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Title: The Behavioral Responses of Western Toad (*Anaxyrus boreas*) Larvae to Simultaneous Stressors

Abstract Approved:

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Animals can be naturally exposed simultaneously to multiple stressors. These include habitat changes, contaminants, diseases, invasive species, parasitism, and predation. Exposure to various combinations of biotic and abiotic stressors may induce behavioral changes that affect the way an individual interacts within its environment.

Like other groups of organisms, amphibians are exposed to a wide array of both natural and introduced stressors. However, due to their life history and utility in experimental studies, amphibians can serve as useful models to examine how an animal behaves when encountering various stressors. Unlike other vertebrates, amphibians have a biphasic life cycle and are aquatic or terrestrial during different times of their life cycle. In their aquatic phase, amphibians may use chemical cues to detect the presence of stressors. For example, predators may emit chemical cues that an amphibian could detect, or another amphibian that has been captured may emit chemical cues that alert nearby

conspecifics of a threat. Moreover, amphibians may use chemical cues to detect parasites when parasites are at certain life stages.

In this thesis, I experimentally tested the behavioral reactions of the larvae of a model amphibian species to the presence of alarm cues emitted by amphibian larvae, a pathogen, and a parasite. The pathogen and parasite were an emerging infectious chytrid fungus and a trematode, respectively. Although the interaction between anti-pathogen, anti-parasite and anti-predator responses in larval amphibians has received some attention, results have shown strong interspecific variation in behavioral responses by amphibians to pathogens, parasites, and predators. Moreover, there is less information about the response of amphibians to simultaneous stressors. To examine the behavioral responses of an amphibian to predation cues, pathogens, and parasites I exposed western toad larvae to combinations of the chytrid fungus, *Batrachochytrium dendrobatidis* (Bd), a trematode parasite, and conspecific alarm cues in a fully factorial experiment. Based on the findings of Han et al. (2011), I predicted that prior exposure to Bd would result in tadpoles having increased activity levels when in the presence of echinostome trematode ocercariae and conspecific alarm cues. Based on the findings of Hews and Blaustein (1985), I also predicted that tadpoles exposed to echinostome cercariae and conspecific alarm cues would avoid the conspecific alarm cues. I found that prior Bd exposure did not influence activity levels of tadpoles exposed to echinostome cercariae and conspecific alarm cues but it did influence avoidance behaviors. Tadpoles that had been previously exposed to Bd displayed increased avoidance behaviors when in the presence of echinostome cercariae and conspecific alarm cues when compared to tadpoles that had only been exposed to echinostome cercariae and conspecific alarm cues. The results from this research provide more information about the interactions of commonly co-occurring stressors and an emerging pathogen and their effects on amphibian behavior.

The Behavioral Responses of Western Toad (*Anaxyrus* boreas) Larvae to Simultaneous Stressors

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Emily R. Reynolds, Author

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CONTRIBUTION OF AUTHORS

Paul Snyder, Adam Augenstein, and Jeffrey Bierman assisted in data collection for Chapter 2. Paul Snyder assisted with the interpretation and writing of Chapter 2. Dr. Andrew Blaustein served as my graduate school advisor in Integrative Biology and contributed to the writing and editing of this thesis. He also contributed to the design and writing of Chapter 2.

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DEDICATION

To Mom and Pops

The Behavioral Responses of Amphibians to Simultaneous Stressors

Emily R. Reynolds

CHAPTER 1: GENERAL INTRODUCTION

All animals experience a range of stressors in their lifetime. Stressors can be abiotic or biotic and often cause animals to change their behaviors (Chivers et al. 2001; Wiles et al. 2003; Relyea, Rick 2005; Johnson and McKenzie 2009; Rall et al. 2009; Rodewald et al. 2011; Ferrari et al. 2011; Harley 2011). Individually, stressors such as contaminants (Fleeger et al. 2003; Rohr and Crumrine 2005), interspecies interactions (Brönmark et al. 1991; Davis 2003; Raffel et al. 2010), and disease (Hart 1990; Loehle 1995) may have variable impacts on species. However, an animal's response to a simultaneous combination of stressors may differ compared to being exposed to a single stressor (Darling and Côté 2008; Holmstrup et al. 2010).

An animal may react to stressors in various ways and these reactions may be coupled with its ability to assess the environment. Thus, for aquatic organisms, chemical cues are especially important for assessing biotic and abiotic environmental parameters (Chivers and Smith 1998; Brönmark and Hansson 2000). For example, fishes often use chemical signaling to avoid predation (Wisenden and Chivers 2006). Alarm cues are examples of chemical cues (Watson et al. 2004; Schoeppner and Relyea 2005, 2009; Wisenden and Chivers 2006; Sutrisno et al. 2014) that may be released as predators capture prey (Schoeppner and Relyea 2005, 2009; Wisenden and Chivers 2006). Prey that are not able to detect a predator may be caught and consumed, but they may be able to warn other nearby conspecifics of the predator. When attacked and consumed, some aquatic species will release alarm cues that are detectable by nearby conspecifics (Wood et al. 2008; van de Nieuwegiessen et al. 2009; Gonzalo et al. 2012; Meuthen et al. 2016;

Stephenson 2016). Upon detection of chemical cues, an animal may alter its behavior in attempt to avoid predation (Petranka and Hayes 1998; Relyea 2001a; Hoverman et al. 2005; Maag et al. 2012). Behavioral responses to these cues are varied and often species specific (Ferrari et al. 2010).

Infection by a pathogen or parasite is another type of interspecies interaction that can be used to assess responses to multiple stressors (Lenihan et al. 1999; Coors and De Meester 2008; Holmstrup et al. 2010). Infection may induce behavioral responses in a host (Hart 1992; Klein 2003; Daly and Johnson 2011; Reisinger and Lodge 2016). Behavioral responses to the threat of infection vary by species and presence of other stressors (Dobson 1988; Hart 1990; Poulin 1995; Altizer et al. 2003). These behavioral changes do not always result in host death, but when combined with other stressors sublethal affects may occur (Baker and Smith 1997; Berven et al. 2001; Gourbal et al. 2001; Marcogliese and Pietrock 2011; Luong et al. 2011; Hesse et al. 2012).

Amphibians are one particular group of aquatic organisms that encounter numerous stressors during their life cycle. Many amphibians are particularly unique because of their biphasic life cycle: they encounter stressors in aquatic as well as terrestrial life stages (Blaustein 1994; Blaustein and Bancroft 2007). Most anuran amphibians have shell-less eggs and persist in ponds, lakes, and streams as larvae after hatching. When they reach a certain point in their development, the larvae metamorphose into juvenile frogs (Gosner 1960). Frogs have thin permeable skin and can therefore readily absorb contaminants and chemical cues from their aquatic environment (Blaustein 1994). Oftentimes, amphibians are referred to by scientists as indicator species of ecosystem health because of this sensitivity (Blaustein and Wake 1995; Daszak et al. 1999).

AMPHIBIAN STRESSORS

Amphibians are subjected to many stressors (Koprivnikar 2010; Salice 2012; Buck et al. 2012; Haislip et al. 2012). Adult and larval amphibian serve as prey to many different predators (Relyea 2003; Ferrari et al. 2009; Bowerman et al. 2010) and are hosts to a variety of pathogens and parasites (Densmore and Green 2007; Szuroczki and Richardson 2009; Fisher et al. 2009). Predation, pathogen infection, and parasitism affect population densities (Hatcher et al. 2006), individual fitness (Pfennig 2000; Berven et al. 2001; Johnson et al. 2008), and may alter disease susceptibility and transmission (Lefcort and Eiger 1993; Belden and Harris 2007; Orlofske et al. 2012, 2014; Rohr et al. 2015).

Predators induce a variety of responses from amphibians (Lima and Dill 1990; Hoverman et al. 2005; Szuroczki and Richardson 2012). When a larval amphibian is attacked by a predator, it may release an alarm cue that can be recognized by other conspecifics in the immediate area (Hews and Blaustein 1985; Kiesecker et al.1996). The detection of an alarm cue can result in decreased time to metamorphosis (Chivers et al. 1999) as well as changes in behavior (Lima and Dill 1990; Relyea 2001b; Ferrari et al. 2009; Gonzalo et al. 2012; Chivers and Ferrari 2013). The responses vary depending on exposure time and the presence of other stressors (Ferrari et al. 2010).

Parasites are another type of stressor amphibians are exposed to (Johnson et al. 2004; Johnson and Chase 2004; Szuroczki and Richardson 2009). For example, trematode worms commonly parasitize amphibians. Trematodes have complex life cycles that vary by species (Huffman and Fried 1990). Trematodes are laid as eggs in freshwater and hatch in 2-3 weeks as freely swimming miracidia (Huffman and Fried 1990). The miracidia infect the first intermediate host (a snail), and develop into their cercarial form (Huffman and Fried 1990). After 4-6 more weeks, cercaria emerge from the snail and seek out their second intermediate host, which is sometimes an amphibian larva (Huffman and Fried 1990). Trematodes can cause morphological (Johnson et al. 1999, 2002) as well as behavioral (Dobson 1988; Sears et al. 2013) changes in amphibians. Some species of trematodes cause severe limb malformations upon metamorphosis (Johnson et al. 2002; Johnson and Chase 2004). Such morphological changes can lead to a higher susceptibility to predation, by impairing an individual's ability to avoid or escape the threat thereby reducing individual fitness (Johnson et al. 2006a; Goodman and Johnson 2011b; Blaustein et al. 2012). In experimental studies, behavioral responses to trematode infection varies with trematode species as well as host species (Blaustein et al. 2012; Preston et al. 2014) (Appendix Table S1).

Chytridiomycosis, a disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (hereafter Bd), has been implicated in the worldwide decline of amphibian species (Skerratt et al. 2007; Fisher et al. 2009; Vredenburg et al. 2010; Olson et al. 2013; Xie et al. 2016). Bd can infect amphibians as well as fish and crawfish (Brannelly et al. 2015; Liew et al. 2017). The World Health Organization (WHO) describes emerging infectious diseases as diseases that have infected a new population, or diseases that have recently increased in incidence or geographic range. Bd is an asexual aquatic fungus with a complex life cycle with two main forms, the motile zoospore form and the sessile zoosporangium (Berger et al. 2005a). The motile zoospores infect amphibian skin in adults and mouthparts in larvae (Marantelli et al. 2004). Once a host is located, the zoospore sprouts a germination tube through which it inserts its cellular components into host cells (Longcore et al. 1999). The infected host cells develop into a zoosporangium and the zoosporangium acts as the reproductive stage, producing new zoospores through multiple rounds of mitosis (Berger et al. 2005a). The new zoospores then exit the zoosporangium into the surrounding water through papillae (Longcore et al. 1999; Berger et al. 2005a). Chytridiomycosis can lead to death in some amphibian species, but effects vary between host species (Gervasi et al. 2017), host populations (Briggs et al. 2005; Tobler and Schmidt 2010; Bradley et al. 2015), Bd infection load (Gervasi et al. 2014; Ohmer et al. 2015), and Bd strain (Berger et al. 2005b; Retallick and Miera 2007; Dang et al. 2017). Some common symptoms of infected amphibian hosts include lethargy, reduced foraging, excess skin sloughing, reduced righting reflexes, and cardiac arrest (Voyles et al. 2009; Venesky et al. 2009). Although the exact cause of mortality is unknown, in adults it is thought to interrupt the osmotic balance within the body (Voyles et al. 2007) or release toxins as a result of infection (Daszak et al. 2001), and larvae are unable to feed efficiently (Venesky et al. 2009). may have originated in Africa (Weldon et al. 2004) but has been detected on every continent where amphibians can be found (Skerratt et al. 2007; Fisher et al. 2009; Olson et al. 2013; Xie et al. 2016). There have been a multitude of studies assessing the effects of Bd, but a severe deficit when it comes to combinations of factors such as Bd and other stressors (Voyles et al. 2009; Kilpatrick et al. 2010; Blaustein et al. 2011).

MULTIPLE STRESSORS

Whereas individual stressors take their toll on amphibians, combined stressors often represent a larger threat to populations (Kiesecker et al. 2001; Sih et al. 2004; Blaustein et al. 2011; Salice 2012). The causes of amphibian population declines are likely due to a combination of stressors (Alford and Richards 1999; Blaustein et al. 2003, 2011; Salice 2012; Battaglin et al. 2016). The effect of individual stressors on amphibians has been well studied, but studies of combined effects of stressors are not as common. However, recent studies of the interaction between predators, pathogens, and parasites have become prominent (Relyea 2003; Ferrari et al. 2010; Groner et al. 2013). Predators, pathogens, and parasites often work together to have important impacts on food webs (Fenton and Rands 2006; Groner et al. 2013; Orlofske et al. 2015). In a laboratory setting, predators, pathogens, and parasites individually have variable effects on prey species, but when combined, the result is often dissimilar to the individual effects (Taylor et al. 2004; Raffel et al. 2010; Szuroczki and Richardson 2012; Marino and Werner 2013; Preston et al. 2014).

There have been several studies investigating amphibian responses to simultaneous predator, pathogen, and parasite exposure (Appendix Table S1). Simultaneous combined exposures can have extensive effects on behavior and survival (Parris and Beaudoin 2004; Parris et al. 2006; Marino et al. 2014; Orlofske et al. 2014; Preston et al. 2014; Koprivnikar and Penalva 2015), as well as many other characteristics. Amphibians often respond to threats of predation, pathogen infection, and parasitism through altered activity (Thiemann and Wassersug 2000; Szuroczki and Richardson

2012; Preston et al. 2014), avoidance behaviors (Goodman and Johnson 2011a; Preston et al. 2014; Koprivnikar and Penalva 2015), and refuge use (Han et al. 2011). The combination of stressors can have variable effects on survival. While infection with a parasite sometimes results in increased predation rates (Koprivnikar 2010; Belden and Wojdak 2011; Marino and Werner 2013), there are instances when infection can decrease predation risk (Parris et al. 2006; Han et al. 2011). Depending on the infecting organism, a behavioral response such as activity may increase or decrease in magnitude after exposure (Szuroczki and Richardson 2012; Marino et al. 2014; Preston et al. 2014). This change in activity could be the difference between becoming prey and surviving. For example, larval *Rana cascadae* exposed to Bd showed a reduction in activity (Han et al.2011). However, the decrease in activity presumably made them less conspicuous to a predatory newt and the tadpoles had higher rates of survival when compared to uninfected conspecifics. In a different study, larval *Lithobates clamitans* displayed increased activity when exposed to a planorbid snail infected with echinostome trematodes (Marino and Werner 2013). Upon exposure to a freely swimming dragonfly larva, a natural predator to many tadpoles (Relyea 2001b), rates of survival in parasite exposed individuals were even lower than those of either parasites alone or the dragonfly alone treatments (Marino and Werner 2013). This is an example of a synergistic interaction. Synergistic, antagonistic, and additive interactions may occur when an animal is exposed to multiple stressors simultaneously. (Vinebrooke et al. 2004; Holmstrup et al. 2010; Przeslawski et al. 2015). Synergistic interactions occur when the response to two variables is greater than the sum of its parts. Antagonistic interactions occur when the response to two combined variables is not as strong in intensity or as large in magnitude as expected based on the individual variables' effects. Additive interactions are those whose end response to combined variables can be predicted based on the individual variables' effects.

In addition to compounding in different ways, responses to multiple stressors can depend on host species (Han et al. 2011; Marino et al. 2014), parasite species (Preston et al. 2014), predator species (Haislip et al. 2012), and there is even some variation within species. For example, one study aimed at assessing the interactions between different species of pathogens and parasites on *Pseudacris regilla* found no interactive effects between Bd exposure and *Ribeiroia ondatrae* (a trematode parasite) exposure (Romansic et al. 2011). This species was also used in two other studies assessing change in tadpole activity to simultaneous predator and parasite exposure (Preston et al.2014 and Orlofske et al.2014). Preston et al. (2014) exposed *P. regilla* to three different species of trematodes: *Echinostoma trivolvis*, *R. ondatrae*, and *Alaria sp*. While none of the trematode species induced a change in tadpole activity in individual exposures, the combination of predator cues and *R. ondatrae* resulted in reduced tadpole activity. Neither *E. trivolvis* nor *Alaria sp.* had any effect alone or combined with predator cues. However, *P. regilla* was used in a separate study conducted by Orlofske et al.(2014) that found simultaneous exposure to *R. ondatrae* and a predator cue did not influence tadpole activity. The same predator was used in both studies. Both studies collected *P. regilla* egg masses in California, but it is possible that they were collected from different populations, which could explain the difference in responses (Preston et al.2014; Orlofske et al.2014).

Many studies have used anuran larvae when assessing the responses of amphibians to predator, pathogen, and parasite cues (Kats and Dill 1998; Ferrari et al. 2010). Typical predators used in these studies include arthropods, fishes, and amphibians. Fish and amphibian predators can also be vulnerable to parasite exposure. Trematodes infect a multitude of fishes and amphibian species (Herrmann and Poulin 2012; Sears et al. 2012). Some studies found that predators were just as susceptible to parasite infection as the prey species, which could complicate the interaction by reducing the predator's efficiency (Belden and Wojdak 2011)

Although *E. trivolvis* has been used to assess the behavioral response to predators and parasites in several species anuran larvae (Huffman and Fried 1990; Kostadinova and Gibson 2000; Taylor et al. 2004), exposure methods often differ between studies. Mesocosm studies generally use snail hosts actively shedding cercariae as their exposure regime (Belden and Wojdak 2011; Marino and Werner 2013; Preston et al. 2014a), and laboratory studies collect cercariae from the snail hosts prior to exposure so the amount of cercariae can be controlled (Thiemann and Wassersug 2000; Szuroczki and Richardson 2012; Koprivnikar and Penalva 2015). The difference in methods is sometimes used to evaluate the impact of the snail host presence, which can be a better representation of the interaction in an ecological setting. However, the amount of parasites or snails used in exposures varies by study (Thiemann and Wassersug 2000; Koprivnikar 2010; Raffel et al. 2010; Belden and Wojdak 2011; Szuroczki and Richardson 2012; Marino et al. 2014; Preston et al. 2014a). Studies aimed at replicating natural interactions may benefit from including snails, as trematodes would not be present without their first intermediate host, but snail presence could become a confounding variable in studies interested in the physiological or developmental effects of parasite infection.

Even if the methods of multiple stressor experiments are considerably different, the results are important to our understanding of species interactions. Combining natural stressors with anthropogenic stressors allows researchers to better understand the changing climate and predict what will happen in the future (Lenihan et al. 1999; Vinebrooke et al. 2004; Christensen et al. 2006; Salice 2012; Przeslawski et al. 2015). With amphibians declining faster than most vertebrates (Stuart et al. 2004), these interactions must be considered. A summary of amphibian responses to combined predator and parasite exposures can be found in the Appendix (Table S1).

IMPORTANCE OF BEHAVIORAL STUDIES

Unfortunately, behavioral studies are often overlooked when assessing the roles of multiple stressors on animals. Behavioral measures have always been an important part of investigating predator and prey interactions, but this parameter is also crucial to disease related studies, but which are fewer in number (Hoverman and Searle 2016). As sick animals display different behaviors than healthy animals, overlooking behavioral changes could miss significant patterns in how animals react to stressors (Hart 1988). Some behavioral changes are specifically facilitated by a parasite so that it can complete its life cycle (Poulin 1995). While it is important to understand physiological responses to stressors, behavior is also an important part a species response. Behavior of animals integrates everything from individual gene expression to community biology (Sih et al.

2010). Morphological and physiological responses can require a span of time, while behavior is often an immediate response (Sih et al. 2010). This immediate response has become a critical component in all animals lives, as human encroachment into the environment has never been higher (Vitousek et al. 1997; Scheffer et al. 2001; Foley et al. 2005).

AMPHIBIANS AS A STUDY MODEL

Amphibians have been used as a study model in ecological studies for decades (Hopkins 2007). For behavioral studies, amphibians are especially useful. They are easy to care for in a laboratory or outdoor mesocosm setting and can be obtained through simple measures (i.e. collecting eggs from ponds). Their biphasic life cycle allows for assessment of both terrestrial and aquatic ecosystems and stressors unique to either (Blaustein 1994; Daszak et al. 1999; Blaustein et al. 2003; Blaustein and Bancroft 2007; Hopkins 2007). Widespread occurrence of amphibians provides relevance for research across the globe (Duellman 1999).

My research focused on western toads (*Anaxyrus boreas*, family Bufonidae), a pond-breeding species with a native range that extends from the southern coast of Alaska to the northern borders of California, Nevada, and Utah (Stebbins 2003). While amphibian populations are rapidly declining, amphibians that belong to the family Bufonidae are disappearing particularly quickly (Stuart et al. 2004). The reasons behind the disappearance of toads is complex but toad habitats are being negatively affected by many anthropogenic stressors (Christensen et al. 2006). For example, western toads are particularly susceptible to ultraviolet-B radiation (Blaustein et al. 2003), Ranavirus (Daszak et al. 1999), and Bd (Blaustein et al. 2005) among other stressors.

OBJECTIVES

Predators, pathogens, and parasites co-occur in ecosystems across the globe (Lafferty et al. 2008; Rohr et al. 2015). Understanding the responses to the interaction between these stressors gives a more complete view of a naturally occurring interaction. Amphibians are the prey to many predators (Relyea 2003; Bowerman et al. 2010; Ferrari et al. 2010), the hosts to many pathogens and parasites (Densmore and Green 2007; Szuroczki and Richardson 2009; Fisher et al. 2009), and an important ecologically relevant model study organism (Blaustein and Wake 1995; Hopkins 2007). Bd is a fungal amphibian pathogen that has been implicated in the declines of amphibian species on every continent where they can be found (Skerratt et al. 2007; Fisher et al. 2009; Vredenburg et al. 2010; Olson et al. 2013; Xie et al. 2016).

I experimentally exposed western toad tadpoles to the combination of conspecific alarm cues (to simulate a general predation event), trematodes of the genus *Echinostoma*, and the emerging infectious fungal pathogen Bd. Western toads have steadily declined throughout some of their historic range (Corn et al. 1989; Carey 1993; Drost and Fellers 1996; Scherer et al. 2005). They are particularly susceptible to Bd (Blaustein et al. 2005; Carey et al. 2006). Their behavioral response to conspecific alarm cues has been previously studied (Hews and Blaustein 1985) and researchers found that larvae increased activity in response to these cues. There have been no published studies regarding the behavioral response of Western toads to echinostome parasites although researchers hypothesize this species can serve as a second intermediate host to the parasite (Johnson and McKenzie 2009). Han et al. (2011) investigated the behavior of western toad larvae when exposed to Bd and predator cues and found that larvae exhibited increased activity and refuge use. This is the first study to document the behavioral effects of larval *A. boreas* to Bd, an echinostome parasite, and conspecific alarm cues simultaneously.

I predicted that the combination of prior Bd exposure, echinostome exposure, and conspecific alarm cue exposure would elicit higher levels of activity in tadpoles when compared to a control treatment. Previous studies involving these stressors have shown that *A. boreas* exhibit increased activity levels when exposed to Bd and in the presence of conspecific alarm cues (Hews and Blaustein 1985). There have been no published studies on *A. boreas* behavioral response to *Echinostoma sp.*, but studies have shown that the presence *Echinostoma* parasites increased activity in larval anurans (Szuroczki and Richardson 2012; Marino and Werner 2013), and a study on a different species of toad observed an increase in activity to an *Echinostoma* parasite as well (Raffel et al. 2010). Hews and Blaustein (1985) found that when exposed to conspecific alarm cues, western toad larvae avoided the source of the cues. Hence, I also predicted that when simultaneously exposed to conspecific alarm cues and a planorbid snail shedding echinostome cercariae, larvae would avoid the conspecific alarm cues.

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CHAPTER 2:

The Behavioral Responses of Western Toad (*Anaxyrus* boreas) Larvae to Simultaneous Stressors

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Abstract

Animals are naturally exposed to multiple stressors in nature. Studies on behavioral responses of animals to simultaneous naturally occurring stressors show different results depending upon context. To examine how behavior may be influenced by naturally occurring stressors of different types, we examined how western toad larvae behaviorally responded to exposure to a pathogen, a parasite, and conspecific alarm cues in a fully factorial experimental design. When comparing the overall effects of these stressors, conspecific alarm cues elicited a strong response from larvae with exposure to a planorbid snail shedding trematode cercariae; exposure to the pathogenic fungus*,* *Batrachochytrium dendrobatidis* (Bd) had less of an effect. Combinations of the three stressors resulted in different behavioral responses than those observed from individual stressors, illustrating that the dynamics between multiple stressors can result in varied behavioral responses.

Significance statement

This study describes previously undocumented behavioral responses by western toads, *Anaxyrus boreas,* an amphibian whose populations are in decline, to co-occurring stressors. Toad larvae responded to conspecific alarm cues more strongly compared with responses to trematode cercariae (a macro-parasite), and the pathogenic fungus, *Batrachochytrium dendrobatidis* (a micro-parasite). Combinations of stressors produced varied responses that differed from overall individual stressor effects. This study emphasizes the complexity of host responses to multiple stressors, which are commonly encountered in nature.

Introduction

In nature, animals are exposed to more than one stressor at a time. When an animal is subjected to numerous stressors simultaneously, complex and varied responses may result, especially when the stressors are novel (Davis et al. 1998; Folt et al. 1999; Sih et al. 2004; Harley 2011). Both biotic and abiotic, natural and anthropogenic stressors such as competition with novel competitors (Chivers et al. 2001; Wiles et al. 2003), changes in rates of predator-prey interactions (Rall et al. 2009; Rodewald et al. 2011; Ferrari et al. 2011; Harley 2011), and exposure to diseases and contaminants (Relyea 2005; Johnson and McKenzie 2009) may affect an animal's behavior. Furthermore, although each stressor alone may affect an animal one way, an animal exposed to several stressors simultaneously may be affected in other ways (Hatch and Blaustein 2000; Johnson et al. 2006b; Townsend et al. 2008).

In aquatic systems, chemical cues are a major form of communication and are often used to perceive and react to a variety of stressors such as disease, contaminants, predators and prey (Dodson et al. 1994; Kats and Dill 1998; Petranka and Hayes 1998; Relyea 2001b; Marquis et al. 2004; Hoverman et al. 2005; Rohr et al. 2009; Ferrari et al. 2010; Steiger et al. 2011; Cummins and Bowie 2012; Cárdenas et al. 2012; Sharp et al. 2015). The use of chemical cues to detect conspecifics and heterospecifics has been documented in many aquatic taxa regarding key ecological interactions (Lima and Dill

1990; Kats and Dill 1998; Tollrian and Harvell 1999; Ferrari et al. 2010). For example, cues used by predators or prey may induce behavioral changes in the way animals forage or hide from predators (Hassell and Southwood 1978; Lima and Dill 1990)**.** Such chemically-based predator-prey interactions may be altered if one or more additional stressors were present. For example, if one or more pathogens were infecting a host species during a predator-prey interaction, the host may display changes in behavior that would not occur if it were only exposed to predator cues (Milinski 1985; Murray et al. 1997; Han et al. 2011; Orlofske et al. 2014).

Our study was designed to experimentally examine the effects of simultaneous stressors on the behavior of amphibian larvae. These stressors include a pathogen and a parasite that have detrimental effects on amphibian larvae and may alter predator cues. Chemical cues are especially important in larval amphibians. For example, when an amphibian larva is captured by a predator, it may release a signal that elicits an alarm reaction in conspecifics (Hews and Blaustein 1985; Kiesecker et al. 1996; Schoeppner and Relyea 2005; Rohr et al. 2009; Fraker et al. 2009). Behavioral responses to these alarm cues differ interspecifically (Ferrari et al. 2010). For example, Wood frogs (*Rana sylvaticus*) did not respond to water treated with macerated conspecifics (Petranka and Hayes 1998), Common frogs (*R. temporaria*) and Common toads (*Bufo bufo*) decreased their activity levels when exposed to conspecific alarm cues (Marquis et al. 2004), Western toads increased activity levels (Hews and Blaustein 1985; Kiesecker et al. 1996), Pacific treefrogs (*Pseudacris regilla*) actively avoided conspecific alarm cues (Chivers et al. 2001), and American toads (*Anaxyrus americanus*) reduced activity levels as well as avoided the source of the cue (Petranka and Hayes 1998). Whereas behavioral responses to conspecific alarm cues have been well documented, the addition of other stressors, such as pathogens or parasites, may further alter predator-prey behavior (Milinski 1985; Murray et al. 1997; Thiemann and Wassersug 2000; Johnson et al. 2006b; Han et al. 2011; Ferrari et al. 2011; Luong et al. 2011; Orlofske et al. 2014)

In our study, we exposed amphibians to conspecific alarm cues a pathogen, and a parasite. The pathogen was the aquatic fungus *Batrachochytrium dendrobatidis* (Bd). Bd is associated with worldwide amphibian population declines (Skerratt et al. 2007; Fisher et al. 2009; Vredenburg et al. 2010; Olson et al. 2013; Xie et al. 2016). Bd causes the disease chytridiomycosis and can infect all developmental stages of amphibians with keratinized body parts (Marantelli et al. 2004). Common symptoms of chytridiomycosis include decreased activity, loss of appetite, loss of righting reflex, excess skin sloughing, reduced foraging, and death (Voyles et al. 2009; Venesky et al. 2009), but the response to Bd infection differs among host species, populations, and life stages (Briggs et al. 2005; Blaustein et al. 2005; Bielby et al. 2008; Kilpatrick et al. 2010; Tobler and Schmidt 2010; Bancroft et al. 2011; Searle et al. 2011; Gervasi et al. 2013, 2017; Bradley et al. 2015). We used trematode cercariae (*Echinostoma spp*) shed from planorbid snails as the macro parasite. Snails serve as the trematode’s first intermediate host, where they progress from their miracidial form to their cercarial form (Huffman and Fried 1990). After this transformation has occurred, the trematodes emerge from the gonads of the snails in search of their second intermediate host, which can be fishes, other snails, other invertebrates, or amphibian larvae (Huffman and Fried 1990). In tadpoles, the echinostome cercariae enters through the cloaca and encysts on the kidneys (Martin and Conn 1990; Johnson and McKenzie 2009). Prior research involving echinostomes has shown larvae may have the ability to detect the presence of echinostome cercariae via chemical cues from the cercariae in the water (Rohr et al. 2009). A common behavioral response to the detection of cercariae is an increase in activity (Thiemann and Wassersug 2000; Taylor et al. 2004; Koprivnikar et al. 2006). This may be an attempt to avoid cercarial infection and prevent encystment (Koprivnikar et al. 2006; Rohr et al. 2009; Sears et al. 2013). However, this increase in activity could cause a larva to become more conspicuous to a nearby predator (Baker and Smith 1997; Berven et al. 2001; Gourbal et al. 2001; Luong et al. 2011; Hesse et al. 2012), resulting in increased predation rates. As the echinostome requires the second intermediate host to be ingested by a definitive host to complete its life cycle (Huffman and Fried 1990; Toledo et al. 2007; Johnson and McKenzie 2009), this altered behavior could be especially beneficial for the echinostome parasite.

Herein, we focused on larvae of the western toad (*Anaxyrus boreas*, Family Bufonidae) due to their susceptibility to multiple stressors. Among amphibians, toads within the family Bufonidae have the highest number of “rapidly declining” species, (Stuart et al. 2004). Larval western toads are particularly susceptible to Bd, sometimes dying within two days of exposure (Blaustein et al. 2005). Exposure to chemical cues from injured conspecifics increases activity levels and refuge seeking behaviors in western toad larvae (Hews and Blaustein 1985; Kiesecker et al. 1996; Han et al. 2011). The effects of *Echinostoma sp.* on *A. boreas* behavioral responses is not well documented but as this genus of trematode infects larval hosts encysting in the kidneys, it is thought that the larva will increase its activity and exhibit a thrashing behavior to impede infection (Koprivnikar et al. 2006; Johnson and McKenzie 2009)**.** Although few studies have assessed western toad behavioral responses to conspecific alarm cues with additional stressors, behavioral responses to cues emitted by predators and additional stressors have been studied. When exposed to both predator cues and Bd, *A. boreas* larvae displayed anti-predatory behaviors through increased refuge seeking and increased activity levels (Han et al. 2011). Larval activity levels significantly decrease in the presence of a predator and trematodes (Thiemann and Wassersug 2000). As there have been no studies considering the combined effects of these three stressors on *A. boreas*, we provide new insights into the unique behaviors of western toads to three common co-occurring stressors they may contribute to understanding the overall population declines of this species (Corn et al. 1989; Carey 1993; Drost and Fellers 1996; Daszak et al. 1999; Blaustein et al. 2003, 2005; Stuart et al. 2004; Scherer et al. 2005; Christensen et al. 2006).

To assess changes in behavior of larval *A. boreas*, we exposed larvae to all possible combinations of Bd and a sham Bd inoculum, a planorbid snail shedding echinostome cercariae or an uninfected planorbid snail, and conspecific alarm cues or no conspecific alarm cues. We predicted that tadpoles exposed to echinostome cercariae and conspecific alarm cues would have higher activity levels if they had been previously exposed to Bd based on previous findings by Han et al. (2011), and larvae exposed to both echinostome cercariae and conspecific alarm cues would avoid the conspecific alarm cues based on the alarm response assessed by Hews and Blaustein (1985).

Methods

Animal Husbandry and Batrachochytrium dendrobatidis inoculation

All *A. boreas* were collected as egg masses from Todd Lake (Deschutes County, OR, elevation 1,870 m; latitude/longitude 44.03/-121.69), in the Cascade Range on 15 June 2016. In the laboratory, egg masses were housed in aerated 40L glass aquaria filled with 38L of dechlorinated water treated with Novaqua and AmQuel and maintained in a temperature controlled room (13-15C) with a natural photoperiod. Eggs were screened daily for signs of decomposition. Upon hatching, animals were fed a 3:1 mixture of ground rabbit chow and fish flakes daily, *ad libitum*. Animals remained in these tanks until the beginning of the experiment, ten weeks after egg masses were collected. Prior to the start of the experiment, animals were moved into 10L glass aquaria filled with 9L of dechlorinated water treated with Novaqua and AmQuel, so that groups of 12 tadpoles could be exposed to Bd.

Approximately 100 snails (*Planorbella trivolvis*) were collected from the Finley National Wildlife Refuge (Benton County, OR; elevation 78m; latitude/longitude 44.41/-123.32) on 6 July 2016. After being brought into the laboratory, each snail was individually screened for *Echinostoma sp.* parasites between 1130 and 1400 hours by placing under a heat lamp for 20 minutes and using a dissecting microscope. Once infection was confirmed, snails were housed in 40L aquaria filled with 38L of dechlorinated water treated with Novaqua and AmQuel in an incubator kept at 25-27C. Snails were fed frozen spinach daily, *ad libitum*. Immediately prior to the beginning of each trial the cues produced by the snail shedding echinostome cercariae and the conspecific alarm cue were prepared. To produce the echinostome cercariae, six snails that were confirmed to be shedding the echinostome parasite were arbitrarily selected from the incubator and placed in individual containers. The containers were then set under a 60 W light to stimulate shedding. After 20 minutes, snails were again individually screened to confirm trematode infection. Infected snails were then placed back into the incubator until needed for a trial.

Culture techniques for Bd follow the methods of Gervasi *et al.* .2013 and Searle *et al.* 2013 and began 10 days prior to the start of the experiment. Culture plates (100 mm x 15 mm plates containing 1% tryptone and agar) were flooded with 10mL of deionized water and allowed to sit undisturbed for 15 minutes to allow adequate release of zoospores from zoosporangia. Plates were then gently scraped and the liquid content from each plate was pooled, and released zoospores were counted using a hemocytometer. Larvae in Bd exposure treatments were inoculated with an intermediate sublethal dose of Bd zoospores (62.5 zoospores mL^{-1} of JEL 646 isolated from *Pseudacris regilla* tadpoles from Point Reyes, CA by J. Longcore at the University of Maine) in stock tanks beginning two days before day one of the experiment. We decided on this concentration because a previous experiment found that *A. boreas* exposed to the same concentration became infected and showed disease symptoms, but did not result in mortality (Gervasi et al. 2013). We chose a two-day exposure period because *A. boreas* has been shown to die within 48 hours of exposure to a high-dose of Bd (Blaustein *et al.* 2005). Inoculations continued for 10 consecutive days, resulting in a total of 10 groups of 12 tadpoles exposed to Bd zoospores. For the sham Bd exposure stock tanks, agar plates without Bd were flooded and an equal amount of inoculum was added. Inoculums were added directly to each tank.

Experimental Design

We used a 2x4 randomized block design. Groups of 12 tadpoles were exposed to Bd or a sham-Bd inoculum two days prior to their use in experimental trials. These two groups were crossed with exposure to four others to yield eight treatments: (1) conspecific alarm cues and a planorbid snail shedding echinostome cercariae; (2) conspecific alarm cues; (3) a planorbid snail shedding echinostome cercariae; or (4) an uninfected planorbid snail (control treatment). The experiment took place over 10 consecutive days (31 August to 9 September 2016) between 1130 and 1400 hours, and the behavior of larvae in each of the eight treatment groups were observed each day. Each treatment was assigned three 40L glass aquaria (51 x 25 x 32 cm) that served as the experimental tanks. Using a window paint marker, we drew two intersecting lines on one of the outer longitudinal sides of each experimental tank creating four equal sized quadrants (Figure 1). To minimize observer bias, all experimental tanks were fitted with tank blinders on all sides except for the side with the quadrant lines. Eight 10L glass aquaria were assigned to be stock tanks for Bd exposures. Four were used for actual Bd exposure and four were used for the sham Bd exposure. At the end of each day's trials, each experimental and depleted stock tank was emptied and cleaned by soaking in a solution of 10% bleach and deionized water for 1 min, which has been shown to inactivate the zoospores (Gold et al. 2013). Tanks were then thoroughly rinsed, allowed to dry for 48 hours, and 8 new experimental tanks were set up for the following day. The position of each of the 8 experimental tanks on the laboratory bench was randomized daily. One additional 40L glass aquarium filled with 38 L of dechlorinated water treated with Novaqua and AmQuel was used as the alarm cue stock tank.

For treatments that included the presence of the trematode parasite, infected snails were placed into cue cages on the top of the experimental tank. Cue cages were constructed out of a plastic slab and plastic mesh (6.5 x 6.5 x 5 cm). Treatments that did not include the echinostome parasite exposure were given an uninfected snail to assure behavioral changes were due to the cercariae and not the presence of a snail host. Cercariae alone were not used in this experiment, as in nature, cercariae would not be present without the second intermediate host (the planorbid snail). Directly opposite of the cue cage containing the snail was a cue cage for a conspecific alarm cue. For treatments that included the presence of the alarm cue, two *A. boreas* larvae were arbitrarily chosen from the alarm cue stock tank and macerated via decapitation and homogenization. The mixture was then added to the alarm cue cage and the trial could commence. The snail and alarm cue cage sides were altered randomly throughout the experiment to reduce position bias.

At the beginning of each trial, three larvae were arbitrarily chosen from the stock tank and put into the experimental tank where they could acclimate within an acclimation cage for 10 minutes. The acclimation cage was a plastic mesh cylinder (11cm diameter and 7.5 cm height) placed horizontally on the top of the experimental tank between the two cue cages. One of the sides contained a hole to add and release tadpoles when the acclimation period was over. Immediately following the acclimation period, animals were released from the cage to swim freely and data collection began. Each trial was video recorded from the longitudinal side with the quadrant lines using a SJCam SJ4000 in 1080p video and ran for a total of 15 minutes. Following the 15-minute mark, larvae were euthanized in a MS-222 bath and frozen.

We tallied the number of quadrant lines crossed by larvae in the experimental tank throughout the 15-minute trial to quantify activity. Avoidance behavior was quantified by tracking the amount of time spent on either the half of the tank containing a snail or the half of the tank containing the presence or absence of an alarm cue. After the experiment had concluded, an additional measure of activity was collected by watching the first 5-minutes of each trial and recording the number of seconds each larvae was actively swimming.

Statistical analyses

All statistical analyses were performed using R statistical software (version 3.2.2). All data was checked for normality by using the Shapiro-Wilk test of normality from the stats package and Levene's test for equality of variances from the lawstat package. We ran multiple two-way analysis of variance (ANOVA) tests from the stats package to determine any significant effects of treatment on the response variables (number of quadrant lines crossed, time spent swimming, and time spent on snail half of tank). If an ANOVA resulted in a significant p-value, we used the post-hoc Fisher's LSD (least significant difference) test to specify which pairwise comparisons had different means. We also ran post-hoc two-sample t-tests from the stats package to evaluate the overall effects of individual stressors. All 80 trials were assessed using two-sample t-tests to compare a stressor’s overall effects on the response variables. Each stressor had 40 replicates with the presence of the stressor and 40 replicates with the absence of the stressor. To assess larval activity, we analyzed the number of quadrant lines crossed as well as the amount of time tadpoles spent swimming during the first five minutes of each trial. To assess avoidance behaviors, we analyzed the amount of time (in seconds) each larvae spent on the snail half of the tank. For clarity, a three-letter code was assigned to each treatment. The code includes a B or b (Bd), T or t (echinostome cercariae), and A or a (alarm cues). A capital letter indicates the presence of a stressor and a lowercase letter indicates the absence of a stressor, the control treatment would be “bta.”

Results

Tadpole Activity

There was no effect of treatment on the time tadpoles spent swimming ($F = 0.786$, $df = 7, 72$, $P = 0.601$), but treatment had an effect on the number of quadrant lines tadpoles crossed during the 15-minute trial ($F = 2.220$, $df = 7, 72$, $P = 0.042$). Post-hoc pairwise comparisons of treatments revealed that alarm cue exposure resulted in increased quadrant lines crossed in tadpoles exposed to the sham Bd inoculum and echinostome cercariae (bTa v. bTA, Fisher’s LSD $P = 0.012$). Compared to the control treatment, larvae exposed to a sham Bd inoculum, echinostome cercariae, and alarm cues showed an increase in quadrant lines crossed (bta v. bTA, Fisher’s LSD $P = 0.035$). Larvae exposed to Bd, echinostome cercariae and alarm cues crossed more quadrant lines than tadpoles exposed to only echinostome cercariae (BTA v. bTa, Fisher’s LSD $P = 0.049$). Exposure to Bd alone resulted in a lower number of quadrant lines crossed compared with tadpoles exposed to echinostome cercariae and alarm cues (Bta v. bTA, Fisher's LSD $P = 0.007$). Larvae exposed to Bd and echinostome cercariae also crossed fewer numbers of quadrant lines compared to larvae exposed to echinostome cercariae and alarm cues (BTa v. bTA, Fisher's LSD $P = 0.007$). Larvae exposed to Bd had higher numbers of quadrant lines crossed when exposed to echinostome cercariae and alarm cues (BTA v. Bta, Fisher's LSD $P = 0.031$). When tadpoles were exposed to both Bd and echinostome cercariae, the addition of alarm cues resulted in an increase in the number of quadrant lines crossed (BTa v. BTA, Fisher's LSD $P = 0.032$).

Overall treatment effects showed exposure to Bd did not have an effect on the number of quadrant lines crossed ($t_{78} = 0.502$, $P = 0.617$), but decreased the amount of time tadpoles spent swimming during the first five minutes of each trial when compared to tadpoles exposed to a sham Bd inoculum ($t_{78} = 1.923$, $P = 0.058$). Alarm cue exposure increased the number of quadrant lines crossed in experimental tanks ($t_{78} = -3.600$, $P < 0.001$), but exposure did not change the amount of time spent swimming in the first five minutes of the trial ($t_{78} = 0.267$, $P = 0.790$). Exposure to echinostome cercariae did not affect time spent swimming ($t_{78} = 0.027$, $P = 0.979$) or the number of quadrant lines crossed ($t_{78} = -0.778$, $P = 0.439$).

Alarm Cue Avoidance

There was some evidence that treatment had an effect on the time spent on the snail half of the tank ($F = 1.967$, $df = 7, 72$, $P = 0.071$; Table S2). Larvae exposed to alarm cues spent a significantly higher number of seconds on the snail half of the experimental tank when compared to: the control (btA v. bta, Fisher's LSD $P = 0.0.048$), tadpoles exposed to echinostome cercariae (btA v. bTa, Fisher's LSD *P* = 0.056), and simultaneous exposure to echinostome cercariae and alarm cues (btA v. bTA, Fisher's LSD *P* = 0.056) (Table 2). When tadpoles were exposed to Bd, echinostome cercariae, and alarm cues, they spent more time on the snail half of the tank when compared to tadpoles exposed to: the control treatment (BTA v. bta, Fisher's LSD *P* = 0.025), echinostome cercariae and alarm cues (BTA v. bTA, Fisher's LSD *P* = 0.029), Bd and echinostome cercariae (BTA v. BTa, Fisher's LSD *P* = 0.077), and only Bd (BTA v. Bta, Fisher's LSD *P* = 0.035) (Table 2). Exposure to Bd resulted in larvae tending to spend less time on the snail half of the tank when compared to tadpoles exposed to alarm cues (Bta v. btA, Fisher's LSD *P* = 0.067).

Overall treatment effects showed that neither exposure to Bd (t_{78} = -1.248, *P* = 0.216), nor exposure to echinostome cercariae (t_{78} = 0.492, *P* = 0.624) had an effect on the amount of time spent on the snail half of the tank (Table 4). Exposure to alarm cues increased the amount of time larvae spent on the snail half of the tank (t_{78} = -2.719, *P* = 0.008; Table 4).

Discussion

Our results demonstrate the importance of considering multiple stressors on host behavior. The behavioral responses to the pathogen, parasite, and conspecific alarm cues alone were different than the responses from combined treatments, which is an interactive effect that has been shown in various other studies on amphibian behavior to multiple stressors (Taylor et al. 2004; Szuroczki and Richardson 2009; Raffel et al. 2010; Marino and Werner 2013; Preston et al. 2014a). Exposure to the generalist trematode parasite did not have any significant effects overall on the behaviors we measured (Table 4), but when combined with the other stressors, the echinostome cercariae had varying effects on behavioral responses (Figure 1, Figure 2). This interaction has been previously observed with other species of anurans and trematodes (Thiemann and Wassersug 2000; Koprivnikar 2010; Preston et al. 2014a).

Exposure to Bd did not affect tadpole activity when combined with echinostome cercariae and alarm cues (BTA v. bTA, Table 1), contrary to our predictions. Han et al. (2011) found that *A. boreas* increased activity rates when exposed to Bd and combined predator and alarm cue compared to the individual stressors. However, differences between Han et al. (2011) and our study may be a result of generally different methodology and differing zoospore concentrations, as exposure concentration plays an important role in infection dynamics (Garner et al. 2009; Gervasi et al. 2013) Another explanation of this lack of tadpole activity response to Bd exposure in combination with other stressors could be due to interspecific variation in response to *Echinostoma sp.* A common anti-parasite response to *Echinostoma sp.* is an increase in activity (Thiemann and Wassersug 2000; Taylor et al. 2004; Koprivnikar et al. 2006), but there have been no published studies regarding the exposure of *A. boreas* larvae directly to *Echinostoma sp.* cercariae. Previous research suggested that other anuran larvae increase activity levels in an attempt to avoid cercarial infection, as the echinostome must enter the cloaca to eventually encyst on the kidney (Huffman and Fried 1990; Johnson and McKenzie 2009). However, results from our study show that this species may not exhibit a behavioral response when no other stressors are present. Johnson and McKenzie (2009) shows evidence that this species of toad co-occurs with the *P. trivolvis*, which serves as the first intermediate host of these parasites, but further research should be conducted to determine the response to direct cercarial exposure. Interestingly, we observed a non-additive interaction between echinostome cercariae exposure and alarm cue exposure on activity (Figure 1). When the two stressors were assessed alone and compared to the control treatment, neither produced a different change in activity. However, the combination resulted in the highest mean of quadrant lines crossed out of all eight treatments (Table 2). As discussed above, the addition of Bd to these two stressors did not alter larval activity levels when compared to tadpoles exposed to only echinostome cercariae and alarm cues, but this treatment resulted in the second highest mean out of all eight treatments (Table 3). For both Bd- and sham Bd-exposed animals, the additional combination of echinostome cercariae and alarm cues resulted in the highest mean number of quadrant lines crossed (BTA and bTA), followed by the addition of only alarm cues (BtA and btA), and then the addition of only echinostome cercariae (BTa and bTa) (Figure 1). The lack of response to the echinostome cercariae contrasts with previously reported responses (Thiemann and Wassersug 2000; Taylor et al. 2004; Koprivnikar et al. 2006), but the combined effects have been seen in other species of anurans (Thiemann and Wassersug 2000; Koprivnikar 2010; Preston et al. 2014a). The disparity between our study and others is likely due to interspecific or inter-populational variance in responses (Holland 2010; Koprivnikar et al. 2014; Marino et al. 2014).

Larvae exposed to echinostome cercariae and alarm cues did not display avoidance behaviors when compared to the control, but showed a significant decrease in avoidance when compared to the alarm cue only treatment (Figure 2). A similar pattern was found by Preston et al.(2014), who exposed *A. boreas* to a different species of trematode. There was also no difference between avoidance behaviors of tadpoles exposed to echinostome cercariae and alarm cues and echinostome cercariae alone, suggesting that the combination of echinostome cercariae and alarm cues resulted in an antagonistic response. The decreased avoidance of conspecific alarm cues in tadpoles exposed to both conspecific alarm cues and echinostome cercariae could be the result of the trematode's effects on tadpole behaviors, as the trematode requires the tadpole to be ingested by the definitive host to complete its life cycle (Huffman and Fried 1990; Toledo et al. 2007; Johnson and McKenzie 2009). Surprisingly, tadpoles exposed to Bd when combined with echinostome cercaria and alarm cues showed a strong increase in avoidance behavior when compared to tadpoles exposed to only echinostome cercariae and alarm cues (Table 2). Parris et al.(2006) conducted a similar study with northern leopard frog tadpoles and found Bd exposure increased avoidance behaviors when tadpoles were exposed to chemical predator cues. This increase in avoidance behavior could be a byproduct of tadpoles succumbing to chytridiomycosis, which has been shown to decrease activity in infected tadpoles (Voyles et al. 2009; Venesky et al. 2009). Tadpoles exposed to Bd were swimming around the tank less (Figure 1), and as a result of decreased activity, they could have ending up on the snail half of the tank after initial avoidance.

Conspecific alarm cue exposure had an overall effect on behavior— similar to Hews and Blaustein (1985), *A. boreas* tadpoles increased their movement throughout the tank and avoided the source of the alarm cue (Figure 3). Han et al.(2011) observed this pattern in Bd-exposed *A. boreas* as well. This increased activity in response to an alarm cue could be a result of tadpoles seeking refuge (Sih et al. 1992; Chivers et al. 1996; Han et al. 2011). Consistent with finding from previous experiments, Bd exposure resulted in reduced activity in *A. boreas* larvae when all Bd- and sham Bd-exposed animals were pooled and analyzed (Figure 4) (Voyles et al. 2009; Venesky et al. 2009).

Conclusions

Overall, this experiment exemplifies the need to include combinations of stressors when assessing behavior of animals. We saw a consistent pattern of individual stressors having different effects than combined stressors. As behavior of animals, especially sick animals, can play a crucial role in disease systems (Hoverman and Searle 2016), we argue that the use of multiple stressors should be implemented in more experiments. The varied responses to the stressors could provide conservationists with a better understanding of the decline of species, as the combinations of stressors is often the reason for species declines (Kiesecker et al. 2001; Sih et al. 2004; Blaustein et al. 2011; Salice 2012). We suggest that future research should evaluate the interaction of these stressors in a longitudinal study to assess the developmental effects of the three stressors when combined as well as a study that investigates *A. boreas*' behavioral response to direct contact with *Echinostoma sp*.

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performed in this study were in accordance with the Oregon State University Institutional Animal Care and Use Committee (ACUP 4924).

TABLE 1. Results of Fisher's LSD (least significant difference) test for comparisons between all treatment combinations for western toad (*Anaxyrus boreas*) activity behaviors (number of tank quadrant lines crossed when exposed to eight treatments). All values in the table are the corresponding *P*-values produced when comparing a treatment on the first row of the table to a treatment on the first column of the table. All treatment names include the letters B/b, T/t, or A/a. A capital letter indicates the presence of a treatment factor (Bd, trematodes, and conspecific alarm cues) while a lowercase letter indicates the absence of a treatment factor.

Treatment	*bta*	*btA*	*bTa*	*bTA*	*Bta*	*BtA*	*BTa*
btA	0.5051	-	-	-	-	-	-
bTa	0.6687	0.2752	-	-	-	-	-
bTA	0.0351 **	0.1436	0.0120 **	-	-	-	-
Bta	0.5295	0.1972	0.8403	0.0069 ***	-	-	-
BtA	0.3215	0.7434	0.1576	0.2540	0.1074	-	-
BTa	0.5419	0.2037	0.8551	0.0073 ***	0.9849	0.1115	-
BTA	0.1200	0.3692	0.0489 **	0.5671	0.0306 **	0.5671	0.0321 **

(*) indicates significance at α=0.1
(**) indicates significance at α=0.05
(***) indicates significance at α=0.01

TABLE 2. Results of Fisher's LSD (least significant difference) test for comparisons between all treatment combinations for western toad (*Anaxyrus boreas*) avoidance behavior (time spent on the snail half of tank when exposed to eight treatments). All values in the table are the corresponding *P*-values produced when comparing a treatment on the first row of the table to a treatment on the first column of the table. All treatment names include the letters B/b, T/t, or A/a. A capital letter indicates the presence of a treatment factor (Bd, trematodes, and conspecific alarm cues) while a lowercase letter indicates the absence of a treatment factor.

Treatment	*bta*	*btA*	*bTa*	*bTA*	*Bta*	*BtA*	*BTa*
btA	0.048 **	-	-	-	-	-	-
bTa	0.947	0.056 *	-	-	-	-	-
bTA	0.947	0.056 *	1.000	-	-	-	-
Bta	0.882	0.067 *	0.934	0.934	-	-	-
BtA	0.077*	0.830	0.088 *	0.088 *	0.104	-	-
BTa	0.620	0.135	0.667	0.667	0.728	0.199	-
BTA	0.025 **	0.778	0.029 **	0.029 **	0.035 **	0.620	0.077 *

(*) indicates significance at α=0.1
(**) indicates significance at α=0.05

TABLE 3. Means and standard errors of behavioral responses of western toad (*Anaxyrus boreas*) larvae when exposed to eight treatment combinations. Both measures of time were measured in seconds. All treatment names include the combinations of B/b which represents Bd exposure, T/t which represents trematode exposure, or A/a which represents conspecific alarm cue exposure. A capital letter indicates the presence of a treatment factor while a lowercase letter indicates the absence of a treatment factor.

Treatment	Quadrant lines crossed	Time (s) spent swimming	Time (s) on snail half of tank
BTA	109.2 ± 14.67	497.4 ± 51.52	1860.0 ± 102.86
BTa	74.6 ± 12.59	450.8 ± 49.54	1536.0 ± 164.37
BtA	100.1 ± 12.11	450.4 ± 47.65	1770.0 ± 129.92
Bta	74.3 ± 9.02	514.2 ± 64.12	1473.0 ± 107.37
bTA	118.3 ± 13.53	572.3 ± 51.08	1458.0 ± 111.01
bTa	77.5 ± 11.71	534.0 ± 51.50	1458.0 ± 145.00
btA	94.9 ± 6.53	516.0 ± 62.96	1809.0 ± 111.27
bta	84.3 ± 6.13	578.0 ± 53.41	1446.0 ± 136.66

TABLE 4. Results of two-sample t-tests to compare overall treatment effects on the avoidance behavior of western toad (*Anaxyrus boreas*) larvae (time spent in seconds on the snail half of the tank when exposed to eight treatments) and activity behaviors of western taod (*Anaxyrus boreas*) larvae (time spent swimming in the first 5-minutes and number of quadrant lines crossed when exposed to eight treatments). Treatment names are "B", "T", or "A", where "B" represents exposure to Bd, "T" represents exposure to echinostome cercariae, and "A" represents exposure to conspecific alarm cues.

Response Variable	Treatment	t-statistic	DF	p-value
Avoidance	B	-1.2482	78	0.2157
Avoidance	T	0.49194	78	0.6241
Avoidance	A	-2.7189	78	0.0081 ***
Activity (time swimming)	B	1.9227	78	0.05816*
Activity (time swimming)	T	0.026792	78	0.9787
Activity (time swimming)	A	0.26739	78	0.7899
Activity (lines crossed)	B	0.50181	78	0.6172
Activity (lines crossed)	T	-0.77837	78	0.4387
Activity (lines crossed)	A	-3.6005	78	0.0006 ***

(*) indicates significance at α=0.1
(***) indicates significance at α=0.01

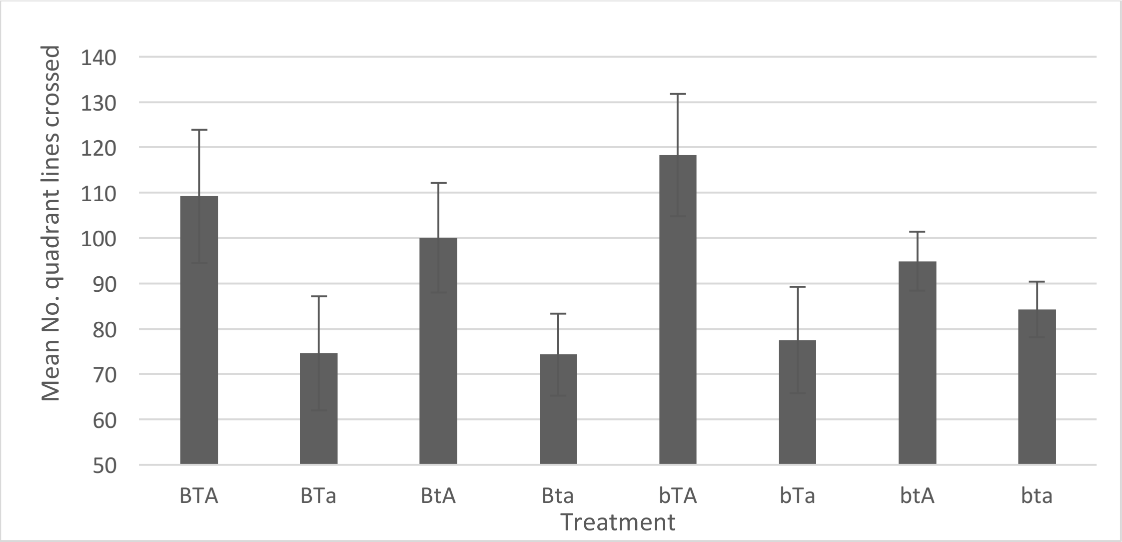

FIGURE 1. The mean number of tank quadrant lines crossed by western toad (*Anaxyrus boreas*) larvae in eight treatments. Error bars represent the standard error for a treatment. All treatment names include the letters B/b, T/t, or A/a. A capital letter indicates the presence of a treatment factor while a lowercase letter indicates the absence of a treatment factor.

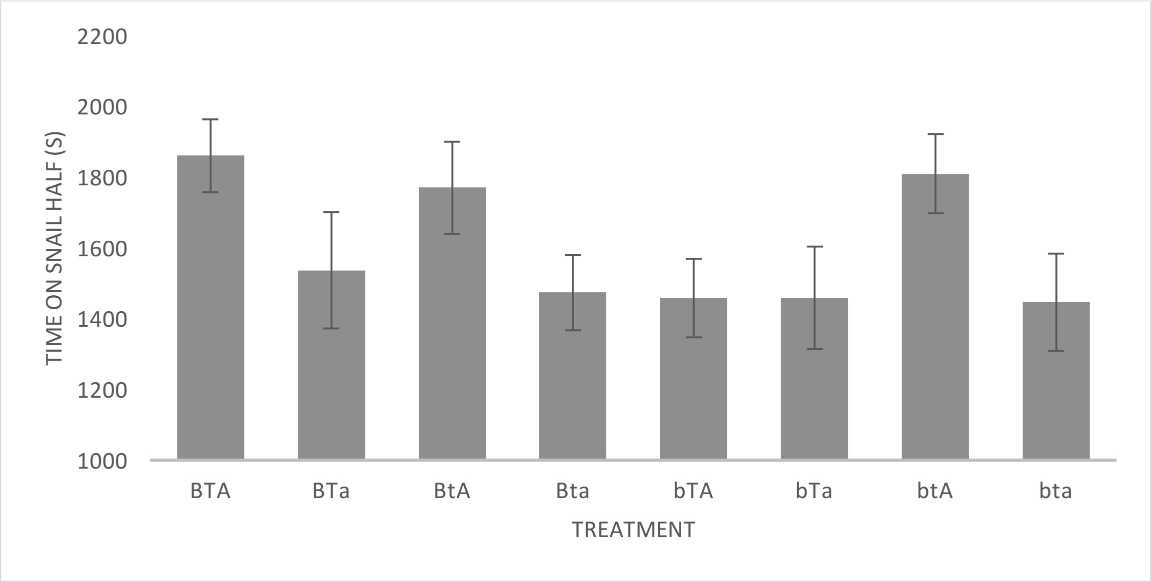

FIGURE 2. The average time by western toad (*Anaxyrus boreas*) larvae spent on the snail half of the tank by treatment. Error bars represent the standard error for a treatment. All treatment names include the letters B/b, T/t, or A/a. A capital letter indicates the presence of a treatment factor while a lowercase letter indicates the absence of a treatment factor.

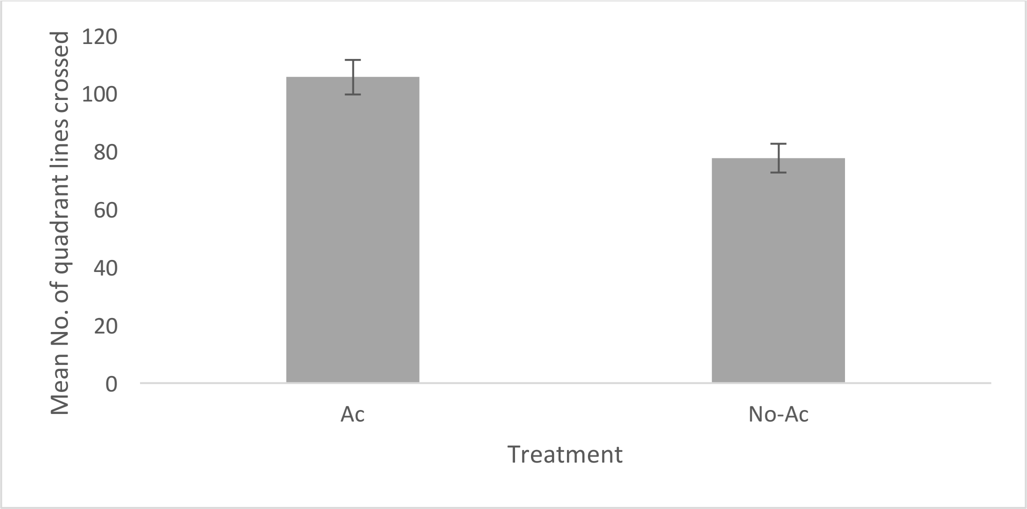

FIGURE 3. The mean number of quadrant lines crossed by western toad (*Anaxyrus boreas*) larvae in alarm cue treatments. The “Ac” treatment includes all four treatments with alarm cue exposure while the “No-Ac” treatment includes all four treatments without alarm cue exposure. Error bars represent the standard error for a treatment.

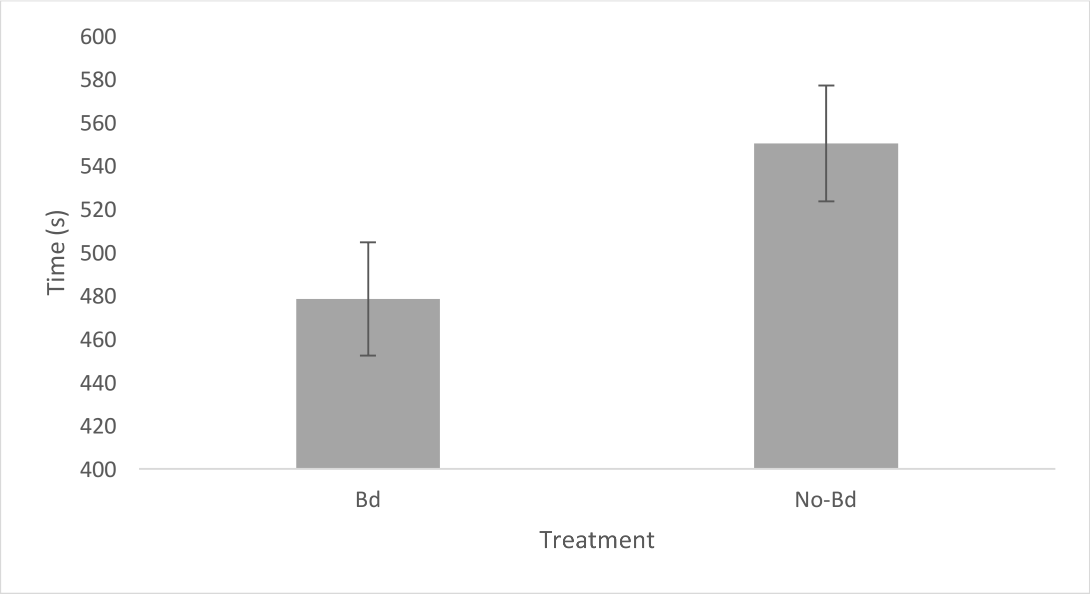

FIGURE 4. The mean time (seconds) western toad (*Anaxyrus boreas*) larvae spent swimming by *Batrachochytrium dendrobatidis* (Bd) exposure treatment. The “Bd” treatment includes all four trials where tadpoles were exposed to Bd, while the “No-Bd” treatment includes all four trials where tadpoles were exposed to a sham Bd inoculum. Error bars represent the standard error for a treatment.

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CHAPTER 3: CONCLUSIONS

The interactions between multiple stressors can be varied and unpredictable (Darling and Côté 2008; Holmstrup et al. 2010; Sih et al. 2011). The objective of my thesis was to assess behavioral response of western toad tadpoles to simultaneous, ecologically relevant stressors. To investigate the behavioral response of western toads when exposed to a macro-parasite, a micro-parasite, and conspecific alarm cues, I ran a 2x4 fully factorial experimental design. Treatments included all possible combinations of exposure to: *Batrachochytrium dendrobatidis* (Bd), *Echinostoma sp*., and conspecific alarm cues. Bd has been implicated in amphibian population declines and extinctions worldwide (Skerratt et al. 2007; Fisher et al. 2009; Vredenburg et al. 2010; Olson et al. 2013; Xie et al. 2016) and there have been relatively few studies involving the concurrent exposure of Bd with echinostome cercariae and alarm cues although they co-occur and are detrimental to amphibian hosts (Martin and Conn 1990; Berger et al. 1998; Skerratt et al. 2007; Holland 2010; Hoverman et al. 2012). I found that activity and avoidance behavior were affected by different combinations of the three stressors. Overall, exposure to alarm cues had a more pronounced effect on activity and avoidance behaviors than Bd or echinostome. Increased activity in response to alarm cues has been observed previously in western toad larvae (Hews and Blaustein 1985; Han et al. 2011). There was some evidence that Bd decreased activity levels, which corroborates previous findings on experimental Bd exposures to amphibian larvae (Voyles et al. 2009; Venesky et al. 2009). Echinostome cercariae exposure alone did not influence tadpole behavior, but combinations involving echinostome cercariae had certain effects on tadpole behavior.

Activity generally increased in the presence of conspecific alarm cues and the response was enhanced in the presence of echinostome cercariae. The addition of Bd had little effect on activity when combined with the two other stressors. Tadpoles avoided conspecific alarm cue significantly more when they had been previously exposed to Bd.

In my first chapter, I reviewed studies from the Web of Science database that included at least one behavioral measurement of a larval amphibian's response to simultaneous parasite or pathogen and predator or alarm cue exposure (Appendix 1, Table 1). There is little information about behavioral responses to these stressors in the field, and I was only able to find 17 studies that fit the criteria. From this summary, I found that the responses to individual stressors differed than compared with responses to combined stressors 59 percent of the time. Various studies illustrated additive, synergistic, and antagonistic responses from combined stressors. Interspecific variation between host species as well as response variation to parasite and predator species was observed. This in conjunction with my findings from chapter 2 provide further evidence that multiple stressors produce complex responses compared to responses to individual stressors.

My research provides additional data into how multiple stressors may affect amphibian behaviors. Moreover, the western toad is declining throughout its range (Corn et al. 1989; Carey 1993; Drost and Fellers 1996; Scherer et al. 2005) and my study suggests that the three stressors I investigated may at least contribute indirectly to some of the declines. To implement efficient conservation strategies, it is important to have an accurate inclusive view of the ecosystem as a whole, which includes behavioral responses to species interactions (Hoverman and Searle 2016). Behavioral responses are an integral part of disease dynamics and understanding the interactions between disease induced behaviors and behavioral responses from additional stressors will allow us to develop better strategies to combat multiple stressor related species declines (Hoverman and Searle 2016).

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APPENDIX

Supplementary materials to Chapter 1

INTRODUCTION AND METHODS

Previous studies of amphibian larval behavioral responses to various stimulus cues show variable findings (Wassersug and Seibert 1975; Relyea 2001b; Blaustein and Kiesecker 2002; Watling et al. 2011). In particular, in studies examining responses to a parasite or pathogen and a predator or alarm cue by different amphibian species, numerous outcomes are evident (Table S1).

All publications were collected using the Web of Science database. Two combinations of keywords were used - “parasite predator amphibian” and “pathogen predator amphibian.” The keyword “pathogen” was used to include interactions involving ranavirus (a commonly studied amphibian virus) and *Batrachochytrium dendrobatidis* (Bd), as many researchers use parasite and pathogen interchangeably when referring to the chytrid fungus. Only publication that included a simultaneous exposure to parasites or pathogens and predators or alarm cues were used. Theoretical modeling articles and studies that did not include a simultaneous exposure treatment were excluded. Survival or the type of behavioral measurements are reported, whether that included activity, refuge use or avoidance of a cue. Instances that tracked the location of an animal in relation to a cue were treated as avoidance behaviors. Studies that utilized multiple species were broken up so each species was given its own row. Type of parasite or pathogen used as well as dose, predator species or alarm cue and method of cue exposure, and effects of simultaneous exposure to the stressors are reported in the table. As these publications were all collected from a single database, this table should not be considered a comprehensive summary of amphibian responses to a predator and parasite or pathogen.

RESULTS AND DISCUSSION

Only 17 articles from the Web of Science database met the selection criteria. There were 12 laboratory studies, six mesocosm studies, and one field study. Twenty-eight groups of individuals were tested that included one caudata species and 11 anuran species. Four different simulated predators were used - amphibian cues (either macerated conspecifics or a species of salamander) were used 28% of times, fish cues were used 15% of times, arthropod cues (dragonflies, damselflies, or giant water bugs) were used 54% of times, and humans were used 3% of times. Parasites were either trematode species (57%), an amphibian virus (21.5%), or an infectious fungus (21.5%). All studies recorded combinations of four different response variables: survival, avoidance, activity, and refuge use. Fifty-one total individual measurements were taken. The measurements were 35% survival, 12% avoidance behaviors, 43% activity behaviors, and 10% refuge use behaviors.

The effects of simultaneous exposure to predators and parasites or pathogens were complex and varied. In the majority of instances (61%), survival was not impacted by the combined presence of a predator and a parasite or pathogen. Survival was reduced in 33% of the studies and increased in 6%. Avoidance behaviors were decreased in half of the trials, increased in a third of trials, and were not affected the rest of the time. Activity was most often decreased (45%) but almost equally non-affected (41%) and increased in only three trials (14%). Simultaneous exposure to predator and parasite or pathogen increased refuge use in one instance (out of five) and otherwise had no effect. Out of the 51 measurements taken, 59% of responses to individual stressors differed from the responses to combined stressors and 41% of responses to individual stressors did not differ from the response to the combined stressors. Additive, synergistic, and antagonistic responses were observed in 20% of responses to multiple stressors.

Methods of predator and parasite or pathogen exposures were varied. Predator exposures included everything from actual predation events to visual cues alone. When actual predators were used in non-survival trials, there were no standard methodologies. Some experiments chose to use water from predators that had recently fed on conspecifics, while some used predator cues in addition to conspecific alarm cues. Only four studies investigated survival in the manner of allowing the predator to ingest the study species individual. Out of the four, only one study standardized predator hunger by starvation before exposure. The remaining three studies fed predators *ad libitum*, and there was a chance that predators had eaten immediately prior to the beginning of the exposure. This lack of standardization could have had significant effects on time to prey capture.

Parasite exposure methods also varied by study. This is to be expected however, as some researchers were interested in assessing the effects of direct cercarial exposure as opposed to exposure to an infected snail shedding cercariae. Direct cercarial exposure allows the researchers to assess the effects of echinostome infection on tadpoles, whereas exposure to an infected snail shedding cercariae provides ecological context, as the cercariae would not be present in the water if there are no snails present.

Although the methodologies between these studies were considerably different, it is still possible to draw general conclusions. Responses to multiple stressors often differ when compared to responses to individual stressors, as seen in the table below. The summary table also exemplifies the interspecific variation in responses between parasite species, host species, as well as predatory species. From the summary, empirical evidence is clearly required to definitively describe a host species' response to a parasite or pathogen, as generalized speculations can be contradicted with different hosts. It should also be noted that there is a severe lack of information regarding the behavioral responses to these stressors in salamander species. I recommend an increase in salamander species use as hosts in future studies involving similar stressors to get a better understanding of amphibian responses.

TABLE S1

Species	**Experiment Type**	**Parasite/ Pathogen**	**Type of Predator**	**Behavioral Measurement**	**Behavioral Measurement Method**	**Combined Effect**	**Individual effect same as combined effect?**	**Reference**
Lithobates sylvaticus	Mesocosm	*Echinostoma trivolvis* infected snail into mesocosm	Jefferson salamander	Survival	None, survival	Reduced survival	No	Belden and Wojdak 2011
Lithobates sylvaticus	Mesocosm	*Ribeiroia ondatrae* infected snail into mesocosm	Jefferson salamander	Survival	None, survival	Reduced survival	No	Belden and Wojdak 2011
Pseudacris regilla	Field	*Ribeiroia ondatrae* adults showing limb malformations	Humans	Avoidance	Distance between frog and simulated threat; Escape distance	Reduced avoidance	Yes, uninfected had normal response	Goodman and Johnson 2011
Lithobates clamitans	Laboratory	Ranavirus, FV3 10^3 PFU/mL	Larval dragonfly, giant water bugs	Activity, survival	Activity (scan sampling), survival	Reduced activity, no difference on survival	Yes/No, predator alone reduced activity to a lesser degree, more active when just virus	Haislip et al. 2012
Lithobates sylvaticus	Laboratory	Ranavirus, FV3 10^3 PFU/mL	Larval dragonfly, giant water bugs	Activity, survival	Activity (scan sampling), survival	Reduced activity, no difference on survival	Yes/No, predator alone reduced activity to a lesser degree	Haislip et al.2012
Pseudacris feriarum	Laboratory	Ranavirus, FV3 10^3 PFU/mL	Larval dragonfly, giant water bugs	Activity, survival	Activity (scan sampling), survival	Reduced activity, no difference on survival	Yes/No, predator alone reduced activity to a lesser degree	Haislip et al. 2012
Hyla chrysoscelis	Laboratory	Ranavirus, FV3 10^3 PFU/mL	Larval dragonfly, giant water bugs	Activity, survival	Activity (scan sampling), survival	Reduced activity, no difference on survival	Yes/No, predator alone reduced activity to a lesser degree, less active to virus alone	Haislip et al. 2012

Anaxyrus boreas	Laboratory	*Batrachochytrium dendrobatidis* 6.18x10^6 zoospores/mL	Rough-skinned newt + macerated conspecific	Activity, refuge use	Gridlines crossed and refuge use (in 30 s intervals)	Increased activity, increased refuge use	Yes/Yes, activity levels were not as high as combined treatment, neutral cue and uninfected used refuge less	Han et al. 2011
Rana aurora	Laboratory	*Batrachochytrium dendrobatidis* 6.18x10^6 zoospores/mL	Rough-skinned newt + macerated conspecific	Activity, refuge use, survival	Gridlines crossed and refuge use (in 30 s intervals)	No effect on activity, no effect on refuge use, increased survival	Yes/No/NA, Predator cues reduced activity, uninfected individuals were not eaten as much.	Han et al. 2011
Rana cascadae	Laboratory	*Batrachochytrium dendrobatidis* 6.18x10^6 zoospores/mL	Rough-skinned newt + macerated conspecific	Activity, refuge use, survival	Gridlines crossed and refuge use (in 30 s intervals)	No effect on activity, no effect on refuge use, no effect on survival	No/No/No	Han et al. 2011
Pseudacris regilla	Laboratory	*Batrachochytrium dendrobatidis* 6.18x10^6 zoospores/mL	Rough-skinned newt + macerated conspecific	Activity, refuge use, survival	Gridlines crossed and refuge use (in 30 s intervals)	No effect on activity, no effect on refuge use, no effect on survival	No/No/No	Han et al. 2011
Ambystoma tigrinum melanostictum	Laboratory	Ranavirus, ATV 1x10^4 PFU	Larval green darner dragonfly	Activity, refuge use, survival	Activity (estimating distance moved in 5 s intervals), refuge use (proportion of time in refuge)	No effect on activity, no effect on refuge use, reduced survival	Yes/No/Yes, predator alone reduced activity, increased mortality	Kerby et al. 2011
Lithobates pipiens	Laboratory	*Echinostoma trivolvis* 15 cercariae	Larval dragonfly	Survival	None, survival	Reduced survival	Yes, parasites only treatment did not reduce survival as much	Koprivnikar 2010
Lithobates pipiens	Laboratory	*Ribeiroia ondatrae* 25 cercariae	Larval dragonfly	Avoidance	Avoidance of cue while foraging (location of tadpole every 30 s)	Increased avoidance of predator cue	Yes, avoided predator cue most but avoided	Koprivnikar and Penalva 2015

							trematode when no predator	
Lithobates sylvaticus	Laboratory	*Echinostoma spp*. No direct contact, contained in mesh container with snail	Larval dragonfly	Activity, survival	Activity (counted number of tadpoles swimming in 5 s intervals) and tadpole location	Activity reduced, but not as much compared to individual treatments, no effect on survival	Yes/No, individual stressors reduced activity	Marino et al. 2014
Lithobates clamitans	Laboratory	*Echinostoma spp.* 250 cercariae	Larval dragonfly	Activity, avoidance, survival	Activity (counted number of tadpoles swimming in 5 s intervals) and tadpole location	No effect on activity, no effect on avoidance, no effect on survival	Yes/No/No, predators reduced activity	Marino et al. 2014
Lithobates clamitans	Mesocosm	*Echinostomatidae* infected *Planorbella trivolvis*	Larval dragonfly, larval damselfly	Activity, survival	Activity (scan sampling), predation rate	No effect on activity, reduced survival when exposed to free predator treatment	Yes/Yes, parasites increased activity levels, survival was higher without parasites	Marino and Werner 2013
Pseudacris regilla	Mesocosm	*Ribeiroia ondatrae*, taken off snails and added to the mesocosms	Larval dragonfly, larval damselfly	Activity	Activity (scan sampling, number of tadpoles moving)	No effect on activity	Yes, predators alone reduced activity	Orlofske et al. 2014
Hyla chrysoscelis	Laboratory	*Batrachochytrium dendrobatidis* 7000 zoospores/mL	Eastern newt	Survival	None, survival	No effect on survival	Yes, predators alone reduced survival	Parris and Beaudoin 2004
Lithobates pipiens	Laboratory	*Batrachochytrium dendrobatidis* 7000 zoospores/mL	Bluegill	Activity, avoidance, survival	Activity, location, predation (recorded tadpole location relative to the center divider and activity	No effect on activity, increased avoidance	Yes/Yes/NA Bd reduced activity, Bd reduced avoidance to visual cues, increased	Parris et al. 2006

					level- swimming any time during the interval at 20 s intervals for 20 min)	with chemical predator cues, increased survival	avoidance to visual and chemical cues. N/A because both uninfected and infected individuals exposed to predator with not alternative	
Pseudacris regilla	Laboratory, mesocosm	*Ribeiroia ondatrae, Echinostoma trivolvis, or Alaria sp. 40 cercariae*	Larval green darner dragonfly	Activity, avoidance	Activity (scan sampling for movement) and position in water column (lab only)	Reduced activity, reduced avoidance	Yes/Yes, no change in activity when exposed to any of the different parasite treatments	Preston et al. 2014
Anaxyrus boreas	Mesocosm	*Ribeiroia ondatrae* infected *Planorbella trivolvis*	Larval green darner dragonfly	Activity, avoidance	Activity (scan sampling for movement)	Reduced activity, reduced avoidance	Yes/Yes, no change in activity when exposed to any of the different parasite treatments	Preston et al. 2014
Bufo americanus	Mesocosm	*Echinostoma trivolvis*	Eastern newt	Activity, survival	Activity (proportion of tadpoles moving in a 10s period)	No effect on activity, no effect on survival	Yes/Yes, increased activity from parasites and decreased activity from predators, parasites increased activity with no effect from predator exposure	Raffel et al. 2010
Lithobates sylvaticus	Laboratory, mesocosm	Ranavirus, FV3 isolate AEC37 1.25x10^4 PFU/mL	Larval diving beetle, larval dragonfly	Survival	Activity (velocity), ranavirus susceptibility	No effect on survival	No	Reeve et al. 2013
Lithobates clamitans	Laboratory	*Echinostoma sp.* 36 cercariae alone	Pumpkinseed sunfish	Activity	Activity (Total time active in both the 15 minute baseline and 15 minute post-treatment period, defined as any movement of the tadpole through the water). Three other measurements-	Increased activity	Yes, parasites increased activity and predators decreased activity	Szuroczki and Richardson 2012

					extreme swimming, body twisting, tail flicking.			
Lithobates catesbeianus	Laboratory	*Echinostoma sp.* 36 cercariae alone	Pumpkinseed sunfish	Activity	Activity (Total time active in both the 15 minute baseline and 15 minute post-treatment period, defined as any movement of the tadpole through the water). Three other measurements- extreme swimming, body twisting, tail flicking.	Increased activity	Yes, parasites increased activity and predators decreased activity	Szuroczki and Richardson 2012
Lithobates sylvaticus	Laboratory	*Echinostoma sp.* 36 cercariae alone	Pumpkinseed sunfish	Activity	Activity (Total time active in both the 15 minute baseline and 15 minute post-treatment period, defined as any movement of the tadpole through the water). Three other measurements- extreme swimming, body twisting, tail flicking.	Reduced activity	Yes, parasites increased activity and predators decreased activity	Szuroczki and Richardson 2012
Lithobates clamitans	Laboratory	*Echinostoma trivolvis* 340 cercariae/tank	Banded killifish	Activity	Activity (number of tadpoles moving was counted and recorded as a proportion)	Reduced activity	No	Thiemann and Wassersug 2000
Lithobates sylvaticus	Laboratory	*Echinostoma trivolvis* 120 cercariae/tank	Banded killifish	Activity	Activity (number of tadpoles moving was counted and recorded as a proportion)	Reduced activity	Yes, parasites and predators decreased activity, combination decreased activity further	Thiemann and Wassersug 2000