Increased Mortality Rates of Anurans Due to Limb Malformations Caused by Parasitic Flukes

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**Introduction**

It is apparent that, in aquatic ecosystems in North America, overall anuran survival rates are in decline. Additionally, the frequency of observed limb malformations like supernumerary limbs, extra digits, malformed bone bridges, and malformed skin webs have increased dramatically (Schotthoefer et al, 2003). According to the 2012 publication by Johnson and Lunde in *Amphibian Declines: The Conservation Status of United States Species*, “Since they are increasing in frequency, abnormalities in amphibians produce an emerging threat to future anuran populations” (Johnson and Lunde, 2012). Due to this fact, and because everything in an ecosystem is connected through energy transfers, food webs, niche competition, etc., the decline of anuran populations could have serious detrimental effects on the environment. Biodiversity in aquatic ecosystems across the United States could decline thus yielding habitats with low complexity, low diversity, low stability, and, in turn, poor overall habitat health (Pfäffle et al, 2015).

The important question at hand is: what is specifically causing the abnormalities in the anurans that are resulting in decreasing populations? Research suggests that these malformations are primarily caused by parasitic trematodes like *Ribeiroia ondatrae* (Schotthoefer et al, 2003). Research also shows that the method in which these trematodes cause these anomalies involves interfering with developmental processes during metamorphosis, resulting in supernumerary limbs that make predator evasion more difficult (Schotthoefer et al, 2003). The limb-malformation-causing effects of parasitic trematodes on anurans are increasingly detrimental to anuran populations and the biodiversity of the anuran’s surrounding ecosystems.

**Anuran Metamorphosis**

To begin, it is beneficial to examine the chemical/hormonal and mechanical components of the metamorphic cycles of the anurans. During metamorphosis, anuran tadpoles undergo changes in almost every part of their bodies. Consequently, tadpole metamorphosis is one of the most rigorous, intense metamorphic processes in the animal kingdom due to the numerous, total-body regressive and constructive changes that take place (Gilbert, 2000). This process involves a gradual shift from aquatic organism characteristics and tendencies to terrestrial: internal gills to fully operating lungs, aortic arches to carotid arches, herbivorous diets and long gut systems to carnivorous diets and short gut systems, ammonotelic processes to ureotelic processes, and tail fins to tetrapods (Gilbert, 2000).

Developmental morphogens called Thyroid Hormones (TH) are responsible for the majority of organogenesis, appendage changes, and tail changes (Brown and Cai, 2007). The thyroid gland itself produces two different major types of thyroid hormones: thyroxine (T₄) and triiodothyronine (T₃) (Gilbert, 2000). The function of T₄ is to create a negative feedback loop between the thyroid and pituitary glands to induce metamorphic climax and convert to the more active T₃. The T₃ hormone is the primary hormone responsible for inducing the metamorphic processes because lower concentrations are required for metamorphosis induction (Brown and Cai, 2007).

The triiodothyronine hormone stimulates gland changes in the head and body as well as muscle cell apoptosis in the tail, resulting in the mechanical changes of limb development, organ development, and the loss of the tail and internal gills. The T₃ is also responsible for bone growth. Its dosage is proportional to the complexity and level of bone throughout metamorphosis. Lower dosages of T₃ towards earlier stages aid in the development of the earliest-forming bones and higher dosages for the later forming bones (Gilbert, 2000). This process is known as the threshold concept: as certain stages of development progress at increasing hormone levels, different events should occur in succession to each other. Certain bones need to be reformed in succession to others in order for development to maintain stability and consistency. The anuran tadpole’s tail should not begin apoptosis in the muscle cells until other appendages meant for motility begin to develop. Pituitary and thyroid glands work conjointly to make this occur (Gilbert, 2000).

An experiment was reported by Gilbert in the *Metamorphosis: Hormonal Reactivation of Development* regarding the relationship between various thyroid hormone levels and metamorphosis rates in tadpoles. The first part of the experiment involved feeding the tadpoles sheep thyroid glands that had been crushed into a powder. Upon ingesting the powdered thyroid, the tadpoles began developing and metamorphosing prematurely compared to tadpoles that did not receive any powdered sheep thyroid. The second leg of the experiment involved removing or altogether destroying the thyroid glands in tadpoles via a thyroidectomy procedure. These tadpoles never underwent metamorphosis at all; they never grew legs or experienced any of the extensive organogenesis. They merely grew into giant tadpoles in the absence of any thyroid glands or hormones (Gilbert, 2000). Research and experiments like the aforementioned information prove that hormones like T₄ and T₃ are the primary element responsible for controlling anuran metamorphosis.

**Potential Causes of Limb Malformations**

The mortality rates of various anuran communities in North America depend on a wide variety of factors including human activity, environmental stressors, predation, and parasitism (Johnson and Lunde, 2012). A few other specific potential factors include pesticide/chemical contamination and retinoids (Johnson et al, 2002). The next question is: which of these factors have the highest influence on anuran mortality rates in the form of mortality due to limb malformation? Upon examining human activity, overall water quality, chemicals in the form of pesticides and herbicides, excessive retinoids in the environment, and parasites, parasites appear to be the primary answer to this question.

**Human Activity**

One potential cause of anuran decline that is arguably common knowledge is the devastation of natural habitats by increased human activity. While the ultimate, long-term effects of human activity on the epidemiology of various biomes are relatively unknown and unstudied, it is safe to assume that an effect of some kind is definitely taking place (Johnson and Lunde, 2012). According to Pfäffle, Littwin, and Petney’s 2015 publishing in the scientific journal *Research and Reports in Biodiversity Studies*, “the rate of extinction has been accelerated due to human intervention in ecosystems by a factor of approximately 1,000–10,000” (Pfäffle et al, 2015). Forces that affect this acceleration include human population growth, socio-economic development, major pollution, and changes and increases in land usage (Pfäffle et al, 2015). These result in habitat fragmentation, climate change (Pfäffle et al, 2015), the detriment to and decline of wetland habitats (Relyea, 2009), increased levels of orthophosphate in aquatic ecosystems (Johnson et al, 2002), and increased water contamination with chemicals like pesticides (Johnson and Lunde, 2012). As detrimentally effective as human activities are on decreasing the health of ecosystems, they can only be counted as an indirect means of anuran population decline because, in most cases, humans are not directly interacting with and harming the anurans themselves, just primarily the environment around them.

**Water Quality**

 The quality of the water in the aquatic ecosystem that the tadpoles are developing could very well play a role in the improper development of anuran tadpoles. An unbalanced pH, various chemicals, and other harmful substances in the water could result in negative effects on the growth of the organisms in that habitat. An experiment was done by Johnson et al and published in the *Ecological Society of America* in 2002 regarding how, if at all, water quality affected anuran development. In the experiment, the concentrations of over 60 pesticides, orthophosphate levels, nitrate levels, and pH levels were compared to parasites in the ecosystem. Other aquatic ecosystem threats like biotic agents and abiotic agents were questioned and researched as well. Examples of biotic agents included parasite infection and predation. Abiotic agent examples were UV-B radiation, retinoid exposure, and pesticides (Johnson et al, 2002). Johnson’s published experiment was beneficial to the study of anuran mortality rates because it helped spur attempts to answer why anuran mortality rates were increasing due to limb malformations. It was concluded that none of the measured water quality variables were functionally related to malformations in anurans in any way (Johnson et al, 2002). While various contaminants in this experiment did not play a role in anuran limb malformations, the potential for negative effects on anurans in contaminated aquatic ecosystems is still present and will be further discussed.

***Chemicals***

Another potential anuran mortality-impacting source is contamination from chemicals and pesticides. Chemicals and pesticides are the first examined potential cause of mortality due to limb malformations in anurans. Data collected across North America has shown that 60-95% of streams and 30-60% of shallow groundwater contain at least one pesticide contaminant (Relyea, 2009). An experiment testing the effects of insecticide and herbicide chemical mixtures on aquatic communities, specifically the metamorphosis and mortality rates of Leopard frogs and gray tree frogs, was conducted in 2008 and published in the scientific journal *Oecologia* in 2009. Researchers placed pre-metamorphosis anuran larvae in controlled habitats with various combinations of the primary chemicals in common herbicides and insecticides. The habitats, after a period of time, dried up which simulated the real environments of which the anurans are natives. According to the study, direct contact with a mixture of herbicides and insecticides resulted in high mortality rates in Leopard frogs and significant growth differences in gray tree frogs. The high mortality was due to failure to undergo metamorphosis in a timely manner before the water in the habitat dried up. It was concluded that the pesticide chemicals affected the neurotransmitters in the tadpoles, staving off metamorphosis for a fatal, prolonged period of time. The only significant effect the pesticides had on gray tree frogs was increased body mass after metamorphosis (Relyea, 2009). This study, in congruence with the thyroid hormone study reported by Gilbert in the *Metamorphosis: Hormonal Reactivation of Development* shows that chemical changes to the tadpole interfere with the regulation of the thyroid hormones and affect the rate at which the tadpoles develop if they develop at all (Gilbert, 2000). However, the chemical contamination in anuran habitats does not induce mortality specifically due to limb malformation.

# *Retinoids*

A third important potential cause to consider is retinoids. Retinoids, specifically retinoic acids, are chemical compounds that are responsible for intracellular messaging, intracellular regulators, suppressing and activating certain genes, and many other factors and processes in the body (Riley, 1998). Retinoids also have significant effects on limb development and various tissues due to their binding proteins. These retinoid protein receptors combine with TH dimers (Gilbert, 2000). The retinoid dimers then bind to other hormones so the hormones can be transported into the cell nuclei for transcription (Szuroczki and Richardson, 2009). Often, in amphibians, retinoids are activated by external, environmental factors that affect retinoic acid protein synthesis and degradation. This is important because environmental stressors that cause retinoids to be present in excess can lead to imbalances in retinoic acid in the body (Riley, 1998). Imbalances of retinoic acid could potentially lead to certain developmental malformations in the amphibian. However, multiple sources agree that the effects of even harmful levels of retinoids on amphibians are not significant enough to stimulate the degree of limb malformations that have been observed (Riley, 1998), (Johnson et al, 2001), and (Johnson et al, 2002).

**Parasites**

 A final potential source of limb malformations in declining anuran populations is parasites. Parasites are functional groups in food webs since they have a large impact on the energy flow in their ecosystems (Thompson et al, 2005). The relationship between parasites and their hosts are ubiquitous in nature, adding to the complex nature of their interactions with other organisms and the productivity of the ecosystem as a whole (Goodman and Johnson, 2011). Unfortunately, parasites are often neglected in most ecological studies regarding food webs and ecosystems (Szuroczki and Richardson, 2009). Due to this reason, there is little information about the direct effects of parasites in ecosystems in general. Nevertheless, there are countless studies about parasites, specifically parasitic trematodes like *Ribeiroia ondatrae*, in aquatic ecosystems, their effects on the organisms around them, and their various hosts for their life cycles. One of the most important intermediate hosts for parasites in aquatic ecosystems is anurans (Johnson et al, 2002). Each of the studies discussed in this paper has come to the same conclusion: “*Ribeiroia* infection has been linked indirectly to decreasing amphibian population sizes and increased incidences of limb deformities,” (Szuroczki and Richardson, 2009) and “*R. ondatrae* infection induced high frequencies… of severe limb malformations in surviving toads, [and] survivorship declined significantly with increasing parasite exposure” (Johnson et al, 2001). Later, the specific effects of the trematodes on the anuran limbs will be discussed.

***Detecting Parasites***

In 2015, Huver et al conducted a study on an extracellular DNA (eDNA) method of analysis for determining the presence of the parasitic trematode *Ribeiroia ondatrae* in perished, deformed anuran hosts that was published in *Ecological Society of America*. This study built upon the previously existing polymerase chain reaction (PCR) test with the hopes that it would improve identification accuracy and further develop disease-risk monitoring among populations in a habitat (Huver et al, 2015). According to the study, the eDNA method was intended to be a superior, sensitive, specific test to finding and identifying extracellular DNA in organisms that died after subjection to pathogenic parasites. The goal was to gain a better understanding of the pathogenicity of parasites in the wild by affirming the presence of *R. ondatrae* in afflicted anurans with accurate and precise eDNA testing on deceased hosts and water samples. They also tested gastropods for the presence of the parasites. It was found that the eDNA test was indeed sensitive and extremely effective, proving to be more accurate than the previously accepted PCR-based tests. The eDNA tests confirmed the presence of *R. ondatrae* in deceased anuran host with limb deformities, the gastropods, and the water samples from the sample area (Huver et al, 2015). This further solidifies the direct cause of limb malformations in anurans.

**Parasitic Trematodes**

Because parasitic trematodes seem to be the primary underlying cause of anuran population decline due to limb-malformations, it is important to examine the trematode’s life cycle as well. Trematodes, specifically *Ribeiroia ondatrae*, are members of the Platyhelminthes phylum, and they have complex life cycles that involve multiple different hosts (Huver et al, 2015). Eggs are commonly found in avian feces. Here, they embryonate and develop into miracidia, the infectious, free-swimming version of the parasite. The first level of intermediate hosts that the miracidia infect are healthy aquatic gastropods that feed on and decompose the feces that the eggs were hatched in. From the gastropods, the trematodes experience several cycles of asexual reproduction until they develop into the next stage: cercariae. The cercariae stage is important because this free-swimming version is what infects primarily anurans (Huver et al, 2015). *Ribeiroia ondatrae* make their way into the tadpoles by penetrating the soft skin and tissue surrounding the area where limb buds will form. This is where the cercariae develop large cysts that interfere with development (Johnson et al, 2002). After encysting in the tadpoles, which will momentarily be discussed, the cercariae then develop into metaercaria. From here, the anuran is preyed upon by an avian species thus ingesting the metaercaria. In the gut of the bird, the metaercaria fully mature and engage in sexual reproduction. Eggs are laid and dispensed by the bird, and the cycle begins again (Schotthoefer et al, 2003).

The circular nature of the parasitic trematode’s life cycle and the specific intermediate hosts needed to reach their definitive host are also responsible for creating “loops” of energy transfer in food webs (Thompson et al, 2005). These loops are harmful to the rest of the organisms in the trematode’s ecosystem because it weakens the links in the food chains within the food web and interferes with the balance of the flow of energy through the ecosystem (Thompson et al, 2005). Parasites also shift the commonly accepted dominant species in higher trophic levels around thus increasing the number of intermediate predators in lower trophic levels of energy transfer (Thompson et al, 2005). The aforementioned effects of parasitic trematodes in their environments ultimately throws food webs and aquatic ecosystems out of whack due to the upset in the original, natural balance.

**Effect of Parasitic Trematodes on Anurans**

It is known that the *Ribeiroia ondatrae* parasitic trematodes do affect anurans and cause limb malformations, but it is lesser known how the limb malformations are brought to fruition by the trematodes. The cercariae affect anurans in a very specific way. As the anuran’s thyroid hormones are working throughout their bodies, the cercariae get into the tadpole and encyst near the sites where limb budding occurs (Johnson et al, 2002). However, it is unclear exactly how the *R. ondatrae* specifically cause the limb malformations. Two primary hypotheses have been suggested: 1) the cysts caused by the trematode creates a mechanical disruption of the developing limb cells’ organization which leads to abnormal cellular growth around the cyst; 2) the trematode produces or releases a growth factor that interferes with the molecular signals being sent by the various thyroid hormones in charge of limb development (Szuroczki and Richardson, 2009). Experiments for these two specific untested theories have not been conducted so far. Nonetheless, many experiments have been conducted regarding what kind of physical and physiological effects the trematodes have on the anurans. Each of the experiments executed revealed related and informative results regarding this topic.

**Types of Malformations**

 Of the different reports of malformations due to trematodes, anurans all tend to exhibit approximately the same common malformations (Johnson and Lunde, 2012). Limb abnormalities most commonly reveal themselves around the hind limbs in developing anurans in the form of ectromelia, ectrodactyly, polymelia, polydactyly, and cutaneous fusions. Ectromelia and ectrodactyly is the absence or improper/underdevelopment of limbs and digits. Polymelia and polydactyly is the presence of extra limbs and digits. Cutaneous fusions are the irregular growth patterns of skin webbing (Johnson et al, 2002). These are all results of the cysts formed by the trematodes. Another common malformation seen in trematode infections in anurans is bony triangles or bony bridges. Bony triangles or bony bridges are the results of bones that have grown in abnormal ways, sometimes resulting in the bones fusing together, which causes the bones to be dysfunctional (Szuroczki and Richardson, 2009). Each of these different types of deformities has detrimental effects on the locomotion of the infected anurans.

**Magnitude of Effect**

 Another element of the overall question is how much do the deformed, supernumerary limbs affect the infected anurans locomotion? In the experimental report “Disease and the Extended Phenotype” by Goodman and Johnson published in 2011, various common tasks and abilities performed by normal anurans were compared to those performed by anurans that have malformations due to exposure to *R. ondatrae*. The jumping distance, swimming distance, swimming speed, swimming endurance time, and amount of prey captured by both groups of anurans were compared. It was found that the differences in performance due to deformities or lack thereof were significant and ultimately quantifiable. Infected and mutated anurans jumped 41% shorter distances, swam 76% shorter distances, swam 37% slower, and swam with 66% lower endurance (Goodman and Johnson, 2011). In regards to prey capture, the anurans with abnormalities were overall less successful because it took them much longer to capture and even consume their prey than the “normal” anurans (Goodman and Johnson, 2011). The quantifiable differences between healthy anurans and infected anurans are crucial in this limb malformation study because it provides numerical evidence that the impairments caused by *R. ondatrae* are significant in the natural world.

**Infectious Dose**

The amount or infectious dose of parasitic trematode the anurans are exposed to, also plays a role in the severity of the deformities. An experiment reported by Johnson et al in the Canadian Journal of Zoology in 2001 examined the effect of the level of exposure of anuran larvae to the parasitic trematode *Ribeiroia ondatrae*. The growth, metamorphic development, and mortality rates of differently exposed tadpoles were compared to a control group that received no exposure to the trematodes. The control larvae had normal development and very low mortality rates recorded. For those exposed to various doses of *R. ondatrae*, survivorship declined directly with increased dosage. Furthermore, limb malformation frequency and amount in the developing anurans increased directly with the dosage. The reported limb malformations were polymely, ectromely, and polydactyly among other forms (Johnson et al, 2001). Another component of the experiment was how the larvae were exposed to the trematodes. It was found that the anuran tadpoles who were infected by the trematodes that had developed naturally in gastropod hosts had an even higher frequency of unilateral or asymmetrical bilateral and missing limbs, missing digits, extra limbs, extra digits, cutaneous fusion, and taumely after metamorphic development (Johnson et al, 2001).

The overall effects of trematodes like *Ribeiroia ondatrae* on anurans was summarized succinctly by Johnson and Lunde in *Amphibian Declines,* “Field and laboratory studies of *Ribeiroia* infection suggest this trematode may substantially reduce amphibian survivorship through two mechanisms: direct mortality due to infection and indirect mortality resulting from impaired fitness associated with limb malformations,” (Johnson and Lunde, 2012).

**Impact on Environment**

 The final question remains: what impact does the decline in anuran populations due to limb malformations from parasitic trematodes have on their ecosystems? The answer lies in the detriment to biodiversity. Goodman and Johnson’s formerly-discussed report from 2011 concluded that the quantifiable and differential vulnerability that the defective anurans with supernumerary limbs played an impactful role on the anurans’ mortality rates via predation (Goodman and Johnson, 2011). If anuran populations decline because their ability to evade predators decreases, the flow of energy in and diversity of their ecosystems would become unstable.

On the topic of biodiversity, Pfäffle’s discusses the relationship between biodiversity and ecosystem health as well as the dilution effect in the 2015 publication in *Research and Reports in Biodiversity Studies.* As stated in the introduction, biodiversity is directly linked to the health, stability, and complexity of an ecosystem. Without biodiversity, a decrease of just one organism in a crucial trophic level in an ecosystem could lead to the imbalance of multiple species. Any species that the parasite’s target previously preyed upon would grow out of control without an important predator keeping those population levels in check. Conversely, species that previously preyed upon the parasite’s target would have lost an important food source resulting in their populations declining as well (Pfäffle et al, 2015). The dilution effect refers to the relationship between infection rates and diversity. If communities have high diversity with non-competent hosts, the rates of disease transmission and infection between organisms, also known as vectors, are “diluted” or reduced. When parasites use their ability to alter various functions in their hosts resulting in increased mortality of the host, the diversity in that ecosystem goes down. This decreases “dilution” as well as biodiversity (Pfäffle et al, 2015).

## Conclusion

In recent years, the frequency at which reported limb and digit deformities in amphibians has increased significantly. The severity of the deformities have also increased. These increases have surpassed the normally expected, base-line levels of mutation occurrence in anuran populations in North America (Johnson et al, 2001). Parasitic trematode species like *Ribeiroia ondatrae* are to blame for said increases because they use anurans as intermediate hosts in their life cycles, causing cysts that disrupt limb development during anuran metamorphosis. Increased biodiversity tends to indicate smaller population sizes and a large number of different populations. If there are fewer, less-diverse populations of animals due to insufficient habitat availability, low water quality, human activity, etc., the risk of spreading infection by parasites increases (Schotthoefer et al, 2003). When there are more gastropods available for parasitism, there are also more parasitic trematodes. Higher numbers of parasitic trematodes result in higher infectious doses for anurans. As analyzed earlier, higher infectious doses in anurans lead to more frequent and severe limb abnormalities in anurans. Increased frequency and severity results in decreased locomotive function, increased probability of predation, and decreased population sizes. The wide-scale effects of limb-malformation-causing parasitic trematodes on anurans are increasingly detrimental to anuran populations as well as the biodiversity of the anuran’s surrounding ecosystems.

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