCTTCCAGCCATCCTTCTTGGGTATGGAATCATGCGGNATTCACGAAACCAC  240
                      |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
R. catesbeiana 216    AGAGGCCCTCTTCCAGCCATCCTTCTTGGGTATGGAATCATGCGGCATTCACGAAACCAC  275

L. sylvaticus  241    ATTCAACTCAATCATGAAGTGCGACGTANATATCCGTAAGGACCTGTATGCCAACACTGT  300
                      |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
R. catesbeiana 276    ATTCAACTCAATCATGAAGTGCGACGTAGATATCCGTAAGGACCTGTATGCCAACACTGT  335

L. sylvaticus  301    GCTGTCTGGAGGCACCACCATGTACCC  327
                      |||||||||||| ||||||||||||||
R. catesbeiana 336    GCTGTCTGGAGGTACCACCATGTACCC  362
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C.

```
L. sylvaticus  1      CAGNTTCACCACCACAGCAGAAAGAGAAATCGTGCGTGACATCAAGGAGAAGCTCTGCTA  60
                      ||| |||||||| ||||| ||||||||||||||||||||||||||||||| ||||||||
R. lessonae    636    CAGCTTCACCACAACAGCTGAAAGAGAAATCGTGCGTGACATCAAGGAGAAACTCTGCTA  695

L. sylvaticus  61     CGTCGCCCTGGACTTCGAGCAGGAGATGGCCACCGCTGCCTCCTCCTCATCCCTGGAGAA  120
                      |||||||||||||||||||||||||||||| || ||||||||||||||| ||||||||||
R. lessonae    696    CGTCGCCCTGGACTTCGAGCAGGAGATGGCAACTGCTGCCTCCTCCTCTTCCCTGGAGAA  755

L. sylvaticus  121    GAGCTATGAGCTTCCCGACGGTCAAGTCATCACCATCGGAAACGAGAGGTTCAGGTGTCC  180
                      |||||| ||||| || |||||||| ||||||||||||||||||||||||||||||||||||
R. lessonae    756    GAGCTACGAGCTACCTGACGGTCAGGTCATCACCATCGGAAACGAGAGGTTCAGGTGTCC  815

L. sylvaticus  181    AGAGGCCCTCTTCCAGCCATCCTTCTTGGGTATGGAATCATGCGGNATTCACGAAACCAC  240
                      |||||||||||||||||||| |||||||||||||||||||||| ||||||||||||||
R. lessonae    816    AGAGGCCCTCTTCCAGCCATCTTTCTTGGGTATGGAATCATGCGGTATTCACGAAACCAC  875

L. sylvaticus  241    ATTCAACTCAATCATGAAGTGCGACGTANATATCCGTAAGGACCTGTATGCCAACACTGT  300
                      |||||||||||||||||||||||||| ||| ||||||||||||||||||||||||||||||
R. lessonae    876    ATTCAACTCAATCATGAAGTGCGATGTAGATATCCGTAAGGACCTGTATGCCAACACTGT  935

L. sylvaticus  301    GCTGTCTGGAGGCACCACCATGTACCC  327
                      |||||||||||| ||||||||||||||
R. lessonae    936    GCTGTCTGGAGGTACCACCATGTACCC  962
```

FIGURE 3.3 CONTINUED ON NEXT PAGE

D.

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L. sylvaticus  1      CAGNTTCACCACCACAGCAGAAAGAGAAATCGTGCGTGACATCAAGGAGAAGCTCTGCTA  60
                      ||| ||||||||||||||| || |||||||| || ||||||||||||||||||||||||||
A. baerii      477    CAGCTTCACCACCACAGCCGAGAGAGAAATTGTCCGTGACATCAAGGAGAAGCTCTGCTA  536

L. sylvaticus  61     CGTCGCCCTGGACTTCGAGCAGGAGATGGCCACCGCTGCCTCCTCCTCATCCCTGGAGAA  120
                      |||||||||||||||||||||||||||||||||||||||||| ||||| || ||||||||||
A. baerii      537    CGTCGCCCTGGACTTCGAGCAGGAGATGGCCACCGCTGCTTCCTCTTCCTCCCTGGAGAA  596

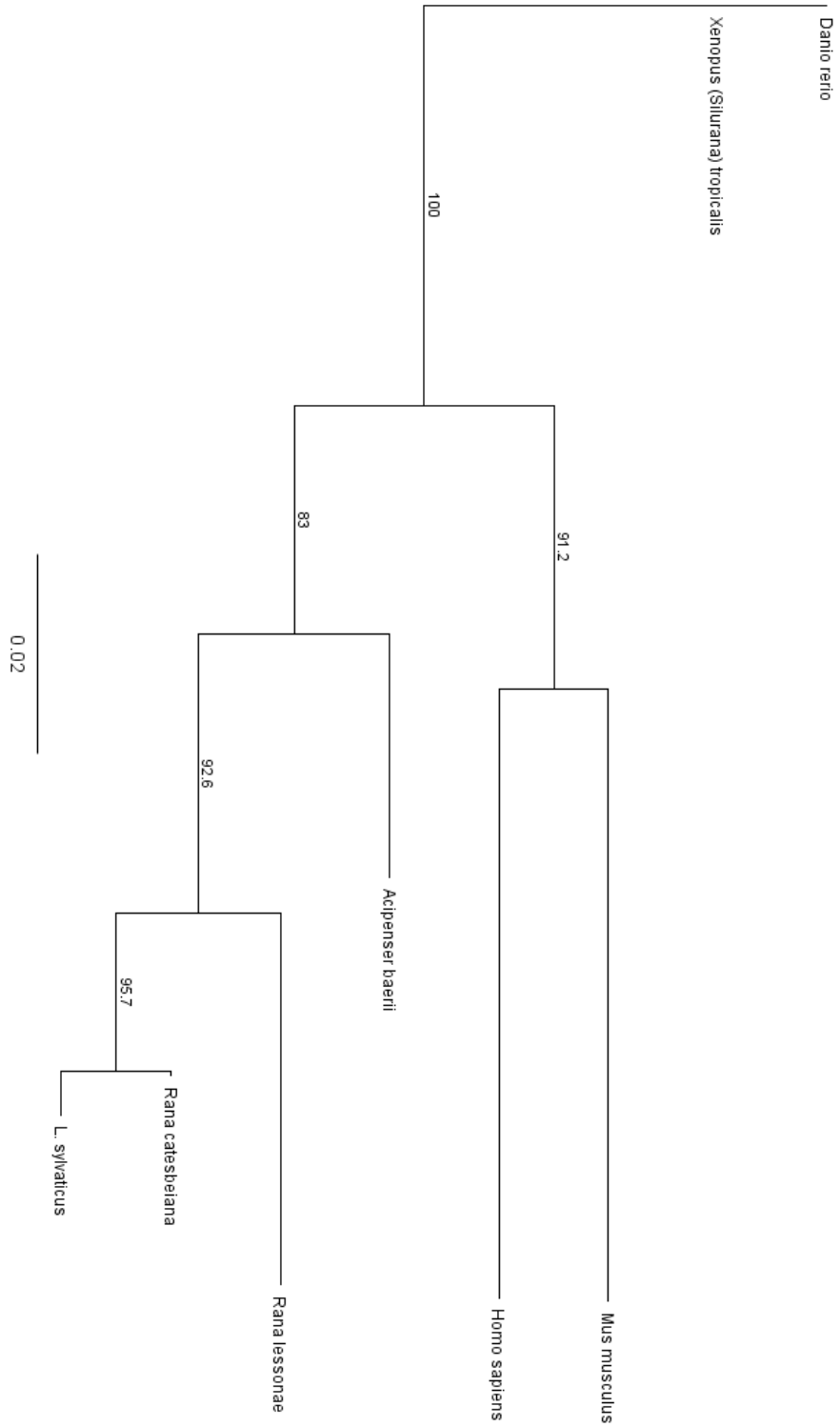
L. sylvaticus  121    GAGCTATGAGCTTCCCGACGGTCAAGTCATCACCATCGGAAACGAGAGGTTCAGGTGTCC  180
                      |||||||||||| |||||||||| |||||||||||||||||||| | |||||||| ||
A. baerii      597    GAGCTATGAGCTGCCCGACGGTCAGGTCATCACCATCGGAAACGAGCGTTTCAGGTGCCC  656

L. sylvaticus  181    AGAGGCCCTCTTCCAGCCATCCTTCTTGGGTATGGAATCATGCGGNATTCACGAAACCAC  240
                      ||||||||| |||||||||||||||||||||||||||| ||||| || || || |||||
A. baerii      657    AGAGGCCCTGTTCCAGCCATCCTTCTTGGGTATGGAATCCTGCGGTATCCATGAGACCAC  716

L. sylvaticus  241    ATTCAACTCAATCATGAAGTGCGACGTANATATCCGTAAGGACCTGTATGCCAACACTGT  300
                       |||||||| |||||||||| |||||  | ||||||||||||||||| ||||||||||
A. baerii      717    CTTCAACTCCATCATGAAGTGTGACGTTGACATCCGTAAGGACCTGTACGCCAACACTGT  776

L. sylvaticus  301    GCTGTCTGGAGGCACCACCATGTACCC  327
                       |||||||||||| ||||||||||||||
A. baerii      777    ACTGTCTGGAGGTACCACCATGTACCC  803
```

Figure 3.3a. BLAST pair wise alignment of the beta-actin primer set amplicon(A) resulted in the following identities beta-actin gene in the following sequences. B. 98% *Rana catesbeiana* (dbj|AB094353.1|) C. *Rana lessonae* (gb|AY272629.1|) and D. *Acipenser baerii* (gb|JX027376.1|).



3.3b. Tamura-Nei Neighbor-Joining tree (1000 Bootstap; Geneious Pro 5.6.5.) of ACTB gene against L. sylvaticus sequence. Sequences compared: Danio rerio (NCBI Reference Sequence: NM_181601.4); Xenopus (Silurana) tropicalis (GenBank: BC167544.1); Mus musculus (NCBI Reference Sequence: NM_007393.3); Homo sapiens (NCBI Reference Sequence: NG_007992.1); Acipenser baerii (GenBank: JX027376.1); Rana lessonae (GenBank: AY272629.1); Rana catesbeiana (GenBank: AB094353.1).

A. The RARa2 primer set amplicon:

GCTTTGAAGTGGGCATGTCCTTCAGGAGTGAGATGAGACAAGCCTTCTTCGTAGGTATAAAACGTGGGGCCTTCACCTAGTCCTCGCTC
ATGAGCCTCCACATCATACTCTTCTCCTTCAAAATCATCATCGGAGTCGTCCAAATCTTCTGGGTCGGGATGCAGAGCCTGGCAGTCAC
ACATGGCAGAGAACATCTCTCCTAAGTCAGTTTTATCCTCTGGAATGAACCGTATTTCTGTGATTGGCCCCTCATCATCACTGTCCTCG
CATTCCTCTTCAGAGTCCTCATCCTTCATCTCGGCTTCGGTTTCATTAACGTCTCCCAGTTTGGCATTGACCATGACATAGAGGTGCTC
CTCGGGGTAAGCCGCGGTGTCCCTGGAGATGGCATGCAGACTGATGGAGGGACATTCCCACTTCAAAGCA

B.

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L. sylvaticus  8      AGTG-GGCATGTCCTTCAGGAGTGAGATGAGACAAGCCTTCTTCGTAGGTATAAAACGTG  66
                      |||| ||| || ||||| |  ||||||||||||||||||||||| || || ||||| |||
X. laevis      637    AGTGTGGCCTGGCCTTCTGTGGTGAGATGAGACAAGCCTTCTTCATAAGTGTAAAATGTG  578

L. sylvaticus  67     GGGCCTTCACCTAGTCCTCGCTCATGAGCCTCCACATCATACTCTTCTCCTTCAAAATCA  126
                      ||  | ||| |  ||||| |||||||||| || |||||||||| ||||||||| | |||
X. laevis      577    GGAACATCAACCTGTCCTTGCTCATGAGCTTCAACATCATACTCGTCTCCTTCATAGTCA  518

L. sylvaticus  127    TCATCGGAGTCGTCCAAATCTTCTGGGTCGGGATGCAGAGCCTGGCAGTCACACATGGCA  186
                      || || |||||  |    |||||||||| ||||| || ||||| || ||||||||||
X. laevis      517    TCGTCAGAGTCTGC---GTCTTCTGGGTCAGGATGGAGGGCCTGACAATCACACATGGCT  461

L. sylvaticus  187    GAGAACATCTCTCCTAAGTCAGTTTTATCCTCTGGAATGAACCGTATTTCTGTGATTGGC  246
                      || ||||||||||||| |||| ||| ||  | ||||  || ||||||||||| || ||
X. laevis      460    GAAAACATCTCTCCTAAATCAGATTTCTCTCCAGGAACAAAACGTATTTCTGTAATAGGT  401

L. sylvaticus  247    CCCTCATCA---TCACTGTCCTCGCATTCCTCTTCAGAGTCCTCATCCTTCATCTCGGCT  303
                       | ||||||   |||   ||||| |  || |||||    |||| |||   ||| |  |||
X. laevis      400    TCTTCATCATCGTCATCATCCTCACTCTCTTCTTC---CTCCTGATCAGCCATATGAGCT  344

L. sylvaticus  304    TCGGTTTCATTAACGTCTCCCAGTTTGGCATTGACCATGACATAGAGGTGCTCCTCGGGG  363
                      ||  | || |    |||| | || ||||  || |||||||| ||||| |||||||| ||
X. laevis      343    TCCTTGTCTTCTTTGTCTGCGAGCTTGGAGTTCACCATGACGTAGAGATGCTCCTCAGGA  284

L. sylvaticus  364    TAAGCCGCGGTGTCCCTGGAGATGGCATGCAGACTGATGGAGGGACATTC  413
                      ||||| || |||||||||||||| ||||| |||||||| || ||| ||||
X. laevis      283    TAAGCAGCCGTGTCCCTGGAGATTGCATGGAGACTGATTGATGGATATTC  234
```

C.

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L. sylvaticus  8      AGTG-GGCATGTCCTTCAGGAGTGAGATGAGACAAGCCTTCTTCGTAGGTATAAAACGTG  66
                      |||| ||| || ||||| |  || ||  |||||||||||||||| || || |||||||||
X. laevis      628    AGTGTGGCCTGGCCTTCTGTGGTTAGGCGAGACAAGCCTTCTTCATAAGTGTAAAACGTG  569

L. sylvaticus   67    GGGCCTTCACCTAGTCCTCGCTCATGAGCCTCCACATCATACTCTTCTCCTTCAAAATCA  126
                      ||  | || ||  ||||| |||||||||| || ||||| ||||| |||||||||| |||
X. laevis      568    GGAACATCGCCCTGTCCTTGCTCATGAGCTTCAACATCGTACTCGTCTCCTTCAAAGTCA  509

L. sylvaticus  127    TCATCGGAGTCGTCCAAATCTTCTGGGTCGGGATGCAGAGCCTGGCAGTCACACATGGCA  186
                      || || |||||  |    |||||||||| ||||| || ||||| || |||||||| ||
X. laevis      508    TCGTCAGAGTCTGC---GTCTTCTGGGTCAGGATGGAGGGCCTGACAATCACACATTGCT  452

L. sylvaticus  187    GAGAACATCTCTCCTAAGTCAGTTTTATCCTCTGGAATGAACCGTATTTCTGTGATTGGC  246
                      || |||||||||||||| || | ||| ||  | ||||  || |||||||||| || ||
X. laevis      451    GAAAACATCTCTCCTAAATCTGATTTCTCTGCAGGAACAAAACGTATTTCTGTAATCGGT  392

L. sylvaticus  247    CCCTCATCATCACTGTCCTCGCATTCCTCTTCAGAGTCCTCATCCTTCATCTCGGCTTCG  306
                       | || || |||   || || |  || |||||    |||| |||   |||    |||||
X. laevis      391    TCTTCGTCGTCATCATCTTCACTCTCTTCTTC---CTCCTGATCGGCCATAGGAGCTTCC  335

L. sylvaticus  307    GTTTCATTAACGTCTCCCAGTTTGGCATTGACCATGACATAGAGGTGCTCCTCGGGGTAA  366
                       | || |    |||||| || ||||  |||||||||| ||||| |||||||| || |||
X. laevis      334    TTGTCTTCTTTGTCTCCGAGCTTGGAGTTGACCATGACGTAGAGATGCTCCTCAGGATAA  275

L. sylvaticus  367    GCCGCGGTGTCCCTGGAGATGGCATGCAGACTGATGGAGGGA  408
                      || || |||||||||||||| ||||| |||||||| || |||
X. laevis      274    GCGGCCGTGTCCCTGGAGATTGCATGGAGACTGATTGATGGA  233
```

FIGURE 3.4 CONTINUED ON NEXT PAGE

D.

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L. sylvaticus  8      AGTG-GGCATGTCCTTCAGGAGTGAGATGAGACAAGCCTTCTTCGTAGGTATAAAACGTG  66
                      |||| ||| || ||||| |  || |  |||||||||||||||| || || |||||||||
X. tropicalis  604    AGTGTGGCCTGGCCTTCTGTGGTTAAGTGAGACAAGCCTTCTTCATACGTGTAAAACGTG  545

L. sylvaticus  67     GGGCCTTCACCTAGTCCTCGCTCATGAGCCTCCACATCATACTCTTCTCCTTCAAAATCA  126
                      ||  | |||||  ||||  |||||||||| || || |||||||| |||||||||| |||
X. tropicalis  544    GGAACATCACCCTGTCCCTGCTCATGAGCTTCAACGTCATACTCGTCTCCTTCAAAGTCA  485

L. sylvaticus  127    TCATCGGAGTCGTCCAAATCTTCTGGGTCGGGATGCAGAGCCTGGCAGTCACACATGGCA  186
                      ||||| |||||  |    |||||||||| ||||| || ||||| || || ||||||||
X. tropicalis  484    TCATCAGAGTCTGC---GTCTTCTGGGTCAGGATGGAGGGCCTGACAATCGCACATGGCT  428

L. sylvaticus  187    GAGAACATCTCTCCTAAGTCAGTTTTATCCTCTGGAATGAACCGTATTTCTGTGATTGGC  246
                      || |||||||||||||| || | ||| ||  | ||||  || |||||||||| || ||
X. tropicalis  427    GAAAACATCTCTCCTAAATCTGATTTCTCTGCAGGAACAAAACGTATTTCTGTAATAGGT  368

L. sylvaticus  247    CCCTCATCATCACTGTCCTC-GCATTCCTCTTCAGAGTCCTCATCCT---TCATCTCGGC  302
                       | |||||||||   || ||  || | ||||||    ||||| | ||    |||    ||
X. tropicalis  367    TCATCATCATCATCATCATCTTCACT-CTCTTCTTCTTCCTCCTGCTCAACCATAGGAGC  309

L. sylvaticus  303    TTCGGTTTCATTAACGTCTCCCAGTTTGGCATTGACCATGACATAGAGGTGCTCCTCGGG  362
                      |||  | || |     ||||| || ||||  || |||||||| ||||| |||||||| ||
X. tropicalis  308    TTCCTTGTCTTCTTTCTCTCCGAGCTTGGAGTTCACCATGACGTAGAGATGCTCCTCAGG  249

L. sylvaticus  363    GTAAGCCGCGGTGTCCCTGGAGATGGCATGCAGACTGATGGAGGGACATTC  413
                       || || || |||||||||||||| ||||| |||||||| || ||| ||||
X. tropicalis  248    ATAGGCGGCCGTGTCCCTGGAGATTGCATGTAGACTGATTGAAGGATATTC  198
```

Figure 3.4. BLAST pair wise alignment of the RARa2 primer set amplicon(A) resulted in the following identity to the *clns1a* regulatory protein in the following sequences. B. 76% identity in *X. laevis* (ref|NM_001088297.1|11161) C.75 % identity in *X. laevis* (ref|NM_001091335.1|9411) D. 75% identity in *X. tropicalis* (ref|NM_001037262.1|9181).

A. RARb1 primer set amplicon:

GCACCCTTGGCGCTGATGCTGGTGCTGTTGGTCCCACCACCACCTACCTCCTCATACATGCTGGCCCCTTC
TGTGGACTCCGCAGAGGAGCTCAAGGTGGGACTGGCTGGCACTTTGGCCACATTGTACTGCCCGGGCTGCT
CCAGGTC

B. The first 41 nucleotides were removed from the blast to produce the following sequence:

ACCTCCTCATACATGCTGGCCCCTTCTGTGGACTCCGCAGAGGAGCTCAAGGTGGGACTGGCTGGCACTTT
GGCCACATTGTACTGCCCGGGCTGCTCCAGGTC

C.

```
L. sylvaticus  1      ACCTCCTCATACATGCTGGCCCCTTCTGTGGACTCCGCAGAGGAGCTCAAGGTGGGACTG  60
                      ||||||||  ||  |  |||  |||||  ||  ||||||||||  ||||||||||||||||  |||
G. gallus      1010   ACCTCCTCGTAGAGGCTCGCCCCCTCGGTGGACTCCGCCGAGGAGCTCAAGGTGGGGCTG  951

L. sylvaticus  61     GCTGGCACTTTGGCCACATTGTACTGCCCGGGCTGCT  97
                      ||  |||||||||||||  ||||||  |||   ||||||
G. gallus      950    GCCGGCACTTTGGCCACGTTGTACCGCCGCAGCTGCT  914
```

D.

```
L. sylvaticus  1      ACCTCCTCATACATGCTGGCCCCTTCTGTGGACTCCGCAGAGGAGCTCAAGGTGGGACTG  60
                      ||||||||  ||  |  |||  |||||  ||  ||||||||||  ||||||||||||||||  |||
G. gallus      1163   ACCTCCTCGTAGAGGCTCGCCCCCTCGGTGGACTCCGCCGAGGAGCTCAAGGTGGGGCTG  1104

L. sylvaticus  61     GCTGGCACTTTGGCCACATTGTACTGCCCGGGCTGCT  97
                      ||  |||||||||||||  ||||||  |||   ||||||
G. gallus      1103   GCCGGCACTTTGGCCACGTTGTACCGCCGCAGCTGCT  1067
```

E.

```
L. sylvaticus   2     CCTCCTCATACATGCTGGCCCCTTCTGTGGACTCCGCAGAGGAGCTCAAGGTGGGACTGG  61
                      |||||||  ||||  |||||  |||||  ||||||||||  ||||||||||  |||  ||  ||||
A. carolinensis 844   CCTCCTCGTACAAACTGGCGCCTTCGGTGGACTCCGCGGAGGAGCTCAGGGTCGGGCTGG  785

L. sylvaticus   62    CTGGCACTTTGGCCACATTGTACTGCCCGGGCTGCTCCAGGT  103
                      |  |||||||||||||  |||||  |||   ||||||  |  |||
A. carolinensis 784   CGGGCACTTTGGCCACGTTGTAGCGCCTCAGCTGCTGCTGGT  743
```

Figure3.5. BLAST (discontiguous megablast) pair wise alignment of the RARb1 primer set amplicon (A). The amplicon was pruned down by 41 nucleotides (B) to obtain a better identity fit in the BLAST of this sequence. The test resulted the following identities to the sex determining region Y box-11(SOX-11) in the following sequences: C.86% identity to *Gallus gallus* (dbj|AB012237.1|15091) D. 86% identity to *Gallus gallus* (ref|NM_205187.1|19771) and E. 82% identity to *Anolis carolinensis* ref|XM_003224245.1|12301.

3.2.5 Determining up/down regulation of the RA pathway

Up/down regulation of the RA pathway could not be performed because sections of *RARα, RARβ, RXRα, RXRβ, RXRγ*, and *Aldh1a2* could not be determined to make identifying primers.

3.3 MALFORMATION RA DISCUSSION

3.3.1 EFFECTS OF TREATMENT ON FROG MORTALITY

The 4.4% (n=45) mortality in control treatments match the ~4% mortality rate in uninfected frogs of the same species previously observed by Johnson *et al.* (2012). Jab controls (6.6%; n=45) showed mortality rates approximately halfway between both controls and bead treatments, indicating that this type of trauma can result in death, perhaps from blood loss or secondary infection. While not as lethal as parasite infection, bead treatments showed a 8.3% (n=60) mortality, indicating that the beads and operation did increase the chance of death relative to either non-treatment controls and jab controls. The 16.7% (n=60) mortality of tadpoles in parasite treatments observed in this project closely reflect the ~18% mortality previously observed for this species of similar parasite loads (Johnson *et al.,* 2012). The RA treatments caused the highest mortality (43.3%; n=60), indicating that the doses administered were too high to reflect parasite induced RA levels (if any). Future RA treatments should adjust dosage levels to reflect parasite level mortality to examine similarities in induced deformities.

3.3.2 EFFECTS OF TREATMENTS ON OBSERVED FROG MALFORMATIONS

In a normal environment, 5% of the amphibian population will exhibit one or more deformities (Roberts and Dickinson, 2012). In the second experiment, no controls exhibited signs of malformation, while the bead treatments showed a 5-10% malformation rate, and the parasite treatments showed a malformation rate of 10%. This indicates that some of the malformations observed in this experiment could be explained by normal background developmental errors and mutations. Previous experiments with *L.*

sylvaticus showed that tadpoles exposed to 20 *R. ondatrae* cercariae during early limb development resulted in a ~60% malformation rate (Johnson *et al.,* 2012). Future experiments should consider administering higher concentrations (~30 cercariae) of parasites each day over the course of 10 days to increase the percentage (~96%) of malformed individuals (Johnson *et al.,* 2012). The drawback of increasing parasite load is that mortality greatly increases as well (~23% mortality with 30 cercariae) (Johnson *et al.,* 2012). However, higher rates of malformation in test subjects will ensure that up- or down-regulation of the RA pathway will be observed using qPCR.

3.3.3 GENES SEQUENCED FOR RETINOIC ACID PATHWAY

In this project I were unable to obtain six of the eight (nine including a house keeping gene for comparison) genes required to accurately determine the effects of parasitic infection on the host's RA pathway. No effective primer sets could be determined for the *RARα, RARβ, RXRα, RXRβ, RXRγ*, and *Aldh1a2* genes. Maden and Corcoran (1996) created primers for *RARα, RARβ, RARγ, RXRα, RXRβ,and RXRγ* genes to examine gene regulation during homeotic transformation of tails into limbs in *Rana temporaria* (Common frog). Of the tested *RAR* suite primers designed by Maden that I tested, only the *RARα* primer set yielded a putative homologue to a *RAR* gene. However, the *RARα* primer set posed by Maden and Corcoran (1996) amplified a sequence in *L. sylvaticus* that showed greatest identity (75%; Figure 3.1a) with a *RARγ-A-like* gene of *X. tropicalis.* Neighbor-joining tree comparisons of *RARγ* with this sequence result in the *L. sylvaticus* sequence as an outlier (Figure 3.1b). While this sequence may represent the *RARγ* homologue for *L. sylvaticus*, it is impossible to confirm without flanking gene

sequences and more closely related species to make valid comparisons. The *Aldh1a3* primer set yielded a sequence that showed greatest identity (83%; Figure 3.2a) to the *Aldh1a3* gene of *X. tropicalis.* The neighbor-joining tree comparisons (Figure 3.2b) of this sequence confirmed that the sequence obtained is most likely a portion of the *Aldh1a3* gene. While not a RA pathway gene, a portion of beta-actin (*ACTB*) was sequenced. This sequence will be useful as a reference gene in any future qRT-PCR analyses to determine up- and down-regulation of the other genes of interest. When compared to others genes within GenBank, this sequence had the greatest identity (98%; Figure 3.3a) to a section of the *ACTB* gene in *Rana catesbeiana.* A neighbor-joining tree comparison (Figure 3.3b) of this sequence confirmed that the sequence obtained is a fragment of the *ACTB* gene. Due to time constraints and failure to produce effective primers to amplify the other RA pathway genes, no further work was performed on this section of the project to determine disruptions in normal RA pathways during parasite infections on the host.

3.3.4 NON-TARGET GENE SEQUENCES DISCOVERED

Through non-specific annealing of the primers, the primers designed to amplify the *RARα* (RARa2 primer set) *and RARβ* (RARb1 primer set) gene sequences also amplified some non-target DNA fragments that had no significant sequence similarity to *RARα and RARβ* gene sequences. Using BLAST, the RARa2 primer product of these non-target sequences had the greatest identity (76%; Figure 3.4) to a fragment of the chloride channel, *clns1a* gene from *X. laevis,* while the RARb1 primer set sequence had the greatest identity (86%; Figure 3.5) to a fragment of the sex determining region Y box-11

(SOX-11) from *Gallus gallus* (chicken). While these sequences may indeed represent the putative homologues to the described genes in *L. sylvaticus*, without acquiring more flanking sequence, it is difficult to make a definitive identity to these non-specific genes. However, as the above fragments possess no similarity to RA biosynthesis genes, they were not pursued any further.

3.3.5 CONCLUDING REMARKS

Due to the lack of effective, gene-specific primers, this portion of the project was not completed. Designing primers for previously undocumented sequences can provide quite a challenge. While this project managed to obtain fragments of *Aldh1a3* and *ATCB,* further research needs to be conducted into sequencing the remaining RA pathway genes in *L. sylvaticus* to better understand the methods by which *R. ondatrae* causes limb deformities. *Lithobates sylvaticus* provides a good model organism to study these parasite-induced deformities due to the host's high rate of malformation under field-level parasite load (Johnson *et al.,* 2012). Once the parasite's effect on the RA pathway has been determined, other host genes involved in limb development (such as *fgf*, *Hoxa*, *and WNT* gene suites, and *shh*) could be examined to determine how other aspects of limb development and regulation may be manipulated/altered by the parasite. Another area of study that may help increase the understanding of host limb development would be to examine the parasite's RA pathway regulation during infection. This can be done using *in-situ* hybridization techniques to examine both localization of RA in tissue and up- or down-regulation of *R. ondatrae* RA pathway genes in tissue samples. *In-situ* hybridization may be used to examine the full scope of host/parasite interactions (up- or

down-regulation) among RA pathways by attaching unique dyes to each primer set. This could allow the researcher to have a full spatial picture of the genes being regulated by which host, and the location of expression within the organism. This information could vastly increase my understanding of the molecular interactions between the two organisms.

4.0 GENERAL DISCUSSION AND FUTURE WORK

This research project aimed to achieve two objectives. Firstly, it has provided new tools to assess the distribution of *R. ondatrae* in North America. This information will allow us to determine which water bodies are at risk and track the spread of this noxious parasite. Having these tools and knowledge will allow us to develop conservation plans such as snail population management and distribution laws concerning movement of planorbid snails and frogs across water-bodies. The qPCR method outlined in this paper has a superior detection threshold when compared to the current *R. ondatrae* PCR methods. The devised method allows us to detect as little as $1.14X10^{-6}$ fg/µl DNA whereas the previous method described by Reinitz *et al.* (2007) had a detection limit of 100fg. The other factor that makes the devised method superior to previous detection methods is that it allows researchers to potentially determine the quantity of parasites in the water-body. Like previous PCR methods, I scored sites (positive or negative) as to whether the parasite was present. Interestingly, some samples showed no evidence of the organism, similar to other eDNA studies (Ficetola *et al.*, 2008; Goldberg *et al.*, 2011), whereas some samples tested positive. Factors such as water quality, including suspended organic matter, may hinder detection during the DNA extraction and PCR amplification. For this reason, I would argue that more samples at each site may be required to increase the accuracy of the test. While this study examined a couple of different temperatures and times for degradation of eDNA in a water-body, these treatments do not adequately reflect the full range of temperature and water conditions occurring in the field, and hence, abiotic factors could still affect the sensitivity of the assay. This information may also help researchers examine why some water-bodies that are infected with *R. ondatrae*

do not show the same levels of malformations as others. Understanding these discrepancies in malformation occurrence between sites can help predict future changes in amphibian risks with future climate, increased agricultural runoff and environmental changes in infested water-bodies. Comparative limnological, toxicological, and ecological studies should be conducted to help root out some of the other causative agents involved in the complex issue of amphibian malformations and decline.

Secondly, I had hoped to determine whether *R. ondatrae* causes limb deformities through interference of retinoic acid signaling. Little is understood of how this parasite interacts with its hosts, and this project aimed to help us better understand some of the molecular mechanisms involved in disruption of limb development. However, failure to acquire species-specific sequence primers resulted in no meaningful results beyond preliminary primer design. While beyond the scope of this MSc project, it is intriguing to consider the full range of interactions this parasite has on its hosts, including how the parasite affects other important limb formation genes, such as *Hox, shh, fgf8,* and *Wnt*. Another intriguing question that may follow from this MSc study is why some species of amphibians show greater levels of tolerance to this parasite when harboring the same number of cysts. This future research could also help explain why some areas are "hotspots" of amphibian malformations if *R. ondatrae* is actually widespread but doesn't always result in a high level of deformities depending on the host species. Further research into the *R. ondatrae* genome and/or transcriptome may open doors to species-specific control methods to lower the risk to my declining amphibian populations.

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APPENDIX I: Cost-effective Hand-made eDNA And Particulate (C.H.E.A.P.) Filtration unit

C.H.E.A.P. Filter Unit Components:

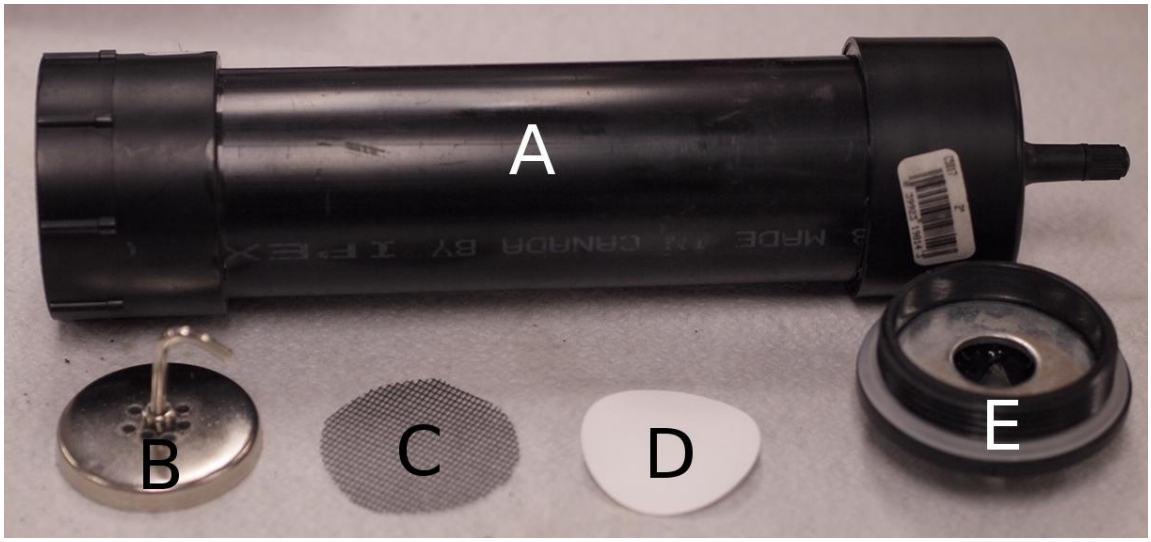

Figure 5.1. C.H.E.A.P. filtration unit components. A. Filter Body Unit. Calibrated for measured collection of water. Left side of the unit has a threaded receiver for Filter Support Unit. Right side has a tire valve for connection to bicycle pump to expel water. B. Filter support retaining magnet. C. Filter support screen. D. Membrane Filter. E. Filter support unit

Materials Required:

- 2” ABS tube
- 2” ABS threaded end cap Adapter, Female (NIBCO 5803 Series ABS DWV Pipe Fitting, Adapter, Schedule 40, Hub x NPT Female: NIBCO 5803)
- 2” ABS threaded end cap (eg. ABS MPT PLUG 2: Bow 602722)
- 2” ABS cap (eg. ABS CAP SCH 40 2: Bow 602557)
- Snap- in tubeless Tire valve (eg. 4 pc Tr 415 Snap-in Tubeless Tire Valves: Power Fist 8341521)

- 3/4” Plate Washer 2” O/D (eg. 3/4 in. Plate Washer - Grade 5: Princess Auto SKU: 8055748)
- 2” round magnet with 3/4” hole (eg. 2 pc 2 in. Magnetic Hooks: Power Fist 8272437)
- ABS solvent cement
- Water resistant 5 minute epoxy (eg. LePage Speed Set Epoxy)
- Door/window screen Fabric or metal (fabric has more flexibility)
- Bicycle pump (preferably with pressure gauge)

Filter Support Unit

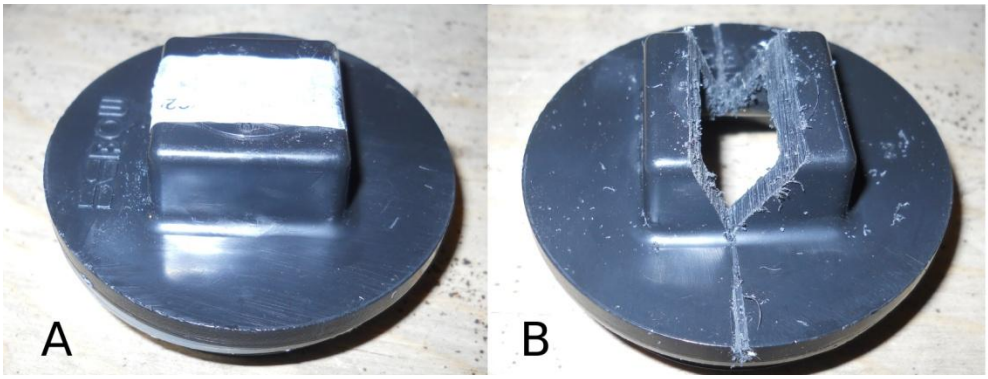

Figure 5.2. V Groove in filter support unit. A. ABS end cap. B. V-Groove cut in cap.

1. Cut 'V' shaped grove across the threaded end cap wrench access block(Figure 5.2). Try to keep the cuts parallel along the face. The apex of the 'V' should touch the base of the cap (Figure 5.2). This will allow the water to drain freely as well as maintain support of the Wrench access if the cap cannot be undone by hand.

2. Glue 3/4” Plate Washer onto the inside of the 2” ABS threaded end cap using Water resistant epoxy (Figure 5.3). Ensure that washer is firmly seated into the Cap. This will allow the magnet to hold the filter in place during pumping. Ensure that no epoxy has oozed onto the face of the washer as this will prevent a proper seal (as can be seen in figure 5.3 A below). If there is extra epoxy on the face of the washer you can chip it off after the epoxy has dried. WARNING: Wear gloves and avoid fumes when working with any Epoxy products. They are known carcinogens.

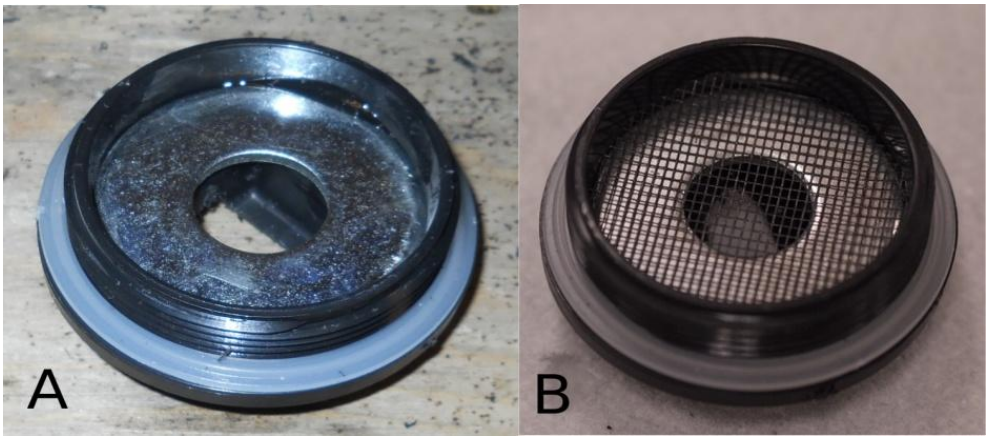

Figure 5.3. Filter support unit. A. Shows the retaining washer. B. Illustrates the filter support screen on the washer.

3. Cut Screen into a 2” diameter circle so that it fits nicely over the washer (As shown in Figure 5.3B above). This will act as the support system for the membrane filter. If cross contamination is a worry, make one for each site sampled. It is a good idea to make extra screens in case of rupture during collection. Avoid going over 40 PSI during pumping to prevent membrane rupture.

4. If the magnets have a cover such as the ones I used holes must be drilled to allow water to pass through. This also works as a grate to prevent large debris from clogging the filter. I made 9 1/8' holes around the hook attachment. The hook is handy to remove the magnet from the filter support system (Figure 5.4).

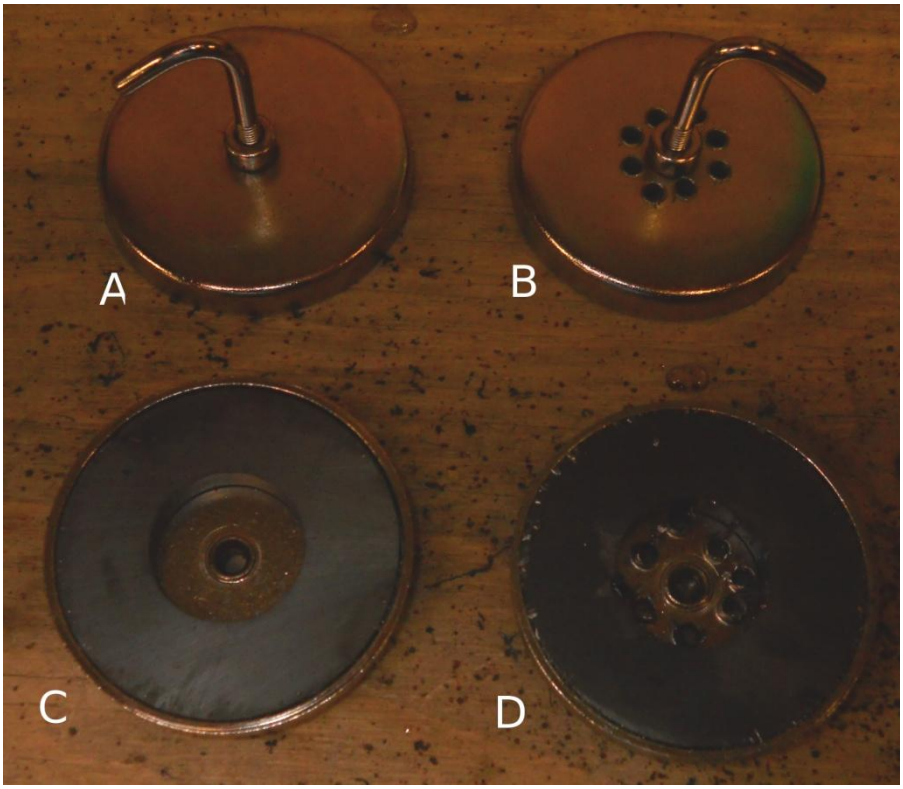

Figure 5.4. Filter retaining magnet detail. Left undrilled magnets. Right drilled magnets. A and B top view of magnet. C and B bottom view of magnet.

Valve installation and Filter body construction

1. Drill a hole in the 2” ABS cap large enough for the tire valve to set into. Insert valve. Seal up with 5 minute epoxy (Figure 5.5). WARNING: Wear gloves and avoid fumes when working with any Epoxy products. They are known carcinogens.

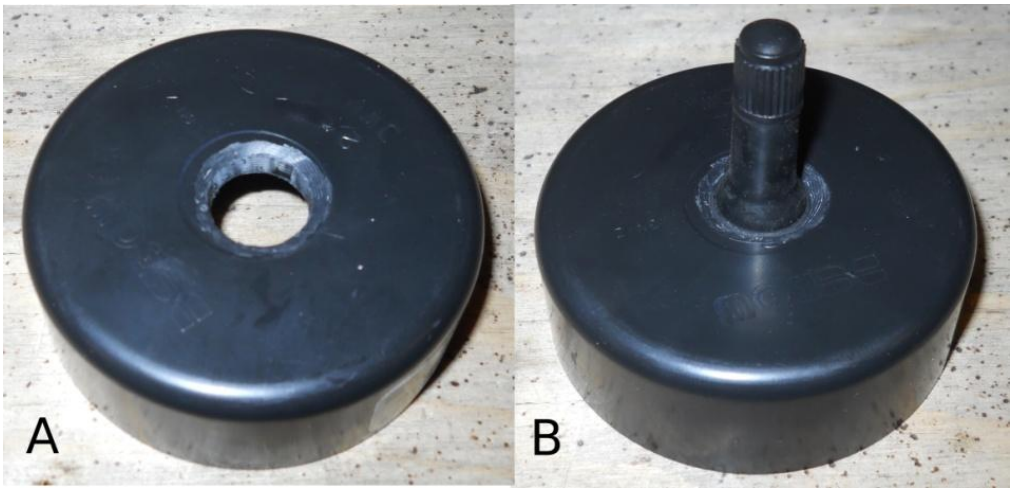

Figure 5.5 Valve instillation. A. detail of hole for valve insertion. B. Valve inserted and glued in place.

2. If making a 500ml pump, cut the tube to about 24.66cm ($V=\pi r^2 h$ or $h=V/\pi r^2$) Adjust length according to desired volume. If your end cap does not rest flush with the pipe (as in NIBCO 2” ABS 5817 DWV cap) when glued in take the extra space into account when cutting your tube if accuracy of the water sampled is of concern. Note: inside diameter of a 2” pipe is about 5.08 cm (Figure 5.6A).
3. Glue 2” ABS cap with attached valve onto pipe section using ABS solvent cement following product directions (Figure 5.6B)
4. Glue female 2” ABS threaded end cap Adapter onto the other end of the tube using ABS solvent cement (Figure 5.6C).

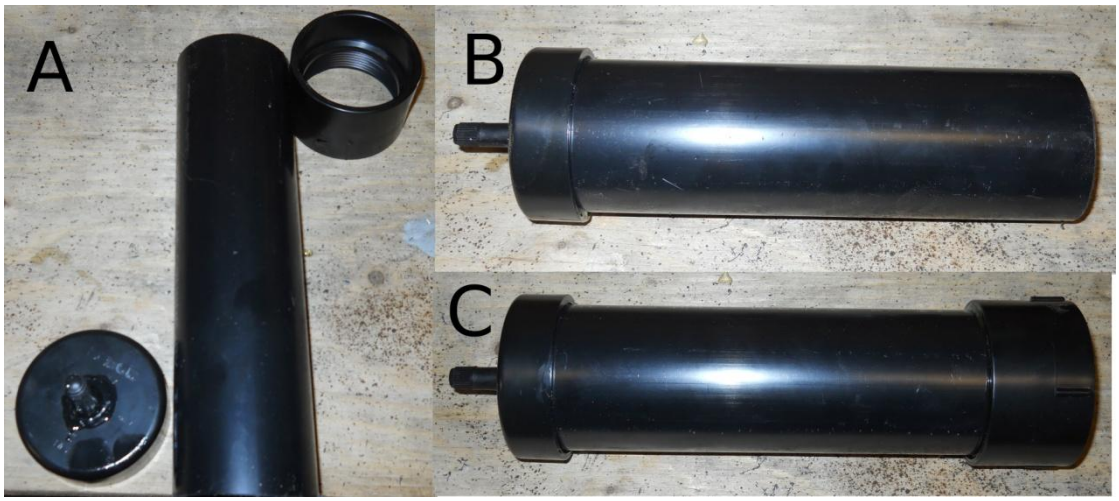

Figure 5.6 Assembly of the filter support unit. A. (From left to right) Valve cap, filter body tube, and female threaded cap adapter. B. Glue valve cap into place. C. Glue female threaded cap adapter into place.

Filter Operation:

Filter setup

This step can be done prior to entering site to save time and reduce contamination on site (Figure 5.7).

Figure 5.7 Filter setup. 1. Place screen on washer in filter support unit 2.Place filter on screen. Note: make sure not to contaminate the other filters with water from the sampling site. 3. Place magnet onto filter, screen, and washer. Press down to ensure that magnet is firmly seated.

Water collection.

1. Scoop up water from site into filter body. Ensure that water is level with the top of the filter body tube to ensure that the sample is the predetermined volume.
2. Screw filter support unit onto filter body
3. Invert pump and attach air pressure pump (eg. Bicycle pump)
4. Provide air pressure to push water through filter.

NOTE: Do not exceed 40 PSI or membrane/screen rupture may occur

NOTE: If filter appears clogged, shake filter system to dislodge debris. If this does not work relieve the pressure and try again. If the filter is still clogged, replace filter and replace with a new one. Keep both filters in the same tube to ensure that sample represents desired volume.

5. Relieve pressure and unscrew filter support unit
6. Remove magnet
7. Remove filter and place in 70% Ethanol

Cleaning maintenance

In Lab cleaning (Optimal)

1. After use clean all parts in warm soapy water
2. Rinse in clean water
3. Fill filter body with clean water
4. Assemble filter support unit (without filter) then connect to filter body
5. Pump water through unit

6. Repeat 3-5 about 5 times to ensure that unit is free of contaminants.
7. Disassemble and allow to air dry

When sampling a new site with a used pump if proper cleaning protocols are impractical

1. Rinse all parts in clean water if available
2. Fill filter body with on site water
3. Assemble filter support unit (without filter) then connect to filter body
4. Pump water through unit
5. Repeat 3-5 about 5 times to ensure that contaminants from previous site are diluted out of pump.
6. Continue sampling as normal

Avoid

1. Cleaning with bleach as this may react with components of the Filter unit and cause DNA to bind to it resulting in cross contamination
2. Do not place unit in UV light to break down DNA as this will break down the epoxy and may damage ABS.

Tips and ideas

1. To help prevent cross contamination make one filter support unit for each unique site to be sampled and set up the filter support unit in a eDNA PCR free zone. Store each in individual Ziploc bags for transport and place them back into the bags after collection. The filter bodies are easily rinsed out so are not as important

to have extras.

2. Add a pressure release valve OR keep a pin by the tire valve to allow quick release of pressure in the filtration unit.