RANGE EXPANSION DYNAMICS AND INTERSPECIFIC INTERACTIONS OF THE INVASIVE CUBAN TREEFROG IN FLORIDA

by

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Abstract

Biological invasions simultaneously represent a threat to native biota and provide an opportunity to test ecological and evolutionary ideas. Increased interest in the field of invasion ecology has greatly improved our understanding of invasion dynamics; however, many ideas and assumptions remain inadequately tested. This dissertation focuses on two major themes: (1) native predator response to a toxic, non-indigenous species, and (2) the processes that may promote range expansion following establishment. Here, I used the Cuban Treefrog (*Osteopilus septentrionalis*) invasion of Florida as a model to explore these aspects of invasive ecology.

Cuban Treefrogs are known prey of native snake predators, despite their possession of a defensive toxin assumed to be novel to their invasive Florida range. I investigated the idea that possession of unique chemical defenses may increase the likelihood of formation of evolutionary traps for predators. Additionally, I explored the possibility that populations of snakes in co-occurrence with Cuban Treefrogs had responded to escape potential trap dynamics. To test these hypotheses, I conducted a series of prey preference assays and feeding trials of Common Gartersnakes (*Thanmonphis sirtalis*) collected from areas of sympathy and allopathy with Cuban Treefrogs. To assess prey preference, we explored the predatory responses of snakes to cues from Cuban Treefrogs, a native treefrog, and positive and negative controls. To evaluate a potential fitness cost of consuming Cuban Treefrogs, I monitored the growth of 61 wild-caught adult snakes fed exclusive diets of either Cuban Treefrogs, or one of two native prey species. Our results suggest that Cuban Treefrogs represent an evolutionary trap for snakes because consumption of these frogs carries fitness costs, and snakes are unable to recognize this prey as being costly. We failed to detect a regional effect of snake growth or prey preference suggesting, snakes are not responding to escape trap dynamics.

The current range of Cuban Treefrogs encompasses the majority of peninsular Florida and expansion of this range likely exacerbates impacts of these frogs on the native biota. Many biological invasions are characterized by rapid range expansion; however, the factors enabling range expansion are poorly understood. Here, I investigated the idea that dispersal ability is promoted at the invasion front by trade-offs facilitating allocation of resources away from immune defense and towards traits that improve dispersal ability. To investigate this idea, I assayed Cuban Treefrog immunity and several characteristics associated with increased dispersal ability in long-colonized and recently-colonized regions of their invasive distribution. Cuban Treefrogs at the invasion front exhibited decreased innate immune responses coupled with greater locomotor endurance, compared with frogs from a long-colonized region. I failed to detect a regional difference in sprint speed or growth rate. These findings provide support for the idea that immune-based trade-offs promote range expansion and invasion success.

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CHAPTER I

INTRODUCTION

Charles Elton’s 1958 book, “The Ecology of Invasions by Animals and Plants” is often cited as the birth of invasion ecology; however, widespread interest in biological invasions is a more recent phenomenon. In terms of number of published studies on invasion ecology, most of the growth of this field has occurred within the last two decades (Lowery et al. 2013). Increased interest has been fueled, in part, by the rapid increase in the number of invasive species worldwide over the past 50 years (D’Antonio et al. 2004; Hulme 2009), likely a consequence of increased global transportation networks and trade of live animals (Meyerson et al. 2007; Romagosa et al. 2009). Additionally, greater impetus to study invasion dynamics has arisen in response to the economic and ecological impacts posed by biological invaders. Invasive species are currently recognized as one of the greatest threats to biodiversity, typically regarded as second only to habitat destruction (Wilcove et al. 1998; Clavero and Garcia-Berthou 2005; Butchart et al., 2010; Simberloff et al., 2013), and are estimated to cost global economies over $100 billion per year (Pimentel et al. 2005).

Study of biological introductions has provided valuable insights into the causes and effects of the invasion process; however, several aspects of the invasion process remain unstudied or unresolved. While many studies have focused on evolutionary responses of non-indigenous species, few have investigated responses of native predators to non-indigenous species (Carlsson et al. 2009). An abundant introduced species can offer a plentiful prey source to native predators (King et al. 2006; Li et al. 2011) and predators may experience pressure to incorporate an introduced species into their diet if that species reduces native prey abundances. Conversely, native predators, absent from a coevolutionary process that shaped characteristics of nonindigenous species, may be at an evolutionary disadvantage during encounters, particularly if an introduced species possesses novel defenses.

Similar to the predator/prey dynamics described above, rapid range expansion observed in many non-indigenous species is poorly understood. Several broad ecological patterns are beginning to emerge concerning what characterizes successful establishment of non-indigenous species, including propagule pressure, climate matching, and high reproductive rates (Bomford et al. 2009; Kolar and Lodge 2001; Lockwood et al. 2005). Conversely, mechanisms promoting range expansion following colonization remain obscure (Kolar and Lodge 2001). Only a small percent of NIS that become established are able to spread beyond areas of initial colonization (Williamson and Fitter 1996), indicating a different suite of selective pressures acting on non-indigenous species during colonization verses range expansion (stages III and IV; Colautti and MacIsaac, 2004). Negative effects on native ecosystems may be exacerbated by rapid range expansion following colonization (White and Perkins 2012). Understanding how species exploit new habitats is a priority for ecologist both in terms of inhibiting invasive species and understanding how distribution shifts in native species may occur in response to anthropogenic perturbations, such as habitat destruction, invasive species, and global climate change.

Early work in invasive ecology, such as Elton (1958), focused on agricultural pests with little attention given to introduced herpetofauna. This view began to change following studies of the Brown Treesnake (*Boiga irregularis*) invasion of Guam that demonstrated the ability of an ectothermic vertebrate to disrupt native communities and directly cause extinction of native species (Savidge 1987; Fritts and Rodda 1998; Campbell III et al. 2012). Currently, Florida is an epicenter of invasive herpetofauna in the United States, with at least 56 established, non-indigenous species (Krysko et al. 2011). For perspective, 43 species of introduced lizards are established in Florida compared with only 22 species native to the state (Krysko et al. 2011).

Among the invasive biota of Florida is the species I chose to use as a model organism, the Cuban Treefrog (*Osteopilus septentrionalis*). Several aspects of the Cuban Treefrog invasion of Florida made it appropriate for testing hypotheses related to predator prey dynamics and range expansion. First, the Cuban Treefrog is an interesting species because it possesses a novel defensive toxin secreted when attacked. Despite chemical defenses, several species of native predators are known, at least occasionally, to consume Cuban Treefrogs, possibly due to their superficial similarity to native prey species. Second, investigation of Cuban Treefrog range expansion dynamics are possible because the expansion chronology through Florida has been well-documented. Native to Cuba, the Bahamas, and the Cayman Islands, Cuban Treefrogs were first introduced to the Florida Keys circa the late 1920’s (Meshaka 2001). By the early 1950’s, Cuban Treefrogs were established in southern, mainland Florida and subsequently spread northward via leading-edge dispersal to their present range encompassing most of the peninsula. Arrival dates relating to the spread of Cuban Treefrogs allowed for the identification of the current invasion front and comparison of populations relative to time since colonization.

Dissertation Outline

Chapter 2 of the dissertation evaluates the hypothesis that toxic Cuban Treefrogs have created an evolutionary trap for native generalist snakes. Evolutionary traps occur when previously beneficial responses become maladaptive. Here, I investigated the prey preferences and growth rate of Common Gartersnakes (*Thamnophis sirtalis*) in response to two native prey species and invasive Cuban Treefrogs. Cuban Treefrogs appear to represent an evolutionary trap because Common Gartersnakes exhibit equal preference of Cuban Treefrogs and native prey, yet consumption of Cuban Treefrogs results in an approximate 50% reduction in growth compared with native prey diets.

Chapters 3 and 4 investigate the idea that rapid range expansion of Cuban Treefrogs may be partially fueled by an immune-based trade-off. Resources typically allocated to immune defense may be directed towards traits that promote dispersal ability and result in an increased rate of range expansion. Foundational to this idea is that mounting an immune response is costly in terms of energetic and nutritional expenditure (Demas et al., ’97; Martin et al., 2003; Demas, 2004; Klasing, 2004); however, there are several arguments that suggest vigorous immune responses may be detrimental or represent wasted effort for invasive species. First, invasive species are often seeded from a small number of propagules, making it likely only a small subset of co-evolved parasites and pathogens will be co-introduced (Torchin et al., 2003; Torchin and Lafferty, 2009). Second, parasites and pathogens that are introduced may be density-dependent, suggesting that they may not be able to become established or will trail behind the leading-edge of range expansion, where host density is likely lower (Torchin et al., 2003; White and Perkins, 2012). Third, many parasites and pathogens native to the invasive range may either be incapable of inducing disease or exhibit reduced virulence in invasive species because they lack a co-evolutionary history and therefore immunosuppression may be a common strategy in invasive species (Rigaud and Moret, 2003; Kopp and Jokela, 2007). Finally, strong immune responses by invasive species to foreign pathogens can be immunopathic, resulting in damage or host death (Raberg et al., 1998; Mims et al., 2001). To test the hypothesis that an immune-based trade-off has facilitated range expansion in Cuban Treefrogs, it is necessary to demonstrate both reduced immune response and increased ability of a trait related to dispersal in frogs at the invasion front.

To evaluate possible immune-based trade-offs related to the Cuban Treefrog invasion, in chapter 3, I investigated possible differences in innate immune response strength relative to time since colonization. I compared the bactericidal ability and metabolic response to an immune challenge of frogs from populations in long-colonized areas and at the invasion front. In both measures, I found evidence of reduced investment at the invasion front. In chapter 4, I used a similar experimental design and compared populations of Cuban Treefrogs relative to time since colonization. I detected a downstream benefit to reduced innate immune response in that frogs from the invasion front had greater locomotor endurance compared with frogs from long-colonized areas. I failed to detect a difference relative to time since colonization in sprint speed or growth rate.

LITERATURE CITED

Bomford M, Kraus F, Barry SC, Lawrence E. 2009. Predicting establishment success for alien reptiles and amphibians: a role for climate matching. Biological Invasions 11:713–724.

Butchart SHM, Walpole M, Collen B, van Strien A, Scharlemann JPW, Almond REA, Baillie JEM, Bomhard B, Brown C, Bruno J, Carpenter KE, Carr GM, Chanson J, Chenery AM, Csirke J, Davidson NC, Dentener F, Foster M, Galli A, Galloway JN, Genovesi P, Gregory RD, Hockings M, Kapos V, Lamarque J-F, Leverington F, Loh J, McGeoch MA, McRae L, Minasyan A, Morcillo MH, Oldfield TEE, Pauly D, Quader S, Revenga C, Sauer JR, Skolnik B, Spear D, Stanwell-Smith D, Stuart SN, Symes A, Tierney M, Tyrrell TD, Vie J-C, Watson R. 2010. Global Biodiversity: Indicators of Recent Declines. Science 328:1164–1168.

Carlsson NO, Sarnelle O, Strayer DL. 2009. Native predators and exotic prey –an acquired taste? Frontiers in Ecology and the Environment 7:525–532.

Clavero M, García-Berthou E. 2005. Invasive species are a leading cause of animal extinctions. Trends in ecology & evolution 20:110.

Colautti RI, MacIsaac HJ. 2004. A neutral terminology to define “invasive”species. Diversity and distributions 10:135–141.

D’Antonio CM, Jackson NE, Horvitz CC, and Hedberg R. 2004. Invasive plants in wildland ecosystems: merging the study of invasion processes with management needs. Frontiers in Ecology and the Environment 2: 513–521.

Demas GE, Chefer V, Talan MI, Nelson RJ. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. Am J Physiol-R I 273:R1631–R1637.

Demas GE. 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Hormones and Behavior 45:173–180.

Earl III WC, Adams AAY, Converse SJ, Fritts TH, Rodda GH. 2012. Do predators control prey species abundance? An experimental test with brown treesnakes on Guam. Ecology 93:1194–1203.

Elton CS. 2000. The ecology of invasions by animals and plants. University of Chicago Press ed. Chicago: University of Chicago Press.

Fritts TH, Rodda GH. 1998. The role of introduced species in the degradation of island ecosystems: a case history of Guam. Annual review of Ecology and Systematics 29:113–140.

Hulme PE. 2009. Trade, transport and trouble: managing invasive species pathways in an era of globalization. Journal of Applied Ecology 46:10–18.

King RB, Ray JM, Stanford KM. 2006. Gorging on gobies: beneficial effects of alien prey on a threatened vertebrate. Canadian Journal of Zoology 84:108–115.

Klasing KC. 2004. The costs of immunity. Acta Zool Sinica 50:961–969.

Kolar CS, Lodge DM. 2001. Progress in invasion biology: predicting invaders. Trends in ecology & evolution 16:199–204.

Kopp K, Jokela J. 2007. Resistant invaders can convey benefits to native species. Oikos 116:295–301.

Krysko KL. 2011. Verified non-indigenous amphibians and reptiles in Florida from 1863 through 2010 outlining the invasion process and identifying invasion pathways and stages. Auckland, N.Z.: Magnolia Press.

Li Y, Ke Z, Wang S, Smith GR, Liu X. 2011. An exotic species is the favorite prey of a native enemy. PLoS One 6:e24299.

Lockwood JL, Cassey P, Blackburn T. 2005. The role of propagule pressure in explaining species invasions. Trends in Ecology & Evolution 20:223–228.

Lowry E, Rollinson EJ, Laybourn AJ, Scott TE, Aiello-Lammens ME, Gray SM, Mickley J, Gurevitch J. 2013. Biological invasions: a field synopsis, systematic review, and database of the literature. Ecology and Evolution 3:182–196.

Martin LB, Scheuerlein A, Wikelski M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? Proceedings of the Royal Society of London B: Biological Sciences 270:153–158.

Meshaka WE. 2001. The Cuban treefrog in Florida: life history of a successful colonizing species. Gainesville: University Press of Florida.

Meyersonl LA, Mooney HA. 2007. Invasive alien species in an era of globalization. Front Ecol Environ 5:199–208.

Mims CA, Nash A, Stephen J. Mims' Pathogenesis of infectious disease. 5th ed. San Diego: Academic Press, c2001.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecological Economics 52:273–288.

Raberg L, Grahn M, Hasselquist D, Svensson E. 1998. On the adaptive significance of stress-induced immunosuppression. Proceedings of the Royal Society of London B: Biological Sciences 265:1637–1641.

Rigaud T, Moret Y. 2003. Differential phenoloxidase activity between native and invasive gammarids infected by local acanthocephalans: differential immunosuppression? Parasitology 127:571–577.

Romagosa CM, Guyer C, Wooten MC. 2009. Contribution of the Live-Vertebrate Trade toward Taxonomic Homogenization. Conservation Biology 23:1001–1007.

Savidge JA. 1987. Extinction of an Island Forest Avifauna by an Introduced Snake. Ecology 68:660–668.

Simberloff D, Martin J-L, Genovesi P, Maris V, Wardle DA, Aronson J, Courchamp F, Galil B, García-Berthou E, Pascal M, others. 2013. Impacts of biological invasions: what's what and the way forward. Trends in ecology & evolution 28:58–66.

Torchin ME, Lafferty KD. 2009. Escape from Parasites. In: Rilov G, Crooks JA, editors. Biological Invasions in Marine Ecosystems. Vol. 204. Berlin, Heidelberg: Springer Berlin Heidelberg. p 203–214. Available from: http://link.springer.com/10.1007/978-3-540-79236-9_11

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM. 2003. Introduced species and their missing parasites. Nature 421:628.

White TA, Perkins SE. 2012. The ecoimmunology of invasive species. Functional Ecology 26:1313–1323.

Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E. 1998. Quantifying threats to imperiled species in the United States. BioScience 48:607–615.

Williamson M, Fitter A. 1996. The varying success of invaders. Ecology 77:1661–1666.

CHAPTER II

TOXIC, INVASIVE TREEFROG CREATES EVOLUTIONARY TRAP FOR NATIVE GARTERSNAKES

ABSTRACT

Possession of unique defensive toxins by nonindigenous species may increase the likelihood of creating evolutionary traps for native predators. We tested the hypothesis that nonindigenous, toxic Cuban Treefrogs (*Osteopilus septentrionalis*) have created an evolutionary trap for native, generalist snakes. Additionally, we explored the possibility that populations of snakes that co-occur with Cuban Treefrogs have responded in ways that allow them to escape potential trap dynamics. To evaluate a potential fitness cost of consuming Cuban Treefrogs, we monitored growth of 61 wild-caught Common Gartersnakes (*Thamnophis sirtalis*) fed exclusive diets of either Cuban Treefrogs, native Green Treefrogs (*Hyla cinerea*), or native Golden Shiners (*Notemigonus crysoleucas*). Snakes in the Cuban Treefrog diet treatment gained less than half the mass of those consuming native prey, and Cuban Treefrogs were significantly less digestible than native prey. There was no difference in the response of gartersnakes to prey scent cues of Cuban Treefrogs and Green Treefrogs. Our results indicate that Cuban Treefrogs likely represent an evolutionary trap for snakes because consumption of these frogs carries fitness costs, yet snakes fail to recognize this prey as being costly. We found no difference in growth or response to prey cues between snakes from invaded and non-invaded regions, suggesting snakes have not responded to escape trap dynamics. Interactions of native snakes and Cuban Treefrogs support the idea that introduced species with novel toxins may increase the likelihood of evolutionary trap formation.

INTRODUCTION

Ecological or evolutionary traps can develop in situations where the environment is altered such that previously-dependable cues no longer result in adaptive outcomes. (Schlaepfer et al. 2002). Demonstration of a trap necessitates fulfillment of two requirements: (1) a novel resource must be preferred (a severe trap) or equally attractive (a mild trap) relative to an existing resource, and (2) the novel resource must be of poorer quality, resulting in reduced fitness (Schlaepfer et al. 2002; Robertson et al. 2013). For example, disturbance-dependent Indigo Buntings (*Passerina cyanea*) rely on structural habitat cues to select breeding habitat. Edge habitat created from anthropogenic forest fragmentation mimics the structural cues of natural forest gaps and was expected to attract nesting birds. As predicted, Indigo Buntings preferentially nested along artificial edges, however, these areas were found to concentrate nest predators, such that, birds nesting on artificial edges suffered reduced fecundity (Weldon and Haddad 2005). An ecological trap formed because previously reliable habitat cues became maladaptive in the altered environment. Ecological traps are a specific type of evolutionary trap, which are not limited to habitat decisions but include behavioral or life-history choices

(Schlaepfer et al. 2002). Thus, evolutionary traps can arise from a variety of environmental alterations, including shifts in predator-prey dynamics.

Predator-prey arms races can be viewed as reciprocal evolutionary responses. In a cyclic relationship, adaptation by one participant drives selection and evolutionary responses from the opponent (Brodie and Brodie 1999). Evidence of reciprocity in arms races can be inferred from interactions involving toxic prey and resistant predators. Level of predator resistance to chemical defenses has been demonstrated to covary with toxicity of co-occurring prey populations, suggesting tight evolutionary relationships (Hanifin et al. 2008; Rowe and Rowe 2008). However, nonindigenous species may subvert the normal step-wise progression of coevolution by introducing evolutionarily-derived characters that are unique to native food webs, such as novel chemical defenses. Native predators, absent from a coevolutionary process that shaped characters of nonindigenous species, may be expected to be at an evolutionary disadvantage during encounters. Additionally, native predators can be misled if nonindigenous species mimic evolutionarily-derived cue(s) of suitable native prey, providing a framework for evolutionary trap formation.

Evolutionary traps can be temporary phenomena if phenotypic plasticity or natural selection provides an escape mechanism (Schlaepfer et al. 2002). Behavioral plasticity can facilitate escape via experienced-based learning, assuming a trap is not always fatal, whereas natural selection can lead to a narrowing of the set of cues signaling resource acceptance or to recognition of additional discriminatory cues that restrict choice to non-trap items (Schlaepfer et al. 2002). For example, in less than 70 years, Red-legged Frogs (*Rana aurora*) acquired the previously-absent ability to recognize chemical cues of American Bullfrogs (*Lithobates catesbeianus*), an introduced predator, and now exhibit selectively advantageous predator-avoidance behaviors (Kiesecker and Blaustein 1997). In this case, recognition of a novel predator may result from selection and/or learning. In another example, juvenile Eastern Fence Lizards (*Sceloporus undulatus*) that co-occur with invasive fire ants (*Solenopsis invicta*) are less likely to consume this toxic prey compared with lizards from univaded regions, suggesting an evolutionary response (Robbins and Langkilde 2012). Despite these examples, trap escape appears to be a relatively rare occurrence. It is unclear if escape is truly an uncommon event or if the majority of traps have either formed too recently to allow for evolution of escape mechanisms or the topic is simply understudied.

In this study, we examined predator-prey dynamics between native Common Gartersnakes (*Thamnophis sirtalis*) and nonindigenous Cuban Treefrogs (*Osteopilus septentrionalis*). Common Gartersnakes occur throughout southeastern USA, including the entire invasive range of Cuban Treefrogs in Florida. As generalist predators, Common Gartersnakes are expected to exhibit less discrimination when deciding to attack prey, indicating a low cost of accepting a poor prey option relative to the cost of rejecting a high-quality one (Robertson et al. 2013). Therefore, Common Gartersnakes may be more likely to attack novel prey. Importantly, Cuban Treefrogs may be a poor prey item because they secrete a defensive toxin. The serous secretions of Cuban Treefrogs are chemically unknown, but likely novel to their invasive range because treefrog glands that produce toxins exhibit phylogenetically-related structural differences (Delfino et al.

2002) and Cuban Treefrogs are not closely related to native treefrogs. Cuban Treefrogs are members of an evolutionary unit within the subfamily Hylinae that is not represented in the continental USA (Faivovich et al. 2005, Frost et al. 2006), and Common Gartersnakes evolved in geographic isolation from this group of treefrogs (Rossman et al. 1996; de Queiroz et al. 2002). In a systematic review of Hylidae, Faivovich and colleagues (2005) classified Cuban Treefrogs as members of a South American/West Indian radiaton, whereas all treefrogs native to southeastern USA were placed within a Middle American/Holartic clade. Delfino and colleagues (2002) found the substructure of Cuban Treefrog granules, glands that produce and/or sequester toxins, to be structurally similar to the Veined Treefrog (*Phrynohyas venulosa*), also a member of the South American/West Indian clade, but different from two species belonging to the Middle American/Holartic clade (Italian Treefrog, *Hyla intermedia*; European Treefrog, *Hyla arborea*). Cuban Treefrogs also produce a milky serous product that is similar to secretions of the Veined Treefrog that are thought to serve as a predator deterrent (Leary and Razafindratsita 1998; Manzanilla et al. 1998).

The effect of Cuban Treefrog toxins on non-mammalian taxa is poorly understood. However, toxins are typically a broad defense, effective against a variety of organisms, making it simultaneously likely that chemical deterrents will have a negative effect on novel predators and unlikely that predators will possess an inherent resistance or ability to neutralize ingested toxins. Avian and mammalian predators may bypass skin toxins by eating only nontoxic portions of poisonous prey (Lizana and Mellado 1990; Antczak et al. 2005; Bechmann and Shine 2011). In contrast, snakes ingest prey whole

(Greene 1997), and thus cannot avoid consumption of prey toxins, making them appropriate predator models to investigate potential consequences of consuming toxic, nonindigenous prey. Numerous bird and reptile species native to Florida, including Common Gartersnakes, have been observed to consume Cuban Treefrogs (Love 1995; Meshaka, 2001). However, there has been no effort to investigate post-consumptive effects or prey preference in relation to Cuban Treefrogs.

We hypothesized that Cuban Treefrogs represent an evolutionary trap for Common Gartersnakes. We predicted Common Gartersnakes would fail to exhibit a preference for a native treefrog species over Cuban Treefrogs. Additionally, we predicted snakes consuming Cuban Treefrogs would suffer a fitness cost due to consumption of toxic compounds we suspect to be novel. If the relationship between Common Gartersnakes and Cuban Treefrogs met the requirements of an evolutionary trap, we wanted to explore potential responses to the trap. A well-documented chronology and clear delineation of the invasive range of Cuban Treefrogs allowed us to investigate potential trap responses of native snakes. We compared snakes from populations where Cuban Treefrogs have been present for over 60 years (i.e. populations in southern peninsular Florida) to populations from non-invaded regions. A 60-year period of co-occurrence with Cuban Treefrogs is conservatively equivalent to 20 Common Gartersnake generations as females typically attain sexual maturity by three years of age (Ernst and Ernst 2003). Invasive species are known to induce evolutionary responses to escape traps within this relatively short period of time (Strauss et al. 2006), including adaptations, in less than 65 years, by native Australian snakes in response to toxic Cane

Toads (*Rhinella marina*, Phillips and Shine 2004; Phillips and Shine 2006). We predicted, if snakes were responding to escape the trap, fitness costs associated with consumption of Cuban Treefrogs would be reduced in snakes from the invaded region relative to non-invaded regions.

METHODS

Study Species

Common Gartersnakes are a well-studied and widespread snake species occupying a variety of mesic habitats throughout most of the eastern USA (Ernst and Ernst 2003). As a group, gartersnakes are dietary generalists, consuming a variety of prey taxa including, invertebrates, fish, amphibians, mammals and birds (Rossman et al. 1996). The relative dietary prevalence of these taxonomic groups of prey varies among gartersnake species. However, amphibians represent a major dietary component (>10%) of most gartersnake species, including Common Gartersnakes (Rossman et al. 1996). Common Gartersnakes have been observed to consume Cuban Treefrogs in Florida (Love 1995; Meshaka, 2001), and are known predators of several species of native treefrogs, including Green Treefrogs (*Hyla cinerea*, Ernst and Ernst 2003). Additionally, we considered gartersnakes to be an appropriate model to explore potential trap escape because gartersnakes have demonstrated both the ability to learn to avoid prey that induce illness following a single exposure (Burghardt et al. 1973) and have evolved resistance to other amphibian toxins (Licht and Low 1968; Brodie and Brodie Jr. 1990).

We collected Common Gartersnakes from two regions (Fig. 1), populations within the invaded range of Cuban Treefrogs in south peninsular Florida (Everglades National Park, Florida, USA), and populations from a non-invaded region (Alabama and Georgia, USA). Additional wild-caught snakes from the non-invaded region were obtained as donations from colleagues and a commercial supplier of snakes from Louisiana, USA. All snakes were transported to a live-animal facility at Auburn University within a week of capture and were housed singly in a rack system. All neonate snakes used in prey preference assays were born in captivity at Auburn University and housed individually prior to assays. Preference was assessed in neonate snakes from both invaded and non-invaded regions and adults from the invaded region. Investigation of snake growth included only adults from the two regions.

Cuban Treefrogs are large West Indian hylids, native to Cuba, the Bahamas, and the Cayman Islands. Invasive populations of Cuban Treefrogs are established in Florida (USA), mainland Costa Rica, and on the Caribbean islands of Puerto Rico, St. Croix, St. Thomas, St. Maarten and Anguilla (Meshaka 2001). Cuban Treefrogs were introduced to the Florida Keys sometime in the 1920's, arriving on the southern tip of peninsular Florida by the early 1950's (Meshaka 2001). Currently, Cuban Treefrogs are established throughout most of peninsular Florida. The species is abundant throughout its invasive range, occupying the same habitats as native treefrog species (Rice et al. 2011).

Cuban Treefrogs were collected from Everglades National Park, Florida, USA. Frogs were transported to Auburn University within a week of capture, and housed communally with size-matched conspecifics. Green Treefrogs, a large hylid species native to the southeastern United States, were used as a control in both prey preference and growth assays. Green Treefrogs were collected from Tuskegee National Forest, Alabama, USA, and housed under conditions identical to those of Cuban Treefrogs. Golden Shiners (*Notemigonus crysoleucas*), a small, native, freshwater fish species, were used as a secondary control in the growth assay to ensure that observed effects of diet restricted to Green Treefrogs were general to all non-toxic prey. Golden Shiners were purchased commercially within 4 hours of their use in assays.

Prey Preference

Tongue-flick assays measure interest in prey by taking advantage of observable behavioral responses (Cooper 1998). Lingual protrusion serves to transfer chemicals from the environment to the vomeronasal system, resulting in a positive relationship between tongue-flick rates and snake interest in potential food stimuli (Halpern and Frumin 1979; Halpern 1992). Much of the early work to verify tongue-flick assays as a reliable measure of diet preference used snakes of the genus *Thamnophis*, and tongue-flick rates of these snakes are widely accepted to reflect prey preference (Burghardt 1967; 1969; 1970; Arnold 1981). We assessed prey preference by measuring the chemosensory responses of neonate (digestively-naïve) and wild-caught adult (digestively-experienced) Common Gartersnakes. Neonates were assayed to eliminate potential phenotypic effects of previous prey exposure (Arnold 1978; Cooper et al. 2000; Waters and Burghardt 2013) allowing for the examination of possible evolutionary differences; however, we were unable to control for potential maternal effects. Adult snakes from the invaded region were assayed to explore possible experienced-based avoidance of Cuban Treefrogs.

We assayed snakes from invaded (neonates, n = 28, 2 litters; adults, n = 22) and non-invaded regions (neonates, n = 36, 3 litters). Snakes were presented with four chemical stimuli in a repeated-measures, randomized design, following Cooper (1998). Chemical stimuli included scents extracted from Cuban Treefrogs, Green Treefrogs, a biologically-irrelevant but pungent stimulus (Brut Aftershave, 1:10 dilution, Cooper et al. 2003) and an odorless control (deionized water). Treefrog prey scents were prepared by dipping a cotton-tipped 15 cm wooden applicator in deionized water and then rolling the cotton tip over the dorsal and ventral surfaces of live frogs. Cotton tips were dipped directly into solutions for control stimuli. Trials were initiated by removing all cage materials from each snake’s enclosure. Once snakes ceased moving and did not exhibit a defensive posture, the cotton applicator tip was positioned approximately one centimeter directly in front of a snake’s snout. Number of tongue flicks (TF) directed at a stimulus was recorded for 60 s. If the stimulus was bitten, the trial was terminated and the latency to bite (LA, in s) was recorded because it is assumed that bites indicate a stronger predatory response than tongue flicks and that there is an inverse relationship between response strength and bite latency (Burghardt 1970; Cooper and Burghardt 1990). A Tongue Flick Attack Score (TFAS) for each snake to each stimulus was determined by the equation: $TFAS = TF + (60 - \mathrm{LA})$. Proximity of observer to snakes was decreased by affixing the cotton-tipped applicator to a 10 ft telescoping graphite fishing pole (Cabela’s, USA). Typically, each experimenter maintained a distance of approximately 2 m from each snake. If a snake responded defensively, the trial was terminated and attempted again after a 10- to 30-min interval. Snakes that repeatedly exhibited unresponsive behaviors (e.g. head-hiding), defensive behaviors (e.g. tail rattling, dorso-ventral flattening of the body, or defensive strikes), or otherwise failed to direct at least one tongue flick toward all stimuli were removed from the study (Cooper 1998). Final sample size of neonates from invaded and non-invaded regions was 15 and 27, respectively; final sample size of adults from the invaded region was 15.

Snake Growth

We used growth as a correlate of fitness because there is a significant positive relationship between snake body size and fecundity, measured as both clutch size and clutch mass (Iverson 1987; Seigel and Ford 1987). Specifically, female gartersnake body size is positively correlated with litter size (Fitch 1965; Stewart 1968; Gregory 1977; Siegel and Fitch 1985). Additionally, gartersnake mass is positively correlated with survival (Bronikowski 2000). Therefore, our measure of growth rate likely serves as a suitable proxy for fitness.

Wild-caught, adult Common Gartersnakes (n = 61) were assigned to one of three treatment diets; Cuban Treefrogs (invaded region, n = 12; non-invaded region, n = 9), Green Treefrogs (invaded region, n = 11; non-invaded region, n = 9), or Golden Shiners (invaded region, n = 7; non-invaded region, n = 13). Following a one-week acclimation period, snakes were fasted for 7 d and an initial weight was taken on a digital top-loading balance (± 0.1 g). Snakes were then fed a series of three, pre-weighed, live meals from their assigned treatment group, one meal every 5 days. Individual meals equaled ~15-20% of snake mass. Treefrogs were introduced to snake enclosures and allowed to move unrestrained, while fish were confined to a snake's water bowl. Following the third meal, snakes were weighed again after a 7 day fast to ensure the digestive tract had been fully evacuated.

Prey Value

We investigated potential differences of digestibility among prey species and frog body composition between indigenous and nonindigenous prey species. To determine if ingestion of Cuban Treefrog toxins hindered snake digestion, we compared the prevalence of prey regurgitation or elimination of undigested body parts relative to snake diet. Any potential differences in snake growth could be due to factors other than skin toxins. To eliminate potential confounding differences in water retention and/or skeletal mass, we recorded the wet mass of 12 Cuban Treefrogs and 11 Green Treefrogs. The treefrogs were then immediately euthanized and dried in an oven at 70°C (Thelco GCA Precision Scientific, Model 18) for 48 h until they maintained a constant weight. Dry treefrogs were then skeletonized by partial rehydration in a water bath (24 h) followed by placement within a dermestid beetle colony for 48 h.

Statistical Analysis

For prey preference assays, TFAS data were log-transformed to meet assumptions of normal distribution of residuals and homoscedasticity. We fit data to a linear mixed-effects model with the fixed effect of treatment and random effect of individual included in the model (R, version 3.2.3, 2015). Analysis of neonate snakes included random effects of individual nested within litter.

For the growth assay, we compared mass increases as a factor of prey species with proportional change in snake mass as the dependent variable and relative prey mass as a covariate. Proportional change in snake mass was determined by dividing final snake mass by initial snake mass and relative prey mass was calculated by dividing the total mass of the three meals by initial snake mass. Initially, diet treatments were compared using an ANCOVA. However, relative prey mass was not a significant covariate of growth ($F_{5, 55} = 5.309$, P = 0.065). Therefore, we used ANOVA with a Tukey's post-hoc test to compare differences among prey treatment groups.

The magnitude of the effect of Cuban Treefrogs on snake growth may vary between invaded and non-invaded regions because snakes in co-occurrence with these frogs may have evolved resistance to their toxins. To test potential differences in the relationship between snake growth and region, we used a one-tailed t-test to compare growth of snakes from the two regions when fed Cuban Treefrogs.

During the growth assay, we observed partial regurgitation or elimination of undigested portions of Cuban Treefrogs. To determine if ingestion of Cuban Treefrog toxins hindered snake digestion we analyzed the proportion of snakes that completely digested prey in the final feeding of each diet treatment to those that did not digest prey completely. We used a two-sided Fisher's exact test to compare proportions.

To investigate potential differences in frog body composition, we compared the relative contribution of water and skeletal mass to overall mass in Cuban Treefrogs and Green Treefrogs. To examine water mass, we used an ANCOVA, with species as a fixed factor, dry mass as a dependent variable, and wet mass as a covariate. Additionally, to compare the relative contribution of skeletal mass, we used an ANCOVA with species as a fixed factor, overall dry mass as the dependent variable, and skeletal dry mass as a covariate. We assumed nutrient content of frog species was not a significant factor in snake growth rates because hylid frogs exhibit only minor interspecific variation of whole-body crude protein content (Boyd and Goodyear 1971).

RESULTS

Prey Preference

Linear mixed-effects models revealed a significant difference between treatments in the TFAS assay in all snake groups, including neonates from invaded regions ($F_{3,\,42}$ = 14.7, P < 0.0001; Fig. 2-2A), neonates from non-invaded regions ($F_{3,\,78}$ = 33.2, P < 0.0001; Fig. 2 - 2 B), and wild-caught adults from invaded regions ($F_{3,\,42}$ = 13.8, P < 0.0001; Fig. 2-2C). Reduced interest in the odorless control demonstrates snakes were not simply tongue-flicking in response to the presence of the cotton-tipped swabs. Similarly, a lack of interest towards the pungent control indicates response was not based on scent strength, but rather its biological relevance (Cooper et al. 2003). All three Common Gartersnake experimental groups failed to exhibit a significant preference for the scent of Green Treefrogs over Cuban Treefrogs, supporting the hypothesis that Cuban Treefrogs represent an evolutionary trap for Common Gartersnakes. Specifically, in neonate snakes, Green Treefrog scent resulted in an increase of 0.1 (±3.7, 95% CI, P = 0.99; invaded regions) and 1.6 (±1.6, 95% CI, P = 0.33; non-invaded regions) tongue flicks relative to Cuban Treefrogs. Prey preference did not vary among litters, or region, demonstrating a generalized lack of preference by digestively-naïve snakes. In adult snakes from invaded regions, Green Treefrogs scent resulted in a decrease of 8.1 (±5.3, 95% CI, P = 0.14) tongue flicks relative to Cuban Treefrogs. This suggests co-occurrence with Cuban Treefrogs has not resulted in learned aversion.

Green Treefrog scent elicited bites in one of 15 trials of neonates from invaded regions, two of 27 trials of neonates from non-invaded regions, and six of 15 trials of adults from invaded regions. Cuban Treefrog prey scent elicited bites in four of 15 trials of neonates from invaded regions, 10 of 15 trials of adults from invaded regions, and one of 27 trials of neonates from non-invaded regions. Neither control stimulus elicited a predatory bite, providing additional evidence that snakes differentiated between controls and treefrog stimuli and did not recognize controls as potential prey items.

Snake Growth

Snakes consumed all offered prey items from each of the three diet treatment groups. A majority of the Common Gartersnakes fed Golden Shiners or Green Treefrogs gained mass during the growth assay; exceptions included one of 20 snakes (5%) fed Golden Shiners and three of 20 snakes (15%) fed Green Treefrogs. However, eight of 21 snakes (38%) fed Cuban Treefrogs lost mass during the growth assay. An ANOVA revealed significant growth differences among diet treatments ($F_{2,\,58} = 8.6$, $P < 0.01$; Fig. 2-3) with snakes in the Cuban Treefrog diet treatment gaining less than half the mass of the other two treatment groups. A Tukey’s post-hoc analysis revealed significant differences between the pairwise comparisons of Cuban Treefrogs and Green Treefrogs ($P < 0.01$) and Cuban Treefrogs and Golden Shiners ($P < 0.01$). There was no significant difference between Green Treefrogs and Golden Shiners ($P = 0.63$). These data support the hypothesis that Cuban Treefrogs represent an evolutionary trap for Common

Gartersnakes. A t-test did not reveal significant differences in growth between snakes fed Cuban Treefrogs from invaded and non-invaded regions ($t_{16} = 0.95$, $P < 0.35$; Fig. 2-4). Additionally, consumption of the first two Cuban Treefrog meals did not deter consumption of the final meal. Our findings fail to support the idea that Common Gartersnakes have evolved an escape mechanism from this evolutionary trap.

Prey Value

A two-sided Fisher's exact test revealed a significant difference in digestibility of the final meal between diet treatment groups (Table 1, $P < 0.01$). Consumption of Cuban Treefrogs resulted in incomplete digestion, regurgitation or elimination of portions of undigested prey, in 28.5% of all trials. Incomplete digestions did not occur in snakes fed native prey. An ANCOVA did not reveal a significant difference of dry mass, adjusted for wet mass, between frog species ($F_{3,18} = 22.0$, $P = 0.35$; Fig. 2-5). Similarly, an ANCOVA did not reveal skeletal mass, adjusted for the effect of dry mass, to differ between frog species ($F_{3,18} = 18.6$, $P = 0.17$; Fig. 2-6). These data do not support the idea that decreased weight gain in Common Gartersnakes fed Cuban Treefrogs was due to factors other than skin toxins.

DISCUSSION

This study demonstrates how Cuban Treefrogs likely function as an evolutionary trap for a native predator. Common Gartersnakes exhibited equal preference toward the cues from native Green Treefrogs and nonindigenous Cuban Treefrogs. Snakes ate all offered prey items during the feeding trial, including Cuban Treefrogs; however, consumption of Cuban Treefrogs resulted in a 50% reduction in growth rate, a variable correlated with fitness (Stewart 1968; Seigel and Ford 1987). Interestingly, we did not detect a difference in growth or preference between snakes from invaded and non-invaded Cuban Treefrog regions indicating that snakes co-occurring with Cuban Treefrogs lack evolutionary or learned responses that would allow escape from the trap.

We proposed three possible explanations for why snakes fed Cuban Treefrogs gained less mass. First, we explored possible variation between treefrog species in the amount of water contributing to body mass, which could bias our values of snake mass gain. However, we found no differences in the proportion of body mass attributable to water mass between the two treefrogs. Secondly, because Cuban Treefrogs possess a larger and wider head in relation to snout-vent-length, we hypothesized that a greater proportion of skeletal mass might be contributing to overall mass in this species as compared to Green Treefrogs. Bones have little caloric value; therefore, differences in the skeletal contribution to overall mass could also bias the amount of prey mass fed to snakes. However, we did not find a significant difference in the contribution of skeletal mass to overall dry mass of Cuban Treefrogs and Green Treefrogs. Finally, the possibility that Cuban Treefrog toxins reduced digestion, and thus growth, was supported by observations of fecal discharge. Specifically, snakes fed Cuban Treefrogs often had abnormally dark, oily feces that were typically spread across the enclosures. Additionally, following the final meal in the growth assay, approximately 30% of snakes either regurgitated or eliminated undigested portions of Cuban Treefrogs. Regurgitation or passage of undigested portions of prey was not observed in either of the native diet treatments. Evidence of incomplete digestion of Cuban Treefrogs suggests their toxins may cause an inflammation of the lining of the stomach and intestines in snakes. Inflammation or irritation of the gut may result in the passage of food items prior to breakdown by enzymatic activity and absorption by intestinal villi, as evidenced by the passage of undigested portions of prey. Irregular feces were observed intermittently in the Cuban Treefrog diet treatment, although the expulsion of undigested portions only occurred after the final feeding. These data suggest that toxins may have a cumulative effect. Likely, either repeated consumption of the toxin allows for an accumulation within snakes, or the digestive system becomes increasingly sensitive to the toxins.

The true fitness cost to Common Gartersnakes in the invaded range may depend on abundance of Cuban Treefrogs relative to native prey. Gartersnakes exhibited equal preference towards native and nonindigenous prey, suggesting they would consume Cuban Treefrogs in accordance to their encounter rate. Cuban Treefrogs often occur in dense populations, however their presence does not appear to be spatially homogeneous at a landscape level. For example, Waddle and others (2010) surveyed 107 sites in southern Florida and found Cuban Treefrogs to be present at only 36, fewer than native treefrogs (Green Treefrogs or Squirrel Treefrogs (*Hyla squirrela*); 52 and 77 sites respectively). However, abundance of native treefrog species was inversely correlated with Cuban Treefrog presence (Waddle et al. 2010; Rice et al. 2011). Thus, in patches where Cuban Treefrogs are present, snakes are predicted to experience increased encounter rates with Cuban Treefrogs compared to native treefrogs. The geographic mosaic hypothesis suggests that coevolution occurs at the population level and that coevolved traits may be less likely if there is gene flow between populations experiencing different selection pressures (Thompson 1997, 1999). Spatial variation of Cuban Treefrog presence at the landscape level may fit patterns described by the geographic mosaic hypothesis, including coevolutionary hot spots (Cuban Treefrogs are abundant) and cold spots (Cuban Treefrogs are absent). Gene flow between snake populations within the invaded range may diminish evolution of trap escape mechanisms. Because rates of gene flow and selection are unknown for this system, we can only suggest that heterogeneity of Cuban Treefrog distribution may explain our failure to detect any evidence of trap escape despite an ability of gartersnakes to learn to avoid other dangerous prey or evolve resistance to toxins (Licht and Low 1968; Burghardt et al. 1973; Brodie and Brodie Jr. 1990).

Nonindigenous species are recognized as an important cause of evolutionary trap formation (Schlaepfer et al. 2005; Robertson et al. 2013). Despite this recognition, there has been limited effort to identify characteristics of introduced species that might increase the likelihood of evolutionary trap formation. Herein, we propose that a maladaptive response of Common Gartersnakes to Cuban Treefrogs may fit a previously undescribed, yet common, pattern of evolutionary trap formation resulting from introduction of species with novel chemical defenses. Nonindigenous species protected by exotic toxins likely represent dangerous diet items to generalist consumers lacking a shared evolutionary history (Cornell and Hawkins 2003). The assumption that novel allelopathic chemicals provide a competitive advantage for nonindigenous plant species gave rise to the novel weapons hypothesis (NWH), which predicts that nonindigenous plant species will have increased invasion success if they possess novel secondary metabolites (Callaway and Aschehoug 2000). The NWH has been expanded to include novel chemical defenses against naïve or generalist predators (Schaffner et al. 2011). Accordingly, the opportunity for trap formation should be greater because there is an increased probability of fitness costs if these chemically-defended species are consumed and if there is limited time for consumers to evolve a response. To our knowledge, this phenomenon has not been directly tested; however, examination of exotic plant literature provides evidence that novel chemical defenses can disproportionately impact naïve consumers. For example, plants with potent secondary metabolites that are unique or underrepresented in the introduced range have repeatedly been found to be more likely to become invasive because they suffer reduced damage from herbivores (Cappuccino and Carpenter 2005; Carpenter and Cappuccino 2005; Cappuccino and Arnason 2006; Jogesh et al. 2008). However, because the objective of these studies is to measure overall levels of herbivory and not to document effects on individual native herbivores, it is not clear whether herbivory is reduced because secondary plant metabolites dissuade attack by native herbivores or because these metabolites reduce the ability of native herbivores to process the plant material.

Genetically Modified Plants (GMPs) offer an interesting corollary and provide circumstantial evidence that naïve herbivores will continually feed on biocidal plants. Plants modified to express insecticidal proteins from *Bacillus thuringiensis* (Bt) have proven effective in reducing plant damage from insect herbivores (Betz et al. 2000; Shelton et al. 2002; O’Callaghan et al. 2005). Rates of herbivory are not reduced because the plants are avoided by insects; rather, ingestion of Bt proteins kill attackers before they can inflict substantial damage (Koziel et al. 1993; Yu et al. 1997). It also appears that insects continue to attack GMPs, despite biocidal effects, as evidenced by evolution of Bt-resistant insects and a need to plant refuge plots of non-transgenic crops to reduce selection pressure favoring insect resistance (Tabashnik 1994; Frutos et al. 1999; Huang et al. 1999). Thus, insecticidal GMPs appear to form evolutionary traps for herbivorous insects, and we suspect introduced plants with novel toxins similarly trap native herbivores.

We suggest predators encountering novel animal toxins may fit a pattern similar to herbivore intolerance for unique plant phytochemicals. The greatest support for this argument can be found in the Cane Toad invasion of Australia. Toads are not native to Australia and, as a consequence, native predators failed to cope with toad toxins. A taxonomically-diverse array of Australian predators consumed toads, presumably because of a resemblance to native frog species, and were frequently poisoned. Mass mortality events and population declines attributed to Cane Toads have been documented in turtles, freshwater crocodiles, lizards, snakes, and marsupials (reviewed in Shine 2010). Cane Toads in Australia represent a single example of an introduced animal with novel toxins. However, the widespread and ecosystem-level effects of this introduction provide a cautionary tale.

It is unclear whether Cuban Treefrogs negatively impact native predators other than Common Gartersnakes. However, it would be surprising to find that such effects were restricted to a single native predator. Amphibians play an important role in the ecosystem as prey species because they account for a large amount of available prey biomass, owing in part to efficient conversion of ingested energy to biomass (Pough 1980), and an ability to transfer energy from aquatic to terrestrial systems (Gibbons et al. 2006). High population densities of Cuban Treefrogs, where they are present, coupled with a suppression of native anuran species (Rice et al. 2011), likely act to increase the probability of encounters with native predators. As in the Cane Toad example, a variety of native predators have been documented to consume Cuban Treefrogs. However, unlike Cane Toads, we do not have a good understanding of how Cuban Treefrogs may be impacting the suite of potential native predators likely to incorporate them into their diet.

Invasive species are currently recognized as the second greatest threat to biodiversity after habitat destruction (Wilcove et al. 1998) and have been implicated as a driver of evolutionary trap formation (Schlaepfer et al. 2005). The data presented here indicate the maladaptive response of a native predator to a nonindigenous, toxic prey resulted in an evolutionary trap. We suggest that our results provide support for the idea that unique chemical weapons of invasive species may be engines for evolutionary trap formation. Additionally, we highlight the threat of unique toxins, even if they have sublethal effects, and caution that greater scrutiny should be applied in nonnative risk assessments when evaluating species possessing such defenses.

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LITERATURE CITED

Antczak M, Hromada M, Tryjanowski P (2005) Frogs and toads in the food of the Great Grey Shrike (*lanius excubitor*): larders and skinning as two ways to consume dangerous prey. Animal biology-leiden 55:227–233

Arnold SJ (1978) Some effects of early experience on feeding responses in the Common Garter Snake, *Thamnophis sirtalis*. Anim Behav 26:455–462

Arnold SJ (1981) Behavioral variation in natural populations. I. phenotypic, genetic and environmental correlations between chemoreceptive responses to prey in the garter snake, *Thamnophis elegans*. Evolution 35:489–509

Beckmann C, Shine R (2011) Toad's tongue for breakfast: exploitation of a novel prey type, the invasive Cane Toad, by scavenging raptors in tropical Australia. Biol Invasions 13:1447–1455

Betz FS, Hammond BG, Fuchs RL (2000) Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Regul Toxicol Pharm 32:156–173

Bose KS, Sarma RH (1975) Delineation of the intimate details of the backbone conformation of pyridine nucleotide coenzymes in aqueous solution. Biochem Biophys Res Commun 66:1173–1179

Boyd CE, Goodyear CP (1971) The protein content of some common reptiles and amphibians. Herpetologica 27:317–320

Brodie III ED, Brodie ED (1990) Tetrodotoxin resistance in garter snakes: an evolutionary response of predators to dangerous prey. Evolution 44:651–659

Brodie III ED, Brodie ED (1999) Predator-prey arms races: Asymmetrical selection on predators and prey may be reduced when prey are dangerous. Bioscience 49:557–568

Bronikowski AM (2000) Experimental evidence for the adaptive evolution of growth rate in the garter snake *Thamnophis elegans*. Evolution 54:1760–1767

Burghardt GM (1967) Chemical-cue preferences of inexperienced snakes: comparative aspects. Science 157:718–721

Burghardt GM (1969) Comparative Prey-Attack Studies in Newborn Snakes of the Genus *Thamnophis*. Behaviour 33:77–113

Burghardt GM (1970) Chemical perception in Reptiles. In: Johnston J, Moulton D, Turk A (eds), Advances in Chemoreception. Vol. I. Communication by Chemical Signals. Appleton Century Crofts, New York, pp 241-308

Burghardt GM, Wilcoxon HC, Czaplicki JA (1973) Conditioning in garter snakes: aversion to palatable prey induced by delayed illness. Anim Learn Behav 1:317–320

Callaway RM (2000) Invasive plants versus their new and old neighbors: a mechanism for exotic invasion. Science 290:521–523

Cappuccino N, Arnason JT (2006) Novel chemistry of invasive exotic plants. Biol Letters 2:189–193.

Cappuccino N, Carpenter D (2005) Invasive exotic plants suffer less herbivory than non-invasive exotic plants. Biol Letters 1:435–438

Carpenter D, Cappuccino N (2005) Herbivory, time since introduction and the invasiveness of exotic plants. J Ecol 93:315–321

Cooper Jr WE (1998) Evaluation of swab and related tests as a bioassay for assessing responses by squamate reptiles to chemical stimuli. J Chem Ecol 24:841–866

Cooper Jr WE, Burghardt GM (1990) A comparative analysis of scoring methods for chemical discrimination of prey by squamate reptiles. J Chem Ecol 16:45–65

Cooper Jr WE, Burghardt GM, Brown WS (2000) Behavioural responses by hatchling racers (*Coluber constrictor*) from two geographically distinct populations to chemical stimuli from potential prey and predators. Amphibia-Reptilia 21:103–115

Cooper Jr WE, Pérez-Mellado V, Vitt LJ, Budzynski B (2003) Cologne as a pungency control in tests of chemical discrimination: effects of concentration, brand, and simultaneous and sequential presentation. J Ethol 21:101–106

Cornell HV, Hawkins BA (2003) Herbivore responses to plant secondary compounds: a test of phytochemical coevolution theory. Am Nat 161:507–522

de Queiroz A, Lawson R, Lemos-Espinalet JA (2002) Phylogenetic relationships of North American garter snakes (*Thamnophis*) based on four mitochondrial genes: how much DNA sequence is enough? Mol Phylogenet Evol 22:315–329

Delfino G, Brizzi R, Nosi D, Terreni A (2002) Serous cutaneous glands in new world hylid frogs: an ultrastructural study on skin poisons confirms phylogenetic relationships between *Osteopilus septentrionalis* and *phrynohyas venulosa*. J Morphol 253:176–186

Ernst CH, Ernst EM (2003) Snakes of the United States and Canada. Smithsonian Books, Washington

Faivovich, J, Haddad CF, Garcia PC, Frost DR, Campbell JA, Wheeler WC (2005) Systematic review of the frog family Hylidae, with special reference to hylinae: phylogenetic analysis and taxonomic revision. B Am Mus Nat Hist 294:1–240

Fitch H (1965) An ecological study of the garter snake, *Thamnophis sirtalis*. Univ Kansas Mus Nat His 15:493–564

Frost DR et al (2006) The amphibian tree of life. B. Am Mus Nat Hist 297:1–291

Frutos R, Rang C, Royer M (1999) Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. Crit Rev Biotechnol 19:227–276

Gibbons JW et al (2006) Remarkable amphibian biomass and abundance in an isolated wetland: implications for wetland conservation. Conserv Biol 20:1457–1465

Greene HW (1997) Snakes: the evolution of mystery in nature. University of California Press, Berkeley

Gregory PT (1977) Life-history parameters of the Red-Sided Garter Snake (*Thamnophis sirtalis parietalis*) in an extreme environment, the interlake region of Manitoba. National Museums of Canada

Halpern M, Frumin N (1979) Roles of the vomeronasal and olfactory systems in prey attack and feeding in adult garter snakes. Physiol Behav 22:1183–1189

Halpern M (1992) Nasal chemical senses in reptiles: structure and function. In: Gans C, Crews D (eds) Biology of the Reptilia, Vol. 18, Physiology E, Hormones, Brain, and Behavior. Univ. Chicago Press, Chicago, pp. 423–523

Hanifin CT, Brodie Jr ED, Brodie III ED (2008) Phenotypic mismatches reveal escape from arms-race coevolution. PLoS Biol 6:e60

Huang F (1999) Inheritance of resistance to *Bacillus thuringiensis* toxin (Dipel ES) in the European Corn Borer. Science 284:965–967

Iverson JB (1987) Patterns of relative fecundity in snakes. Florida Sci 50:223–233

Jogesh T, Carpenter D, Cappuccino N (2008) Herbivory on invasive exotic plants and their non-invasive relatives. Biol Invasions 10:797–804

Kiesecker JM, Blaustein AR (1997) Population differences in responses of Red-Legged Frogs (*Rana aurora*) to introduced bullfrogs. Ecology 78:1752–1760

Koivula T, Koivusalo M (1975) Partial purification and properties of a phenobarbital-induced aldehyde dehydrogenase of rat liver. Biochim Biophys Acta 410:1–11

Koziel MG et al (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Nat Biotech 11:194–200

Leary CJ, Razafindratsita VR (1998) Attempted predation on a hylid frog, *Phrynohyas venulosa*, by an indigo snake, *Drymarchon corais*, and the response of conspecific frogs to distress calls. Amphibia-Reptilia 18:442–446

Licht LE, Low B (1968) Cardiac response of snakes after ingestion of toad parotoid venom. Copeia 1968:547–551

Lizana M, Mellado VP (1990) Predation by the otter (*Lutra lutra*) of the toad of the Sierra de Gredos (*Bufo bufo gredosicola*). Donana: acta vertebrata 17:109–112

Love WB (1995) *Osteopilus septentrionalis* (Cuban Treefrog). Predation. Herpetol Rev 26:201–202

Macel M, de Vos RCH, Jansen JJ, van der Putten WH, van Dam NM (2014) Novel chemistry of invasive plants: exotic species have more unique metabolomic profiles than native congeners. Ecol Evo 4:2777–2786

Manzanilla J, La Marca E, Villareal O, Sanchez D (1998) *Phrynohyas venulosa* (Veined treefrog, "*Rana lechosa*"). Antipredator device. Herpetol Rev 29:39–40

Meshaka WE (2001) The Cuban treefrog in Florida: life history of a successful colonizing species. University Press of Florida, Gainesville

O'Callaghan M, Glare TR, Burgess EPJ, Malone LA (2005) Effects of plants genetically modified for insect resistance on nontarget organisms. Annu Rev Entomol 50:271–292

Phillips BL, Shine R (2004) Adapting to an invasive species: Toxic Cane Toads induce morphological change in Australian snakes. P Natl Acad Sci USA 101:17150–17155

Phillips BL, Shine R (2006) An invasive species induces rapid adaptive change in a native predator: Cane Toads and Black Snakes in Australia. P Roy Soc B-Biol Sci 273:1545–1550

Pough FH (1980) The advantages of ectothermy for tetrapods. Am Nat 115:92–112

R Core Team (2016) R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Available online at: https://www.R-project.org/

Rice KG, Waddle JH, Miller MW, Crockett ME, Mazzotti FJ, Percival HF (2011) Recovery of native treefrogs after removal of nonindigenous Cuban Treefrogs, *Osteopilus septentrionalis*. Herpetologica 67:105–117

Robbins TR, Langkilde T (2012) The consequences of lifetime and evolutionary exposure to toxic prey: changes in avoidance behavior through ontogeny. J Evolution Biol 25:1937–1946

Robertson BA, Rehage JS, Sih A (2013) Ecological novelty and the emergence of evolutionary traps. Trends Ecol Evol 28:552–560

Rossman DA, Ford NB, Seigel RA (1996) The garter snakes: evolution and ecology. University of Oklahoma Press, Norman

Rowe AH, Rowe MP (2008) Physiological resistance of grasshopper mice (*Onychomys* spp.) to Arizona bark scorpion (*Centruroides exilicauda*) venom. Toxicon 52:597–605

Schaffner U, Ridenour WM, Wolf VC, Bassett T, Müller C, Müller-Schärer H, Sutherland S, Lortie CJ, Callaway RM (2011) Plant invasions, generalist herbivores, and novel defense weapons. Ecology 92:829–835

Schlaepfer MA, Runge MC, Sherman PW (2002) Ecological and evolutionary traps. Trends Ecol Evol 17:474–480

Schlaepfer MA, Sherman PW, Blossey B, Runge MC (2005) Introduced species as evolutionary traps: Introduced species as evolutionary traps. Ecol Letters 8:241–246

Seigel RA, Fitch HS (1985) Annual variation in reproduction in snakes in a fluctuating environment. J Anim Ecol 54:497–505

Seigel RA, Ford NB (1987) Reproductive ecology. In: Seigel JT, Collins JT, Novak SS (eds) Snakes: ecology and evolutionary biology. Macmillan Publishing Company, New York, pp 210-252

Shelton AM, Zhao JZ, Roush RT (2002) Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu Rev Entomol 47:845–881

Shine R (2010). The ecological impact of invasive Cane Toads (*bufo marinus*) in Australia. Q Rev Biol 85:253–291

Stewart GR (1968) Some observations on the natural history of two Oregon garter snakes (genus *Thamnophis*). J Herpetol 2:71–86.

Strauss SY, Lau JA, Carroll SP (2006) Evolutionary responses of natives to introduced species: what do introductions tell us about natural communities? Ecol Letters 9:357–374

Tabashnik BE (1994) Evolution of resistance to *Bacillus thuringiensis*. Annu Rev Entomol 39:47–79

Thompson JN (1997) Evaluating the dynamics of coevolution among geographically structured populations. Ecology 78:1619–1623

Thompson JN (1999) Specific hypotheses on the geographic mosaic of coevolution. Am Nat 153:S1–S14

Waddle JH, Dorazio RM, Walls SC, Rice KG, Beauchamp J, Schuman MJ, Mazzotti FJ (2010) A new parameterization for estimating co-occurrence of interacting species. Ecol Appl 20:1467–1475

Waters RM, Burghardt GM (2013) Prey availability influences the ontogeny and timing of chemoreception-based prey shifting in the Striped Crayfish Snake, *Regina alleni*. J Comp Psychol 127:49–55

Weldon AJ, Haddad NM (2005) The effects of patch shape on Indigo Buntings: evidence for an ecological trap. Ecology 86:1422–1431

Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E (1998) Quantifying threats to imperiled species in the United States. BioScience 48:607–615

Yu CG, Mullins MA, Warren GW, Koziel MG, Estruch JJ (1997) The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. Appl Environ Microb 63:532–536

Table 2 - 1.— Presence of complete digestion of prey after final experimental feeding by Common Gartersnakes as a factor of diet treatment. Diet treatments include Cuban Treefrogs (*Osteopilus septentrionalis*), Green Treefrogs (*Hyla cinerea*) and Golden Shiners (*Notemigonus crysoleucas*). Incomplete digestion is defined here as regurgitation or elimination of undigested portions of prey. Numbers are observed values, expected frequencies are in parentheses.

Prey Species	**Complete Digestion**		**Total**
	Yes	**No**	
Cuban Treefrogs	**15 (18.58)**	**6 (2.06)**	**21**
Green Treefrogs	**20 (17.66)**	**0 (1.96)**	**20**
Golden Shiners	**20 (17.66)**	**0 (1.96)**	**20**
Total	**54**	**6**	**61**

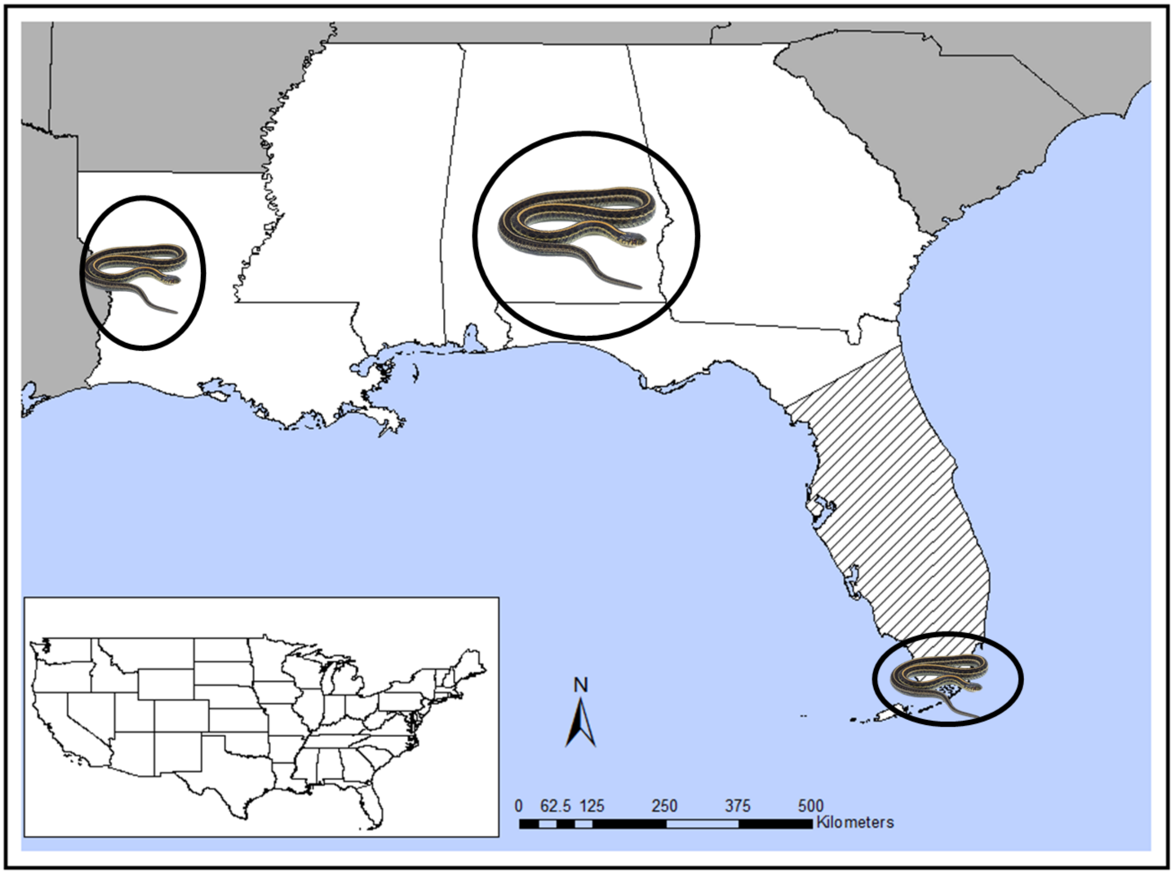
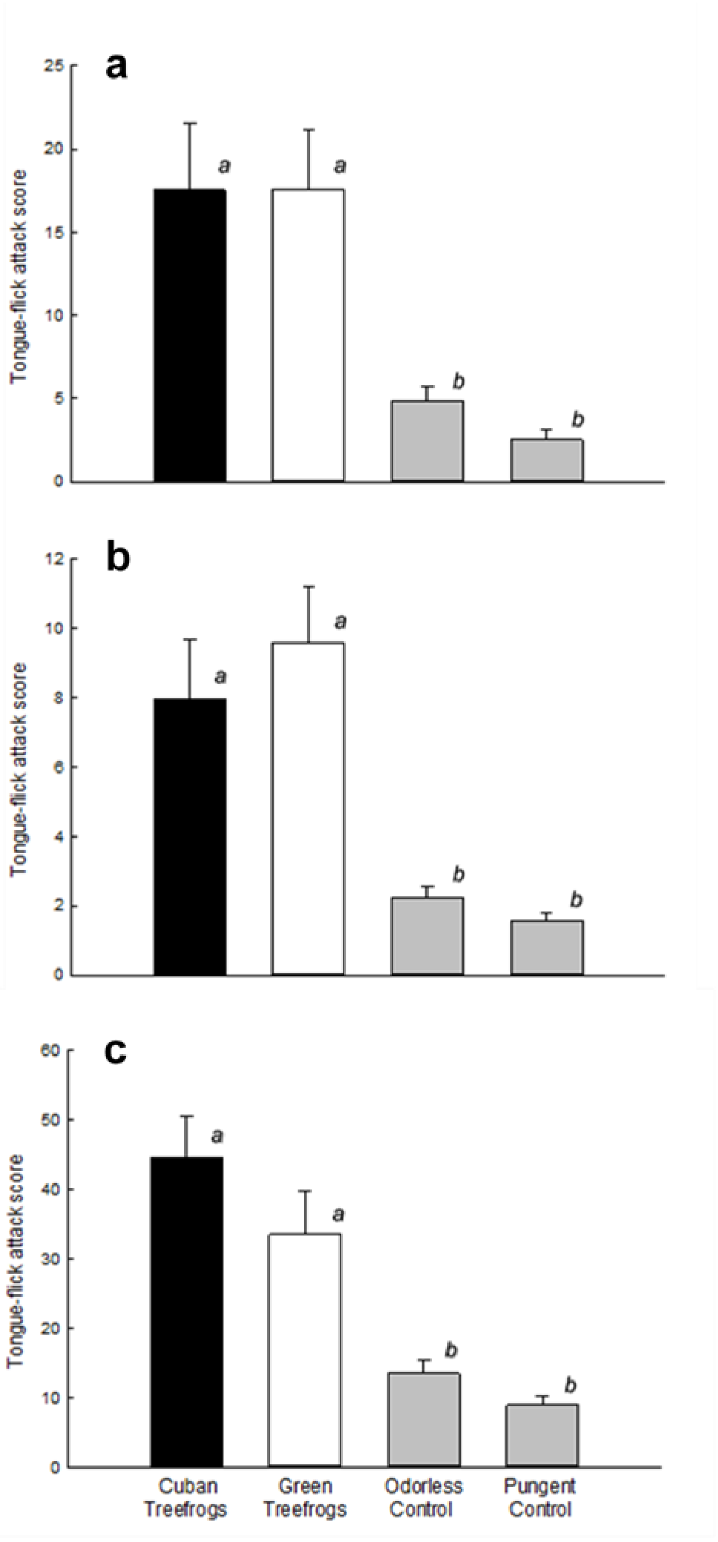


Figure 2 - 1.— Sampling areas in the southeastern United States. Lined portion of the state of Florida, USA, represents approximate invasive distribution of the Cuban Treefrog. Snakes inside black circles represent the three Common Gartersnake collection areas.

Figure 2 - 2.— Observed TFAS of Common Gartersnakes (*Thamnophis sirtalis*) in response to chemical cues of Cuban Treefrogs (*Osteopilus septentrionalis*), Green Treefrogs (*Hyla cinerea*), an odorless control (deionized water), and a pungent control (diluted cologne). None of the snake treatment groups exhibited a significantly different response between Green Treefrogs and Cuban Treefrogs, including (A) neonatal snakes from within invaded regions 0.1 tongue flicks (±3.7, 95% CI, P = 0.99), (B) neonates from non-invaded regions 1.6 tongue flicks (±1.6, 95% CI, P = 0.33), and (C) adults from within invaded regions 8.1 tongue flicks (±5.3, 95% CI, P = 0.14). Values are mean ± SE. Different letters represent a significant difference between treatment groups.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Green Treefrogs
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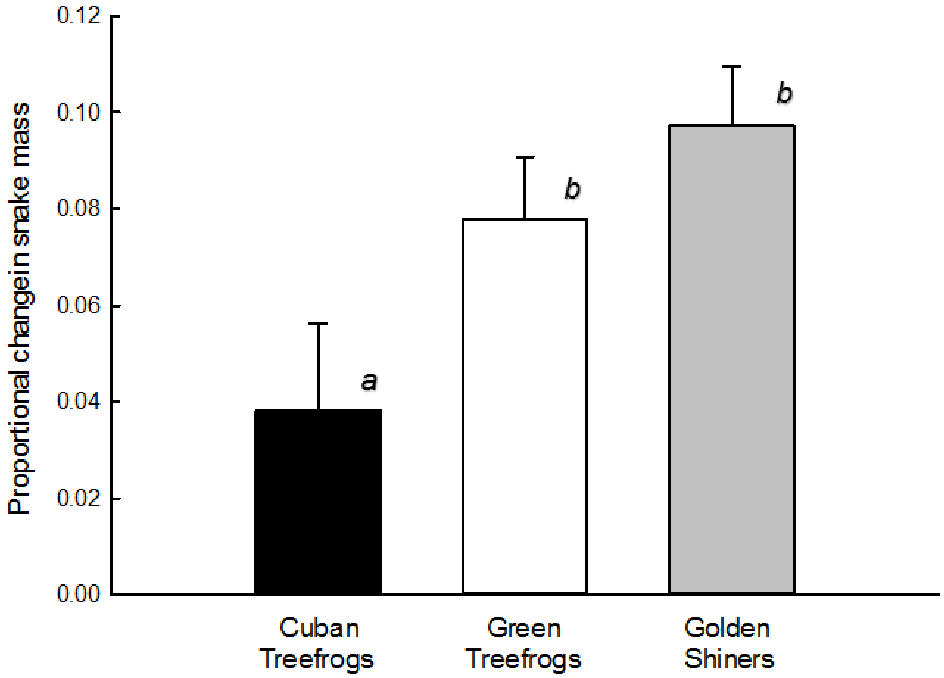

Figure 2 - 3.— Proportional change in body mass of Common Gartersnakes over a 17 d period during which snakes were fed one of three diet treatments. Diet treatments included Cuban Treefrogs (*Osteopilus septentrionalis*), Green Treefrogs (*Hyla cinerea*) or Golden Shiners (*Notemigonus crysoleucas*). Values are mean ± SE. Different letters represent a significant difference between treatment groups based on a Tukey post hoc test (Cuban Treefrogs–Green Treefrogs, P < 0.01, Cuban Treefrogs–Golden Shiners, P < 0.01).

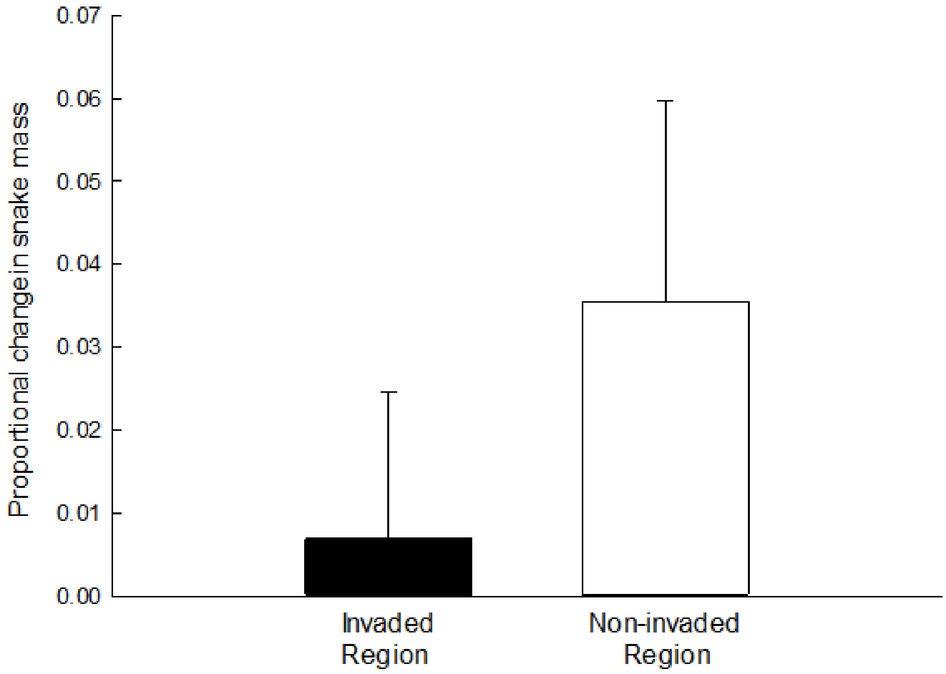

Figure 2 - 4.— Mean ± SE values of proportional change in body mass of Common Gartersnakes (n = 21) fed a diet of Cuban Treefrogs over a 17 d period. Snakes were either collected from regions within the invaded range of Cuban Treefrogs or from non-invaded regions. There was not a significant growth difference between regions.

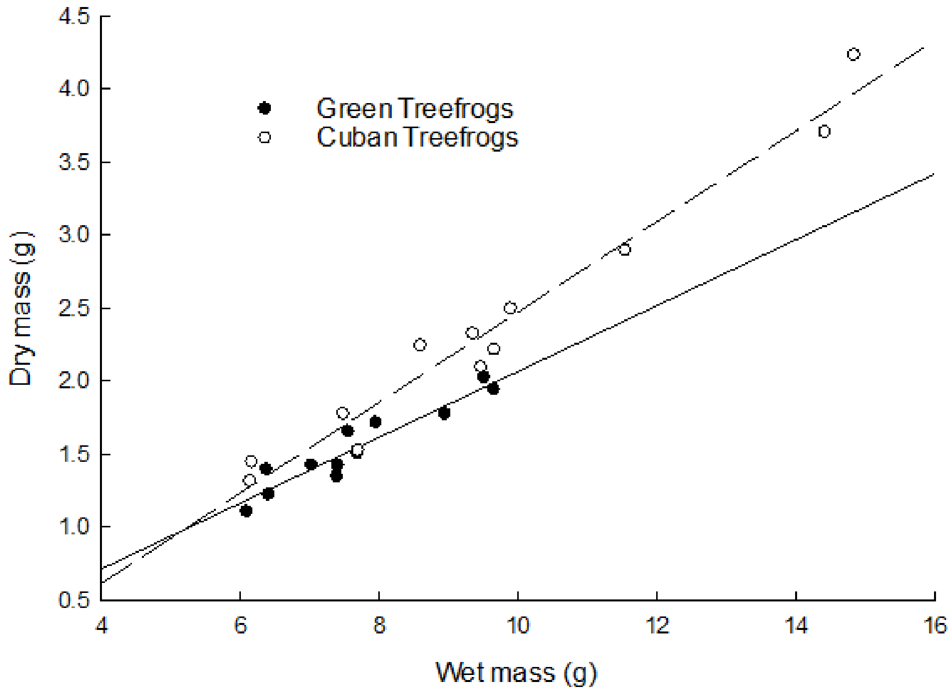

Figure 2 – 5.—Relationship of dry mass (g) as a factor of treefrog species with a covariate of wet mass (g).

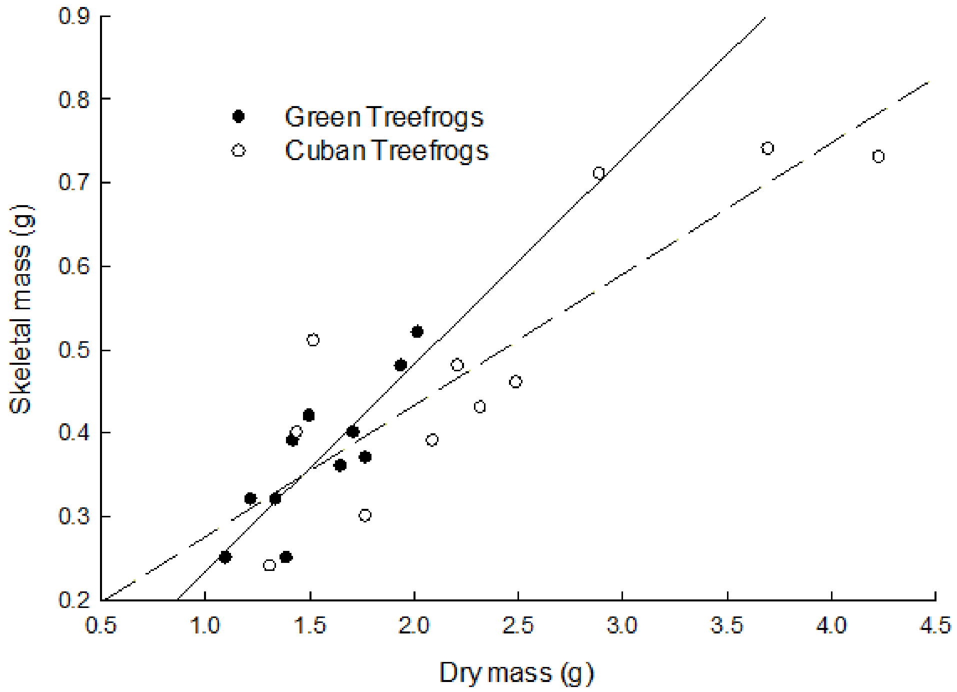

Figure 2 - 6.—Relationship of skeletal mass (g) as a factor of treefrog species with a covariate of dry mass (g).

CHAPTER III

INNATE IMMUNITY POSITIVELY COVARIES WITH TIME SINCE COLONIZATION IN CUBAN TREEFROGS

ABSTRACT

Immunological trade-offs provide the basis of many ideas seeking to explain the success of biological invasions. It has been suggested that invasive species at the leading-edge of expanding distributions should direct resources away from costly immune functions and allocate them to life-history traits associated with dispersal. We investigated differences in innate immunity of invasive Cuban Treefrogs in relation to time since colonization. We used two techniques to compare immune responses between a long-colonized region (core) and a recently colonized region (leading-edge). We compared metabolic responses of frogs injected with an endotoxin that induces an inflammatory response, lipopolysaccharide (LPS), to sham-injected controls. We detected a sex-related difference in mass-independent metabolism of sham-injected frogs, with males exhibiting a significantly higher metabolic rate ($F_{1, 21} = 29.02$, $P < 0.001$) than females. We observed significantly higher metabolic rates in both males ($P < 0.041$) and females ($P < 0.007$) from the core region when compared to control frogs. Conversely, metabolism of LPS injected frogs did not differ significantly from control frogs in males ($P = 0.195$) or females ($P = 0.132$) from the leading-edge region. We also measured bacterial killing ability of frog blood plasma and observed a significant increase of bactericidal ability of frogs from the core compared with the leading edge ($F_{1, 26}$ = 28.67, $P < 0.001$). Our results suggest Cuban Treefrogs at the leading edge of their expanding distribution have reduced innate immunocompetence. These findings support hypotheses predicting a relationship between invasion success and immunological trade-offs.

INTRODUCTION

Biological invasions are one of the greatest current threats to biodiversity (Wilcove et al., ’98; Butchart et al., 2010; Simberloff et al., 2013), and are costly to global economies (Pimentel et al. 2005). Despite the recognized consequences of bioinvasions, an understanding of how species successfully invade new areas, and specifically, why only a proportion of introduced species become invasive (i.e., widespread and locally dominant; Colautti and MacIsaac, 2004) remains elusive. Several broad ecological patterns are beginning to emerge concerning what characterizes successful establishment of non-indigenous species, including propagule pressure, climate matching, and high reproductive rates (Kolar and Lodge, 2001; Lockwood et al., 2005; Bomford et al., 2009). However, mechanisms that facilitate range expansion following colonization are not well understood (Kolar and Lodge, 2001). Only a small percentage of non-indigenous species that become established spread beyond the area of initial colonization (Williamson and Fitter, ’96), signifying a different suite of selective pressures acting on introduced species during colonization verses range expansion (stages III and IV; Colautti and MacIsaac, 2004). Introduced species are likely subjected to ecological constraints that differ from those present in their native range and populations undergoing range expansion have an added burden of increased physiological costs related to dispersal activities.

Increased invasiveness may be characterized by life-history trade-offs that allow exploitation of new ecological and environmental conditions. An ability to allocate finite resources to select traits in response to changing conditions can maximize fitness; however, increased investment in one trait is likely to be associated with decreased investment in a competing trait (Stearns, ’89; Zera and Harshman, 2001). Blossey and Notzold (’95) formulated the hypothesis of evolution of increased competitive ability (EICA) to explain, in part, the observation that plants tend to be more vigorous and grow taller in areas where they have been introduced compared with conspecifics in native areas. Predicated on optimal defense theory and assuming introduced species have escaped many natural enemies, EICA predicts that non-indigenous plants maximize fitness by reducing investment in herbivore defense and allocating greater resources towards growth or reproduction. The corollary for herbivore defense in animals is the immune system, and trade-offs between immune defense and competing life-history traits may enhance the invasiveness of non-indigenous species (Lee et al., 2005; White and Perkins, 2012; Bailly et al., 2016).

Hypotheses of immune-based trade-offs are driven, in part, by a growing recognition of a high cost of mounting an immune response, in terms of both increased energetic and nutritional expenditure (Demas et al., ’97; Martin et al., 2003; Demas, 2004; Klasing, 2004). Because immunity is costly, it represents a large reservoir of resources that can potentially be allocated to competing processes. Components of the vertebrate immune system can be broadly categorized as one of three interdependent branches, including innate, cell-mediated, and humoral responses (Janeway et al., 2005). These three types of immune responses are thought to differ in their relative costs (Janeway et al., 2005) and, therefore, differ in potential amount of resources that may be gained from reduced investment. Because innate and cell-mediated responses induce systemic inflammation, they are considered more energetically costly than humoral responses (Lee and Klasing, 2004). It is unclear whether immunological trade-offs result from a system-wide reduction of investment in immune function, or a selective decrease of specific branches of the immune system (Lee and Klasing, 2004). In either scenario, a downregulation of innate and/or cell-mediated responses may be expected. For example, an overall reduction of immune function would necessarily include a reduction of both branches. If only specific immune components are downregulated, innate and cell-mediated responses are the most likely to be reduced because these components have the potential to yield the greatest energetic benefit for alternative activities.

Reduced investment in immune function could decrease fitness by rendering an organism more vulnerable to infection; however, this may not accurately characterize interactions between non-indigenous species and pathogens. First, pathogens native to the introduced range may either be unable to produce disease or exhibit reduced virulence because they lack a co-evolutionary history with non-indigenous hosts (Rigaud and Moret, 2003; Kopp and Jokela, 2007). In this scenario, immune responses to native pathogens may provide little benefit to a non-indigenous host and be costly in terms of energy and nutrients (Bonneaud et al., 2003; Lee et al., 2005). Second, vigorous immune responses to novel pathogens can be immunopathic, resulting in damage to the non-indigenous host organism (Raberg et al., ’98; Mims et al., 2001). Immunopathology can occur when pathogens lack mechanisms to temper host immune responses due to an absence of a shared evolutionary history (Mansfield and Oliver, 2002). Unchecked immune responses can create a positive feedback loop resulting in continual escalation of the immune response, particularly systemic inflammation, potentially resulting in host tissue damage or mortality (Mims et al., 2001). Third, invading species often leave behind many co-evolved parasites and pathogens during the invasion process. Non-indigenous species are often seeded from a relatively small number of individuals, making it probable that only a subset of the total diversity of natural parasites and pathogens will be co-introduced (Torchin et al., 2003; Torchin and Lafferty, 2009). Pathogens that are co-introduced may not become established because either initial host abundances are too low to facilitate density-dependent transmission, or because an obligate intermediate host is absent in the invasive range (Torchin et al., 2003; White and Perkins, 2012). For example, Roche and colleagues (2010) reported lower parasite species richness and lower parasite abundance in an introduced cichlid compared with a native cichlid fish. Similarly, a recently-introduced population of goby had lower parasite species richness compared with two native fish species that occupy a similar ecological niche, although this difference was dampened in a longer-established population of goby (Gendron et al., 2012). Gendron and colleagues (2012) not only provide an example of enemy release, but also illustrate how a threat from parasites may positively correlate with time since colonization.

Immunological investigations of introduced species have used primarily one of two approaches, either biogeographical comparisons between native and introduced populations, or comparisons among introduced populations that differ in time since colonization (Cornet et al., 2016). There is broad overlap in what can be learned from each approach; however, comparisons based on time since colonization may be more applicable to examinations attempting to explain how trade-offs increase invasiveness. Time-since-colonization studies compare populations that differ in their likelihood to be undergoing dispersal activities related to range expansion, one of two attributes that define an invasive species (Colautti and MacIsaac, 2004). Activities related to dispersal, including increased locomotor demands and risk of starvation (Bonte et al., 2012), may, in part, trigger immunological trade-offs because exercise stress and food limitations are associated with reduced immune response (Glick et al., ’83; Alonso-Alvarez and Tella, 2001; Husak et al., 2016).

In this study, we investigated innate immune responses in relation to time since colonization in invasive Cuban Treefrogs (*Osteopilus septentrionalis*) in Florida, USA. The invasion chronology of the currently-expanding range of Cuban Treefrogs is well documented and we compared immune responses from regions representing two extremes in terms of time since colonization. We predicted that Cuban Treefrogs in recently colonized regions would exhibit reduced innate immune response compared with frogs from long-colonized regions.

METHODS

Study Species

Cuban Treefrogs are West Indian hylids exhibiting sexual size dimorphism, native to Cuba, the Bahamas, and the Cayman Islands. Invasive populations of Cuban Treefrogs are established in Florida (USA), mainland Costa Rica, and on the Caribbean islands of Puerto Rico, St. Croix, St. Thomas, St. Maarten and Anguilla (Schwartz and Henderson, 1991; Powell et al., 1992; Townsend et al., 2000; Meshaka, 2001; Savage, 2002). It is unclear when a viable population of Cuban Treefrogs first became established in the USA; however, the first individual was reported in the Florida Keys by the late 1920's and a reproductive population was documented on the southern tip of peninsular Florida in 1954 (Meshaka, 2001). Once established, Cuban Treefrogs spread northward via leading-edge dispersal to their current invasive distribution, encompassing most of peninsular Florida (Fig. 3-1). The current northern limit of their range extends from Jacksonville on the Atlantic coast, through Alachua county, to Cedar Key on the Gulf coast (McGarrity and Johnson, 2009; Rodder, 2009). We designated populations of Cuban Treefrogs occurring in southern Florida (Monroe, Miami-Dade, and Collier counties) as "core" populations because they have been established for > 60 years and are surrounded by areas already occupied by conspecifics. Populations occurring at the vanguard of range expansion (Alachua County, FL) are designated as "leading-edge" populations.

Bacterial Killing Assay

We assessed innate immune responses using a bacterial killing assay (BKA) that measures the ability of blood plasma complement to kill bacteria, *Escherichia coli*, in vitro. Blood samples were collected in heparinized capillary tubes via puncture of an ulnar vein or brachial artery within 10 min of capture. Blood samples were collected from frogs in the leading-edge region (N = 17) in October, 2016 and core region in November, 2016 (N = 13). Collection dates were staggered with sampling occurring earlier in the year in the leading-edge region to match seasonal temperature shifts such that daily high and low temperatures at the time of collection were similar in both regions (assessed from average monthly temperature data accessed at www.usclimatedata.com). Blood plasma was separated from whole blood via centrifugation within 1 h of collection. Plasma samples were stored in cryogenic tubes, initially in a liquid nitrogen dewar, and, subsequently, in a -80°C freezer. Bactericidal ability of plasma samples was assessed within two weeks of collection and samples were only thawed at the time of use to avoid sample degradation (Liebl and Martin, 2009). We diluted plasma to 1:10 in RPMI-1640 media (VWR, USA) and separately diluted *E. coli* bacteria to 1:200 in sterile phosphate buffered solution. A total of 140 µl of diluted plasma was mixed with 10 µl of diluted bacteria and incubated at 22°C for 1 h. Once incubated, two 50 µl samples of plasma and bacteria mixture for each frog were spread onto trypticase soy agar plates (BD Diagnostic systems, USA). Four control plates were prepared, each consisting of a mixture of 140 µl of media and 10 µl of diluted bacteria, but no plasma. All plates were incubated at 37°C for 12 h, after which bacterial colonies were counted and average number for each plasma sample was determined. We quantified percent bacteria killed according to the equation ((# of sample colonies / mean # of control colonies) * 100). We compared regional differences of bactericidal ability in a two-way ANOVA with sex and region as factors.

LPS Challenge

Metabolic response to a mitogen-induced immune challenge was measured to assess innate immune responses of Cuban Treefrogs in core and leading-edge regions. Frogs used in the in vivo immune challenge were collected in July and August, 2016 when daily high and low temperatures between the two regions are similar (www.usclimatedata.com). Treatment frogs were challenged with Lipopolysaccharide (LPS), an endotoxin that induces an innate immune response and associated systemic inflammation. Frogs were housed individually in plastic shoeboxes at an indoor facility at Auburn University and challenged following a 10 d acclimation period. Measurements of O_2 consumption and CO_2 production of frogs were obtained using closed-system respirometry. Individual frogs were weighed on an electronic balance to the nearest 0.01 gm, and body volume was determined by placing frogs in a 200 ml graduated cylinder and measuring water displacement. Frogs were injected with 2 µl per gm of body mass of either phosphate buffered solution (PBS; control treatment; core N = 12; leading-edge N = 13) or LPS (Sigma Aldrich, L2630; challenge treatment; core N = 15; leading-edge N = 13) at a concentration of 1mg/1ml of PBS. Following measurements, frogs were returned to their individual enclosures and placed in an environmental chamber set to 26°C with a 12:12 L:D photoperiod for a 24 h period prior to measurement of gas exchange. Frogs were fasted for two days prior to determination of metabolic rates to ensure frogs were in a post-absorptive state. Measurements of gas exchange were collected 24 h post injection in the afternoon, the non-activity period of Cuban Treefrogs. Each frog was placed in a 250 ml mason jar modified to serve as a closed-system respirometer. Jar lids were fitted with two, two-way stopcocks. Frogs were placed in a jar with both stopcocks open and the lid secured with a metal band. A single stopcock was then connected to a manifold through which dry, CO_2-free air was force at a rate of ca. 230 ml/min. Air was allowed to flow through the jars for a duration of four min to purge the chambers of ambient air. Once purged, jars were sealed by closing both stopcocks and returned to the environmental chamber. Jars were incubated for approximately 10 min prior to initial measurement, and approximately 30 min prior to final measurement. Rates of gas exchange were calculated by subtracting initial from final measurements.

We used a Sable Systems TR-3 respirometry system (Sable Systems, Henderson, NV, USA) to determine O_2 depletion and CO_2 enrichment in the jar respirometers. Briefly, outside air was scrubbed of CO_2 and H_2O, drawn through a computer-controlled baselining system, a Li-Cor CO_2 and H_2O analyzer (LI-6262), a water vapor and CO_2 scrubber, a Sable Systems FC-1 Oxygen Analyzer, and a Sable Systems Mass Flow System (pump and mass flow controller) set to pull air through the system at 150 ml/min at STP. A 4 ml sample of air from each jar was collected in a 5 ml syringe tipped with a two-way stopcock. The stopcock on the syringe was attached to one of the stopcocks on the jar, both stopcocks were opened and the chamber of the syringe was flushed by drawing in and expelling 4 ml of air three times. A 4 ml sample was then collected from the jar, the stopcock on the syringe was replaced with a needle and a 2 ml sample was injected into an injection port installed anterior to the CO_2-H_2O analyzer, and passed through the system for analysis. Exact time of incubation (i.e., from sealing of the syringe to injection of the sample) was noted for each frog. Respirometry data were recorded and analyzed using Datacan V (Sable Systems, Henderson, NV, USA). Analysis was performed by converting data into units of ml h^{-1} and subsequently integrating peaks to calculate the total CO_2 production or O_2 consumption per syringe and finally converting the values to ml per jar. For specific equation and more details, see Lighton ('91).

We investigated sex-related differences of metabolism by regressing log body mass of all sham-injected control frogs from both regions ($N = 25$) versus log metabolic rate as a factor of sex in an ANCOVA. The regression lines of males and females were parallel ($F_{1, 21} = 0.293$, $P = 0.99$; Fig. 3-2), but intercepts differed significantly indicating a sex-related effect in metabolism of sham-injected control frogs such that males exhibited a significantly higher metabolic rate ($F_{1, 21} = 29.02$, $P < 0.001$; Fig. 3-2). Because intercepts differed, it was necessary to compare respiration separately for both sexes; however, sex-specific slopes generated in the ANCOVA were not significantly different (males, 0.808; females, 0.807), therefore we used a mass scaling coefficient of 0.81 for all frogs. We converted all metabolic rates, including those from challenged frogs, to mass-independent values by dividing the amount of oxygen consumed by incubation time and the body mass raised to the scaling coefficient. We pooled sex-specific, sham-injected controls from both regions because two-tailed t-tests did not reveal significant differences for males ($t_{12} = 0.21$, $P < 0.83$) or females ($t_{11} = 1.09$, $P < 0.30$). We examined sex-specific regional differences by comparing observed metabolic rates from leading-edge and core regions to pooled controls using one-tailed ANOVAs with a Tukey's post-hoc test to compare differences among treatment groups. Statistical analyses were completed using R version 3.2.4 (R Core Team, 2016).

RESULTS

Region of frog origin explained a significant amount of the variation in bacterial killing ability ($F_{1, 26} = 28.67$, $P < 0.001$; Fig. 3-3). Frogs at the leading edge exhibited a significant reduction in their ability to kill bacterial colonies compared with individuals from the core region. There was no main effect of sex ($F_{1, 26} = 0.02$, $P < 0.88$) and no significant sex*region interaction ($F_{1, 26} = 1.43$, $P < 0.24$).

In both sexes, we found a significant increase in metabolism of LPS injected frogs from the core region compared to sham-injected controls, but failed to detect a significant increase in frogs from the leading-edge region relative to controls. For female frogs, ANOVA revealed a significant difference in metabolic rates between treatment groups ($F_{2, 24} = 4.76$, $P < 0.001$; Fig. 3-4a). A Tukey's post-hoc analysis revealed a significant difference between the pairwise comparisons of sham-injected control females and females from the core region ($P < 0.007$), however, there was no significant difference between control frogs and frogs from the leading-edge ($P = 0.132$) or between core and leading-edge frogs ($P = 0.18$). Similarly, for male frogs, ANOVA revealed a significant difference in metabolic rates between treatment groups ($F_{2, 23} = 2.38$, $P = 0.048$; Fig. 3-4b). A Tukey's post-hoc analysis revealed a significant difference between the pairwise comparison of sham-injected control males and males from the core region ($P < 0.041$), however, there was no significant difference between control males and males from the leading-edge (P = 0.195) or between core and leading-edge males (P = 0.306).

DISCUSSION

The objective of this study was to investigate Cuban Treefrog immunocompetence in relation to time since colonization. Specifically, we used techniques providing a measure of two aspects of innate immunity, including response to an in vitro challenge (i.e. BKA) and an in vivo challenge (i.e. metabolic response to LPS). Our data support assumptions based on the ideas of EICA and related hypotheses predicting decreased immune investment at the leading-edge of range expansion. Further, we found that a pattern of greater immune response in long-colonized areas did not differ between reproductive and non-reproductive seasons.

The interdependence of components of the immune system adds complexity to the task of assigning energetic costs to specific types of responses. Energetic costs are incurred when mounting an immune response (Lochmiller and Deerenberg, 2000; Bonneaud et al., 2003), and innate responses are predicted to be more costly than other types of immune response, in part, because they induce systemic inflammation (Lee and Klasing 2004). Endotoxins, such as LPS, induce an inflammatory response by initiating a cytokine release that nonspecifically activates a variety of effector immune cells (Janeway et al., 2005). For ectothermic vertebrates, incapable of physiologically inducing a fever response, the price of mounting an immune response is primarily attributable to mobilizing and fueling the activity of immune cells (Klasing, 2004). As expected, Cuban

Treefrogs exhibited higher metabolism when injected with LPS in comparison to sham-injected controls. However, the response strength was greater in frogs from long-colonized regions, suggesting a more robust inflammatory response in that region. Our results are similar to those of Llewellyn et al. (2012) who reported reduced immune function in the offspring of Cane Toads (*Rhinella marina*) collected at the leading-edge of their expanding distribution in comparison to longer-established populations.

By examining innate immunity in terms of the metabolic response to an endotoxin, we captured the whole-animal response, including a potentially broad array of interacting immune functions. Additionally, we challenged live, wild-caught animals, which to our knowledge is the first time this approach has been used to investigate immune differences relative to time since colonization. We assayed wild-caught individuals to capture potential facultative responses to both biotic and abiotic factors that may have influenced immune function (Pedersen and Babayan, 2011). For example, we suspect Cuban Treefrogs at the leading-edge of their expanding range may move more often and/or for greater distances than conspecifics in longer-colonized areas, a result that is similar to the increased locomotor activity that has been reported for Cane Toads (Phillips et al., 2008; Alford et al., 2009). Components of the immune response may be dampened because of strenuous exercise, such as, an increase in the amount of energetically-demanding movements (Freidenreich and Volek, 2012; Brown and Shine, 2014; Husak et al., 2016). Dispersers may also be more likely to face food limitation and increased stress caused by movement through unfamiliar habitats, two factors also known to suppress immune response (Glick et al., ’83; Raberg et al., ’98; Alonso-Alvarez and

Tella, 2001). In this study, Cuban Treefrogs were held in captivity for a 10 d acclimation period, during which frogs were kept in conditions that likely did not mirror natural conditions (i.e. movement limited by enclosures, food provided *ad libitum*). However, we suggest this period of captivity was likely too brief to alter possible physiological effects related to being at the invasion front. Further, any captivity effect would have acted to dampen, not increase, observed differences between leading-edge and core frogs.

We collected frogs for the LPS challenge during the active season because this is when frogs are most likely to be engaged in activities associated with range expansion (i.e., increased movement, reproduction, and growth) and when differences in immune investment between regions may be the most pronounced. Because we detected a sex-related difference in metabolic rates, we compared sex-specific metabolic responses between regions. Interestingly, sex is rarely considered as a source of potential variation in studies of amphibian metabolism (Gatten et al., ’92; Wells, 2007). Dunlap (’69) reported a higher metabolic rate in male Northern Cricket Frogs (*Acris crepitans*) just prior to the start of the breeding season; however, all other sex-related differences of amphibian metabolism have been shown to occur when one gender is actively engaged in energetically-expensive reproductive behaviors. For example, metabolism increases in male frogs when they are actively calling (Taigen et al., ’85; Pough et al., ’92). Similarly, gravid female salamanders may exhibit higher metabolic rates near the time of oviposition, likely due to energetic costs associated with gamete production (Finkler and Cullum, 2002; Finkler et al., 2003). It’s unlikely that our detection of a sex-related difference was attributable to differential investment in reproductive activities because frogs were not collected at breeding sites and males were not observed calling at the time of collection or any time thereafter. All females used in this study possessed ova in early stages of development; however, if gamete production influenced metabolism we would predict it to raise, not lower, female metabolism. Alternatively, sex-related effects on metabolism may be related to the sexual-size dimorphism exhibited in Cuban Treefrogs. Smaller males of this species likely have more lean body mass and a higher muscle-to-body-mass ratio that has been suggested to explain higher metabolism of the males of some endothermic species (Klausen et al., ’97). Unfortunately, only two previous studies investigated metabolism of Cuban Treefrogs and they did not report sex of individuals, although we suspect a male sampling bias because mean frog mass was approximately 5 gm (Taigen et al., ’82; Taigen, ’83). Regardless of the mechanism causing the observed sex difference, we suggest that future studies of amphibian metabolism consider sex-related effects as a potential source of variation.

We also observed a stronger immune response in frogs from the long-colonized region in the BKA; however, we did not find a sex-related difference in bactericidal ability. It is unclear if the absence of a sex difference is the result of collecting blood plasma samples outside of the typical breeding season or because bactericidal ability is not a sex-influenced response in Cuban Treefrogs. We included the BKA in our experimental design because it provides a direct measure of the ability of immune components to clear actual pathogens and may be superior to in vivo immune challenges that measure response to artificial mitogens (Demas et al., 2011). The pattern of greater immune response from frogs in the core region fits with our *a priori* prediction; however, we were surprised by the large effect size between regions. Because we obtained blood plasma late in the active season, when most frogs are in a post-reproductive state, we expected to find a significant, yet smaller, effect of time since colonization relative to the LPS challenge. We cannot place our findings in the context of other studies because, to our knowledge, this is the first assessment of the bactericidal ability of Cuban Treefrog blood plasma. We considered that greater food limitation in the more northern leading-edge region might cause greater immune suppression at this time of year. However, we infer that food availability is unlikely to have influenced responses because Cuban Treefrogs are generalist predators, consuming wide variety of invertebrate and vertebrate prey (Glorioso et al., 2012) that are unlikely to vary in abundance between the two regions.

Expansion of Cuban Treefrogs in Florida is occurring along a latitudinal gradient, granting us a unique opportunity to consider how latitudinal differences may influence immune responses in relation to time since colonization. The core and leading-edge regions compared in this study are separated by approximately 480 km, the equivalent of nearly five degrees of latitude. Intraspecific latitudinal patterns predict animals at lower latitudes will exhibit higher metabolic rates and greater prevalence of parasite infection. T-tests revealed the metabolic rates of sham-injected frogs were not significantly different between regions for males or females, suggesting latitudinal differences were not present or were too minor to cause a detectable shift in metabolism. Lower latitudes are also associated with greater parasite abundance and the “adjustment-to-parasite-pressure” hypothesis suggests that species living in parasite-rich areas may increase investment in immune defenses because they experience higher infection rates throughout their life (Hasselquist, 2007). However, previous exposure to parasite pressure is expected to have a much greater effect on acquired immune responses that exhibit delayed-type hypersensitivity, rather than the constitutive innate immune functions examined here. The few studies that have investigated how latitude might influence innate immune responses have not found latitude to have a significant effect on response strength (Martin et al., 2006; Mendes et al., 2006).

Allocation of resources away from costly immune functions to the benefit of life-history traits associated with successful invasion is a central tenant of ideas that seek to explain invasion success, such as evolution of increased competitive ability (EICA) and related hypotheses. Yet, a recent review by Cornet and colleagues (2016) revealed only seven studies of vertebrate bioinvasions that have investigated immunological differences in relation to time since colonization, and only four that directly measured some aspect of immune response. Beyond a small literature record, description of broad patterns explaining the role of immunological trade-offs in invasion success is further hampered by the complexity and interdependence of the vertebrate immune system and the diversity of techniques available to measure immunity. Despite these potentially confounding factors, we believe our results fit a general pattern of reduced immune investment at the invasion front. For example, investigations of captive-raised Cane Toads have reported lower immune response at the invasion front or correlated increased dispersal movements with dampened immunity (Llewellyn et al., 2012; Brown and Shine, 2014; *but see* Brown et al. 2015). This relationship between dispersal and immunosuppression is supported by the observation that only toads at the leading-edge of the expanding distribution suffer from spinal arthritis, caused by bacterial infection (Brown et al. 2007; Skelly 2007). Diagne and colleagues (2017) provide an interesting counterpoint to the time since colonization literature, describing an opposite trend in two non-indigenous species of rodents in which inflammatory (i.e. haptoglobin) and/or humoral (i.e. natural antibodies) responses were elevated at the invasion front. To assess inflammatory responses the authors measured circulating levels of haptoglobin proteins (Hp) in non-experimentally challenged individuals. While Hp is a strong anti-inflammatory agent, it also serves numerous, non-immune related functions that can influence whether Hp levels are elevated (reviewed in Martin et al., 2010). Thus, the results reported by Diagne and colleagues (2017) may be more indicative of increased investment in constitutive humoral responses, rather than innate inflammatory responses.

We have documented a crucial first step toward understanding how eco-immunological parameters influence range expansion by documenting that there are differences of immune investment between regions within the invaded distribution. We have also found increased locomotor endurance, a trait that likely increases dispersal ability, in Cuban Treefrogs collected from the invasion front (Goetz et al. in prep). Consideration of reduced investment in innate immunity and increased dispersal ability at the invasion front is strongly suggestive that the ongoing range expansion of Cuban Treefrogs is promoted by an immunological trade-off. Determining how immunological, physiological, and ecological factors influence range expansion and dispersal ability are crucial to modeling how non-indigenous species may respond to their introduced environment and predicting responses of native species to environmental changes such as habitat alterations and global climate change.

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LITERATURE CITED

Adamo SA, Jensen M, Younger M. 2001. Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. Anim Behav 62:417–425.

Alford RA, Brown GP, Schwarzkopf L, Phillips BL, Shine R. 2009. Comparisons through time and space suggest rapid evolution of dispersal behaviour in an invasive species. Wildlife Res 36:23.

Alonso-Alvarez C, Tella JL. 2001. Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. Can J Zool 79:101–105.

Bailly J, Garnier S, Khimoun A, Arnoux E, Eraud C, Goret J-Y, Luglia T, Gaucher P, Faivre B. 2016. Reduced inflammation in expanding populations of a neotropical bird species. Ecol Evol 6:7511–7521.

Blossey B, Notzold R. 1995. Evolution of increased competitive ability in invasive nonindigenous plants: a hypothesis. J Ecol 83:887.

Bomford M, Kraus F, Barry SC, Lawrence E. 2009. Predicting establishment success for alien reptiles and amphibians: a role for climate matching. Biol Invasions 11:713–724.

Bonneaud C, Mazuc J, Gonzalez G, Haussy C, Chastel O, Faivre B, Sorci G. 2003. Assessing the cost of mounting an immune response. Am Nat 161:367–379.

Bonte D, Van Dyck H, Bullock JM, Coulon A, Delgado M, Gibbs M, Lehouck V, Matthysen E, Mustin K, Saastamoinen M, Schtickzelle N, Stevens VM, Vandewoestijne S, Baguette M, Barton K, Benton TG, Chaput-Bardy A, Clobert J, Dytham C, Hovestadt T, Meier CM, Palmer SCF, Turlure C, Travis JMJ. 2012. Costs of dispersal. Biol Rev 87:290–312.

Brown GP, Shilton C, Phillips BL, Shine R. 2007. Invasion, stress, and spinal arthritis in cane toads. P Natl Acad Sci 104:17698–17700.

Brown GP, Shine R. 2014. Immune response varies with rate of dispersal in invasive Cane Toads (*Rhinella marina*). PloS one 9:e99734.

Brown GP, Phillips BL, Dubey S, Shine R. 2015. Invader immunology: invasion history alters immune system function in Cane Toads (*Rhinella marina*) in tropical Australia. Ecol Letters 18:57–65.

Butchart SHM, Walpole M, Collen B, van Strien A, Scharlemann JPW, Almond REA, Baillie JEM, Bomhard B, Brown C, Bruno J, Carpenter KE, Carr GM, Chanson J, Chenery AM, Csirke J, Davidson NC, Dentener F, Foster M, Galli A, Galloway JN, Genovesi P, Gregory RD, Hockings M, Kapos V, Lamarque J-F, Leverington F, Loh J, McGeoch MA, McRae L, Minasyan A, Morcillo MH, Oldfield TEE, Pauly D, Quader S, Revenga C, Sauer JR, Skolnik B, Spear D, Stanwell-Smith D, Stuart SN, Symes A, Tierney M, Tyrrell TD, Vie J-C, Watson R. 2010. Global biodiversity: indicators of recent declines. Science 328:1164–

Colautti RI, MacIsaac HJ. 2004. A neutral terminology to define “invasive” species. Divers Distrib 10:135–141.

Cornet S, Brouat C, Diagne C, Charbonnel N. 2016. Eco-immunology and bioinvasion: revisiting the evolution of increased competitive ability hypotheses. Evol Appl 9:952–962.

Demas GE, Chefer V, Talan MI, Nelson RJ. 1997. Metabolic costs of mounting an antigen-stimulated immune response in adult and aged C57BL/6J mice. Am J Physiol-R I 273:R1631–R1637.

Demas GE. 2004. The energetics of immunity: a neuroendocrine link between energy balance and immune function. Horm and Behav 45:173–180.

Demas GE, Zysling DA, Beechler BR, Muehlenbein MP, French SS. 2011. Beyond phytohaemagglutinin: assessing vertebrate immune function across ecological contexts: assessing vertebrate immune function across ecological contexts. J Anim Ecol 80:710–730.

Diagne C, Gilot-Fromont E, Cornet S, Husse L, Doucouré S, Dalecky A, Bâ K, Kane M, Niang Y, Diallo M, Sow A, Fossati-Gaschignard O, Piry S, Artige E, Sembène M, Brouat C, Charbonnel N. 2017. Contemporary variations of immune responsiveness during range expansion of two invasive rodents in Senegal. Oikos 126:435–446.

Dunlap DG. 1969. Influence of temperature and duration of acclimation, time of day, sex and body weight on metabolic rates in the hylid frog, *Acris crepitans*. Comp Biochem Physiol 31:555–570.

Finkler MS, Cullum KA. 2002. Sex-related differences in metabolic rate and energy reserves in spring-breeding Small-Mouthed Salamanders (*Ambystoma texanum*). Copeia 2002.

Finkler MS, Sugalski MT, Claussen DL. 2003. Sex-related differences in metabolic rate and locomotor performance in breeding Spotted Salamanders (*Ambystoma maculatum*). Copeia 2003:887–893.

Freidenreich DJ, Volek JS. 2012. Immune responses to resistance exercise. Exercise Immunology review 18:8–41.

French SS, DeNardo DF, Moore MC. 2007. Trade-offs between the reproductive and immune systems: facultative responses to resources or obligate responses to reproduction? Am Nat 170:79–89.

Gatten RE, Miller K, Full RJ. 1992. Energetics at rest and during locomotion. In: Feder ME, Burggren WW, editors. Environmental physiology of the amphibians. Chicago: University of Chicago Press. p 314–377.

Gendron AD, Marcogliese DJ, Thomas M. 2012. Invasive species are less parasitized than native competitors, but for how long? The case of the round goby in the Great Lakes-St. Lawrence Basin. Biol Invasions 14:367–384.

Glick B, Taylor RL, Martin DE, Watabe M, Day EJ, Thompson D. 1983. Calorie-protein deficiencies and the immune response of the chicken II. Cell-mediated immunity. Poultry Sci 62:1889–1893.

Glorioso BM, Waddle JH, Crockett ME, Rice KG, Percival HF. 2010. Diet of the invasive Cuban Treefrog (*Osteopilus septentrionalis*) in pine rockland and mangrove habitats in South Florida. Caribb Journal Sci 46:346–355.

Hasselquist D. 2007. Comparative immunoecology in birds: hypotheses and tests. J Ornithol 148:571–582.

Hasselquist D, Lindstrom A, Jenni-Eiermann S, Koolhaas A, Piersma T. 2007. Long flights do not influence immune responses of a long-distance migrant bird: a wind-tunnel experiment. J Exp Biol 210:1123–1131.

Husak JF, Ferguson HA, Lovern MB. 2016. Trade-offs among locomotor performance, reproduction and immunity in lizards. Funct Ecol 30:1665–1674.

Janeway C. ed. 2005. Immunobiology: the immune system in health and disease. 6th ed. New York: Garland Science.

Klasing KC. 2004. The costs of immunity. Acta Zool Sinica 50:961–969.

Klausen B, Toubro S, Astrup A. 1997. Age and sex effects on energy expenditure. Am J Clin Nutr 65:895–907.

Kolar CS, Lodge DM. 2001. Progress in invasion biology: predicting invaders. Trends Ecol Evol 16:199–204.

Kopp K, Jokela J. 2007. Resistant invaders can convey benefits to native species. Oikos 116:295–301.

Lee KA, Klasing KC. 2004. A role for immunology in invasion biology. Trends Ecol Evol 19:523–529.

Lee KA, Martin LB, Wikelski MC. 2005. Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (*Passer domesticus*), than its less-invasive congener. Oecologia 145:243–250.

Liebl AL, Martin II LB. 2009. Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry: Antimicrobial capacity and spectrophotometry. Funct Ecol 23:1091–1096.

Lighton JRB. 1991. Insects: measurements. In: Payne PA, editor. Concise Encyclopedia of Biological and Biomedical Measurement Systems. New York: Pergamon Press. p 201–208.

Llewellyn D, Thompson MB, Brown GP, Phillips BL, Shine R. 2012. Reduced investment in immune function in invasion-front populations of the Cane Toad (*Rhinella marina*) in Australia. Biol Invasions 14:999–1008.

Lochmiller RL, Deerenberg C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98.

Lockwood JL, Cassey P, Blackburn T. 2005. The role of propagule pressure in explaining species invasions. Trends Ecol Evol 20:223–228.

Mansfield JM, Olivier M. 2002. Immune evasion by parasites. In: Kaufmann SHE, Sher A, Ahmed R, editors. Immunology of Infectious Diseases. Washington: ASM Press. p 379–392.

Martin LB, Scheuerlein A, Wikelski M. 2003. Immune activity elevates energy expenditure of house sparrows: a link between direct and indirect costs? P Roy Soc B-Biol Sci 270:153–158.

Martin LB, Hasselquist D, Wikelski M. 2006. Investment in immune defense is linked to pace of life in house sparrows. Oecologia 147:565–575.

Martin LB, Alam JL, Imboma T, Liebl AL. 2010. Variation in inflammation as a correlate of range expansion in Kenyan house sparrows. Oecologia 164:339–347.

Martin LB, Brace AJ, Urban A, Coon CAC, Liebl AL. 2012. Does immune suppression during stress occur to promote physical performance? J Exp Biol 215:4097–4103.

McGarrity ME, Johnson SA. 2009. Geographic trend in sexual size dimorphism and body size of *Osteopilus septentrionalis* (Cuban Treefrog): implications for invasion of the southeastern United States. Biol Invasions 11:1411–1420.

Mendes L. 2006. Variation in the innate and acquired arms of the immune system among five shorebird species. J Exp Biol 209:284–291.

Meshaka WE. 2001. The Cuban Treefrog in Florida: life history of a successful colonizing species. Gainesville: University Press of Florida.

Mims CA, Nash A, Stephen J. 2001. Mims' Pathogenesis of infectious disease. 5th ed. San Diego: Academic Press.

Owen JC, Moore FR. 2006. Seasonal differences in immunological condition of three species of thrushes. Condor 108:389–398.

Owen JC, Moore FR. 2008. Swainson's thrushes in migratory disposition exhibit reduced immune function. J Ethol 26:383–388.

Pedersen, AB, Babayan, SA. 2011. Wild immunology. Mol Ecol 20:872–880.

Phillips BL, Brown GP, Travis JMJ, Shine R. 2008. Reid's paradox revisited: the evolution of dispersal kernels during range expansion. Am Nat 72:S34–S48.

Pimentel D, Zuniga R, Morrison D. 2005. Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ 52:273–288.

Pough H, Magnusson WE, Ryan MJ, Wells KD, Taigen TL. 1992. Behavioral energetics. In: Feder ME, Burggren WW, editors. Environmental physiology of the amphibians. Chicago: University of Chicago Press. p 395–436.

Powell R, Passaro RJ, Henderson RW. 1992. Noteworthy herpetological records from Saint Maarten, Netherlands Antilles. Caribb J Sci 28:234–235.

Raberg L, Grahn M, Hasselquist D, Svensson E. 1998. On the adaptive significance of stress-induced immunosuppression. P Roy Soc B-Biol Sci 265:1637–1641.

R Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, Available online at: https://www.R-project.org/.

Rigaud T, Moret Y. 2003. Differential phenoloxidase activity between native and invasive gammarids infected by local acanthocephalans: differential immunosuppression? Parasitology 127:571–577.

Roche DG, Leung B, Mendoza Franco EF, Torchin ME. 2010. Higher parasite richness, abundance and impact in native versus introduced cichlid fishes. Int J Parasitol 40:1525–1530.

Rödder D, Weinsheimer F. 2009. Will future anthropogenic climate change increase the potential distribution of the alien invasive Cuban Treefrog (Anura: Hylidae)? J Nat Hist 43:1207–1217.

Savage JM, Fogden M, Fogden P. 2005. The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas. Bristol: University of Chicago Press.

Schwartz A, Henderson RW. 1991. Amphibians and reptiles of the West Indies: descriptions, distributions, and natural history. Gainesville: University of Florida Press.

Simberloff D, Martin JL, Genovesi P, Maris V, Wardle DA, Aronson J, Courchamp F, Galil B, García-Berthou E, Pascal M, others. 2013. Impacts of biological invasions: what's what and the way forward. Trends Ecol Evol 28:58–66.

Shilton CM, Brown GP, Benedict S, Shine R. 2008. Spinal Arthropathy Associated with *Ochrobactrum anthropi* in Free-ranging Cane Toads (*Chaunus* [*Bufo*] *marinus*) in Australia. Vet Pathol 45:85–94.

Stearns SC. 1989. Trade-Offs in Life-History Evolution. Funct Ecol 3:259–268.

Taigen TL. 1983. Activity metabolism of anuran amphibians: implications for the origin of endothermy. Am Nat 121:94–109.

Taigen TL, Emerson SB, Pough FH. 1982. Ecological correlates of anuran exercise physiology. Oecologia 52:49–56.

Taigen TL, Wells KD, Marsh RL. 1985. The enzymatic basis of high metabolic rates in calling frogs. Physiol Zool 58:719–726.

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM. 2003. Introduced species and their missing parasites. Nature 421:628–630.

Torchin ME, Lafferty KD. 2009. Escape from Parasites. In: Rilov G, Crooks JA, editors. Biological Invasions in Marine Ecosystems. Vol. 204. Berlin, Heidelberg: Springer Berlin Heidelberg. p 203–214.

Townsend, JH, Eaton JM, Powell R, Parmerlee Jr JS, Henderson RW. 2000. Cuban Treefrogs (*Osteopilus septentrionalis*) in Anguilla, Lesser Antilles. Caribb J Sci 36:326–328.

Wells KD. 2007. The ecology and behavior of amphibians. Chicago: The University of Chicago Press.

White TA, Perkins SE. 2012. The ecoimmunology of invasive species. Funct Ecol 26:1313–1323.

Wilcove DS, Rothstein D, Dubow J, Phillips A, Losos E. 1998. Quantifying threats to imperiled species in the United States assessing the relative importance of habitat destruction, alien species, pollution, overexploitation, and disease. BioScience 48:607–615.

Williamson M, Fitter A. 1996. The varying success of invaders. Ecology 77:1661–1666.

Zera AJ, Harshman LG. 2001. The physiology of life history trade-offs in animals. Annu Rev Ecol Syst 32:95–126.

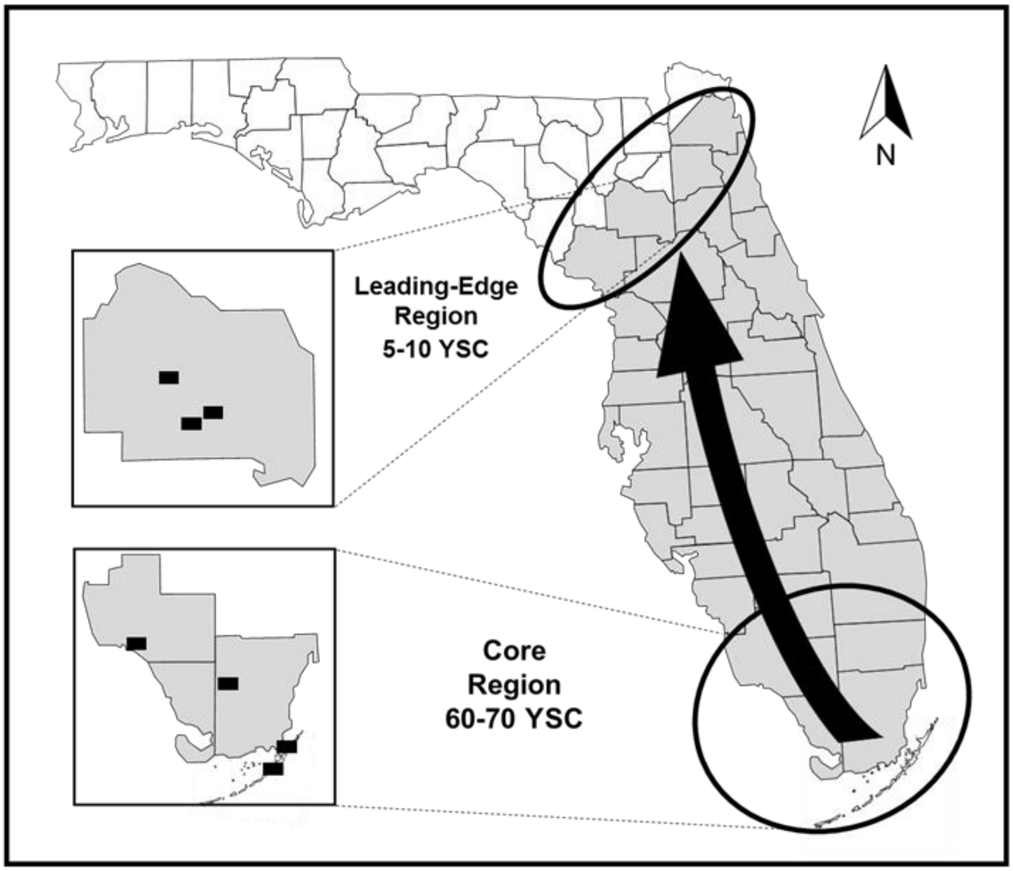


Figure 3 - 1. — Invasive distribution of Cuban Treefrogs in Florida, USA. Gray areas represent counties with established populations, black arrow indicates invasion route starting in the Florida Keys, and circled areas correspond respectively to core region and leading-edge region (YSC = Years Since Colonization). Sampling sites are indicated by black rectangles.

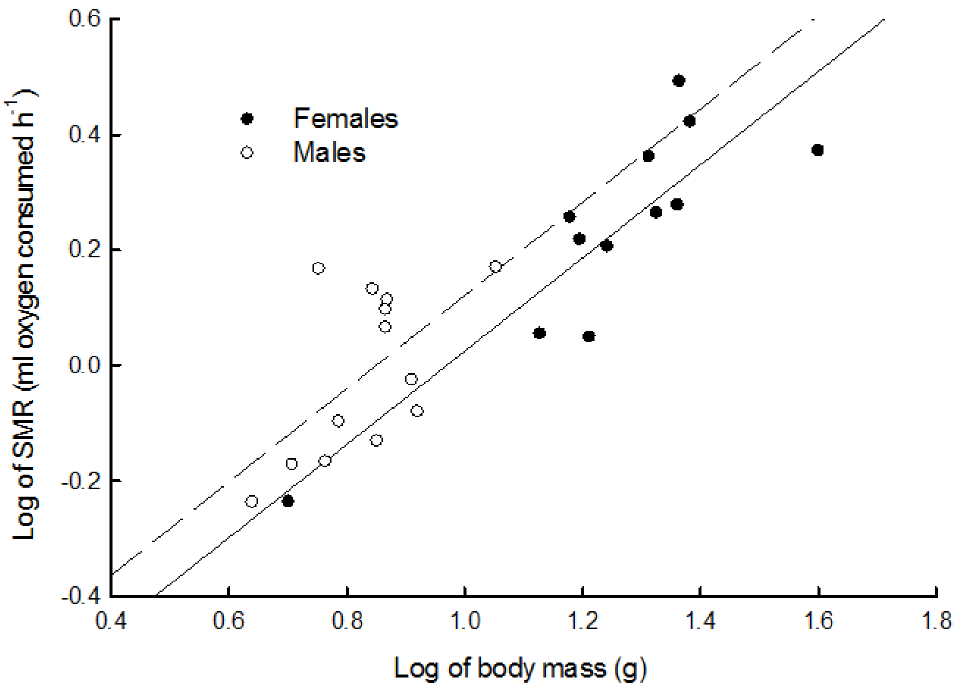


Figure 3 - 2. — Allometric relationship of log-transformed mass and log-transformed metabolic rate as a factor of sex in Cuban Treefrogs. Dashed line represents male frogs (coefficient = 0.808) and solid line represents females (coefficient = 0.807).

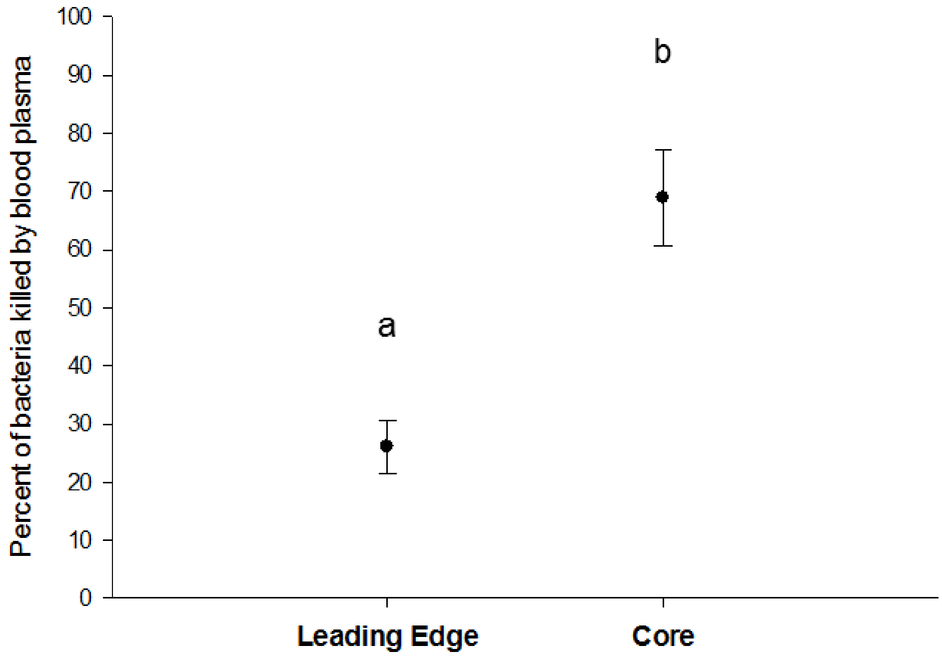
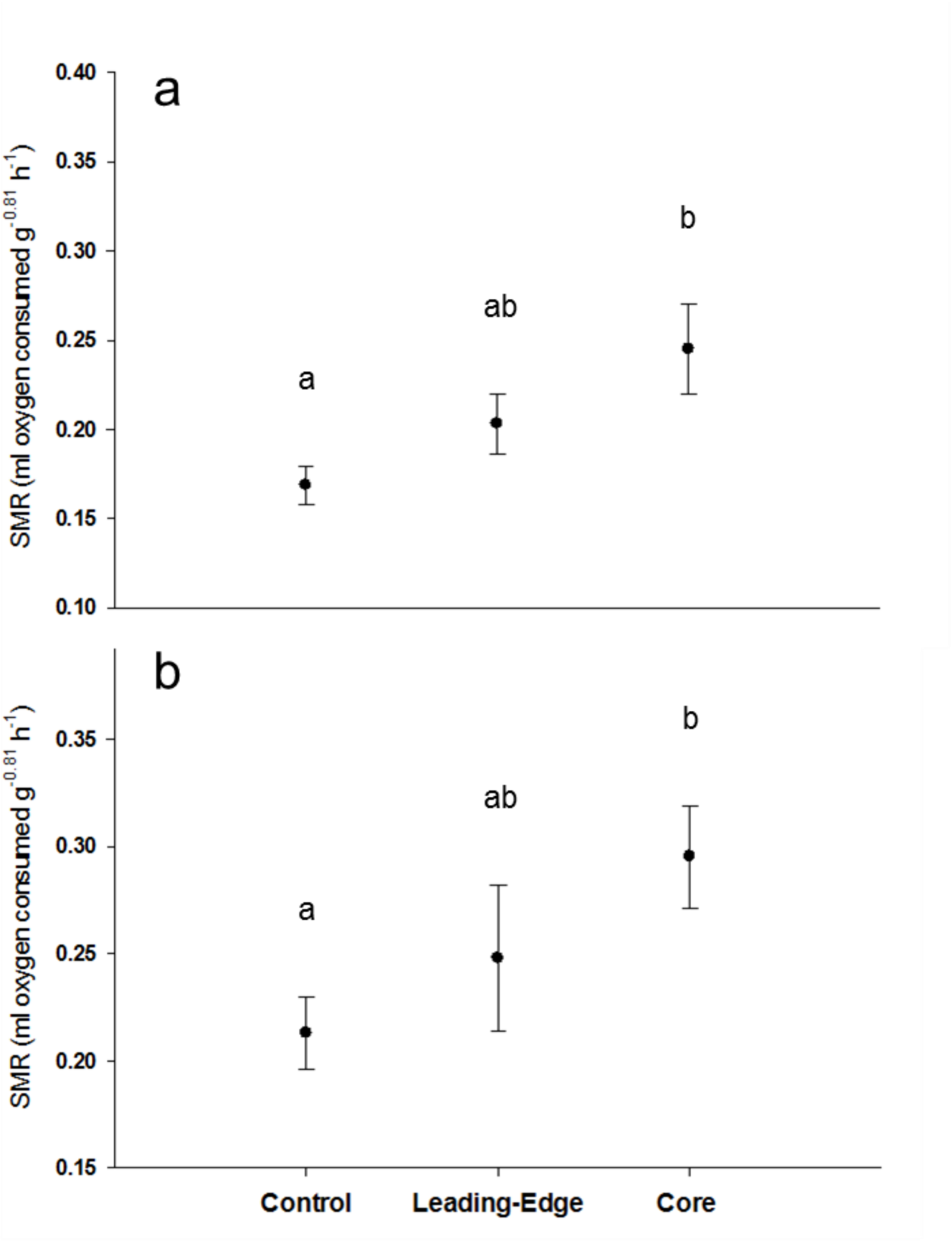


Figure 3 - 3. — Comparison of percent of *E. coli* bacteria colonies killed (mean ± SE) following incubation with blood plasma from Cuban Treefrogs in leading-edge and core regions. Different letters represent a significant difference between regions based on a two-way ANOVA ($P < 0.001$).

Figure 3 - 4. — Mean ± SE values of mass-independent standard metabolic rate of (a) females and (b) males for Cuban Treefrogs collected from the leading-edge or core region. Frogs received either a sham-injection (controls pooled from both regions) or a LPS injection. Different letters represent a significant difference between treatment groups based on a Tukey post hoc test in female frogs (control injection–core LPS injection; P = 0.007) and male frogs (control injection–core LPS injection; P = 0.041).


a
SMR (ml oxygen consumed g$^{-0.81}$ h^{-1})
0.40
0.35
0.30
0.25
0.20
0.15
0.10
a
ab
b
b
SMR (ml oxygen consumed g$^{-0.81}$ h^{-1})
0.35
0.30
0.25
0.20
0.15
a
ab
b
Control
Leading-Edge
Core

CHAPTER IV

INVASION-FRONT CUBAN TREEFROGS HAVE GREATER ENDURANCE BUT NOT SPRINT SPEED OR GROWTH RATE

ABSTRACT

Rapid range expansion of invasive species may, in part, be explained by trade-offs involving allocation of resources away from immune defenses and towards traits that enhance dispersal ability. Cuban Treefrogs have spread via leading-edge dispersal through their invasive range in Florida and previous investigations have found reduced investment of innate immune defenses at the invasion front. We investigated potential downstream dispersal-related benefits of reduced immune investment. Specifically, we compared sprint speed, locomotor endurance, and growth rates between populations in long-established and invasion-front regions. Using a vertical, enclosed racetrack, frogs from both regions had similar sprint speeds; however, frogs from the invasion front traveled more than 25% farther prior to exhaustion compared with frogs from the long-established region. Greater endurance of invasion-front frogs was correlated with a significant increase in blood lactate levels. We performed a skeletochronology analysis of femurs to determine the age of female frogs from both regions. We then fit age and snout-vent length to a von Bertalanffy growth model to compare growth rates. There was

no significant difference in growth rates for female Cuban Treefrogs between the two regions. Our finding of increased locomotor endurance at the invasion front, coupled with reduced investment in innate immune defenses by frogs from this same region, supports the idea that Cuban Treefrog range expansion is facilitated by an immune-based trade-off.

INTRODUCTION

The invasiveness of a species is determined by its ability to spread and become abundant (Colautti and Maclsaac 2004); however, identification of the underlying processes that facilitate range expansion remains an unresolved question in invasion ecology. Increasingly, investigations into range expansion, and invasiveness in general, have considered the influence of enemy release and ecological immunity (White and Perkins 2012). Originally proposed to explain observations of reduced herbivory of nonindigenous plants, the enemy-release hypothesis has been broadened to include escape from predators, parasites, and pathogens in introduced animals (Crawley 1987; Keane and Crawley 2002; Torchin et al., 2003). Over time nonindigenous animals may acquire new parasites and pathogens within the invasive range (Gendron et al. 2012); however, the risk of infection is expected to be lower during the initial stages of introduction and establishment because many coevolved enemies are lost during the invasion process (Mitchell and Power 2003; Torchin et al. 2003; Torchin and Lafferty 2009). A loss of these natural enemies may lessen the necessity of a robust immune defense and liberate energy that could be directed to other processes such as growth and locomotion.

Building on the idea that parasite and pathogen pressure may be reduced, Lee and Klasing (2004) proposed the hypothesis that dampened immune response in nonindigenous species may positively influence successful establishment. Specifically, they suggested a potential benefit from the suppression of immune responses that induce resource-costly systemic inflammation (i.e. components of innate and cell-mediated responses), while simultaneously increasing investment in less-costly humoral defenses (Lee and Klasing 2004). This life-history trade-off may be adaptive because suppression of costly immune responses could allow additional resources to be directed toward fitness-related traits including, reproduction and growth (Stearns ’89; Lochmiller and Deerenberg 2000; Zera and Harshman 2001). In a logical extension of the hypothesis proposed by Lee and Klasing (2004), reduced investment in inflammatory components of the immune system may also enable range expansion by increasing dispersal ability. Because activities associated with dispersal are expected to be resource-demanding, costs may be offset by an immune-based trade-off. Similar to the reduced parasite pressure predicted during the establishment stage, individuals at the invasion-front of range expansion may have fewer parasites and pathogens compared to conspecifics in longer-established areas (Phillips et al. 2010). Even if the threat of infection is not reduced at the invasion front, immune-mediated trade-offs may still occur because selection during range expansion is expected to favor riskier dispersal strategies (i.e. immunosuppression) to increase the likelihood that an individual or its progeny will be represented at the edge of the expanding front (Barton et al. 2012).

Beyond reduced threat from infection, populations at the invasion front of range expansion are also expected to be under different selective pressures than conspecifics from longer-established areas (Phillips et al. 2010). Dispersing organisms are likely to experience increased energetic and temporal costs directly tied to dispersal activities and increased stress as a consequence of encountering novel environments (Burton et al. 2010). For example, movement through novel environments may increase the risk of mortality if dispersers must traverse areas with hostile environmental conditions and/or make long-distance movements that increase exposure to predators (Bonte et al. 2012). Direct energetic costs are predicted to include increased expenditure of metabolic energy due to locomotor activities, and/or the development of morphological features specifically related to dispersal (Phillips et al. 2006; Forsman et al. 2011). Additionally, temporal costs may be incurred because time invested in dispersal activities is unavailable for other activities.

If immune-based trade-offs are, in part, promoting range expansion we should predict two associated responses (1) immune responses should be weaker at the invasion front compared with longer-colonized areas and (2) individuals at the invasion-front should exhibit a trait(s) that enhances dispersal ability and/or the likelihood to disperse, including phenotypes that may differ in at least one aspect of physiology, locomotor performance, behavior, and/or morphology. Despite increased recognition regarding the role immune trade-offs may play in the invasion process, few studies have addressed both of the aforementioned predictions within the same invasion. A recent review by Cornet and colleagues (2016) highlights the relatively small number of studies on vertebrates that examine the strength of immune responses with respect to range expansion. Discerning a general pattern based on these studies is complicated by variation of the immunological techniques used, study species examined, and the immune components assessed. Despite these difficulties, this literature suggests a weak, positive correlation between invasiveness and decreased response of at least one component of the immune system. A second body of literature, largely independent from investigations of immunity, provides support for the presence of dispersal phenotypes in populations at the invasion-front of range expansion (reviewed in Chaung and Peterson 2016). For example, round gobies at the invasion front are bolder, have a greater tendency to disperse, exhibit higher resting metabolism, and grow more rapidly compared with conspecifics in established areas (Myles-Gonzalez et al. 2015). Consideration of both bodies of literature (i.e. studies of immunosuppression and dispersal ability) suggests immune-mediated trade-offs occur during range expansion; however, it is difficult to correlate reduced immunity with increased dispersal ability because these studies do not explore both aspects of the potential trade-off within the same model organism or invasion scenario. For example, immune responses may differ between regions for a variety of reasons, unrelated to dispersal. Similarly, dispersal phenotypes may arise independent of immune-based trade-offs, possibly because of demographic processes at the invasion front, including spatial sorting and allele surfing (White and Perkins 2012).

Notably, investigations of the Cane Toad (*Rhinella marina*) invasion of Australia may provide the best support for the idea that immune-based trade-offs promote range expansion. In comparison to conspecifics in longer-colonized areas, experimental investigations have revealed that toads at the invasion front exhibit greater endurance (Llewelyn et al. 2010) coupled with a decreased inflammatory response (Llewelyn et al. 2012). Additionally, consistent with the prediction that increased dispersal cost may drive immunosuppression, greater distance moved by individual toads is correlated with decreased innate immunity (Brown and Shine 2014). Alternatively, other studies failed to find physiological enhancements in invasion-front toads, including endurance (Tracy et al. 2011) and immune response (Brown et al. 2015). However, observational studies appear to support the presence of immune-based trade-offs. For example, toads at the invasion front appear to be superior dispersers, as evidenced by the increasing rate of range expansion, possibly driven by the adaptation of longer legs (Phillips et al. 2006). Additionally, immunosuppression at the invasion front is supported by observations of spinal arthritis, caused by bacterial infection that is only present in toads from the invasion front (Brown et al. 2007; Skelly 2007).

The dynamics of Cane Toad range expansion in Australia provide compelling support for the hypothesis that immune-based trade-offs promote range expansion; however, investigations of other invasions are needed to evaluate the generality of this hypothesis. Here, we investigate potential differences in the dispersal ability of Cuban Treefrogs (*Osteopilus septentrionalis*) within their invasive range in Florida, USA. Previous investigations have revealed significant variation of Cuban Treefrog immune response relative to time since colonization (Goetz et al. *in review*). Specifically, frogs from the invasion-front region exhibited reduced bactericidal ability and a reduced metabolic response to an inflammatory challenge compared to individuals from long-colonized (core) areas (Goetz et al. *in review*). To investigate potential downstream effects from this reduced investment in immune defense, we examined a series of physiological and performance measures that likely influence dispersal ability. We compared growth rate, sprint speed, and locomotor endurance, coupled with measurement of blood lactate levels, between Cuban Treefrogs from long-established and invasion-front populations. Based on the hypothesis that immune-based life-history trade-offs promote dispersal ability, we predicted frogs from the invasion-front region would exhibit reduced investment in immune response and at least one physiological enhancement correlated with dispersal ability.

METHODS

Study Species

Cuban Treefrogs, native to Cuba, the Bahamas, and the Cayman Islands, were first documented in the USA in the Florida Keys circa 1930. Following establishment on the southern tip of mainland Florida, USA by 1954, Cuban Treefrogs have continued to expand their range via leading-edge dispersal northward, such that, their distribution now encompasses the majority of peninsular Florida (Fig. 4-1). The current northern limit of their invasive range roughly extends from Jacksonville on the Atlantic coast, through Alachua county, to Cedar Key on the Gulf coast (McGarrity and Johnson 2009; Rodder

2009). We designated populations of Cuban Treefrogs occurring in southern Florida (Monroe, Miami-Dade, and Collier counties) as “core” populations because they have been established for over 60 years and are surrounded by areas already occupied by conspecifics. Populations occurring at the vanguard of range expansion (Alachua County, FL) are designated as “invasion-front” populations.

Locomotor performance

Cuban Treefrog sprint speed and endurance was measured in a vertically-oriented raceway consisting of a 185cm clear acrylic pipe. To ensure frogs climbed up the raceway, rather than vertically leaping, we used a raceway with a 4 cm internal diameter for frogs < 9 g and a raceway with a 6.3 cm internal diameter for frogs > 9 g. When necessary, movement was encouraged with gentle prodding with a padded wire. Both measurements of sprint speed and endurance were assessed in blind trials, such that the observer did not know the identity or collection locality of the frog being assessed. Trials occurred in the evening during the normal activity period of Cuban Treefrogs at ambient temperature (23 °C). Frogs were collected from three sites, separated by at least 5 km, from the core region and four sites in the invasion-front region. All animals were individually housed for two weeks following collection to acclimate to their enclosures. Speed and endurance were modeled as a factor of geographic locality (grouping variable; invasion-front vs core populations).

For sprint speed, 87 frogs (core = 30; invasion-front = 57) were raced up the vertical raceway through four sets of infrared sensors placed at 25 cm intervals. As a frog passed through each sensor gate, the time was automatically relayed and recorded on an attached laptop computer. The time to traverse each interval was determined by subtracting the time to reach a sensor gate by the time to reach the subsequent gate. Each frog was raced three times with an hour-long break between, resulting in a total of nine sprint speeds per individual. The fastest time to traverse the 25cm distance out of the nine intervals was used as maximum sprint speed. We compared sprint speed using an analysis of covariance (ANCOVA) with region as a factor, snout-vent-length (SVL) as a covariate and speed in seconds as the dependent variable.

Similar to sprint speed, the endurance of 39 frogs (core = 11 females, 9 males; invasion-front = 9 females, 10 males) was assessed by determining the total vertical distance treefrogs would climb before exhaustion. When treefrogs reached the top of the pipe, they were moved back to the starting position at the bottom of the pipe. Exhaustion was determined when an individual refused to move following three consecutive taps with the padded wire. We measured blood lactate levels immediately prior to the endurance assay, one-hr post-exhaustion, and 24-hr post-exhaustion. Lactate was measured using a commercially purchased lactate meter (Lactate Plus Meter, Nova Biomedical). All blood samples were collected in heparinized capillary tubes via puncture of an ulnar vein or brachial artery and transferred to a disposable test strip already inserted in the meter. We compared endurance using a one-tailed analysis of covariance (ANCOVA) with region as a factor, snout-vent-length (SVL) as a covariate and distance prior to exhaustion as the dependent variable. For blood lactate levels, we fitted data to a general linear model and used a one-tailed, repeated measures analysis of variance (ANOVA) to compare the pre-exercise, 1-hr post-exercise, and 24-hr post-exercise lactate levels with region and sex as factors.

Growth Rate

We hand-captured Cuban Treefrogs from core and invasion-front populations between June and August of 2015 and 2016. Age of individual frogs was determined through skeletochronology analysis of the femur bones of 98 frogs (core = 55; invasion-front = 43). The sex of frogs was determined by the presence of nuptial pads on males and SVL was measured prior to euthanization. The left femur was extracted from each frog, all soft tissue was removed, and the bones were placed in labeled envelopes for accurate identification. Femurs were then placed in labeled cassettes and decalcified by submersion in decalcification solution (Cal Ex II, Fisher Scientific) for 24-hr. Following decalcification, the epiphyses of bones were removed with a razor blade, leaving only the 5 mm center portion of the diaphysis and samples were placed back in cassettes and washed in a running bath of tap water for one hr. Samples were then serially dehydrated in ethanol prior to consecutive baths in a clearing agent (CitriSolv, Decon Labs Inc.) and embedded with paraffin (Tissue Prep II, Fisher Scientific). Immediately following internal embedding, samples were removed from cassettes and externally embedded in metal block forms. Wax blocks were cooled in a refrigerator for 24 hr, forms were removed, and bones were sectioned to 6 µm using a rotary microtome (American Optical Company). Sections were affixed to subbed microscope slides and stained with Harris's hematoxylin and eosin. Bone sections were photographed on a microscope-mounted camera at 10x and 32x magnification. The number of lines of arrested growth (LAGs) were counted to determine the age of individual frogs (Fig. 4-2).

The age and SVL of frogs were fitted to the von Bertalanffy growth model to compare growth rates between core and invasion-front regions. Because we suspect that an indeterminable number of LAGs had either been reabsorbed or became too faint to distinguish in older individuals, we only included frogs that were determined to be ≤ 5 years of age in our comparison of regional growth rate.

RESULTS

Sprint Speed

Frogs from both regions had a similar mean SVL (Core = 58.4 mm; invasion-front = 57.3 mm) and mass (Core = 11.5 g; invasion-front = 12.0 g). Average sprint speed of frogs from the core region was slightly faster than invasion-front frogs; however, the effect of region was not significant ($F_{1,\,81} = 2.04$, P = 0.157; Fig. 4-3). The effect of SVL was significant ($F_{1,\,81} = 6.63$, P = 0.011), such that larger frogs were faster. There was no interaction between region and SVL ($F_{1,\,81} = 0.26$, P = 0.609).

Endurance and Lactate

Frogs used to assess endurance and blood lactate response had a similar mean SVL by region (Core = 58.9 mm; invasion-front = 57.0 mm). Frogs from the invasion-front region had significantly greater endurance than frogs from the core region ($F_{1,\,31} = 8.61$, P = 0.006; Fig. 4-4). There was no effect of SVL ($F_{1,\,31} = 0.002$, P = 0.967) or interaction between region and SVL ($F_{1, 31} = 0.009$, P = 0.923). Mean distance traveled prior to exhaustion for females from both regions was 476.5 cm and 473.5 cm for males.

There was a significant increase in lactate 1-hr post endurance trials compared with lactate levels prior to trials ($F_{1, 35} = 282.13$, P < 0.001; Fig. 4-5). There was a significant interaction between lactate levels and region ($F_{1, 35} = 3.55$, P < 0.034), such that frogs from the invasion-front region had a greater increase in lactate (11.75 mmol L^{-1}) following endurance trials compared with frogs from the core region (9.38 mmol L^{-1}). There were non-significant interactions between lactate and sex ($F_{1, 35} = 0.001$, P < 0.949) and between lactate, region, and sex ($F_{1, 35} = 0.001$, P < 0.979). There was no significant difference between pre-exercise and 24-hr post-exercise lactate levels ($F_{1, 32} = 1.71$, P = 0.201).

Growth Rate

Lines of arrested growth were counted in 101 Cuban Treefrogs (25 males and 77 females). Sample size was reduced to include only individuals that were determined to be ≤ 5 years of age (23 males and 55 females). For regional comparisons, male sample size was determined to be insufficient. Female age ranged from zero to eleven years with a mean age was 3.9yr with a mean SVL of 60.1mm (SVL min = 41.3mm, max = 85.3mm). Female age and SVL were significantly correlated ($F_{4, 49} = 1908.28$, P < 0.001); however, there was no significant difference in growth rates between regions ($F_{4, 49} = 0.13$, P = 0.882; Fig. 4-6).

DISCUSSION

Dampened immune responses have been suggested as a possible mechanism to promote dispersal ability of invasive species, in part, explaining an ability of some introduced species to quickly spread through non-indigenous environments. We previously demonstrated weakened innate immune responses in Cuban Treefrogs at the invasion-front of their expanding geographic range (Goetz et al. *in review*). We hypothesized that energy and resources not allocated to immune defenses will have a downstream benefit, such that, traits associated with enhanced dispersal ability may be promoted at the invasion front. Here, we show that treefrog locomotor endurance is greater at the invasion front compared to core regions; however, sprint speed and growth rate did not vary by region.

Greater endurance of frogs at the invasion front is the primary difference that we found in our regional comparison. Blood lactate levels provided a physiological variable that appears to support our finding that invasion front frogs have greater endurance capabilities. Initial lactate levels were similar for frogs from both regions, indicating that all frogs were in a similar pre-exercise state. It was important to ensure that movements prior to the assessment of endurance did not negatively influence locomotor capabilities because invasion front animals may have a greater tendency to move. During strenuous exercise, demand for ATP increases and oxygen supply to muscles decreases, resulting in a switch from aerobic to anaerobic respiration; however, anaerobic metabolism is not sustainable for prolonged periods of time causing fatigue and ultimately exhaustion (Hillman et al. 2009). Lactate is an end-product of anaerobic respiration and post-exercise lactate levels can be interpreted multiple ways. For instance, higher lactate levels could suggest a quicker switch to anaerobic metabolism, and thus decreased aerobic capabilities. Conversely, higher lactate may indicate a greater ability sustain anaerobic activity prior to exhaustion. In this study, we suggest the later explanation is occurring because increased lactate levels were coupled with a greater than 25% increase in distance moved prior to exhaustion in invasion front frogs. At 24 hr, post-exercise lactate in frogs from both regions had returned to pre-exercise levels, indicating no difference in the speed that lactate is cleared at this time scale. The ability to clear lactate quickly might enhance dispersal ability by reducing recovery time between bouts of long-distance movements. We considered measuring lactate at 12-hr post-exercise; however, any variation at this time scale would likely not be biologically relevant to crepuscular/nocturnal Cuban Treefrogs that are unlikely to make diurnal movements regardless of blood lactate levels.

The wave-like, northward expansion of the Cuban Treefrog geographic range over the last 60yrs is indicative of invasion-front dispersal, likely facilitated by long-distance dispersal movements. In general, there is a paucity of information regarding the movement capabilities of treefrogs; however, several recent studies suggest treefrogs can make long-distance movements over short time periods. For example, Johnson and McGarity (2010) reported a 3 m mean distance per movement for radiotracked Cuban Treefrogs that were relocated to old-field, presumably sub-optimal, habitat. Similarly, individual native Gray Treefrogs (*Hyla chrysoscelis*), have been observed moving 80 m (Johnson et al. 2008) and 200 m (Johnson et al. 2007) within a single 24-hr period, and 330 m over a longer period of time between breeding and foraging areas (Johnson et al.

2008). Increased endurance of Cuban Treefrogs documented here may represent an adaptation granting greater dispersal ability to frogs at the invasion front.

We did not find any evidence to support predictions of greater sprint speed in frogs at the invasion front. Similarly, multiple investigations of sprint speed in Cane Toads failed to find differences relative to time since colonization (Llewelyn et al. 2010; Tracy et al. 2011; *but see* Phillips et al. 2006). Failure to detect differences in sprint speed may indicate that this variable is under similar selection pressure in both regions. Sprint speed may have a more direct tie to predator escape and prey capture than dispersal ability. However, because risk of predation and starvation are predicted to be greater in dispersing organisms (Bonte et al. 2012), we argue that sprint speed should continue to be considered in investigations of locomotor capabilities linked to enhanced dispersal ability.

As with sprint speed, we failed to detect a difference in growth rates by region. It is not known whether Cuban Treefrogs at the invasion front disperse throughout their life or only during discrete age/size periods. Regardless of when dispersal occurs, increased growth rates should enhance dispersal ability. Faster growth would enable increased fecundity by facilitating reproduction at an earlier age and greater reproductive output by females. Intuitively, locomotor performance capabilities increase following the transition from metamorph to adult sizes. Additionally, the ratio of surface area to volume decreases as amphibians increase in size making them less vulnerable to desiccation. Thus, larger frogs are better able to sustain movements through hostile environments. However, our failure to detect growth differences in Cuban Treefrogs was not surprising.

In a previous study, I investigated metabolic response of Cuban Treefrogs from core and invasion-front regions to an immune challenge. The controls for this experiment, sham-injected frogs, exhibited no regional metabolic difference of metabolism. If Cuban Treefrogs were allocating more energy towards growth at the invasion-front, we would have expected to find increased standard metabolic rates of sham-injected frogs from the invasion-front region.

We find no direct link between our previous finding of decreased innate immune defenses at the invasion front (Goetz et al. in review) and our finding, here, of greater locomotor endurance. However, consideration of both studies suggests a link between investment in immune defenses and range expansion in the Cuban Treefrog invasion of Florida. Alternatively to an immune-mediated trade-off, it is possible that greater endurance is the result of other processes related to range expansion, such as, spatial sorting (Shine et al. 2011). In this study, we also made directional predictions regarding other traits, including sprint speed and growth rate. We did not expect all traits investigated to differ regionally; rather, we lacked an *a priori* basis to determine which traits were more likely to be under selection pressure at the invasion front. While further research is necessary to better understand the relationship between investment in immune defenses and range expansion, these results add to a growing body of literature highlighting the possible role of immune-based trade-offs in mediating rapid range expansion.

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LITERATURE CITED

Barton, K.A., Hovestadt, T., Phillips, B.L. & Travis, J.M.J. (2012) Risky movement increases the rate of range expansion. *Proceedings of the Royal Society B: Biological Sciences*, **279**, 1194–1202.

Brown, G.P., Phillips, B.L., Dubey, S. & Shine, R. (2015) Invader immunology: invasion history alters immune system function in cane toads (*Rhinella marina*) in tropical Australia (ed J Byers). *Ecology Letters*, **18**, 57–65.

Brown, G.P., Shilton, C., Phillips, B.L. & Shine, R. (2007) Invasion, stress, and spinal arthritis in cane toads. *Proceedings of the National Academy of Sciences*, **104**, 17698–17700.

Brown, G.P. & Shine, R. (2014) Immune response varies with rate of dispersal in invasive cane toads (Rhinella marina). *PLoS One*, **9**, e99734.

Burton, O.J., Phillips, B.L. & Travis, J.M.J. (2010) Trade-offs and the evolution of life-histories during range expansion: Evolution during range expansion. *Ecology Letters*, **13**, 1210–1220.

Chuang, A. & Peterson, C.R. (2016) Expanding population edges: theories, traits, and trade-offs. *Global Change Biology*, **22**, 494–512.

Colautti, R.I. & MacIsaac, H.J. (2004) A neutral terminology to define “invasive”species. *Diversity and distributions*, **10**, 135–141.

Cornet, S., Brouat, C., Diagne, C. & Charbonnel, N. (2016) Eco-immunology and bioinvasion: revisiting the evolution of increased competitive ability hypotheses. *Evolutionary Applications*, **9**, 952–962.

Crawley, M.J. (1987) What makes a community invasible? Colonization, Succession and Stability (eds A. J. Gray, M. J. Crawley & P. J. Edwards), pp. 429-453. Blackwell Scientific Publications, Oxford.

Gendron, A.D., Marcogliese, D.J. & Thomas, M. (2012) Invasive species are less parasitized than native competitors, but for how long? The case of the round goby in the Great Lakes-St. Lawrence Basin. *Biological Invasions*, **14**, 367–384.

Hillman, S.S., Withers, P.C., Drewes, R.C. & Hillyard, S.D. (2009) Ecological and Environmental Physiology of Amphibians. Oxford University Press, New York.

Keane, R.M. & Crawley, M.J. (2002) Exotic plant invasions and the enemy release hypothesis. *Trends in ecology & evolution*, **17**, 164–170.

Lee, K.A. & Klasing, K.C. (2004) A role for immunology in invasion biology. *Trends in Ecology & Evolution*, **19**, 523–529.

Llewellyn, D., Thompson, M.B., Brown, G.P., Phillips, B.L. & Shine, R. (2012) Reduced investment in immune function in invasion-front populations of the cane toad (Rhinella marina) in Australia. *Biological Invasions*, **14**, 999–1008.

Llewelyn, J., Phillips, B.L., Alford, R.A., Schwarzkopf, L. & Shine, R. (2010) Locomotor performance in an invasive species: cane toads from the invasion front have greater endurance, but not speed, compared to conspecifics from a long-colonised area. *Oecologia*, **162**, 343–348.

Lochmiller, R.L. & Deerenberg, C. (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos*, **88**, 87–98.

Mitchell, C.E. & Power, A.G. (2003) Release of invasive plants from fungal and viral pathogens. *Nature*, **421**, 625.

Myles-Gonzalez, E., Burness, G., Yavno, S., Rooke, A. & Fox, M.G. (2015) To boldly go where no goby has gone before: boldness, dispersal tendency, and metabolism at the invasion front. *Behavioral Ecology*, **26**, 1083–1090.

Phillips, B.L., Brown, G.P. & Shine, R. (2010) Concepts & Synthesis. *Ecology*, **91**, 1617–1627.

Skelly, D.K. (2007) The ailing invader. *Proceedings of the National Academy of Sciences*, **104**, 17561–17562.

Stearns, S.C. (1989) Trade-Offs in Life-History Evolution. *Functional Ecology*, **3**, 259.

Shine, R., Brown, G.P. & Phillips, B.L. (2011). An evolutionary process that assembles phenotypes through space rather than through time. *Proceedings of the National Academy of Sciences*, **108**, 5708–5711.

Torchin, M.E. & Lafferty, K.D. (2009) Escape from Parasites. *Biological Invasions in Marine Ecosystems* (eds G. Rilov & J.A. Crooks), pp. 203–214. Springer Berlin Heidelberg, Berlin, Heidelberg.

Torchin, M.E., Lafferty, K.D., Dobson, A.P., McKenzie, V.J. & Kuris, A.M. (2003) Introduced species and their missing parasites. *Nature*, **421**, 628.

Tracy, C.R., Christian, K.A., Baldwin, J. & Phillips, B.L. (2012) Cane toads lack physiological enhancements for dispersal at the invasive front in Northern Australia. *Biology Open*, **1**, 37–42.

White, T.A. & Perkins, S.E. (2012) The ecoimmunology of invasive species (ed A Dunn). *Functional Ecology*, **26**, 1313–1323.

Zera, A.J. & Harshman, L.G. (2001) The physiology of life history trade-offs in animals. *Annual review of Ecology and Systematics,* **32**, 95–126.

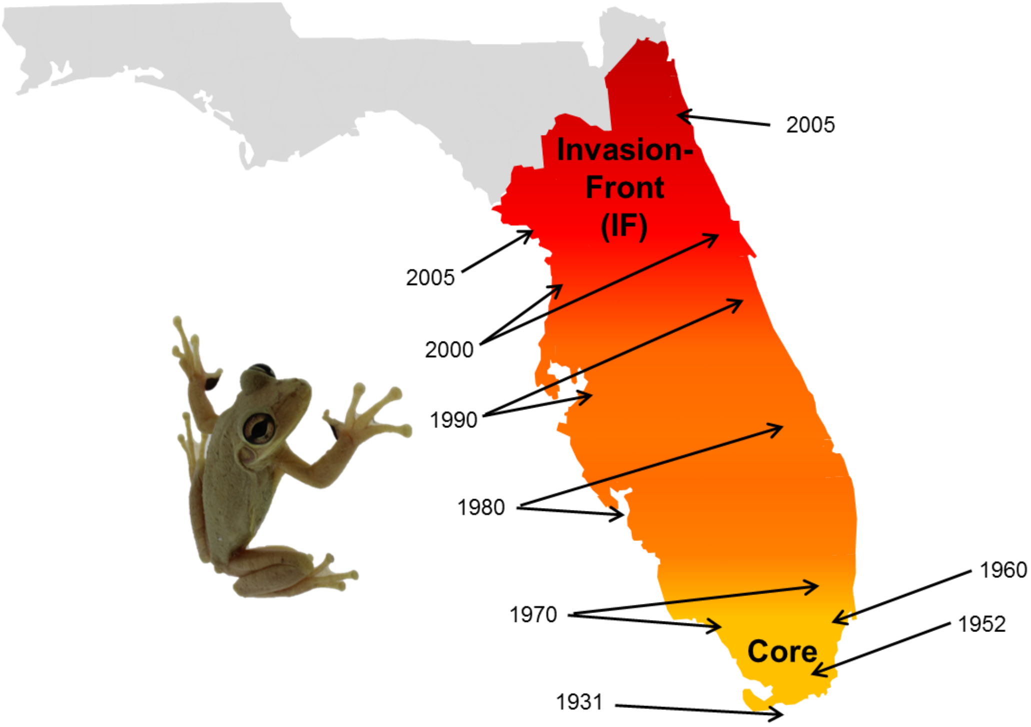

Figure 4 - 1.— Map showing the invasive distribution (colored region) of Cuban Treefrogs in Florida, USA. Darker colors indicate more recent colonization. Dates denote approximate first report of breeding populations of Cuban Treefrogs in areas indicated by arrows.

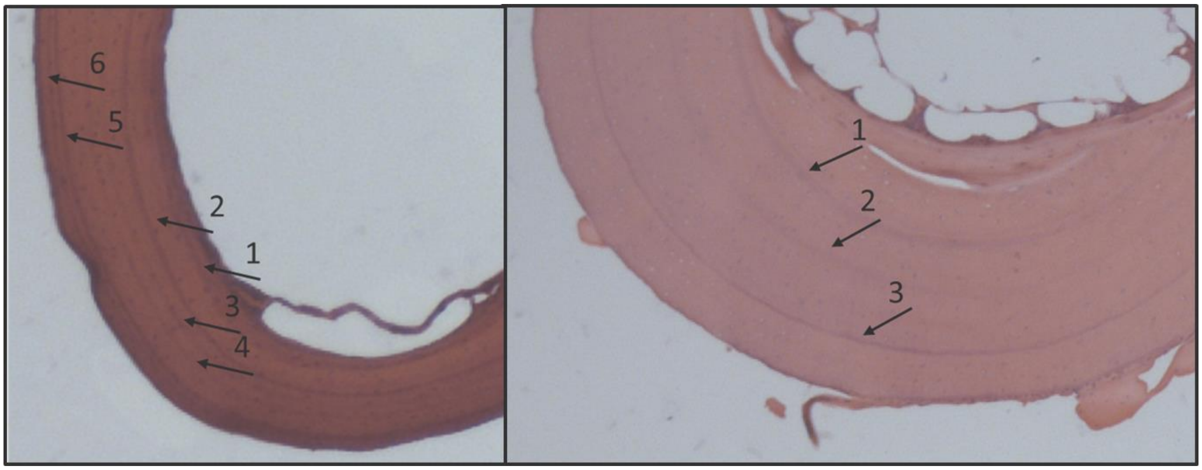

Figure 4 - 2.— Examples of hematoxylin-stained cross-sections of the femur of Cuban Treefrogs (*Osteopilus septentrionalis*) used in analysis of growth rate. Frogs were collected in core and invasion-front regions of Florida. A = six year old male at 32x magnification; B = three year old Female at 10x magnification. Arrows indicate lines of arrested growth.

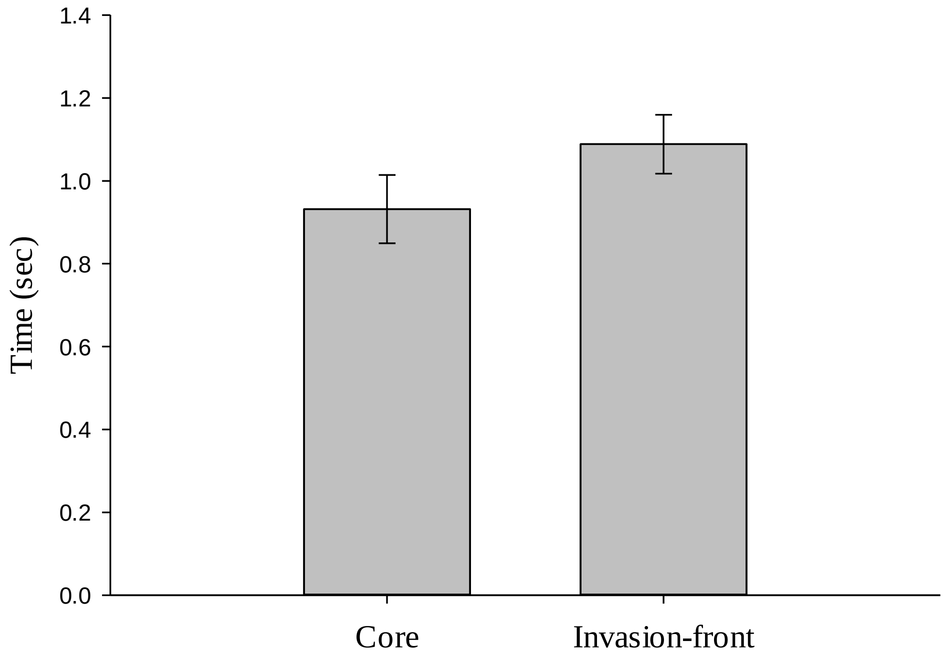

Figure 4 - 3.— Comparison of locomotor sprint speed of Cuban Treefrogs from core and invasion-front regions. Bars represent mean ± SE speed to move 25 cm. The fastest time from nine trails per individual was used in the analysis.

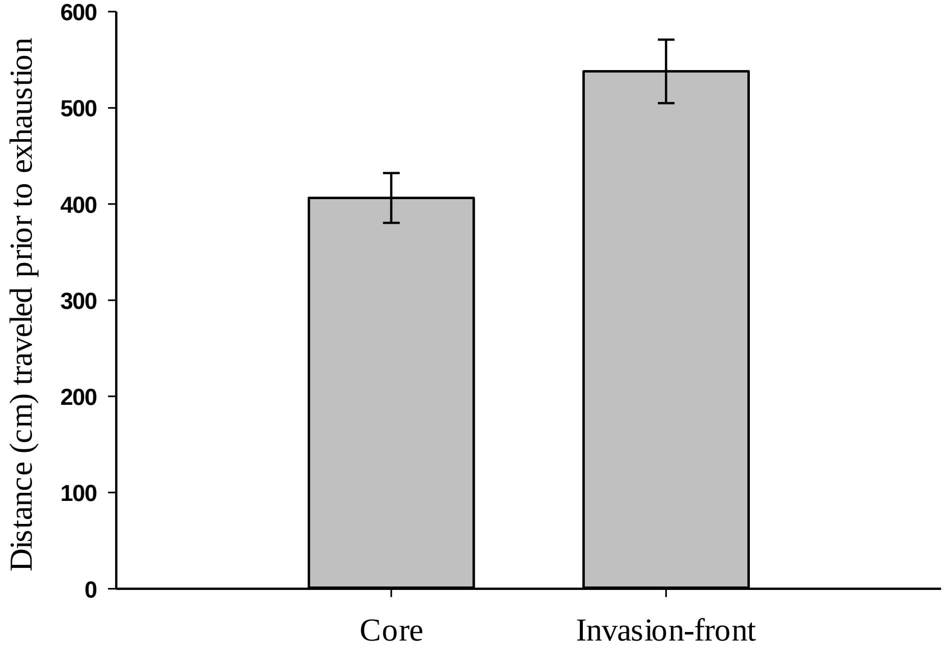

Figure 4 - 4.— Comparison of mean ± SE locomotor endurance of Cuban Treefrogs from core and invasion-front regions.

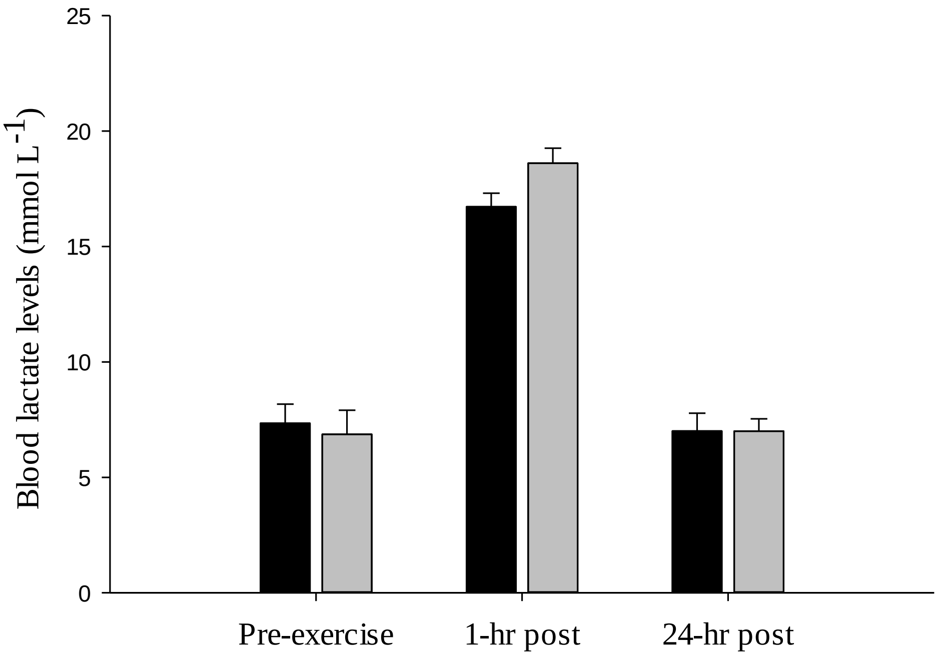

Figure 4 - 5.— Mean ± SE values of blood lactate levels of Cuban Treefrogs measured immediately prior to test of locomotor endurance (Pre-exercise), one hour post-exercise (1-hr post), and 24 hours post-exercise (24-hr post). Black bars represent the core region and gray bars represent the invasion-front region.

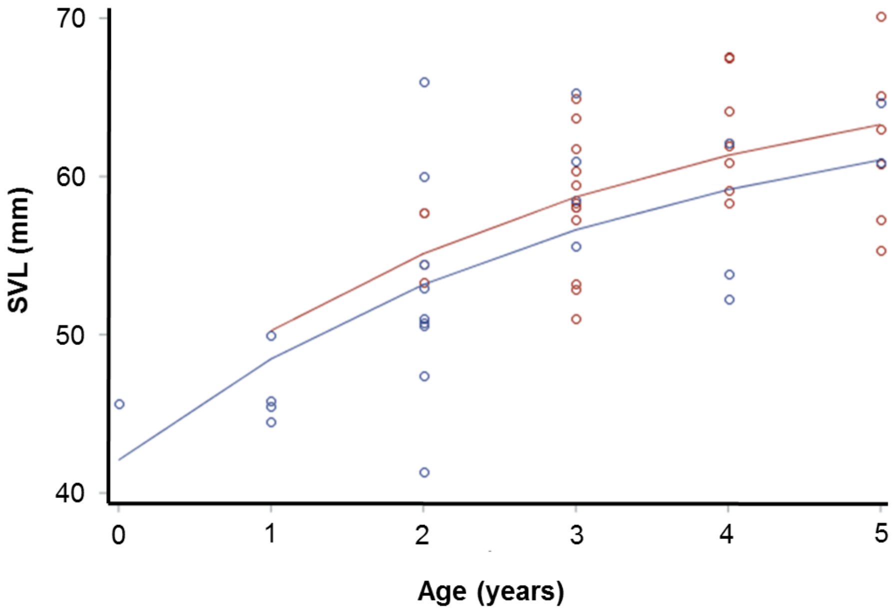

Figure 4 - 6.— Snout-vent length vs. age of female Cuban Treefrogs fit to von Bertalanffy growth curves. Lines represent growth curves determined for frogs from the core region (red line) and the invasion-front region (blue line).

CHAPTER V

CONCLUSIONS

Interest in the study of biological invasions is rapidly increasing, causing our understanding of invasion dynamics to be broadened and reshaped. In this dissertation, I address two aspects of invasion ecology that are understudied or unresolved. I investigated effects of an introduced species on native predators, and tested a prediction that life-history trade-offs may promote spread of non-indigenous species. The research presented herein focuses on Cuban Treefrogs (*Osteopilus septentrionalis*); however, the results of this research advance our general understanding of biological invasions and are intended to be broadly applicable.

Chapter 2 describes a series of experiments demonstrating Cuban Treefrogs likely act as an evolutionary trap for native Common Gartersnakes (*Thamnophis sirtalis*). Introduced species are recognized as a cause of evolutionary traps; however, there has been no effort to determine if characteristics of introduced species predict the likelihood of trap formation. Here, I suggest that Cuban Treefrogs fit a pattern associating novel chemical defenses with increased invasiveness. This idea is supported by native herbivore response to introduced plant species possessing defensive metabolites; plants suffer reduced damage from herbivores and are more likely to become invasive. Similarly, the success of the Cane Toad (*Rhinella marina*) invasion of Australia is largely attributed to possession of novel defensive toxins. In this chapter I suggest that an additional consequence of novel chemical defenses is an increased likelihood of evolutionary trap formation. Similar to Common Gartersnakes, I also suggest that other native predators may exhibit maladaptive predatory responses to Cuban Treefrogs.

Chapters 3 and 4 present evidence to support the idea that range expansion of non-indigenous species is promoted by immune-based trade-offs. There are several lines of reasoning as to why non-indigenous species may not benefit from vigorous immune defenses. Resources saved from suppressed immune responses may be allocated toward dispersal-related activities or traits. Specifically, chapter 3 documents reduced investment in innate immune responses by Cuban Treefrogs at the leading-edge of their expanding distribution. I found both a decreased metabolic response to an immune challenge and reduced bactericidal ability in frogs collected at the invasion front compared with those from a long-established region.

Chapter 4 compares several aspects of Cuban Treefrog dispersal ability in populations from a long-established region and the leading-edge region. Frogs from the leading-edge exhibited increased locomotor endurance coupled with an associated increase in blood lactate levels. Lactate levels in frogs from both regions returned to normal levels after 24 hrs. I failed to find a regional difference in frog sprint speed or growth rate. The ability to induce immune-based trade-offs may explain, in part, the ability of certain introduced species to quickly spread in their introduced range. Additionally, greater understanding of the role of trade-offs and dispersal ability may increase our ability to forecast responses of native species to environmental changes such as habitat alterations and global climate change.

Overall, this dissertation highlights the complicated interspecific interactions that can occur as a result of a single biological invasion. Additionally, this aspect of the dissertation outlines a new hypothesis that non-indigenous species possessing chemical defenses unique to their introduced range are more likely to cause an evolutionary trap for native predators. This dissertation also addresses intraspecific processes that may explain why some non-indigenous species are able to move beyond the establishment stage and become invasive. Further, for the first time, the role of immune-based trade-offs in relation to range expansion were assessed in wild-caught individuals. Because we did not use captive reared frogs, we were able to capture potential facultative responses to both biotic and abiotic factors that may have influenced immune function and dispersal ability.