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Parasites and sexual selection in a sexually dichromatic toad

By

Nicole Erin Shangi

A Thesis

Submitted to the Faculty of Graduate Studies
through the Department of Integrative Biology
in Partial Fulfillment of the Requirements for
The Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2021

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Declaration of Co-authorship

I hereby declare that this thesis research incorporates material that is the result of joint research as follows:

I am the sole author of Chapters 1 and 3 under the editorial guidance of Dr. Daniel Mennill and Dr. Stéphanie Doucet. I am the principal author of Chapter 2. Chapter 2 incorporates data collected by Katrina Gardner under the supervision of Dr. Daniel Mennill and Dr. Stéphanie Doucet. In all three chapters the key ideas, primary contributions, experimental designs, data analysis, interpretation, and writing were performed by myself; Katrina Gardner contributed to the data collection and Dr. Daniel Mennill and Dr. Stéphanie Doucet contributed to analyses, writing, and funding of this research.

I am aware of the University of Windsor Senate Policy on Authorship and verify that I have properly acknowledged the contributions of other researchers to my thesis and have obtained written permission from each of the co-authors to include the above materials in my thesis.

I declare that, with the above qualification, this thesis and the research to which it refers is the product of my own work.

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I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

Abstract

Parasitism is among the most common and successful life history strategies on Earth, leading to constant coevolution between parasites and hosts. Parasites continuously pressure hosts to evolve more effective parasite resistance, fueling interest in the relationships between expression of sexually selected host ornamentation and parasite resistance. By studying parasite-mediated sexual selection we gain unique insights into the evolution of animal traits. In this thesis, I attempt to further our understanding of a sexually dichromatic neotropical anuran by studying its parasites in the context of sexual selection. Firstly, in the General Introduction (Chapter 1), I reviewed important background information key to understanding anuran host-parasite relationships, as well as the literature studied so far on anuran parasite-mediated sexual selection. In my data chapter (Chapter 2), I used neotropical yellow toad blood samples to identify blood parasites, which have never been explored in this species. I identified four groups of parasites infecting neotropical yellow toads: Apicomplexa, Nematoda, frog erythrocytic virus, and bacteria. Furthermore, I used blood samples, colour, and morphometrics to explore parasite-mediated sexual selection in neotropical yellow toads. I found evidence that some blood parasites of neotropical yellow toads are correlated with colour and body condition, suggesting that parasite resistance and expression of sexual traits may be related in this species. In the General Discussion (Chapter 3), I reviewed the findings of Chapter 2 and their implications for parasite-mediated sexual selection in anurans. Motivating this thesis is my interest in amphibian conservation due to recent population declines and extinctions owing to climate change and disease. My aim is for this research to further our understanding of anuran host-parasite relationships and sexual selection and to contribute to amphibian conservation efforts.

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Chapter 1: General Introduction

Introduction

The co-evolutionary arms race between hosts and parasites has led to an incredible diversity of parasite species. Parasites have evolved to have diverse life cycles, infection strategies, and pathogenic effects on their host. Anurans are hosts for a wide variety of parasites, from microscopic single celled organisms to macroscopic arthropods. In this General Introduction, I will review multiple topics relevant to my data chapter (Chapter 2) regarding parasite-mediated sexual selection in the neotropical yellow toad (*Incilius luetkenii*). First, I will describe the parasites that infect anurans, including helminths, chytrid fungi, protozoans, frog erythrocytic virus, bacteria, and ectoparasites. To close, I will review the literature studied so far of parasite-mediated sexual selection in anurans and introduce my thesis objectives.

Helminths

Nematoda. Nematoda are parasitic round worms, and are likely the most numerous of all helminth groups to infect anurans (Anderson, 2000; Campião et al., 2015). These species are generalists that inhabit multiple areas of an anuran's body including the intestines, lungs, stomach, and blood (Campião et al., 2015). Although most Nematoda species are macroscopic and infect body cavity organs of anurans, some species such as those from the family Onchocercidae have larval stages called microfilaria that inhabit the blood or other tissues of their host (Anderson, 2000; Netherlands, Svitin, et al., 2020). The Nematoda parasites that infect anurans are very diverse, and so too are the pathogenic effects they cause. Generally, infection by Nematoda parasites can cause skin ulcers, reduced organ function, neurological complications, and loss of skin pigmentation; and heavy infection may lead to death (Densmore & Green, 2007; Kelehear et al., 2009; Selechnik et al., 2017).

Acanthocephala. Acanthocephala is a phylum of parasitic “spiny-headed” helminths (Greiner et al., 2006; Margulis & Chapman, 1998). Most species parasitize fish; however, Acanthocephala also parasitize mammals, reptiles, and amphibians (Greiner et al., 2006; Margulis & Chapman, 1998). Using their spiny proboscis, Acanthocephala parasites burrow into the walls of the stomach and intestines of anuran hosts, which can introduce secondary infection of bacteria and can be life-threatening (Burrowes et al., 2004).

Trematoda. Trematoda, commonly known as flukes, are parasitic flatworms (Densmore & Green, 2007). Trematoda are known for their complex life histories often requiring three host species to complete their life cycle (Poulin & Cribb, 2002; Szuroczki & Richardson, 2009). Some species of Trematoda, such as *Echinostoma trivolvis* and *Riberioia ondatrae*, cause an interesting abnormality in anuran hosts (Szuroczki & Richardson, 2009): infection has been shown to cause polydactyly in adult anurans, most commonly leading to the growth of 1-3 extra hind limbs (Johnson, Lunde, Ritchie, & Launer, 1999; Szuroczki & Richardson, 2009).

Monogenea. Like Trematoda, Monogenea is a class of parasitic flatworms belonging to the phylum Platyhelminths (Badets et al., 2013). Only members of the family Polystomatidae infect amphibians (Chaabane et al., 2019; Park et al., 2007; Woo, 2006). These parasites most commonly reside in the bladder of anuran hosts, where they feed on host blood; however, they may also reside in the skin or gills of larval anurans (Badets et al., 2013; Chaabane et al., 2019; Nugaraite et al., 2017; Theunissen et al., 2014; Vásquez & Pfennig, 2007; Woo, 2006). Monogenea parasites feeding on the blood of anuran hosts can cause anemia in severe instances, and infection of the skin can increase the chances of secondary infection (Densmore & Green, 2007; Woo, 2006).

Chytrid fungus

The chytrid fungus, *Batrachochytrium dendrobatidis*, is a fungal parasite that infects anuran species worldwide (Kilpatrick et al., 2010). This fungus causes the disease chytridiomycosis, and has led to extreme population declines and extinctions of many species (Kilpatrick et al., 2010). Fungal spores require water to reach anuran hosts, and once infected, the fungus causes keratinization of the skin in adult anurans, and of the mouthparts of larval anurans. Keratinization resulting from chytridiomycosis causes lethargy, dehydration, lesions, and ultimately death (Densmore & Green, 2007; Kilpatrick et al., 2010). Interestingly, anuran species have variable responses to infection. For example, chytridiomycosis caused rapid mass die offs in the cane toad (*Bufo marinus*) and great barred frog (*Mixophyes fasciolatus*; Berger et al., 1998), but the bullfrog (*Rana catesbeiana*) experienced no clinical signs of infection and little keratinization (Daszak et al., 2004). *B. dendrobatidis* is found on all continents except Antarctica (Fisher et al., 2009), and has caused many extinctions since 1980. For example, at least 67 species of the genus *Atelopus* have gone extinct likely due to chytridiomycosis (Rohr et al., 2008). Many studies have linked the prevalence of *B. dendrobatidis* to climate, due to the dependence of the fungus on warm temperatures and moisture, making the fungus one of the most prevalent and worrying parasites infecting anurans worldwide (Cohen et al., 2019; Horner et al., 2017; Pounds et al., 2006).

Protozoans

Apicomplexa. Apicomplexa is a diverse phylum of unicellular protists that parasitize diverse animals (Morrison, 2009). They share an evolutionarily unique structure called an apical complex which contains organelles allowing the invasion of host cells (Sibley, 2010). This

phylum infamously includes the malaria-causing parasite, *Plasmodium falciparum*, which is transmitted by infected mosquitos (*Anopheles*) to humans (Mbogo et al., 2003). In reptiles and amphibians, mosquitos and other blood sucking arthropods such as ticks (Ixodidae) are common vectors for Apicomplexa parasites (Netherlands et al., 2014; Rubini et al., 2009; Viana et al., 2012). Interestingly, it was observed that although mosquitos (*Culex*) were found to transmit the Apicomplexa parasite, *Hepatazoon caimani*, to caimans (*Caiman yacare*), the main source of caiman infection by these parasites comes from consumption of infected anurans such as *Leptodactylus chaquensis* and *Scinax nasicus* (Viana et al., 2012). Anurans serve as hosts for many different species of Apicomplexa, many of which reside in the blood; however, various stages of these parasites can also be found in organs such as the lungs and liver (Barta & Desser, 1984; Bolek et al., 2003; Smith et al., 2000). There are few studies on the pathogenic effects of Apicomplexa parasites on anurans; however, studies conducted on reptiles have documented contradictory results. For example, water pythons (*Liasis fuscus*) exhibited correlations between host fitness and parasitism such as slower growth rates and lower juvenile survival in more parasitized individuals, but in the sympatric species, keelback snakes (*Tropidonophis mairii*), there were no correlations between parasitism and measures of host fitness (Madsen et al., 2005; Selechnik et al., 2017).

Trypanosoma. *Trypanosoma* is a genus of parasitic protozoan parasites that infect the blood of their vertebrate hosts (Kutz et al., 2012). Although species of *Trypanosoma* have diverse morphologies which can make identification challenging, many share identifying characteristics such as flagella and undulating membranes which allow them to move freely throughout the blood plasma (Desser, 2001; Greiner et al., 2006; Spodareva et al., 2018). One study found a fascinating relationship between túngara frogs (*Engystomops pustulosus*) and the vector for a

previously undescribed *Trypanosoma* species, *Trypanosoma tungarae* (Bernal & Pinto, 2016). The vector for *T. tungarae* is a frog-biting midge (*Corethrella*) which intercepts the calls of túngara frogs, and because only the males of the species produces calls, the authors found a sexual difference in *T. tungarae* prevalence with less than 1% of females infected (Bernal & Pinto, 2016). The general term for diseases caused by *Trypanosoma* infection is trypanosomiasis; one well known trypanosomiasis is Chagas disease in humans, spread through the bites of kissing bugs (*Triatoma infestans*) infected with *Trypanosoma cruzi* (Schofield & Dujardin, 1997). In anurans, trypanosomiasis can cause a range of pathogenic effects, from anemia and anorexia to organ necrosis and death (Densmore & Green, 2007).

Frog erythrocytic virus

Viruses are infectious agents that require a host cell to replicate, and are sometimes considered parasites in parasitology research (Aisien et al., 2015; Desser, 2001; Netherlands., 2015). As the name suggests, frog erythrocytic virus infects the blood cells of anurans. More specifically, they are found in the cytoplasm, surrounded by a protective vacuole (Gruia-Gray et al., 1989). Individual frog erythrocytic viruses are extremely small (~450 nm) and therefore challenging to identify (they are often difficult to distinguish from bacteria found in the erythrocyte cytoplasm; Densmore & Green, 2007; Gruia-Gray et al., 1989; Netherlands et al., 2015). This virus is transmitted to anurans through biting midges (*Ceratopogonidae*) and mosquitos (*Culex*) when the arthropods bite and feed on anuran blood (Ferguson & Smith, 2012; Gruia-Gray & Desser, 1992). No clinical effects of frog erythrocytic virus have been shown in anuran hosts; however, the virus has been found in the presence of other parasites such as those belonging to

Apicomplexa and *Trypanosoma* (Desser, 2001; Graczyk et al., 1996), and confounding effects of these parasites have not been investigated.

Bacteria

There have been few studies of bacteria in anuran blood because they are small and difficult to identify without magnification higher than x1000. Bacteria have been recorded in the blood of species such as the Mozambique-ridged frog (*Ptychadena mossambica*); however, species-level identification or classification is often not reached (Netherlands et al., 2015). Bacterial infections in anurans such as red-leg syndrome are life-threatening diagnoses and present similar symptoms to the deadly fungal pathogen *B. dendrobatidis* (Densmore & Green, 2007). Bacteria commonly infect anurans after traumatic injury or secondarily after viral infection (Densmore & Green, 2007).

Ectoparasites

An ectoparasite is any parasitic organism that lives on the outside of the host's body. Many ectoparasites are blood-feeding arthropods that spend much of their life parasitizing a host, such as ticks (Ixodidae; Coêlho et al., 2019). Mosquitos (Culicidae) are generally not regarded as ectoparasitic organisms because they consume blood relatively quickly and they do not live on their prey (Centers for Disease Control and Prevention, 2020). The most studied ectoparasites of anurans are leeches (Glossiphoniidae), ticks, and mites (Trombiculidae), all of which spend the majority of their lives feeding on the host's blood (Coêlho et al., 2019; Mendoza-Roldan et al., 2020; Siddall & Bowerman, 2006). Other ectoparasites of anurans are flies (Diptera) and fish lice (*Argulus*). Adult tachinid flies are known to deposit eggs on the bodies of anuran hosts, which

hatch into parasitic larvae, and where extreme infestations can kill the host anuran (Densmore & Green, 2007; Duellman, 1994). As the name suggests, fish lice are common parasites of fish; however, they have been known to parasitize aquatic stages of anurans (Densmore & Green, 2007; Duellman, 1994). Some ectoparasites are innately involved in the transmission of parasites mentioned in the above sections because they can also act as vectors transporting parasites from one host to another (Oliveira et al., 2020; Siddall & Dessler, 1992). Leeches, ticks, and mites acquire parasites by feeding on an anuran infected with parasites such as helminths or *Trypanosoma*; the infected arthropods are then capable of transmitting those parasites if they feed on another anuran host (Dessler, 2001; Mendoza-Roldan et al., 2020; Oliveira et al., 2020; Siddall & Dessler, 1992).

The Hamilton-Zuk Hypothesis

The co-evolution between hosts and parasites as well as parasite-induced pathogenic effects on hosts have gathered interest for possible implications on host sexual selection. In 1982, Bill Hamilton and Marlene Zuk introduced the hypothesis of parasite-mediated sexual selection (Hamilton & Zuk, 1982). Now known as the “Hamilton-Zuk hypothesis”, these two biologists proposed that females choose mates based on the quality of their sexual characteristics and ornaments and that their quality is limited by their ability to resist parasitism. Females can therefore use the degree of sexual ornament expression as an honest indicator of the health and vigor of a potential mate (Hamilton & Zuk, 1982). Females indirectly benefit from discriminating between mates by passing on better genes for parasite and disease resistance to offspring, and it has also been proposed that choosiness allows a direct benefit to females by avoiding parasites transferred from a lower quality mate (Borgia & Collis, 1989; Hamilton &

Zuk, 1982). Although the Hamilton-Zuk hypothesis predicts that the degree of ornament expression signals the host's degree of parasite resistance, scientists most often use parasite load as a proxy for parasite resistance, with the assumption that fewer parasites reflect higher parasite resistance (Hausfater et al., 1990; Pfennig & Tinsley, 2002; Tinsley, 1990; Vásquez & Pfennig, 2007).

Since the Hamilton-Zuk Hypothesis was proposed, it has been investigated in diverse species across multiple taxa, accumulating results both supporting and contradicting its predictions. For example, evidence supporting the Hamilton-Zuk hypothesis has been observed in the three-spined stickleback (*Gasterosteus aculeatus*), where parasitism caused a decrease in the bright red breeding colouration of males, which females used as an honest indicator of health and as a consequence, chose mates expressing more red colouration indicative of fewer parasites and good condition (Milinski & Bakker, 1990). Similarly, in experimentally parasite-infected red junglefowl (*Gallus gallus*), male feathers were duller and shorter than those of uninfected males, and females used these differences in sexual characteristic expression to choose a healthier mate (Zuk et al., 1990). Some species have shown no association between parasitism and the degree of sexual ornament expression, such as in the mandrill (*Mandrillus sphinx*), where no relationship was found between the degree of male facial breeding colouration and parasitism (Setchell et al., 2009), and in the red bishop (*Euplectes orix*), where no relationship was found between the number of nests built by males, territory establishment, and parasitism (Edler et al., 2004).

Few investigations of the Hamilton-Zuk hypothesis have been conducted on anurans despite the fact that many anuran species exhibit sexual ornamentation, which is a fundamental requirement of the hypothesis (Hamilton & Zuk, 1982; Shine, 1979). In Table 1.1, I review all

the studies of the Hamilton-Zuk hypothesis in anurans. Interestingly, the majority of studies have shown positive relationships between parasitism and anuran sexual characteristics. For example, in male spadefoot toads (*Scaphiopus couchii*), call duration and body condition increased with parasitism (Table 1; Pfennig & Tinsley, 2002), and in male Puerto Rican rock frogs (*Eleutherodactylus cookii*), brightness increased with parasitism (Table 1; Longo et al., 2020). Although much less commonly, the expected negative association between parasites and sexual characteristics has been documented in some anurans, such as in Burmeister's treefrog (*Hypsiboas prasinus*), where call rate increased in less parasitized males (Table 1; Madelaire et al., 2013). Of the few studies including female preference for parasitized males or traits indicative of parasitism, the majority have found that females either have no preference for parasitized or unparasitized males, or they have found a positive relationship; that is, females preferring more parasitized males. For example, in the gray treefrog (*Hyla versicolor*), females more often chose males that were parasitized over those that were not (Table 1; Hausfater et al., 1990). In spadefoot toads, females had no preference for males which had longer call durations or were in better body condition, which, as mentioned above, was indicative of higher parasitism (Table 1; Pfennig & Tinsley, 2002). Given the mixed results of the Hamilton-Zuk hypothesis in anurans, more research is needed with more anuran and parasite species to determine the possible role of parasite-mediated sexual selection in anurans.

Study Species and Habitat

Incilius luetkenii, neotropical yellow toads, are found in tropical dry forests from southern Mexico to central Costa Rica (Savage, 2002). The tropical dry forest is a unique environment experiencing two starkly different seasons: a dry season from December to May with no rainfall

followed by a rainy season from May to December with substantial rain (Murphy & Lugo, 1986). The majority of tree species found in dry forests are deciduous, and they lose their leaves during dry seasons (Bullock et al., 1995; Janzen, 1988; Murphy & Lugo, 1986). The Area de Conservación Guanacaste in Costa Rica is the largest stand of tropical dry forest on Earth and is recognized as a UNESCO world heritage site given its special importance (Fedigan & Jack, 2012).

Neotropical yellow toads emerge from dry season aestivation immediately after the first rainfall at the start of the rainy season and proceed to breeding pools (Doucet & Mennill, 2010). Ephemeral ponds and streams are breeding aggregation sites for up to several hundred neotropical yellow toads (Rehberg-Besler et al., 2015). These toads exhibit a scramble competition mating system wherein males will compete with each other over access to females for one to four days, with the majority of mating occurring on the first day (Nicolas Rehberg-Besler et al., 2015). Neotropical yellow toads are dynamically sexually dichromatic; during the short breeding period males alter their colouration from a female-like brown to bright yellow. Previous research has shown that colour varies between individual males (Figure 1; Doucet & Mennill, 2010; Gardner et al., 2020); however, no research has been conducted on the parasites of neotropical yellow toads or their relationship with colour. The rapid onset of dynamic sexual colour change in neotropical yellow toads and their well characterized populations makes them an ideal study species to investigate questions about parasite-mediated sexual selection, such as the Hamilton-Zuk hypothesis.

Thesis Objectives

In this thesis, my objectives were to identify the blood parasites of neotropical yellow toads and uncover the possible role of parasite-mediated sexual selection in this dynamically sexually dichromatic anuran species. To date, no one has investigated the blood parasites of this species, and although male colour change is dramatic and charismatic, only recently has scientific attention focused on why neotropical yellow toads change colour dynamically. Amongst the recent climate-driven declines and extinctions of anuran species worldwide, it is important to monitor parasitizing species of anurans because fluctuations of parasites are predicted to occur as climate changes (Blaustein et al., 2010; Harvell et al., 2002). These fluctuations in parasitism may play an unpredictable role in sexual selection of vulnerable anuran species, making investigations into parasite-mediated sexual selection important for anuran conservation as climate changes.

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Figures and Tables



Figure 1.1. Three male neotropical yellow toads (*Incilius luetkenii*) in their breeding colouration. Male neotropical yellow toads dynamically change colour from dull female-like brown colours to bright lemon yellow; however, extent of yellow breeding colouration can vary between individual males.

Table 1.1 Summary of studies of the Hamilton-Zuk hypothesis in anurans. These studies investigate male traits in relation to parasitic infection or female choice, or male parasite load in relation to female choice.

+/- Represents whether the authors found a positive or negative relationship respectively.

N/A Represents a factor that was not studied.

*Traits which may indirectly influence male mating success.

Study Species	Male trait examined	Male trait relationship to parasite infection	Male trait relationship to female choice	Male parasite load relationship to female choice	Reference
<i>Hyla versicolor</i>	Call parameters	No relationship	N/A	+	(Hausfater et al, 1990)
	Snout-to-vent length	+	N/A		
<i>Scaphiopus couchii</i>	N/A	N/A	N/A	No relationship	(Tinsley, 1990)
<i>Scaphiopus couchii</i>	Call duration	+	No relationship	No relationship	(Pfennig & Tinsley, 2002)
	Dominant call frequency	No relationship	N/A		
	Condition	+	No relationship		
<i>Scaphiopus couchii</i>	Colouration	No relationship	+	N/A	(Vásquez & Pfennig, 2007)
<i>Oophaga pumilio</i>	Call pulse rate	+	N/A	No relationship	(Pröhl et al., 2013)
	Condition	No relationship	N/A		
	Colour contrast with background	-	N/A		
<i>Hypsiboas prasinus</i>	Call rate	-	N/A	N/A	(Madelaire et al., 2013)
	Call duration	No relationship	N/A		
	Body mass	No relationship	N/A		
<i>Rhinella icterica</i>	Condition	No relationship	N/A	N/A	(Moretti et al., 2014)
	Locomotor performance*	-	N/A		
<i>Hyla japonica</i>	Call rate	+	N/A	N/A	(An & Waldman, 2016)
	Call duration	+	N/A		
<i>Rhinella icterica</i>	Calling effort	No relationship	N/A	N/A	(Moretti et al., 2017)
	Locomotor performance*	-	N/A		
<i>Litoria wilcoxii</i>	Colouration	No relationship	N/A	N/A	(Kindermann et al., 2017)
	Corticosterone*	+	N/A		
	Testosterone*	-	N/A		
<i>Eleutherodactylus cooki</i>	Colouration	+	N/A	N/A	(Longo et al., 2020)

**Chapter 2: The clue in their hue: parasites and sexual selection in a sexually
dichromatic toad**

Chapter Summary

Parasite-host coevolution is a cycle that arises when a parasite evolves to increase infection success while the host evolves to increase infection resistance. Research on anuran parasites often focuses on the description and identification of parasite species, and we know that parasitic infection can have deleterious effects on the health of individual frogs. There is comparatively little research on how parasitism influences evolutionary dynamics such as sexual selection. Here, we investigate anuran blood parasites with a focus on parasite-mediated sexual selection. One widely studied hypothesis, proposed by Hamilton and Zuk in 1982, suggests that parasite-host coevolution drives sexual ornamentation in hosts and that the degree of ornament expression honestly signals heritable parasite-resistance. While the Hamilton-Zuk Hypothesis has received support in a number of animal taxa, it has received scant attention in amphibians. Neotropical yellow toads (*Incilius luetkenii*) are a sexually dichromatic frog with an unusual sexual ornament: males turn from cryptic brown to bright lemon yellow for a very short period during the breeding season. In this study, we identify blood parasites in male neotropical yellow toads, and we investigate the Hamilton-Zuk hypothesis by comparing colour and morphometric data to blood parasite levels. We found four types of parasites; Apicomplexa and frog erythrocytic virus were the most common, infecting 73% and 46% of male neotropical yellow toads respectively, whereas Nematoda and bacteria were present at lower levels. We found that some parasites had a negative relationship with some aspects of male colour and male body condition, while others had a positive relationship. Our results suggest that parasites may influence sexual traits in this species, but that the relationship is complex. This research contributes to our understanding of amphibian host-parasite coevolution and may inform future amphibian conservation efforts.

Introduction

Darwin's theory of sexual selection suggests that conspicuous male ornaments can evolve if they are preferred by females and thereby increase male reproductive success (Darwin, 1871).

However, conspicuous ornaments may be costly to produce or maintain, or they may increase detection by predators, threatening the survival of the bearer (Andersson, 1982; Zahavi, 1977).

Why would females choose more conspicuous males if conspicuousness decreases a male's chance of survival? One explanation involves the honest indicator hypotheses of sexual selection, which suggest that ornamentation represents the displayer's quality, providing a basis for female mate choice (Andersson, 1994; Zahavi, 1977). In particular, the "good genes" hypothesis states that females exhibit preferential choice of high-quality males, thereby allowing females to pass on these higher quality genes to their offspring (Andersson, 1994; Hamilton & Zuk, 1982). For example, in whitefish (*Coregonus lavaretus*), males ornamented with larger breeding tubercles were in better condition, suggesting more efficient foraging, and they produced more viable offspring (Huuskonen et al., 2009). Therefore male breeding tubercles can provide an honest signal to females about traits inherited by offspring such as foraging ability (Huuskonen et al., 2009).

One important indicator of health, and therefore quality, is parasite load (Sheldon & Verhulst, 1996). In 1982, Hamilton and Zuk proposed that parasite and pathogen resistance genes are heritable, and that the more resistant males signal their lower parasite load and therefore superior quality through sexual ornaments such as plumage colouration in birds and fur condition in mammals (Hamilton & Zuk, 1982). In other words, the Hamilton-Zuk hypothesis states that the degree of expression of male courtship traits signal to females their degree of

heritable parasite resistance (Hamilton & Zuk, 1982). Testing the Hamilton-Zuk hypothesis requires: (1) assessing whether a species carries parasites, (2) whether expression of a trait is indicative of parasite resistance (Hamilton & Zuk, 1982; Møller, 1990), and (3) whether females show a preference for the ornamental trait. Currently, most studies use the parasite load of host species as a proxy for parasite resistance because it is assumed that the individual with fewer parasites have improved resistance (Hausfater et al., 1990; Pfennig & Tinsley, 2002; Tinsley, 1990; Vásquez & Pfennig, 2007). To our knowledge, no studies have taken the next step of investigating the genetic basis of parasite resistance in anurans. The relationship between parasitism and the expression of a sexually selected trait has mainly been studied in birds (Buchholz, 1995; Doucet & Montgomerie, 2003; Korpimäki et al., 1995; Thompson et al., 1997). For example, in satin bowerbirds (*Ptilonorhynchus violaceus*) males with duller plumage colouration had higher parasite loads, consistent with the Hamilton-Zuk hypothesis (Doucet & Montgomerie, 2003; Hamilton & Zuk, 1982). In house finches (*Haemorrhous mexicanus*), red colouration in male feathers decreases with parasite load (Brawner et al., 2000), and females prefer redder males indicative of better quality (Hill, 1991).

Many studies have investigated the relationship between male courtship traits and female mate choice in anurans, often showing that females choose more conspicuous traits such as those related to colour and vocal behaviour (e.g., Ryan, 1980; Sullivan & Kwiatkowski, 2007; Sztatecsny et al., 2012; Welch et al., 1998). Few studies have explicitly tested the Hamilton-Zuk hypothesis in anurans, however, despite recent interest on colour variation in this group. Of those studies, some have found support for the Hamilton-Zuk hypothesis in anurans, such as in Burmeister's treefrog (*Hypsiboas prasinus*) where call rate was higher in less infected males (Madelaine et al., 2013). Other investigations have found no relationship between male sexual

traits and parasite load; for example in the gray treefrog (*Hyla versicolor*), there was no relationship between call parameters and parasitism (Hausfater et al., 1990). In *Scaphiopus couchii*, a positive relationship was found between body condition and parasite infection in male toads (Pfennig & Tinsley, 2002). More research is needed to document the parasites of anurans and their sexual ornaments, as well as the relationships between those parasites and ornaments to gain a broader understanding of the Hamilton-Zuk hypothesis in anurans.

In this study, we investigate the relationship between male parasitism and male condition and breeding colouration in the neotropical yellow toad (*Incilius luetkenii*). The neotropical yellow toad is an explosively-breeding species that exhibits dynamic sexual dichromatism: males alter their skin colour from a dull brown to a bright yellow during a very short breeding period lasting only 1-3 days (Doucet & Mennill, 2010). In addition to the dramatic colour change exhibited by males during breeding, males vary considerably in the brightness of their yellow dorsum colour (Gardner et al., 2020; Figure 2.1). The purpose of male colour change is not fully understood, although it has been suggested that possible functions include sex recognition within a busy mating aggregation (Rehberg-Besler et al. 2015) and mate selection by females (Gardner et al., 2020; Gardner et al., 2021). We were interested in investigating whether this variation in male colouration could function as an honest indicator of male parasite load and parasite resistance.

The objectives of this study were twofold. First, we documented the blood parasites of neotropical yellow toads, which had been previously unexplored. Given that many anurans are host to a diversity of blood parasites, we predicted that neotropical yellow toads would host parasites, especially those belonging to Apicomplexa and Nematoda because they are common

anuran parasites in neotropical regions (Burseley & Brooks, 2010; Dessler, 2001). Secondly, we investigated the relationship between male skin colouration and parasite load as a test of the Hamilton-Zuk Hypothesis. In accordance with this hypothesis, we predicted that the most colourful male toads would have the fewest parasites, indicating that more colourful males are more resistant to parasites. We also predicted that males with higher body condition would have fewer parasites, indicating that higher body condition males are more resistant to parasites, or that parasitism reduced body condition. The two objectives of our study seek to investigate the first two steps in testing the Hamilton-Zuk hypothesis: (1) assessing whether *Incilius luetkenii* carries parasites, and (2) whether expression of yellow colouration is indicative of parasite resistance (Hamilton & Zuk, 1982; Møller, 1990).

Methods

General methods

We collected field data in Sector Santa Rosa within the Guanacaste Conservation Area in northwestern Costa Rica as part of an ongoing study of neotropical yellow toads. When males emerged from aestivation at the end of the dry season, we captured male toads as they arrived at the breeding pond that formed with the first large rainfall of the rainy season. In 2018, we captured males as they emerged on May 16 and 17. In 2019, we captured males as they emerged on May 13 to 17. In both years, we captured males beginning in early morning (at approximately 5:30 am) until mid-morning. We captured males by hand and obtained a blood sample and colour measurements within five minutes of capture. We also measured each male's mass (to the nearest g) and snout-vent length (to the nearest mm; Gardner et al., 2020, 2021).

Field measurements

We collected ~20 μ L of blood from each male using a 28-gauge needle and 0.5 mL syringe via a blood vessel behind the knee (blood was used in a separate study of hormones; Gardner et al., 2020, 2021). We placed one drop of blood on a glass microscope slide, and created a blood smear by running a microscope covering slip along the slide (Arikan & Çiçek, 2014). We air dried blood slides in the field. We measured colour of the male toads' dorsum using a field-portable spectrometer (model: JAZ-COMBO; Ocean Optics, Dunedin, FL). We collected colour measurements shortly after capture, immediately after collecting a blood sample. We fit the probe of the spectrometer with a rubber probe holder that allowed us measure colour at a consistent distance of ~5mm from the skin surface while also blocking any external light sources, and all of the reflectance measurements were calculated relative to a white standard for consistency (Doucet & Montgomerie, 2003; Gardner et al., 2020).

Parasite identification

We brought the air-dried blood slides from our field site in Costa Rica to campus at the University of Windsor, where we stained the slides following the Hema 3 staining procedure (Arikan & Çiçek, 2014; Doucet & Montgomerie, 2003). Each slide was observed with a microscope (Olympus EH; Tokyo Japan) under x1000 magnification oil immersion. Within each slide we scanned for intra- and extra-cellular parasites until 10,000 host cells had been surveyed (as in Doucet & Montgomerie, 2003). All slides were scanned by the same observer (NES) who was blind to all colour and morphometric data. Parasites were identified and categorized into

Apicomplexa, Nematoda, frog erythrocytic virus, and bacteria (Barta et al., 1987; Barta & Dessler, 1984; Dessler, 2001; Dessler & Barta, 1984; Netherlands, Cook, et al., 2020; Netherlands et al., 2015; Netherlands et al., 2020; Smith et al., 2000).

Colour measurements

We collected three reflectance measurements from each male's dorsum that we later averaged for use in our analyses (Gardner et al., 2020). We calculated three colour variables from these data (brightness, saturation, and hue) using the program CLR (Montgomerie, 2008). We calculated brightness as the mean reflectance from 400-700 nm; higher values for brightness correspond to more light being reflected off a surface, appearing lighter in colour (Montgomerie, 2008). We calculated saturation as the difference between maximum and minimum brightness values divided by the mean brightness; higher values of saturation correspond to a more prominent spectral curve peak which appear more intensely yellow, compared to lower saturation values which have flatter spectral curves, appearing greyer (Montgomerie, 2008). We calculated hue using the equation: $\{(B_y - B_b) / [(B_r - B_g) / B_1]\}$, where B is brightness, B_1 is the total brightness across the reflectance spectrum from 300-700 nm, and subscripts correspond to parts of the reflectance spectrum as follows: red (r) = 600-700 nm, yellow (y) = 500-600 nm, green (g) = 400-500 nm, and blue (b) = 300-400 nm. Higher values of hue correspond to more reflectance at higher wavelengths which are on the redder end of the visible colour spectrum, whereas lower values of hue correspond with lower wavelengths at the bluer end of the colour spectrum (Montgomerie, 2008).

Statistical Analysis

We collected blood from 52 male toads for parasite analysis (22 males in 2018 and 30 males in 2019). We were able to collect colour data from 50 males (22 males in 2018 and 28 males in 2019), and morphometric data from 49 males (21 males in 2018 and 28 males in 2019). To quantify body condition, we calculated a body condition score as the residuals of a regression between body mass and snout-to-vent length (Jakob et al., 1996; Moretti et al., 2014). The parasite data were not normally distributed and could not be normalized by standard transformation; we therefore used non-parametric tests in all of our analyses.

Results

Blood parasites of neotropical yellow toads

Our examination of blood slides collected from male neotropical yellow toads yielded four categories of parasites: Apicomplexa, Nematoda, frog erythrocytic virus, and bacteria (Table 2.1, Barta et al., 1987; Bursey & Brooks, 2010; Conradie et al., 2017; Dessler, 2001; Dessler & Barta, 1984; Esslinger, 1986; Netherlands et al., 2018, 2015).

We identified Apicomplexa in 73% of the samples. All Apicomplexa were intracellular parasites inhabiting the cytoplasm, ranging from 3-6 μm in size, and stained lightly pink or purple (Fig 2.2a, 2.2b). We could not identify the Apicomplexa parasites below the level of the phylum.

We identified Nematoda parasites in 27% of samples. All Nematoda were found in the microfilaria life stage, they were extracellular and stained dark purple. We found two types of

microfilaria nematodes. The first we identified as *Ochoterenella digiticauda* (Burse & Brooks, 2010; Esslinger, 1986), approximately 100 μm in length (Fig. 2.2c; found in 3.8% of samples). The second we were unable to identify below the level of phylum, approximately 60 μm in length (Fig. 2.2d; found in 23% of samples).

We identified frog erythrocytic virus in 46% of samples. Our observations revealed that frog erythrocytic virus was always intracellular, inside the cytoplasm. We identified these as small (<1-3 μm), dark purple stained inclusions (Fig. 2.2e, 2.2f).

In 36% of our samples, we also identified Bacteria inside the cytoplasm, characterized by light pink staining, and 1-3 μm in size (Fig. 2.2g, 2.2h).

Blood parasites and colour

We found negative correlations between the intensity of parasitism and the saturation and hue of toad colour. The number of Apicomplexa blood parasites was negatively correlated with both saturation (Figure 2.3b Spearman's rho: = -0.36, n = 50, p = 0.01) and hue (Figure 2.3c; Spearman's rho: = -0.32, n = 50, p = 0.02); males with fewer Apicomplexa parasites had higher yellow saturation and longer wavelength hue (i.e., the warmer end of the visible colour spectrum). The number of Nematoda parasites was negatively correlated with hue (Figure 2.3i; Spearman's rho: = -0.33, n = 50, p = 0.01); males with fewer Nematoda had longer-wavelength hue. Bacterial load was negatively correlated with saturation (Figure 2.3k; Spearman's rho: = -0.29, n = 50, p = 0.04); males with less bacterial infection had higher yellow saturation. In contrast, frog erythrocytic virus was significantly positively correlated with saturation (Figure

2.3e; Spearman's rho: = 0.28, n = 50, p = 0.04); males with more viral infection had higher yellow saturation.

We found no correlation between the brightness of neotropical yellow toads and any of the parasites detected (Apicomplexa: Figure 2.3a, Spearman's rho: = 0.16, n = 50, p = 0.24; Frog erythrocytic virus: Fig. 2.3d, Spearman's rho: = 0.15, n = 50, p = 0.29; Nematoda: Fig. 2.3g, Spearman's rho: = 0.26, n = 50, p = 0.05; Bacteria: Fig. 2.3j, Spearman's rho: = 0.15, n = 50, p = 0.27). Likewise, frog erythrocytic virus was not correlated with hue (Figure 2.3f; Spearman's rho: = 0.06, n = 50, p = 0.67), Nematoda was not correlated with saturation (Figure 2.3h; Spearman's rho: = -0.06, n = 50, p = 0.66), and bacteria was not correlated with hue (Figure 2.3i; Spearman's rho: = -0.20, n = 50, p = 0.15).

Blood parasites and body condition

We found that parasite load was correlated with male body condition (calculated as the residuals of a regression between body mass and snout-to-vent length). Only frog erythrocytic virus was negatively correlated with body condition (Figure 2.4b; Spearman's rho: = -0.48, n = 49, p = 0.0004), such that males with less frog erythrocytic virus were in better condition. The other three categories of blood parasites showed positive correlations with body condition (Apicomplexa: Figure 2.4a; Spearman's rho: = 0.33, n = 49, p = 0.02, Nematoda: fig. 2.4c; Spearman's rho: = 0.29, n = 49, p = 0.04, Bacteria: fig. 2.4d; Spearman's rho: = 0.40, n = 49, p = 0.003), such that males infected with more Apicomplexa, Nematoda, and bacteria were in better condition.

Discussion

Male neotropical yellow toads undergo a dramatic transformation from dark brown to bright lemon yellow during their brief mating events. We investigated the blood parasites of neotropical yellow toads and considered their potential role in communicating parasite resistance through colour and body condition. Through analysis of blood collected from male toads, we identified four types of parasites infecting neotropical yellow toads, providing the first description of the blood parasites in this species. We found evidence consistent with predictions of the Hamilton-Zuk hypothesis for parasite-mediated sexual selection. More colourful yellow toads had fewer blood parasites: males less infected with Apicomplexa and bacteria were more saturated yellow, and males less infected with Apicomplexa and Nematoda showed higher values of yellow hue (i.e., more reflectance at longer wavelengths). We also found evidence consistent with our prediction that males in better condition would have fewer blood parasites such that males less infected with frog erythrocytic virus were in better condition. We also found some trends that stood in contrast with our predictions: males with lower frog erythrocytic virus loads had higher colour saturation and males infected with more Apicomplexa, Nematoda, and bacteria were in better body condition. Our study suggests a possible role for parasite-mediated sexual selection in anurans, but further investigation is needed to better understand the relationship between colour, body condition, and parasitism.

Parasite identification in neotropical yellow toads

Our first goal in this study was to identify the blood parasites of neotropical yellow toads, which is the first step in testing the Hamilton-Zuk hypothesis. Through blood slide analysis, we found four types of parasites: Apicomplexa, Nematoda, frog erythrocytic virus, and bacteria.

Apicomplexa and Nematoda are two large phyla containing parasitic organisms that are the most commonly documented parasites in anuran species (Barta & Dessler, 1984; Dessler, 2001; Gruija-Gray et al., 1989; Miller et al., 2009; Netherlands et al., 2020). The phylum Apicomplexa is exclusively parasitic, infecting a wide range of animals from fish, mammals, birds, and amphibians, and exhibiting considerable diversity in size and morphology (Woo, 2006). Many Apicomplexa have been identified in frogs, with the most common being the genus *Hepatozoon* which has been found in the guttural toad (*Amietophrynus gutturalis*), and the marbled reed frog (*Hyperolius marmoratus*) among many others (Conradie et al., 2017; Netherlands et al., 2018; Netherlands et al., 2014; Smith, 1996). Apicomplexa are transmitted to anuran hosts through biting arthropods and consumption of infected organisms or feces (Amino et al., 2006; Reid et al., 2012). There are few studies on the health effects of Apicomplexa on anuran hosts; however, in water pythons (*Liasis fuscus*), infection by *Hepatozoon* sp. caused slower growth rates and reduced condition (Madsen et al., 2005).

All Nematoda found in male neotropical yellow toads were larval stages called microfilaria which reside in the blood until molting and migrating to other areas of the host's body. Microfilaria have been found in anuran hosts such as the natal forest tree frog (*Leptopelis natalensis*), and the cane toad (*Bufo marinus*; Dessler, 2001; Kuzmin et al., 2021). Nematoda's most likely mode of infection is through transmission by an infected mosquito (*Culex* and *Aedes*) (Aisien et al., 2015), and infection can cause loss of skin pigmentation, ulcers, neurological complications and death in anuran hosts (Densmore & Green, 2007). Nematoda parasites are usually larger and more distinctive in shape than the other three parasites identified, which allowed us to identify one species as *Onchoteronella digiticauda* (Burseley & Brooks, 2010; Esslinger, 1986). Interestingly, the only description of neotropical yellow toad parasites

identified Nematoda, including *Onchoteronella digiticauda*, which were studied through dissection of toads and collection of adult worms found in the body cavity and organs (Burse & Brooks, 2010). Although we found an additional Nematoda species, we were not able to identify it precisely.

We also found virus and bacteria in the blood of male neotropical yellow toads. We identified the virus as frog erythrocytic virus; however, we could not identify the bacteria due to the lack of research in this field (Gruia-Gray & Dessler, 1992; Gruia-Gray et al., 1989; Netherlands et al., 2015). Viruses and bacteria are also very small and difficult to identify without magnification greater than x1000. Nonetheless, frog erythrocytic viruses and bacteria have been classified as parasites and documented in some anuran species such as bullfrogs (*Rana catesbeiana*) and the Mozambique ridged frog (*Ptychadena mossambica*; Gruia-Gray & Dessler, 1992; Netherlands et al., 2015). It has been suggested that frog erythrocytic virus is transmitted to anuran hosts through mosquitoes (*Culex*) and frog-biting midges (*Forcipomyia*; Gruia-Gray & Dessler, 1992). Frog erythrocytic virus has not been shown to cause any clinical effects in anuran hosts; however, in this study as well as others, it has been found in the presence of other parasites such as Apicomplexa, and compounding effects of these parasites have not been investigated (Densmore & Green, 2007; Dessler, 2001; Dessler & Barta, 1984; Graczyk et al., 1996). Bacteria have been known to cause many issues in anuran hosts such as red-leg syndrome which has similar clinical signs to the deadly fungal pathogen, *Batrachochytrium dendrobatidis* (Densmore & Green, 2007). Bacteria commonly infects through traumatic injury to the host and can even secondarily infect after viral infections (Densmore & Green, 2007).

Detection of parasites through blood smears is less common than detection through organs or the body cavity (Hausfater et al., 1990; Madelaire et al., 2013; Pfennig & Tinsley, 2002; Tinsley, 1990; Vásquez & Pfennig, 2007). In gray treefrogs, blood smears revealed only 1.5% of males were infected with parasites belonging to the genus *Trypanosoma*, so few that the parasites were not included in analyses (Hausfater et al., 1990). Similarly, a study of spadefoot toads (*Scaphiopus couchii*) revealed only a 5% prevalence of filarial Nematoda in blood smears, which the authors declared too few to include in analyses (Tinsley, 1990). We have shown in this study that blood smears are capable of revealing a diversity of blood parasites, some which may also indicate prevalence of adult parasites found in the organs (eg. microfilaria stages of Nematoda parasites), and collection of blood smears is non-lethal which is important for anuran species experiencing population declines.

Studies which detect blood parasites in anurans report a variety of host species, parasite species, and prevalence of parasites. One study reported that in *Rana vaillanti* in Costa Rica, the prevalence of Apicomplexa species was 47% and the prevalence of a species of Nematoda microfilaria was 42%, while *Eleutherodactylus fitzingeri* captured at the same time and the same location were not found to have these parasites (McKenzie & Starks, 2008). Another study found that 7 species of anurans found in Ontario, Canada, all had Nematoda microfilaria prevalences of 2% or less, and the prevalence's of a species of Apicomplexa ranged from 0% to 49.1% in the same species (Barta & Desser, 1984). Results from these studies show that drawing meaningful comparisons of parasite prevalence between host species is difficult due to various factors influencing parasitism. Such factors include the anuran host species, the parasite, and even the year the study was conducted. More investigations into the diversity of blood parasites in a

diversity of anuran hosts and monitoring parasite prevalence over time is required to compare prevalence between populations of species.

Parasite-mediated sexual selection in neotropical yellow toads

The second goal of this study was to investigate the Hamilton-Zuk hypothesis for parasite-mediated sexual selection in neotropical yellow toads. We predicted that more colourful males would have fewer blood parasites, indicating their higher resistance to parasitism. We found that saturation and hue were negatively correlated with two of the four parasites: saturation with Apicomplexa and bacteria, and hue with Apicomplexa and Nematoda, in accordance with our predictions. These results are the first to show that more colourful male anurans have fewer parasites. In previous studies, it was found that in the sexually dichromatic anuran, *Litoria wilcoxii*, there was no relationship found between male colour and *B. dendrobatidis* infection (Kindermann et al., 2017), and higher tick (*Carios*) parasitism was found on more colourful male *Eleutherodactylus cooki* (Longo et al., 2020). Along with these expected negative associations between parasites and colour, we also revealed one positive correlation, opposite of our predictions, between frog erythrocytic virus and yellow saturation. Although studies of the Hamilton-Zuk hypothesis in anurans are uncommon, investigations have revealed mixed results for other traits. For example, one investigation found that male Burmeister's treefrogs with higher call rates were less parasitized by helminths (Madelaire et al., 2013); however, another investigation found no association between male gray treefrog call rate and parasitism of helminths (Hausfater et al., 1990). Mixed results such as these suggest that parasite resistance may be host-species dependent, but also resistance may depend on the individual parasite species present in hosts, as suggested by our findings.

In accordance with the Hamilton-Zuk hypothesis, we also predicted that male neotropical yellow toads in better condition would have lower blood parasite load, indicating their higher parasite resistance. We found frog erythrocytic virus to be the only parasite negatively associated with body condition. This result is consistent with our predictions because the Hamilton-Zuk hypothesis states that more parasite resistant, and therefore less parasitized males, will advertise their superior health to females through traits such as mass and body length in anurans (Hamilton & Zuk, 1982; Pfennig & Tinsley, 2002). However, we also revealed several positive associations between parasites and male condition, in contrast to our predictions. We found that three of the four parasites were positively associated with body condition: Apicomplexa, Microfilaria, and bacteria. Interestingly, some studies comparing elements of body condition to parasitism in anurans found similar positive correlations, such as a study that found more parasitized male spadefoot toads to have larger testes, longer calls, and larger body sizes (Pfennig & Tinsley, 2002). The authors suggested that the result could be because more parasitized males in better condition are older than less parasitized males, and males in better condition are able to survive longer, giving them more time to accumulate parasites (Pfennig & Tinsley, 2002). Such a pattern could be the reason for the positive association between parasites and body condition in neotropical yellow toads as well.

The next steps testing the Hamilton-Zuk hypothesis in neotropical yellow toads includes investigating the heritability of parasite resistance and investigating female preferences for males based on parasite load and traits indicative of parasite load to test whether parasite-mediated sexual selection is indeed present in this species. No studies have investigated whether innate parasite resistance is heritable in anuran species; however, in a study of domesticated sheep (*Ovis aries*), resistance to Nematoda parasites was found to have a genetic component heritable

to offspring, where more resistant parents passed on their resistance genes to offspring (Gulland et al., 1993; J. A. Smith et al., 1999). Few studies have investigated female preference for less parasitized males, and these studies either directly test the parasite load of chosen males (Tinsley, 1990), test female preference for male traits indicative of parasite load such as call length or mass (Pfennig & Tinsley, 2002; Vásquez & Pfennig, 2007), or both (Hausfater et al., 1990; Pröhl et al., 2013). The results of these studies revealed that females prefer more parasitized males or found no preference for more or less parasitized males (Hausfater et al., 1990; Pfennig & Tinsley, 2002; Pröhl et al., 2013; Tinsley, 1990). However, more investigations should be conducted on more anuran species and more parasite species before conclusions can be made about parasite-mediated sexual selection in anurans. It may also be important to investigate female parasitism and its influence on mate choice. In spadefoot toads, unparasitized females preferred males in better condition but were more parasitized, whereas parasitized females showed no preference, reducing sexual selection on condition-dependent traits (Pfennig & Tinsley, 2002).

In neotropical yellow toads, a recent study showed that when presented with both a dull and colourful model of male toads, females showed no preference for either colouration (Gardner et al., 2021). Results from our study show that Apicomplexa, Nematoda, and bacteria are negatively associated with colouration, while one parasite (frog erythrocytic virus) is positively associated with colour. Therefore our results suggest that parasite-mediated sexual selection may be present in neotropical yellow toads. Nevertheless, mixed associations between parasites and male quality may be sending confusing signals to female neotropical yellow toads, especially when males harbour a diversity of parasites. This may explain their lack of preference in a previous study because females may be unable to distinguish between parasitized and healthy

males. Our findings suggest that because different parasites are associated with various aspects of colour, parasitism by multiple species may require species-specific parasite resistance, and some male toads may be more resistant to some parasites over others. Variations in males' species-specific parasite resistance may interact in a way that make colour an unreliable indicator of parasite resistance in this species. Nevertheless, further female choice experiments based on live males rather than models should be considered before rejecting the Hamilton-Zuk hypothesis in this species.

Conclusion

Currently, there is little research of parasite-mediated sexual selection in anurans. This is the first study to identify blood parasites and investigate the Hamilton-Zuk hypothesis in neotropical yellow toads. In this study we found that neotropical yellow toads are parasitized with Apicomplexa, Nematoda, frog erythrocytic virus, and bacteria. Our results revealed a mix of findings, some which support the Hamilton-Zuk hypothesis in neotropical yellow toads, and some which stand in contrast to it. Male toads that are less parasitized by some parasites are more colourful and in better condition, while male toads that are less parasitized by other parasites are less colourful and in worse condition. Because these results are mixed, future research is required involving whether heritable parasite resistance genes are present in neotropical yellow toads, female preference to observe whether parasite-mediated sexual selection operates in this species, and if females can distinguish between parasitized, non-parasitized and even males infected with multiple parasite species so they may pass on the best genetics to their offspring.

Anuran populations have been declining worldwide due to climate change and habitat loss (Stuart et al., 2004), both of which have been shown to facilitate disease and parasite spread (Tylianakis et al., 2008). Furthermore, parasite infection has already caused major declines in amphibian populations, such as the parasitic chytrid fungus, *B. dendrobatidis* (Wake & Vredenburg, 2008). Climate change and habitat loss is theorized to drive fluctuations in parasitic infection (Tylianakis et al., 2008) which may influence sexual selection in many species where parasite-mediated sexual selection is important (Hamilton & Zuk, 1982). If climate continues to change, increased pressure from parasites on neotropical yellow toads, and introductions of novel parasites, could limit populations in a similar fashion to the chytrid fungus. For anuran conservation, it is important to develop long term data-sets to study and identify the parasites which infect anuran hosts, as well as the influence parasitism may have on host sexual selection, in a continually changing environment.

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Figures and Tables

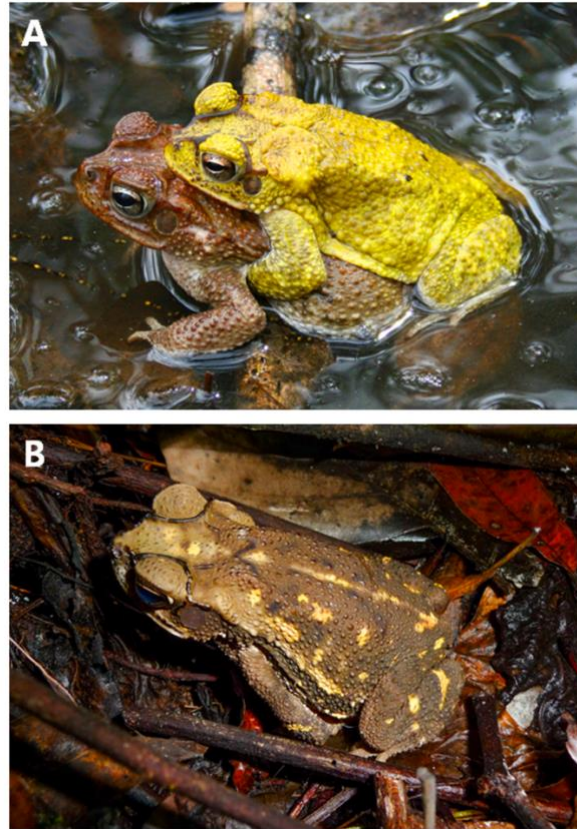


Figure 2.1. Photographs of neotropical yellow toads (*Incilius luetkenii*) exhibiting dynamic sexual dichromatism. (A) A male neotropical yellow toad, displaying bright lemon-yellow mating colouration in amplexus with a brown female. (B) A male neotropical yellow toad displaying dull brown, non-breeding colouration.

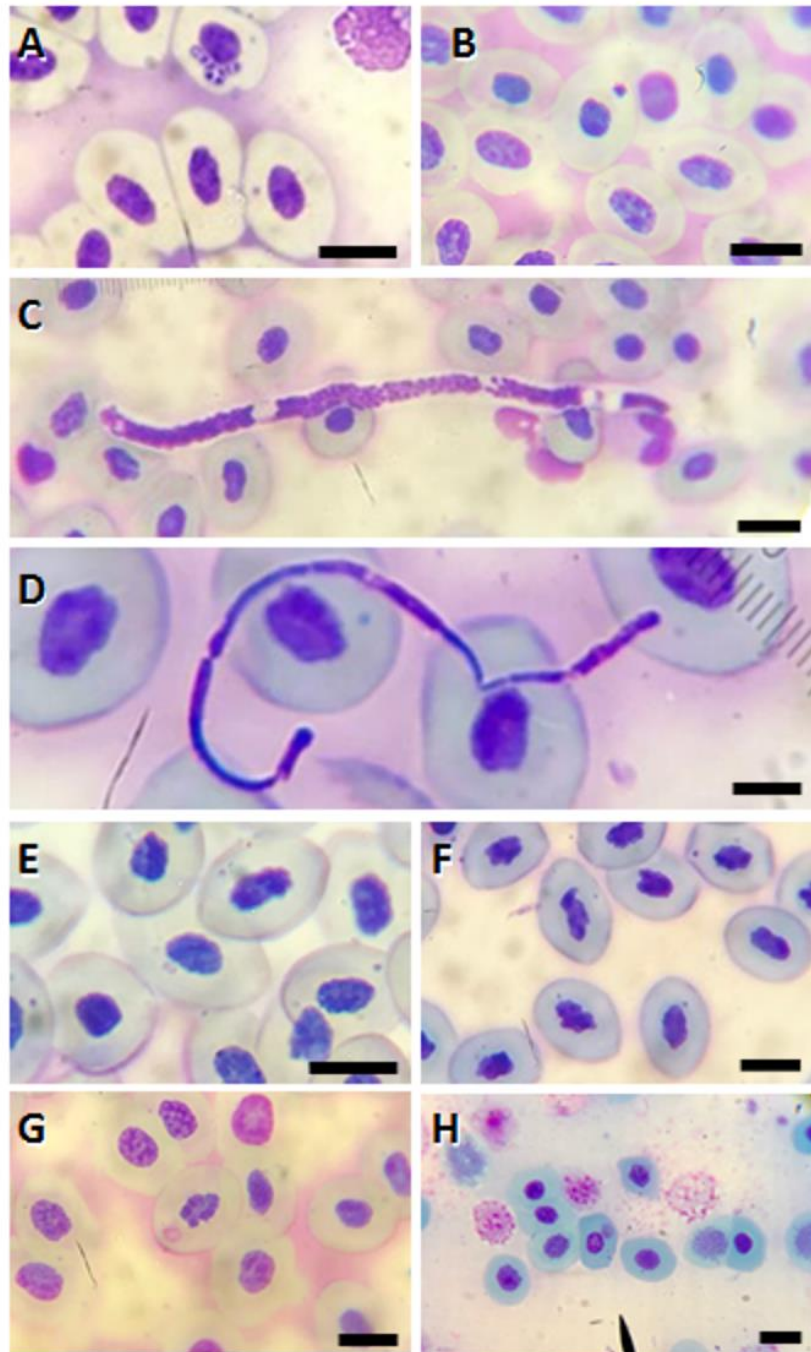


Figure 2.2. Photomicrographs depicting examples of blood parasites observed in the neotropical yellow toad (*Incilius luetkenii*). (A, B) Dark stained, intracellular parasites belonging to the Phylum Apicomplexa. (C) *Ochoterenella digiticauda*, belonging to the phylum Nematoda in the microfilaria life stage. (D) Unidentified species belonging to Nematoda in the microfilaria life stage. (E, F) Dark stained, intracellular frog erythrocytic virus. (G, H) Lightly stained, intracellular bacteria. The scale bar is 10 µm in each image.

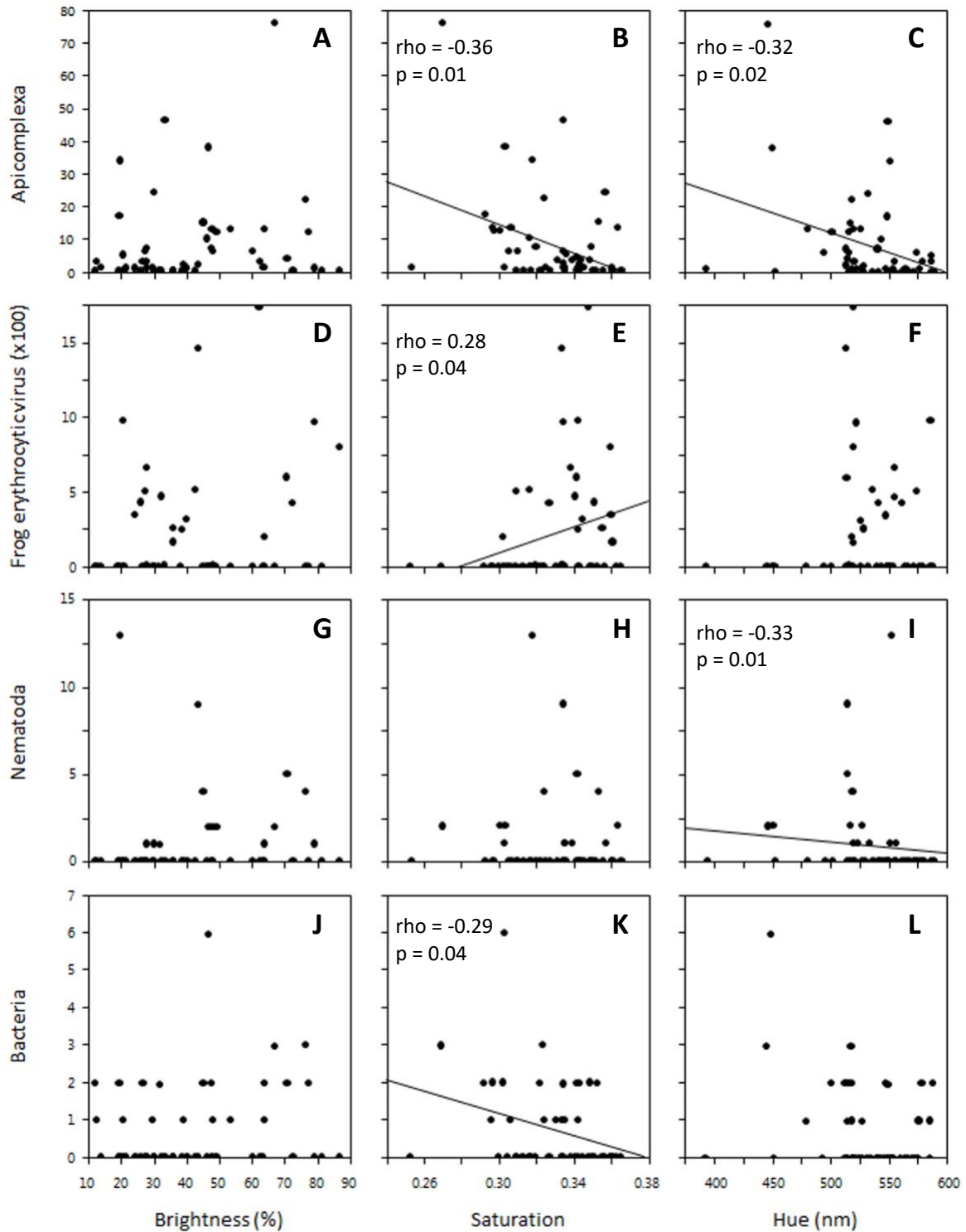


Figure 2.3. Scatterplots of the relationship between four types of blood parasites found in male neotropical yellow toads (*Incilius luetkenii*; Apicomplexa, frog erythrocytic virus, Nematoda, and bacteria) versus colourmetric features of frog skin (brightness, saturation, and hue). Regression lines correspond to significant relationships ($p < 0.05$).

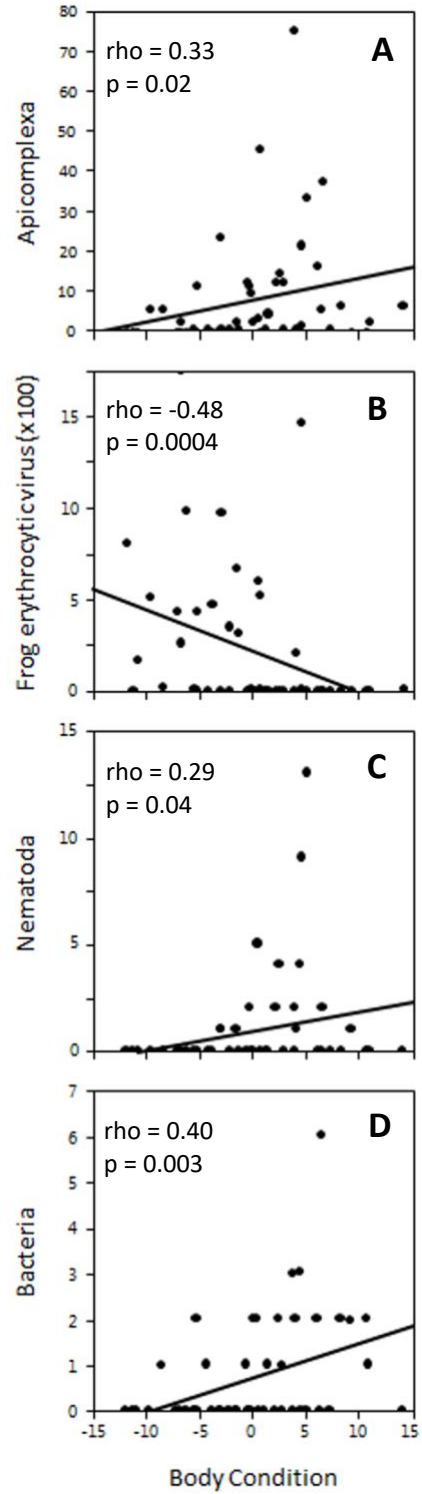


Figure 2.4. Scatterplots of the relationship between four types of blood parasites found in male neotropical yellow toads (*Incilius luetkenii*; Apicomplexa, frog erythrocytic virus, Nematoda, and bacteria) versus body condition (ratio of mass to snout-to-vent length).

Table 2.1 Summary statistics of blood parasites found in male neotropical yellow toads (*Incilius luetkenii*) sampled in northwestern Costa Rica (52 toads were sampled in total).

Type of parasites	Number of toads affected	Number of parasites per individual	
		Range	Mean \pm SE
Apicomplexa	38	0 - 46	8.63 \pm 1.97
Nematoda	14	0 - 13	0.92 \pm 0.32
Frog erythrocytic virus	24	0 - 1735	213 \pm 54
Bacteria	19	0 - 6	0.71 \pm 0.16

Chapter 3: General Discussion

In this thesis, I investigated parasites and parasite-mediated sexual selection in a dynamically sexually dichromatic toad. The objectives of my data chapter were twofold. First, I identified and quantified the blood parasites of neotropical yellow toads (*Incilius luetkenii*) which, until now, has never been investigated. Second, I investigated whether parasite-mediated sexual selection may be operating in this species by testing predictions of the Hamilton-Zuk hypothesis (Hamilton & Zuk, 1982). The Hamilton-Zuk hypothesis suggests that individuals with greater heritable parasite resistance can honestly signal their superior genetic quality to the opposite sex through elaborate ornamentation, since only individuals with low parasitism should be able to produce the most elaborate ornaments. To test this hypothesis in neotropical yellow toads, I needed to confirm the presence of parasites in this species before investigating whether expression of a sexual trait may be indicative of parasite resistance. Neotropical yellow toads provided an excellent study species for testing the Hamilton-Zuk hypothesis because they are dynamically sexually dichromatic; males change colour from female-like brown to bright, lemon yellow during their 1-4 day breeding season, and variation in the extent of yellow colour has been documented between individual males (Doucet & Mennill, 2010; Gardner et al., 2020; Rehberg-Besler et al., 2015). The male neotropical yellow toad's dramatic colour change is an area of ongoing research; however, no one has investigated possible relationships between colour and parasitism in this species. Many anuran species are hosts to blood parasites and therefore I predicted that neotropical yellow toads would be parasitized by Apicomplexa and Nematoda because of their common documentation in the blood of tropical anurans (Bursey & Brooks, 2010; Dessler, 2001). I also predicted that more colourful male toads and males with higher body condition would be the least parasitized, as an indicator of their implied superior parasite resistance.

In addressing my first objective, as predicted, I identified parasites belonging to Apicomplexa and Nematoda. I also identified frog erythrocytic virus and bacteria. Apicomplexa and frog erythrocytic virus were the most prevalent, infecting 73% and 46% of toads respectively. Bacteria and Nematoda were less prevalent, infecting 36% and 27% of toads respectively. Apicomplexa and Nematoda are common anuran parasites (Barta & Dessler, 1984; Dessler, 2001; Gruia-Gray et al., 1989; Miller et al., 2009; Netherlands et al., 2020); frog erythrocytic virus and bacteria have been documented as well, although less commonly (Gruia-Gray & Dessler, 1992; Gruia-Gray et al., 1989; Netherlands et al., 2015). This was the first documentation of blood parasites in neotropical yellow toads.

Secondly, I tested a key prediction of the Hamilton-Zuk hypothesis, that the expression of a sexual trait is indicative of parasite resistance. Given that male neotropical yellow toads dramatically change colour from brown to yellow only during their short breeding season, it has been hypothesized to be a sexual signal (Nicolas Rehberg-Besler et al., 2015). I tested whether expression of yellow colour is related to parasite load, which I used as an indicator of parasite resistance. Body condition as a ratio of mass to snout-vent-length is as an indicator of overall health, and I therefore tested whether body condition is related to parasite load (Arantes et al., 2015; Pfennig & Tinsley, 2002). As predicted, I found negative relationships between parasitism and colour: Apicomplexa was negatively correlated with saturation and hue, Nematoda was negatively correlated with hue, and bacteria was negatively correlated with saturation. However, I also found a positive relationship between parasitism and colour: frog erythrocytic virus was positively correlated with saturation. Also as predicted, I found a negative relationship between parasitism and body condition, that is, males with more frog erythrocytic virus were in lower condition. However, I found positive relationships between Apicomplexa, Nematoda and

bacteria with body condition. Investigating male traits and their relationship with parasitism is an integral step of testing the Hamilton-Zuk hypothesis and exploring parasite-mediated sexual selection. These results show that different parasites are associated with aspects of colour in various ways, indicating that male toads may require species-specific parasite resistance. The relationship between parasite resistance and sexual ornaments proves to be more complex than previously thought; however, I have uncovered evidence that parasites are negatively associated with colour in neotropical yellow toads, and therefore that parasite-mediated sexual selection may be present in this species. Variation in individual male's species-specific parasite resistance may cause colour to be an unreliable signal to females in neotropical yellow toads.

My research contributes a colourful perspective to a field of anuran research mainly dominated by acoustic studies (An & Waldman, 2016; Hausfater et al., 1990; Madelaire et al., 2013; Moretti, Madelaire et al., 2014; Pfennig & Tinsley, 2002; Pröhl et al., 2013; Nick Rehberg-Besler et al., 2016). Although it is known that many anuran species display bright colouration, sexual dimorphism, and mate choice (Hoffman & Blouin, 2000), parasite-mediated sexual selection through colour has received little attention (Kindermann et al., 2017; Longo et al., 2020; Pröhl et al., 2013; Vásquez & Pfennig, 2007). Furthermore, only one prior study has investigated parasites in this species, specifically searching for Nematoda parasites, for which 48 neotropical yellow toads were necropsied (Bursey & Brooks, 2010). By using blood samples, I was able to explore the diversity of parasites infecting neotropical yellow toads, and to do so without sacrificing individuals. Future studies investigating the parasites of anurans and exploring parasite-mediated sexual selection might consider using less invasive approaches especially in such a vulnerable group.

My data show support for a key component of the Hamilton-Zuk hypothesis. However, in order to fully test the Hamilton-Zuk hypothesis in neotropical yellow toads, two more steps should be investigated in future research. First, it is important to investigate female preferences for males' sexual traits that are indicative of parasitism. For example, female *Scaphiopus couchii* were presented with different male call types indicative of male quality (Pfennig & Tinsley, 2002), and in *Hyla versicolor*, males chosen by females were assessed for their parasite levels after a relationship between male call parameters and parasite levels was found (Hausfater et al., 1990). Assessing female preference will show whether females indeed choose males based on traits honestly signalling their parasite resistance. Previously, female neotropical yellow toads were found to exhibit no preference between dull and brightly coloured robotic male toads (Gardner et al., 2021). However, I recommend also studying female preference between live dull and brightly coloured males, as well as determining the male's infection status in order to confidently determine whether females may use male colouration as a signal of health. Secondly, male neotropical yellow toads should be tested for the heritability of parasite-resistance. No studies have taken this step in anurans; however, in domesticated sheep (*Ovis aries*), parasite resistance was found to be heritable through genetic analysis of parents and offspring (Gulland et al., 1993). The Hamilton-Zuk hypothesis requires that ornamented males are able to pass on genes for parasite resistance to their offspring, and therefore determining whether parasite resistance is heritable is important.

Currently, most studies use parasite levels as a proxy for parasite-resistance (Hausfater et al., 1990; Pfennig & Tinsley, 2002; Tinsley, 1990; Vásquez & Pfennig, 2007); however, this assumption would have stronger support if parasite-resistance was also investigated. For example, when Atlantic Salmon (*Salmo salar*) were exposed to the bacteria, *Aeromonas*

salmonicida, broods that were less infected were found to have higher prevalence of an allele associated with disease resistance (Langefors et al., 2001). Similar techniques could be used in neotropical yellow toads to determine a genetic basis for parasite resistance. Lastly, in this study, I found four different groups of parasites, which appeared to influence colour and body condition in various ways. It would be beneficial to find effects of individual parasites without interference from other species, to find the true effect of each parasite on colour and body condition. Although this would prove difficult in the wild, experimental manipulation of naturally occurring parasite species in a laboratory setting could be a promising avenue of future study.

Future studies might also consider climate change as a factor influencing parasite prevalence. Climate change is predicted to cause fluctuations in parasite and disease spread across the globe (Petney et al., 2021; Tylianakis et al., 2008). It is not known whether the parasite levels I discovered in neotropical yellow toads are high, average, or low, for this species and comparisons with other species is difficult because of differences in host species resistance, parasite species variation in different locations, annual variation in parasites, etc. Long-term datasets monitoring parasite prevalence over time and with climate change is required to determine what normal parasitism looks like and how climate change might influence parasite loads is important in vulnerable groups such as anurans. Long-term monitoring of parasite prevalence will also facilitate investigations into parasite-mediated sexual selection through changes in ornamentation and sexual traits in relation to fluctuations in parasitism. For the reference of future scientists who may be interested in changes in parasite prevalence and parasite diversity that may occur as a result of climate change, I include average parasite data, body size data, and colour data of neotropical yellow toads in Table 3.1 and Table 3.2.

In conclusion, through analyses of blood parasites, colour, and body condition, I have provided the first study of blood parasites in neotropical yellow toads and preliminary support for parasite-mediated sexual selection in this species. I have identified several parasites of neotropical yellow toads. As well, I have demonstrated a diversity of parasites can be identified without sacrificing individuals. I have shown that parasites interact with colour and body condition in fascinating manners worthy of further investigation. My research has expanded our scientific knowledge of parasite-mediated sexual selection in anurans and brought us closer to understanding the mystery of neotropical yellow toad colour change.

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Table 3.1. Summary statistics of blood parasites found in male neotropical yellow toads (*Incilius luetkenii*) in 2018 and 2019 sampled in northwestern Costa Rica.

Parasite	Mean \pm SE per year	
	2018 n = 22	2019 n = 30
Apicomplexa	2.13 \pm 0.60 (Present in 13 toads)	13.4 \pm 3.14 (Present in 25 toads)
Nematoda	0.72 \pm 0.45 (Present in 4 toads)	1.06 \pm 0.46 (Present in 10 toads)
Frog erythrocytic virus	494 \pm 99 (Present in 21 toads)	6.96 \pm 6.65 (Present in 3 toads)
Bacteria	0.18 \pm 0.10 (Present in 3 toads)	1.1 \pm 0.25 (Present in 16 toads)

Table 3.2. Summary statistics of morphometric features and colour features of male neotropical yellow toads (*Incilius luetkenii*) in 2018 and 2019 sampled in northwestern Costa Rica (male toads were sampled in total).

	Mean \pm SE per year	
	2018 n = 21	2019 n = 28
Morphometrics		
Mass (g)	47.57 \pm 1.16	56.48 \pm 1.55
Snout-vent-length (mm)	83.57 \pm 1.03	85.17 \pm 0.90
Colour	2018 n = 22	2018 n = 28
Brightness (%)	41.48 \pm 4.34	42.29 \pm 3.86
Saturation	0.33 \pm 0.003	0.32 \pm 0.005
Hue (nm)	539 \pm 4.71	524 \pm 9.11

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