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A Population Genetic Investigation of the Reticulated Flatwoods Salamander (Ambystoma bishopi) on Eglin Air Force Base

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A POPULATION GENETIC INVESTIGATION OF THE RETICULATED FLATWOODS SALAMANDER (AMBYSTOMA BISHOPI) ON EGLIN AIR FORCE BASE

by

ALEXANDER WENDT

(Under the Direction of James H. Roberts)

ABSTRACT

The reticulated flatwoods salamander (RFS) is an endangered salamander with a unique life history. One of the largest known, best studied refuges for RFS is found on Eglin Air Force Base, and these RFS have been sampled and managed extensively since 2010. My thesis seeks to better understand RFS by using genetic techniques to address several unknowns, including: 1) determining the population structuring of RFS and the manageable units for species conservation, 2) estimating the size and status of populations, 3) understanding dispersal of RFS and factors that influence this, 4) exploring the breeding biology and recruitment patterns of RFS and how they affect population sizes, and 5) drawing general conclusions about RFS population biology and recommendations for future management. The first, second, and third objectives are addressed in Chapter 1, by analyzing variation at nuclear microsatellite genetic markers within and among known, extant breeding populations on Eglin to determine the genetic structuring of RFS as well as landscape factors that would influence dispersal between the breeding ponds. The fourth objective is addressed in Chapter 2 and utilizes the same microsatellite markers but

focuses on two ponds and two years in which extensive sampling of adult and larval RFS was conducted. The fifth objective is addressed in the General Conclusion section in which I use data from both chapters to provide management suggestions that can be utilized both on Eglin and elsewhere.

INDEX WORDS: Reticulated flatwoods salamanders, *Ambystoma bishopi*, Population genetics, Landscape genetics, Wildlife management

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Figure 2.6 – Frequency of deduced half-sib family sizes by year (Y1= 2013-2014; Y2= 2015-2016) and pond (P4= pond 4; P5= pond 5).

GENERAL INTRODUCTION

Amphibian Global Patterns of Diversity

Amphibians emerged from early tetrapods 350 million years ago during the Devonian period and now consist of 7469 species distributed globally (AmphibiaWeb 2015). New taxa are being discovered and described every day. The class Amphibia consists of three orders, including Anura (frogs and toads), Caudata (salamanders and newts) and Gymnophiona (caecilians). Of these three orders, Anurans constitute a majority with 6584 (88%) classified species. Next is the order Caudata with 680 (9%) species and then Gymnophiona with 205 (3%) species listed.

The majority of these species are found in equatorial regions across the globe with the highest biodiversity concentrated within the rainforests of South America (Gouveia *et al.* 2013). In fact, amphibian species richness has a strong positive correlation with both temperature and precipitation and these two factors, when accounting for biogeographic realm, explain 88% of the variation in global amphibian richness (Buckley and Jetz 2007). None the less, amphibians have adapted to survive in a variety of climates, from deserts to rainforests, despite the fact they are ectothermic and dependent on environmental moisture for physiological requirements. Many amphibians rely primarily on behavior to regulate body temperature and body moisture with a few exceptions. Amphibians can even be found in some of the northern most latitudes due to special adaptations. The wood frog (*Rana sylvatica*) has adapted to live in the northern area of North America, even in the Arctic Circle. These amphibians are extremely hardy and can survive freezing of 65-70% of their body water. Subarctic populations have been shown to survive temperatures as low as -16°C due to specialized proteins in their blood (Costanzo and Lee 2013a;

Costanzo *et al.* 2013b; Reynolds *et al.* 2014). Some of the immense biodiversity within the class is due to physiological attributes, including the synthesis of poisons, and behavioral attributes, including parental care of offspring (Ringler *et al.* 2013). These evolutionary adaptations have allowed amphibians to occupy several niches even when water is scarce and conditions are not always ideal for a poikilothermic organism.

Most amphibians require water at some stage in their life cycle, which is almost always an aquatic larval stage of some type. The array of water dependence is vast with some species requiring an aqueous environment throughout their entire life while other species need minimal water due to their behavioral and physiological ability to conserve water. Compared to reptiles and mammals, amphibians have a greater number of reproductive modes which include direct development and viviparity, which allow them to exist in many environments (Duellman and Trueb 1994; Kupfer *et al.* 2004).

With this vast amount of biodiversity, amphibians are an integral part of many food webs across the globe. This is mainly due to the fact that amphibians are a mid-trophic level class and they serve as both a food source as well as a predator, and usually encompass a significant portion of the vertebrate biomass in several ecosystems, especially in forests and wetlands (Burton and Likens 1975a; Gibbons *et al.* 2006; Hamer and McDonnell 2008; Clipp and Anderson 2014). Given that amphibians inhabit terrestrial, aquatic, and fossorial habitats, they are vital in connecting energy transfer between these environments by being both a food source as well as a predator (Burton and Likens 1975b; Pough 1980; Clipp and Anderson 2014).

A Fauna in Decline

Over the past two decades, approximately 168 amphibian species have gone extinct. Further, nearly one-fourth (26.7%, 1994 species) of the world's amphibians are listed as vulnerable or higher on the IUCN redlist (IUCNredlist 2015). This is higher than either birds (13.8%, 1375 species) or mammals (21.8%, 1197 species) (Stuart *et al.* 2004; Blaustein *et al.* 2011; IUCNredlist 2015). Approximately 2500 species of amphibians have declining populations as of 2011 (Kiesecker 2011).

Infectious diseases, hazardous chemicals, invasive species, habitat loss, and climate change are perceived to be the primary factors driving amphibian declines globally (Daszak *et al.* 2003; Davidson 2004; Daszak *et al.* 2005; Cushman 2006; Collins 2010; Salice 2012). There are two major diseases within amphibian communities that are reducing amphibian populations globally. Ranavirus is an infection that severely affects amphibians, with eight known strains that can infect multiple species of frogs and salamanders (Collins 2010). Symptoms of ranavirus include edema (swelling) as well as erythema (skin reddening) in the hindlimbs. Ranavirus has caused epidemics in frogs and salamanders in North America as well as amphibians in Europe, South America, Australia, and Asia (Carey *et al.* 2003). Since 1997, over 20 species across five families have been reported reported in die-off events across North America (Torrence *et al.* 2010). In a study performed by Hoverman et al. (2012), it was found that, 83% of ponds tested positive for the detection of ranavirus. Permanent ponds have a higher persistence of ranavirus whereas those ponds that experience pond drying do not since the drying inactivates the virus (Brunner *et al.* 2007). Ranavirus is most prevalent in species that are primarily aquatic and although all life stages can be affected, larvae appear to be most susceptible to the disease (Miller *et al.* 2011; Stark *et al.* 2014).

Batrachochytrium dendrobatidis (*Bd*) is a parasitic fungus that has become an epidemic spreading around the globe. It causes chytridiomycosis and has been linked to amphibian declines since 1998 (Berger *et al.* 1998). Chytrid kills amphibians by interfering with the integrity of the skin which is important in amphibians for gas exchange, hydration, electrolyte balance, and protection from other diseases (Voyles *et al.* 2009; Rosenblum *et al.* 2012). *Bd* has been detected on every continent where amphibians occur and there is a strong correlation of species experiencing enigmatic declines in areas where *Bd* has been detected (Olson *et al.* 2013). It has been shown to transmit from one host to another easily. Chytrid fungus has varying effects, from no clinical disease to 100% mortality depending on the host and the host's microenvironment (Collins 2010). *Batrachochytrium dendrobatidis* also has been shown to be carried via other taxa including on the keratinous tissue on the feet of geese (Garmyn *et al.* 2012). Its spread and prevalence within amphibian communities is of growing concern and the infection has been aided by other stressors, especially human encroachments.

Chemicals such as pesticides can have both direct and indirect effects on amphibian populations. Directly, pesticides can cause both lethal and sub lethal effects to larval and adult forms of amphibians alike. Pesticides have been shown to cause changes in behavior, including reduced locomotion (Relyea 2010). This can have an impact on the feeding and growth of the individuals and subsequently the wellbeing of the population as a whole. Pesticides can interact with the endocrine system of amphibians. Atrazine, which is used to prevent pre and post emergence broadleaf weeds, is known to make genetically male frogs hermaphroditic or even fully feminized (Hayes *et al.* 2006). Indirectly, pesticides can have negative implications on amphibians by disrupting the food web in a way that affects other taxon that larvae are reliant on (Relyea 2010). Malathion, an organophosphate used as an insecticide, has serious negative

effects on zooplankton levels in an ecosystem. With a decrease in the zooplankton levels, phytoplankton levels increase and subsequently choke out periphyton which is the main staple for tadpoles (Relyea and Diecks 2008; Blaustein *et al.* 2011).

Invasive species also have a drastic impact on amphibian populations, especially on recruitment of larvae to the age of reproduction. Amphibians are especially sensitive to the introduction of new species since their life-cycle involves both aquatic and terrestrial stages, with the aquatic eggs and larvae being consumed in high amounts (Gillspie 2001; Polo-Cavia and Gomez-Mestre 2014). Young larvae are unable to detect chemical cues from invasive species and are thus unable to take the appropriate anti-predatory measures needed (Stauffer and Semlitsch 1993; Kats 1998; Chivers and Mirza 1998; Polo-Cavia and Gomez-Mestre 2014). For example, the North American red swamp crayfish, *Procambarus clarkia*, is an invasive species that was introduced to Spain in the 1970's and has caused massive declines of native amphibian populations due to intense predation of eggs and larvae (Ficetola *et al.* 2012; Polo-Cavia and Gomez-Mestre 2014). These naïve tadpoles are unable to detect the chemical cues of *P. clarkia* due to their lack of coevolutionary history, and thus are easy prey for the crayfish. Even if the larvae can detect the invasive predator, the effects of having a non-native species can result in reduced activity and slow growth rates, which can have an impact on other species as well (Miner *et al.* 2005; Arribas *et al.* 2014). Invasive predators are not the only trophic level that can affect amphibians, but also invasive plants. Amur honeysuckle (*Lonicera maackii*) is an Asian shrub that has invaded much of the eastern U.S. and produces phenolic compounds (tannins) in the leaves and roots that are toxic to native flora (Hutchinson and Vankat 1997; Bartuszevige *et al.* 2006; Dorning and Cipollini 2006; Cipollini *et al.* 2008; Watling 2010; Watling *et al.* 2011). These compounds can be toxic to developing frogs and salamanders as well, especially those in

small vernal pools that are obligate gill breathers, and can cause a behavioral shift in larvae that are not obligate gill breathers forcing them to surface more and thus be more exposed to predators (Maerz *et al.* 2005; Watling *et al.* 2011).

Increased urbanization, agriculture and roadways are common outcomes of human population growth and often result in the loss and fragmentation of amphibian habitat. Fragmentation decreases the ability for individuals to migrate between suitable habitats, as well as direct mortality from habitat clearings and roads (Vos and Chardon 1998; Gibbs 2000; Guerry and Hunter Jr. 2002; Riley *et al.* 2004; Stuart *et al.* 2004; McKinney 2006; Harper and Semlitsch 2007; Eigenbrod *et al.* 2008; Hamer and McDonnell 2008; Wilson and Hopkins 2013; Clipp and Anderson 2014). The encroachment on habitat may force populations into closer proximity which can cause an increase in the spread of disease. For example, the trematode parasite *Ribeiroia ondatrae*, which causes limb deformities in frogs, have been found to be more widely distributed in urban and otherwise human-modified environments compared to natural habitats (Holland *et al.* 2006). Anthropogenic changes to habitat also cuts off populations from others, causing reduced immigration and a decrease in genetic diversity, both of which have negative effects (Cushman 2006). This can eventually lead to inbreeding, loss of adaptive potential and local extinctions for the species.

Climate change is another major driver of ecological change, and is progressing at an increased rate due to human impact. Over the past 30 years, the rate of increase in global temperature has been greater than the past two thousand years (Mann *et al.* 2008). Within the past century, the Earth has experienced a mean increase of 0.6 °C and it has had an effect on amphibians (Parmesan 2006; Duarte *et al.* 2012). The increase in temperature has a great effect on precipitation, reducing the amount of rainfall that accumulates, which is important for

maintaining breeding ponds as well as initiating breeding events. Multiple studies have observed a shift in the reproductive timing of many pond-breeding amphibians from Europe, Asia, and North America (Todd *et al.* 2011). These shifts can cause severe consequences to the population in that there can be interactions with species not usually occupying breeding sites at the same time, causing competition (Beebe 2002). For example, earlier arrival of *Bufo calamita* leads to interference with the tadpoles of *Rana temporaria* leading to increased competition and even predation (Beebee, 2002). Reduction in precipitation also has a significant effect on amphibians with long larval periods and the premature drying of breeding ponds can lead to reduced recruitment as well as producing individuals with decreased body size (Li *et al.* 2013). Increasing temperatures also affect amphibian metabolic rates, causing and increased need for food which results in an increase in foraging with a higher chance of desiccation and disease and a decrease in fecundity and recruitment (Martin *et al.* 2010). With climate change becoming more rapid as time persists, amphibians will be unable to adapt and a decline in species is expected with the increase in temperatures worldwide, especially those species with low upper thermal tolerances.

Diversity and Imperilment of Amphibians in the Southeast United States

The Southeastern United States (defined here as: Alabama, Florida, Georgia, Kentucky, Louisiana, Mississippi, North Carolina, South Carolina, Tennessee, and Virginia) contains a large portion of the country's amphibian diversity, with approximately 140 of the 295 species found in the country (Bartlett and Bartlett 2006; Graham *et al.* 2010; IUCNredlist 2015). Of these 140 species, nearly 75% are salamanders in the order caudata. Caudates are represented by seven different families including Ambystomatidae (mole salamanders), Amphiumidae (amphiumas), Cryptobranchidae (giant salamanders), Plethodontidae (lungless salamanders),

Proteidae (aquatic salamanders), Salamandridae (newts), and Sirenidae (sirens). The Southeast United States is a global diversity hotspot for salamanders with 17% of the world's species (Mitchell and Gibbons 2010). Many of the species of salamanders within the Southeast are narrow endemics, which increases vulnerability to extinction. For example, the Peaks of Otter Salamander are found only in Bedford and Botetourt counties in the Blue Ridge province in Virginia and the Pigeon Mountain Salamander is found only on the eastern side of Pigeon Mountain in northwestern Georgia. Within the region, fifteen species of amphibians are listed as vulnerable, five species are listed as endangered, and one species is listed as critically endangered (IUCNredlist 2015). Habitat loss and climate change are the leading causes of population reduction within the Southeast region (Tuberville *et al.* 2005; Milanovich *et al.* 2010; Walls *et al.* 2014). Urbanization and farming within and near habitat for amphibians has caused both direct loss of habitat and fragmentation of amphibian habitat.

Pond Breeding Salamanders in the Southeastern United States

Pond breeding salamanders are a good example of amphibians with complex life cycles that are particularly vulnerable to anthropogenic impacts. These species must migrate to breeding ponds from the surrounding upland habitat, which in the Southeast usually consists of either mixed hardwood forest, pine forests or wetland landscapes. Many of these landscapes are diminishing across the Southeastern region due to anthropogenic land conversion. During the 1800s, extensive sections of old-growth forests were removed for the creation of cropland with much still being managed for plantation based timber production today (Sharitz 2003; Wyman 2003; Fairman *et al.* 2013). Wetland habitats have also been reduced by 20-50% in the Southeast since 1780 (Dahl 1990). These habitats are vital since many pond breeding salamanders are

presumed to be philopatric and will only travel to breeding ponds during the correct environmental conditions, for example warm rainy nights (Selitsch and Bodie 1998; Kinkead and Otis 2007). The breeding ponds can range in both size and hydroperiod, from highly ephemeral to more permanent, and these parameters determine the type and number of species that use the ponds (Wilbur 1980; Wilbur 1990; Semlitsch *et al.* 2015; Chandler *et al.* 2016). Ambystomatidae, Plethodontidae and Salamandridae are all represented by species that use ponds and vernal pools for breeding purposes but are terrestrial during non-mating periods, usually inhabiting moist burrows (Mitchell and Gibbons 2010). The breeding season for each species varies widely, usually in the range of late fall to late spring with several species having overlap in breeding seasons causing larval competition within breeding ponds.

Time needed for larval metamorphosis is highly variable between species as well, with some species needing only a few weeks and others several months to develop into terrestrial juveniles. These juveniles then leave the breeding pond and are presumed to be the primary dispersers between populations (Gamble *et al.* 2007). Most newly metamorphosed individuals, however, stay close to the natal pond. Scott *et al.* (2013) found that 79% of marbled salamanders (*Ambystoma opacum*) remain within 90 meters of the breeding pond and only 2% move beyond 332 meters. Getting to suitable habitat can be difficult for juveniles no matter what the distance, with high mortality due to desiccation (Rothermel and Luhring 2005), predation (Rittenhouse *et al.* 2009), energy depletion (Scott *et al.* 2007) and density effects (Harper and Semlitsch 2007; Pittman and Semlitsch 2013). Rothermel and Semlitsch (2006) found that 83% of spotted salamanders (*Ambystoma maculatum*) do not survive 1 year after metamorphosis due to the causes listed above.

The life after leaving the breeding pond is poorly known for pond breeding species, particularly the mole salamanders (family Ambystomatidae) which live underground a majority of their adult lives. These salamanders find burrows within the surrounding landscape where they presumably consume various invertebrates and migrate to breeding ponds during the winter months on rainy nights. After the breeding season, adults return to their burrows and are seldom seen if at all until the next breeding season (Mitchell and Gibbons, 2010). However, the basic biology of a number of species has been poorly studied within this family with much still to be learned.

Flatwoods Salamander

Against the backdrop of decline and the need for better natural history information, I will focus on the dispersal and reproductive ecology of reticulated flatwoods salamanders, a pond-breeding ambystomatid that exemplifies the family. Flatwoods salamanders were recently divided into two distinct species, the frosted (*Ambystoma cingulatum*) and reticulated (*A. bishopi*) salamander. Both species are fossorial in nature and inhabit crayfish burrows near breeding ponds (Bevelhimer *et al.* 2008). They live in mesic flatwoods and savannahs consisting of longleaf pine (*Pinus palustris*) and wiregrass (*Aristida stricta*), a declining ecosystem of the Southeast United States (Palis 1997a; Bevelhimer *et al.* 2008). The geographic division of the two species is the Apalachicola River (Pauly *et al.* 2007). The frosted flatwoods salamander is currently listed as federally threatened while the reticulated flatwoods salamander is listed as federally endangered by the U.S. Fish and Wildlife Service as of February 10, 2009 (USFWS 2015).

Currently, the two species occupy 27,423 acres of land across three states designated as critical habitat by the U.S. Fish and Wildlife Service. The reticulated flatwoods salamander (RFS) is found in the Southwest region of Georgia and the panhandle region of Florida and consists of 22 populations (Pauly *et al.* 2007). Approximately 4,453 acres across Florida and Georgia are designated as critical habitat for RFS. An additional 2,881 acres are located on military lands in Florida are excluded from this designation due to the fact that the military has Integrated Natural Resource Management Plans (INRMP) intended to protect the salamander and its habitat. A majority of the land (1,880 acres) is found on Eglin Air Force Base located in the panhandle region of Florida.

Flatwoods salamanders have a complex life-cycle dependent upon the natural seasonal variation of longleaf-wiregrass ecosystems. Between October and December, flatwoods salamanders emerge from their burrows and migrate to deposit their eggs terrestrially in a moist microhabitat located in the basin of a dry breeding pond (Anderson and Williamson 1976). Besides the two species of flatwoods salamander, the only other species of ambystomatid salamanders that exclusively lays its eggs terrestrially is the marbled salamander, *Ambystoma opacum* (Petranka 1998). The eggs begin to develop immediately but only hatch after the rains have initiated the hatching response which may take weeks or months to happen. Typically, hatching occurs between the months of December to February (Anderson, and Williamson 1976; Palis 1995; Palis 1997b; Bevelhimer *et al.* 2008). Once the eggs have hatched, it takes 11 to 18 weeks for larval development to complete with metamorphosis believed to be initiated by the drying of the breeding pond (Palis 1995). During this time, larvae feed on aquatic invertebrates including isopods (*Caecidotea*), amphipods (*Crangonyx*), cyclopoid copepods and cladocerans (Whiles *et al.* 2004). Once these individuals metamorphose and leave the breeding pond, they

disperse out into the surrounding landscape and occupy crawfish burrows. Male flatwoods salamanders take 1 year to reach sexual maturity while female flatwoods salamanders typically take about 2 years to reach sexual maturity (Palis 1997b). Flatwoods salamanders have shown emigration orientation in the direction of immigration, which may show the ability for the species to home to and from specific breeding ponds as well as specific terrestrial retreats (Palis 1997a). The basic biology of the species as a whole, especially the time spent within these burrows, is not well known with less information available than most other Ambystomatid species (Anderson and Williamson 1976). This secretive nature of reticulated flatwoods salamanders makes population estimates difficult with the total adult population size presumably at least 1,000, but the actual number is unknown (IUCNredlist 2015). To better understand and manage this species, additional information on the demography and breeding biology is needed.

Eglin Air Force Base Overview

Eglin Air Force Base contains the majority of known, extant RFS populations and habitats. Eglin is the largest Air Force installation in the world, with 464,000 acres of land, as well as 120,000 square miles of water ranges. Within this vast area, there are 106 rare, threatened and endangered species of plants and animals, 63 of which are considered to be rare globally. The landscape itself consists of 34 distinct natural community types, many of which are dependent on periodic fires to maintain biodiversity (Secretary of Defense 2013). Figure 0.1 shows all the natural communities' locations on the base, as well as other important distinguishing areas. This includes the old-growth longleaf pine habitat, of which Eglin is home to the largest contiguous acreage of longleaf pine in the world.

Longleaf pine is the prime habitat for the reticulated flatwoods salamander and Figures 0.2a and 0.2b show confirmed and potential breeding ponds for the species. Work has been done by the Eglin AFB Integrated Natural Resource Team, U.S. Fish and Wildlife Services, and Virginia Tech in association with the reticulated flatwoods salamanders' habitat and breeding ponds on the base. Figure 0.3 shows 27 ponds that have been monitored for RFS occupancy on Eglin by the organizations listed above. Ponds 4 and 5 have been more intensely sampled since 2010 and have also had drift fences installed to collect individuals leaving the breeding sites. Wetland analysis as well as egg laying site preference studies have been performed extensively on Eglin (Gorman *et al.* 2014).

Eglin Air Force Base has 34 distinct habitat types (Figure 0.2a and 0.2b) spread throughout the base. Several habitats are fragmented by water systems and roads that run throughout the base that could hinder the dispersal of flatwoods salamanders on the base. Flatwoods salamanders have been observed to travel more than 1700 meters away from breeding ponds (Ashton Jr. 1992) though a more recent study documented flatwoods salamanders traveling about 300-500 meters to the closest breeding pond (Means *et al.* 1995). It is believed that flatwoods salamanders have the ability to home to and from a particular terrestrial retreat meaning that individuals most likely visit the same breeding pond each breeding season (Palis 1997a). However, some individuals do disperse to other breeding ponds, though how these individuals find these other ponds is still an unknown. One belief is that flatwoods salamanders are able to sense minute changes in topography and can detect where a breeding pond will be based on this (Gorman *et al.* 2014). However, this theory has not been vigorously analyzed.

Another possibility is that the flatwoods salamanders use herbaceous vegetation as an indicator for breeding wetland habitat (Gorman *et al.* 2009). The habitat that flatwoods

salamanders use consists of longleaf pine (*Pinus palustris*) and wiregrass (*Aristida stricta*) (Palis 1997a; Bevelhime *et al.* 2008). In 2013, the National Resources Conservation Team restored 12,200 acres of longleaf pine habitat by removing 150,000 tons of invasive sand pine and 250 acres of new longleaf habitat was created (Secretary of Defense Environmental Awards 2013). This habitat type is dependent on fire during the growing season, which decreases the amount of woody vegetation from occurring and encroaching on the natural herbaceous vegetation (Kirkman 1995). However, prescribed burning on Eglin AFB is implemented during the dormant (winter) season since fires are easier to control (Bishop and Haas 2005). Burning during this time is ineffective in promoting the growth of the native, natural vegetation for two main reasons. First, the winter dormant season overlaps with the recharge of water into the wetlands which inhibits fire from entering the wetlands (Gorman *et al.* 2009). Secondly, dormant winter fires may promote re-sprouting of woody vegetation and increase the density of shrubs in the wetland that subsequently choke out the wiregrass which rely on growing season fires (Outcalt 1994; Drewa *et al.* 2002). Loss and alteration to habitat is considered a main threat and a cause of population decline for flatwoods salamanders (Means *et al.* 1996; Gorman *et al.* 2009).

Purpose of this Study

My thesis seeks to better understand RFS by using genetic techniques to address several unknowns including: 1) Determine the population structuring of RFS and the manageable units for species conservation, 2) Estimate the size and status of populations, 3) Understand dispersal of RFS and factors that influence this, 4) Recognize breeding biology and recruitment of RFS and how this affects population sizes, 5) Draw general conclusions on population declines and provide recommendations for future management. The RFS on Eglin AFB allow for an in depth

look at the species on Eglin. The first, second and third objective are addressed in Chapter 1 by using 9 nuclear microsatellite markers to determine the genetic structuring of RFS as well as landscape factors that would influence dispersal between the breeding ponds. The fourth objective utilizes the same microsatellite markers but focuses on two ponds and two years in which extensive sampling of adult and larval RFS was conducted. Pedigrees were then formed to better address the question. The fifth objective is addressed in the General Conclusion section in which I use data from both chapters to design management plans that can be utilized both on Eglin and elsewhere.

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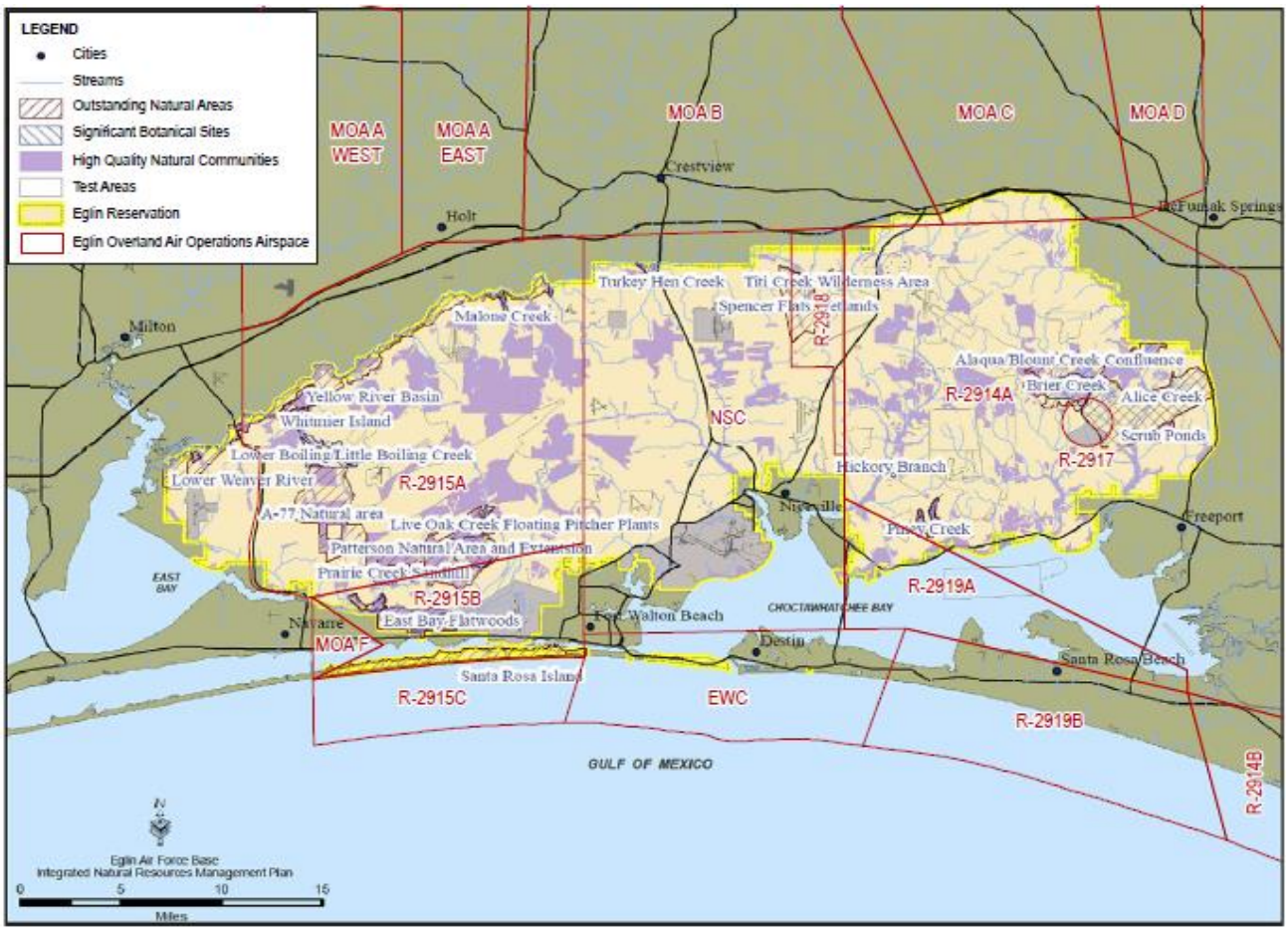

Figure 0.1 – Natural community sites on Eglin AFB. Source: Eglin AFB Integrated Natural Resources Management Plan (U.S. Air Force, 2012).

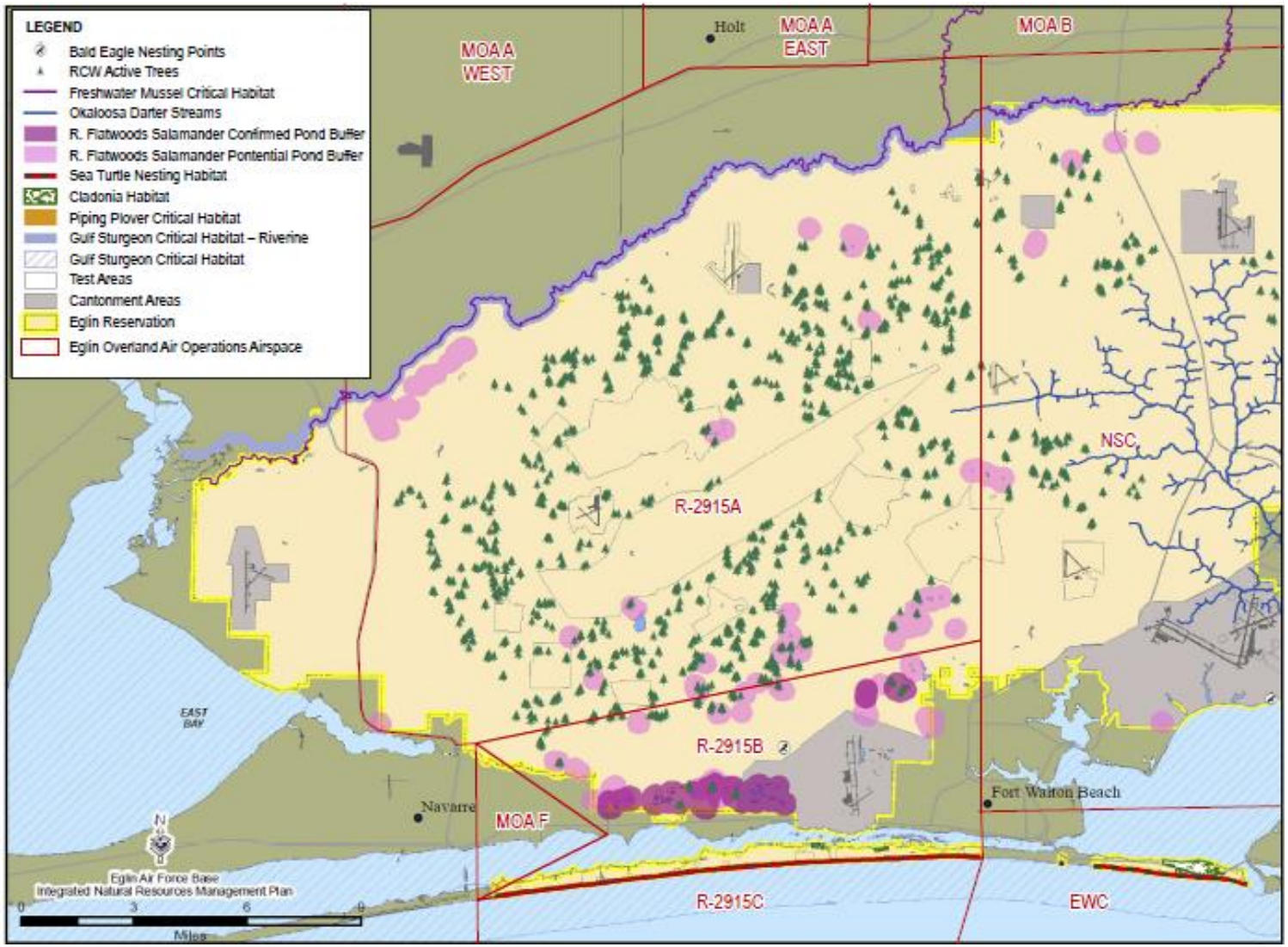

Figure 0.2a – Species and Habitats on Eglin AFB (West). Source: Eglin AFB Integrated Natural Resources Management Plan (U.S. Air Force, 2012).

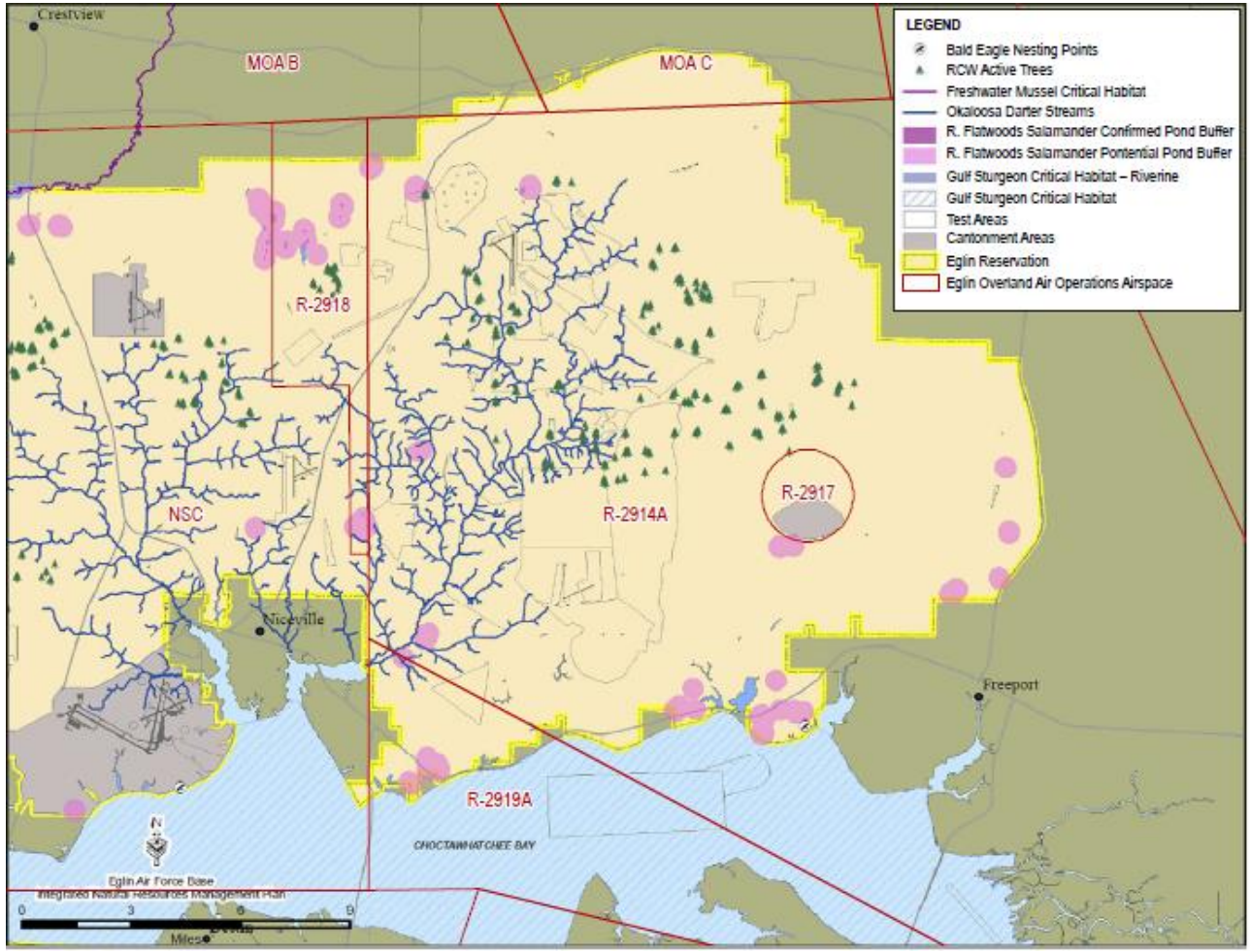

Figure 0.2b – Species and Habitats on Eglin AFB (East). Source: Eglin AFB Integrated Natural Resources Management Plan (U.S. Air Force, 2012).

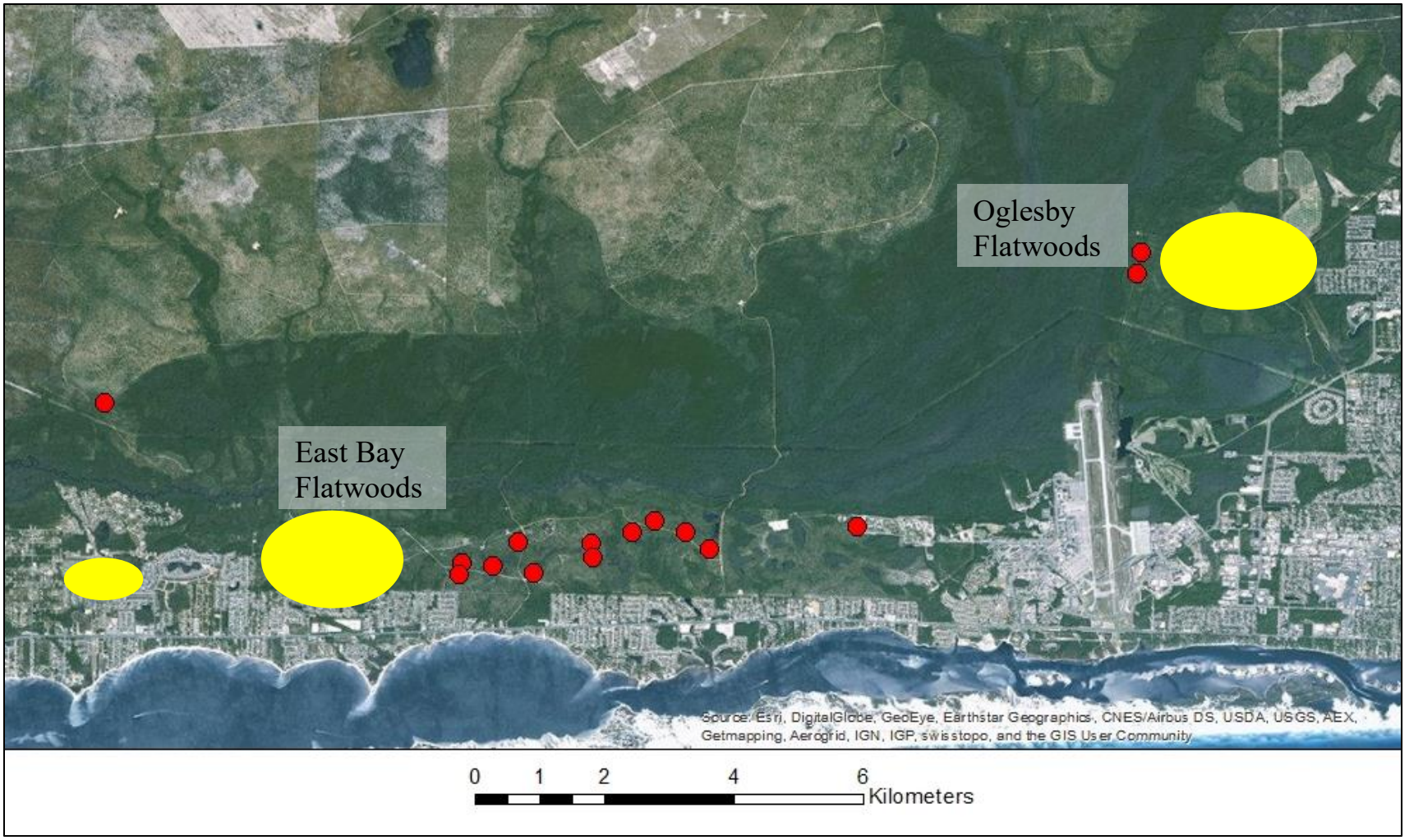

Figure 0.3 – Approximate locations of breeding pond sites for reticulated flatwoods salamanders on Eglin Air Force Base. Yellow ovals show locations of extant populations (6 in East Bay, 6 in Oglesby, and 1 to the west of East Bay) and red dots show locations of ponds presumed to be extirpated since 2006.

CHAPTER 1: FACTORS INFLUENCING POPULATION STRUCTURE AND GENE FLOW OF RETICULATED FLATWOODS SALAMANDERS (*AMBYSTOMA BISHOPI*) ON EGLIN AIR FORCE BASE

Abstract

Population structure and connectivity can be influenced by various characteristics of the landscape, including those stemming from both natural and anthropogenic disturbances. Pond-breeding amphibians exhibit complex life-cycles in environments easily fragmented by human activities. Better understanding of these influences could help improve conservation activities for such species. In this study, I delineated the population structure of an endangered pond breeding salamander, the reticulated flatwoods salamander (*Ambystoma bishopi*), on Eglin Air Force Base, then addressed questions about the size and connectivity of delineated populations, and landscape factors regulating gene flow among them. I analyzed 613 larval and metamorphic individuals using 9 microsatellite nuclear DNA markers. The largest component of genetic variation was among three spatially disconnected regions, yet even ponds spatially close to each other (< 1 km) typically were genetically differentiated, indicating each breeding pond functions as a semi-independent local population. Effective population sizes were less than 50 individuals in most ponds, potentially indicating a need for genetic restoration programs. Land cover, especially urbanization, was the landscape factor most associated with restricted gene flow between populations, whereas elevation and slope had less influence on gene flow. Conservation efforts should focus both on increasing population sizes and maintaining or enhancing habitat quality within and between breeding ponds.

Introduction

Terrestrial landscapes are complex mosaics of habitats that vary in their suitability to organisms. This patchiness places ecological constraints on the distribution of the animals inhabiting them (Forman and Gordon 1986; Turner 1989; Johnson *et al.* 1992). As a result, regional metapopulations tend to be spatially subdivided into local subpopulations occupying patches of suitable habitat, separated from each other by a matrix of less-suitable habitat. These less-suitable areas mediate population dynamics, dispersal, and gene flow that occur between the suitable habitats (Gonzalez *et al.* 1998; Spear *et al.* 2010; Kershenbaum *et al.* 2014).

On the one hand, population structuring and mediated gene flow can benefit the metapopulation as a whole. For example, population structuring can decrease the overall extinction risk, increase standing population genetic diversity, and promote local adaptations (Slatkin 1987; Storfer *et al.* 1999; Hill *et al.* 2002; Zamudio and Wieczorek 2007; Hendrick 2009; Roberts *et al.* 2016). Amphibians have patchy distributions due to their habitat specificity and their various physiological requirements which link distinct environments together for breeding, larval development and adult survival (Dunning *et al.* 1992; Stebbins and Cohen 1995; Pope *et al.* 2000; Zamudio and Wieczorek 2007). Local extinction and recolonization events are quite common in amphibian species (Wilbur 1984) and population structuring allows those local dynamics to occur without having a drastic effect on the metapopulation as a whole (Paine 1988; Wegner and Merriam 1990; Merriam and Wegner 1992; Villard *et al.* 1992; Fahrig and Merriam 1994). With the rise of diseases that affect amphibians, like *Batrachochytrium dendrobatidis* (*Bd*), population structuring may also be helpful in slowing the progression of disease throughout the entire metapopulation. In addition, structuring of populations allows for local adaptations which may eventually be spread to other subpopulations by gene flow.

On the other hand, when population structure is amplified by human activities such as habitat degradation and fragmentation, it can negatively affect persistence and evolution. Isolation of subpopulations decreases the effective population size and decreases the rates of immigration, colonization and gene flow (Young *et al.* 1996; Whitlock and Barton 1997; Gonzalez *et al.* 1998; Pearman and Garner 2006; Spear *et al.* 2006). Without the availability for demographic rescue or genetic rescue from immigrating individuals, a subpopulation must be self-sustaining which requires a large N and N_e (Kanarek *et al.* 2015). As a result, small isolated populations may be more vulnerable to extirpation and loss of genetic diversity due to drift (Sjögren-Gulve 1991; Fahrig 1994). Genetic drift can be detrimental to a population because it constrains adaptive alleles and increases the frequency of deleterious alleles (Conner and Hartl 2004). This in turn reduces the adaptive potential of the metapopulation as a whole which can lead to an extinction event should a sudden shift in environmental conditions occur (Frankham *et al.* 2010). Population structuring is often exacerbated by human activities, which may reduce the overall presence of suitable habitat, increase the contrast between habitat and non-habitat, and create barriers to movement. Urbanization, agriculture and roadways are some of the most direct consequences of human population growth since they remove potential habitat, create edge effects, and cause fragmentation. The encroachment on habitat may increase density by restricting area, which can cause an increase in the spread of disease and cuts off subpopulations from others causing a decrease in genetic diversity, both of which have negative effects (Cushman *et al.* 2006). Fragmentation decreases the ability for individuals to disperse between suitable habitats, as well as direct mortality from habitat clearings and roads (Vos and Chardon 1998; Gibbs 2000; Guerry and Hunter Jr. 2002; Riley *et al.* 2004; Stuart *et al.* 2004; McKinney 2006; Harper and Semlitsch 2007; Eigenbrod *et al.* 2008; Hamer and McDonnell 2008; Wilson

and Hopkins 2013; Clipp and Anderson 2014). Reduced recolonization potential for these isolated subpopulations reduces the likelihood that otherwise suitable habitats will be recolonized, and thus affects metapopulation persistence by decreasing the number of occupied habitat patches.

Studies of the population structuring in pond-breeding amphibians have shown an array of results, from pronounced genetic structuring at small spatial scales (e.g. Rowe *et al.* 2000; Newman and Squire 2001; Palo *et al.* 2004; Jehle *et al.* 2005) to panmixia across a broad geographic area (e.g. Tallmon *et al.* 2000; Burns *et al.* 2004; Zamudio 2007; Purrenhage *et al.* 2009). These differences may be due to the dispersal ability of the different species as well as the habitat types surrounding those species. Using a ponds-as-patches metapopulation model for pond-breeding amphibians provides a starting point to determine population structuring (Marsh 2001; Purrenhage *et al.* 2009). In this case, ponds would demarcate subpopulations with the degree of gene flow between ponds being influenced by the interpond habitat matrix (Joly *et al.* 2001). This makes pond-breeding amphibians an ideal taxon for understanding factors that influence both the size and structure of populations.

In order to understand and mitigate the potential impacts of habitat loss and fragmentation to pond breeding amphibians, one must understand: 1) The size, location, and juxtaposition of subpopulations on the landscape, 2) The degree to which subpopulations are small, isolated, and vulnerable to extirpation and loss of genetic diversity due to drift, and 3) How landscape features influence movement and connectivity between subpopulations. Using population genetic methods, we can infer the answers to all of these questions. For amphibians, metapopulation models, with breeding ponds as subpopulations, have been frequently used (Gill 1978; Sjögren-Gulve 1994; Hecnar and M'Closkey 1996; Driscoll 1997; Hels and Nachman

2002) and are recommended as an important management tool (Semlitsch 2000; Marsh 2008; Greenwald 2010). For example, Zamudio and Wieczorek (2007) performed a study of the genetic structure of spotted salamanders (*Ambystoma maculatum*). Spotted salamanders, have limited dispersal capabilities of 300-500 meters (Madison 1997), with a majority (80%) staying within 90 meters of their natal breeding pond (Semlitsch 1998) and display of high breeding site fidelity (Whitford and Vinegar 1996; Zamudio and Wieczorek 2007). This finding was supported by the genetic data from the 29 breeding ponds sampled by Zamudio and Wieczorek (2007), with ponds greater than 4.8 km apart showing a reduction in gene flow. In contrast, in a study on *Ambystoma maculatum* in Ohio, it was found that the sampled ponds showed evidence of panmixia across a 2100 square km area, including pond to pond distances of >4 km suggesting geographic extensive dispersal (Purrenhage *et al.* 2009).

Among pond-breeding amphibians, reticulated flatwoods salamanders (*Ambystoma bishopi*) make a compelling study species due to their unusual life history strategy of laying eggs singly or in small clusters in ephemeral wetlands prior to inundation, and their endangered status (USFWS, 2015). These salamanders live in mesic flatwoods and savannahs consisting of longleaf pine (*Pinus palustris*) and wiregrass (*Aristida stricta*) (Palis 1997b; Bevelhimer *et al.* 2008). Eglin Air Force Base, located in the panhandle region of Florida, is home to the largest contiguous acreage of old growth longleaf pine in the world and contains several ponds used for breeding by flatwoods salamanders (see General Introduction). During the fall months, flatwoods salamanders emerge from their burrows and migrate to deposit their eggs terrestrially in a moist microhabitat located in the basin of a dry breeding pond (Anderson and Williamson 1976). The eggs begin to develop immediately but hatch only after the rains have initiated the hatching response, which may take weeks or months to happen. Typically, hatching occurs between the

months of December to February (Anderson and Williamson 1976; Palis 1995; Palis 1997b; Bevelhimer *et al.* 2008). Once the eggs have hatched, it takes 11 to 18 weeks for larval development to complete with metamorphosis believed to be initiated by the drying of the breeding pond (Palis 1995). Once these individuals leave the breeding pond, they disperse out into the surrounding landscape and occupy crawfish burrows. Flatwoods salamanders have shown emigration orientation in the direction of immigration, which may show the ability for the species to home to and from specific breeding ponds as well as specific terrestrial retreats (Palis 1997a). With such specific and distinct larval and adult habitat needs, and the possibility of high site fidelity in the species, I hypothesize that local breeding populations of RFS would be highly susceptible to poor pond years (e.g. short hydroperiod of ponds, inundation of ponds mismatched with breeding time) that would reduce recruitment. Further, for individuals attempting to disperse to other ponds, I hypothesize that anthropogenic fragmentation of landscapes (e.g. by roads and urbanization) reduces the likelihood of success and the amount of gene flow between subpopulations, and thus may negatively affect the connectivity and persistence of the metapopulation as a whole.

I used population genetic approaches to test these ideas and better understand the size, structure and connectivity between subpopulations of RFS on Eglin Air Force Base. First, by utilizing gene flow estimates between subpopulations, I constructed models to infer the connectivity between subpopulations as well as the costs of traversing habitat types between subpopulations on Eglin Air Force Base (Wang *et al.* 2009). By doing this, I compared movement costs of different habitat types and other variables to gene flow to obtain an understanding of what factors contribute to the population structure (Michels *et al.* 2001; Coulon *et al.* 2004; Cushman *et al.* 2006; Wang *et al.* 2009). For example, amphibians require wet areas

to prevent desiccation (Duellman and Trueb 1994) and thus the presence of wetlands should be important in maintaining gene flow and anthropogenic barriers like roads and urbanization would hinder gene flow (Spear *et al.* 2005). Also, slope and topography are suggested to be a major predictors of gene flow in amphibians, mainly due to the energetic costs associated with traversing landscapes as well as their potential to facilitate dispersal of aquatic life stages during flooding events (Funk *et al.* 2005; Spear *et al.* 2005; Blank and Blaustein 2012; Blank and Blaustein 2014).

Second, I used estimates of gene flow, immigration, and N_e to evaluate the degree of isolation and vulnerability of local breeding subpopulations on Eglin. Using microsatellite loci, I genotyped salamanders from 13 different breeding ponds and used this data to construct models of population structure. I hypothesized that the ponds on Eglin would have small effective population sizes and there would be population structuring between ponds with anthropogenic disturbances inflating this. The specific questions I sought to address were:

1. What is the nature of reticulated flatwoods salamander population structure on Eglin Air Force Base?
2. To what extent are local breeding subpopulations connected by contemporary dispersal and gene flow?
3. Which local breeding populations are smallest and most vulnerable to genetic drift?
4. How do both natural and anthropogenic landscape features, in particular urbanization and differing landscape variables, affect gene flow among breeding populations?
5. How might conservationists more effectively manage habitats for reticulated flatwoods salamander conservation?

Methods

Sample Collection

Twenty-seven historic RFS breeding ponds have been identified on Eglin, of which thirteen have confirmed RFS breeding activity since the early 1990s (pers. comm. Haas). Personnel from Virginia Tech and Eglin Air Force Base collected 259 larval reticulated flatwoods salamanders by dipnet from the 13 active breeding ponds throughout the base in the 2013-2014 and 2015-2016 breeding seasons (Figure 1.1, exact locations redacted for reasons of confidentiality). Euclidean distances between these ponds (km) can be found in Table 1.1. Personnel also collected 354 metamorphs at ponds 4 and 5 using drift fence surveys conducted at these two ponds in the same seasons. Ponds on Eglin are separated into two main clusters, with the Oglesby flatwoods cluster in the east and the East Bay flatwoods to the west. Dipnet sampling was performed between January and May, when larvae have reached sufficient size for detection and capture (Bishop, 2006). Survey methods were described by Gorman *et al.* (2009). Drift fences used 60-cm high rolls of galvanized steel flashing that were buried 15-20 cm to reduce the chance of escape under the fence. Drift fences had funnel traps that were placed approximately 10 meters apart and were placed in pairs on either side of the fence. Funnel traps were 85-cm x 20-cm with 5-cm openings, and were constructed using aluminum window screening. Wooden stakes were used to hold these funnel traps along the fence and pressed firmly against the ground. A wet sponge was placed in all traps and traps were covered with a 61-cm x 61-cm shade board. Traps were checked every evening to reduce mortality. More information on the methods and time periods for drift fence trapping can be found in Erwin (2016). Upon capture, tail clips of approximately 5-12 mm in length were taken using surgical

scissors that had been sterilized by wiping with alcohol and then burning. Samples were then placed in 95-100% ethanol. Samples were stored at -20°C until DNA extraction.

Laboratory Methods

Whole genomic DNA was extracted from tissue samples using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer protocols. I screened 100 different microsatellite loci that had been developed for *A. bishopi* as well as other ambystomatid species (Table 1.2) for amplification and polymorphism in *A. bishopi*. Each locus was screened for PCR amplification in eight individuals selected from four different ponds. PCR reaction mixes were 25 μL in total volume, and consisted of 12.5 μL GoTaq Mastlermix (Promega, Madison, Wisconsin, USA), 1 μL forward primer, 1 μL reverse primer, 8.5 μL molecular-grade H_2O, and 2 μL of template DNA. Cycling conditions were as follows: initial denaturation at 95°C (160 s), 35 cycles of denaturation at 95°C (30 s), annealing at 56°C (30 s), and extension at 72°C (40 s), followed by a final extension at 72 °C (420 s). PCR products were visualized on 1% agarose gel stained with GelRed (Biotium, Hayward, California, USA).

Loci that showed positive amplification on the gel were then screened for polymorphism across 12 individuals. This was done by ordering loci with a universal M13 primer sequence to forward primers and annealing an M13 labeled tail marked with FAM. For the M13 PCR reaction mixes were 25 μL in total volume, and consisted of 12.5 μL GoTaq Mastlermix (Promega, Madison, Wisconsin, USA), 1 μL forward primer, 1 μL reverse primer, 8 μL molecular-grade H_2O, 0.5 μL M13 labeled tail, and 2 μL of template DNA. Cycling conditions were as follows: initial denaturation at 95°C (300 s), 10 cycles of denaturation at 94°C (30 s), annealing at 57°C (60 s), and extension at 72°C (40 s), followed by 27 cycles of denaturation at

94°C (30 s), annealing at 55°C (60 s), and extension at 72°C (40 s), followed by a final extension at 72 °C (420 s). PCR products were visualized on an ABI 3500 Genetic Analyzer. From the total 100 loci screened, 11 were found to amplify reliably and be polymorphic.

I grouped these loci into three multiplexes for screening individuals, as follows: Multiplex 1- AcroD300, Abp04, AjeD23, Atex65; Multiplex 2- AcroD330, AjeD75, AjeD162; and Multiplex 3- AjeD314, AjeD37, AmaD367, AmmH136. The PCR cycling conditions for multiplex 1 and 2 were as follows: initial denaturation at 95°C (120 s), 35 cycles of denaturation at 95°C (30 s), annealing at 56°C (40 s), and extension at 72°C (90 s), followed by a final extension at 72°C (600 s). For multiplex 3, all conditions were equal except for the annealing step, which was set to 58°C.

Amplified PCR products were visualized on an ABI 3500 Genetic Analyzer with a Genescan 500HD LIZ dye standard (Applied Biosystems). Allele sizes were then scored independently by Dr. Roberts and me in GeneMapper (GeneMapper v4.0; Applied Biosystems). In case of disagreement the GeneMapper output was discussed until a consensus was reached.

Evaluation of Markers

For preliminary tests of microsatellite marker suitability, individuals were separated by year and breeding pond, resulting in 22 sample groups of varying sample size (Table 1.3). Using ARLEQUIN version 3.5 (Excoffier 2010), I tested whether genotype frequencies were at Hardy-Weinberg equilibrium for each locus in each group (10^6 MCMC steps following a burn-in of 10^5 steps) and tested for linkage disequilibrium between each pair of loci within each group (10^5 permutations). Null alleles were also tested for using MICRO-CHECKER (Oosterhout *et al.* 2004).

Delineation of Population Structure

I characterized the genetic structure of sampled ponds by comparing patterns among various methods of genetic analysis. First, in order to determine if the 2013-2014 and 2015-2016 sampling years could be combined for further analyses, I performed an Analysis of Molecular Variance (AMOVA) in ARLEQUIN version 3.5 (Excoffier 2010) using 10^4 permutations. The analysis decomposed variation into each of four hierarchical levels: between breeding seasons, among ponds within breeding seasons, among individuals within ponds within breeding seasons, and within individuals. If minimal variation was found to come from years compared to all other factors, then years could be combined. I also examined STRUCTURE results (see below) to determine if individuals from the same pond but different years clustered together. I then performed a second AMOVA in ARLEQUIN using 10^4 permutations in which I divided ponds into the three flatwoods regions (East Bay, Oglesby, and pond SF) and then decomposed variation into each of four hierarchical levels: among flatwoods regions, among ponds within regions, and within individuals.

Second, I used a Bayesian clustering approach (Pritchard *et al.* 2000) in STRUCTURE 2.3.4 to determine the number (*K*) of hypothetical populations that gave rise to the dataset. The model allowed admixture with parameters set at 10^6 MCMC chains followed by a burn in of 10^5. Ten replicates were run for each *K* value from 1 to 23 (determined from preliminary runs to be a sufficient search of parameter space), and log-likelihood was averaged across all 10 replicates. Some authors consider the *K* value with the highest log-likelihood to be the best representative of population structure (Faubet *et al.* 2007), whereas other authors advocate for Evanno *et al*'s (2005) "delta-*K*" approach, which selects as best the *K* value that maximizes the second-order

rate-of-change in log-likelihood among successive *K* values. I compared results from both methods, as calculated by importing STRUCTURE results in Structure Harvester version 0.6.94 (taylor0.biology.ucla.edu/structureHarvester/).

Third, I estimated pairwise genetic distance (F_{ST}) among all pairs of ponds, to determine genetic relatedness and infer levels of connectivity between ponds. Pairwise F_{ST} values were calculated using ARLEQUIN at the breeding pond level (both years pooled). To determine the relationship between genetic and spatial distance (isolation-by-distance; IBD), I regressed F_{ST} on the spatial distance (km) separating each of the pairs of ponds. I used a Mantel test in IBDWS version 3.23 with 10^4 randomizations to estimate the strength of association (R) between F_{ST} and each of the IBD matrices (Jensen *et al.* 2005).

Fourth, a neighbor-joining tree was constructed based on genetic distances among ponds. Nei's minimum genetic distance (D_m) was calculated between all pairs of ponds (Nei, 1973) and these values were then used to create a neighbor-joining tree, in POPULATIONS 1.2.3 (Langella 1999). This tree was then visualized in Figtree (Rambaut and Drummond 2009).

Determining Dispersal between Ponds

I estimated contemporary rates of dispersal among the five ponds with sample sizes of at least 30 individuals (i.e., ponds 4, 5, 212, 53, and 15). The first four of these were located in Oglesby's flatwoods, whereas pond Pond 15 was located in East Bay flatwoods. To prevent unequal sample size from influencing the analysis, I randomly subsampled 30 individuals each from ponds 4, 5, and 15, such that the analysis was based on 30 individuals from each of the five ponds (n = 150 total). I implemented the Bayesian model of Wilson and Rannala (2003) in BAYESASS 3.0 (www.rannala.org/inference-of-recent-migration/). The approach estimates

asymmetric migration rates among populations over the past 2 generations, and makes the following assumptions: (1) migration rate is constant over this time interval, (2) loci are in linkage equilibrium, (3) sampling is performed immediately after migration, prior to reproduction, and (4) immigration rates are less than 1/3 into any given locality. In BAYEASS models, the prior for each individual pair-wise migration rate was uniform over the interval of 0 to 1/3. I ran 10 replicate models, each starting from a different random seed value, and retained the model with the highest mean log-likelihood (Faubet *et al.* 2007). Each replicate model searched parameter space using 5×10^7 MCMC iterations, following a burn-in of 5×10^7 iterations. To ensure adequate mixing of MCMC chains, I adjusted delta values to obtain acceptance rates between 30% and 60%. Posterior values were sampled every 5×10^3 iterations, resulting in 10^4 sampled values for each of the 20 immigration rate parameters. Examination of trace plots (not shown) indicated that this modeling strategy resulted in adequate mixing and convergence. I then fit a Gaussian density function to each parameter and estimated modes and 95% credible intervals in R 3.1.3 (R Foundation for Statistical Computing). I used mode rather than mean as a measure of central tendency, because posterior distributions were skewed (see Results).

Estimation of Effective Population Size and Genetic Diversity

To determine the size, potential influence of drift, and the viability of subpopulations I used LDNe v1.31 (Waples and Do 2008) to estimate the effective population size (N_e). I estimated N_e separately for each cohort at each pond, and only used cohort-by-pond samples in which 10 or more individuals had been sampled. The linkage-based model underlying LDNe assumes that there is random mating, non-overlapping generations, and sampling of a single

cohort. The second of these assumptions is violated, because RFS are iteroperous. However, all populations should be similarly affected by this violation and thus the variation in N_e estimates should be relatively similar across all populations (Robinson and Moyer 2013). Thus, results should be interpreted as relative measures of gene-pool size that fall between N_e and the effective number of breeders (N_b). Rare alleles can have a large effect on estimating N_e using the LDNe approach. Waples and Do (2010) suggest that the exclusion of alleles occurring at a frequency less than 2% yields the most accurate N_e, so I excluded from calculations alleles that occurred at a frequency < 0.02. I also calculated 95% confidence intervals by jackknifing. I used linear regression to test for a relationship between estimated N_e and pond area (estimated by C. Haas, unpublished data). Both N_e and pond area were natural-log transformed. For ponds where N_e was estimable across two years, I took the harmonic mean of these interannual estimates and used this as my estimate of that pond's N_e for the regression analysis.

Finally, I estimated genetic diversity statistics for each breeding pond (years pooled), including observed heterozygosity (H_O), unbiased gene diversity (H_E), number of alleles (A), and allelic richness (A_R). These metrics were estimated in FSTAT 2.9.3.2 (Goudet 2002).

Landscape Influences on Gene Flow

Using genetic relationships as a surrogate for gene flow, I tested alternate hypothesized models of ways landscape structure influences RFS movement among ponds (Johansson *et al.* 2005; Spear *et al.* 2005; Purrenhage *et al.* 2009). I focused on land cover type, elevation, and slope as the factors most likely to influence movement. Land cover has been shown to be a strong influence on amphibian movement. Several studies have shown that amphibians will avoid open habitats, like roads and fields, and move more through forested areas (Rothermel &

Semlitsch 2002; Spear *et al.* 2005; Wang *et al.* 2008). Amphibians are sensitive to anthropogenic habitat alterations (Guerry and Hunter 2002), so urbanization will dramatically affect connectivity. Elevation and slope were chosen as both slope and topography have been suggested as a major predictor of amphibian gene flow, due to their influence on the energetic cost of movement (Funk *et al.* 2005).

Land cover data were downloaded from the Natural Resources Conservation Service which is available from the United States Department of Agriculture (USDA, 2010) and consisted of 15 different land cover types (Figure 1.2). A separate layer, the TIGER 2015 Roads data layer, with primary and secondary roads, was also added from the U.S. Department of Commerce. Elevation data in the form of LiDAR data were released on August 11, 2016 and were acquired from The National Map (U.S. Department of the Interior, U.S. Geological Survey). Continuous elevation data were binned into biologically meaningful categories. Percent slope data were calculated in ArcGIS 10.4.1, and were likewise binned into categories.

In order to determine which landscape variables were most associated with gene flow, I used expert opinion from researchers at Virginia Tech (Carola Haas, Thomas Gorman, Kelly Jones, and Houston Chandler) to construct a series of landscape resistance layers (i.e. models), each featuring different resistance-value assignments made to different levels of each of the three factors (land cover, elevation, slope), then evaluated which layer(s) most strongly correlated with observed genetic differentiation among breeding ponds. Resistance values were set from 1 to 10, with 10 representing the maximum resistance to RFS movement (Table 1.4-1.6). I parameterized resistance layers in ArcGIS 10.4.1 using the toolbox Gnarly Landscape Utilities version 0.1.9 (McRae *et al.* 2013). Once created, resistance layers were converted to ASCII files using the Export to Circuitscape tool version 1.0.87 (Jenness 2010). I then calculated matrices of pair-wise

resistance between ponds using CIRCUITSCAPE version 4.0 (McRae and Shah 2009), which assesses connectivity based on average resistances using an eight-neighbor connection scheme. To make the addition of layers manageable, elevation and slope rasters were coarsened to 30m x 30m in order to overlay with land cover. It has been shown that resistance inferences are robust to changes in resolution of land cover (McRae and Beier 2007). For assessment, I compared these alternative isolation-by-resistance (IBR) models to both a traditional IBD model using Euclidian distances between ponds, as well as an IBD model for which I set the entire landscape to have a resistance of 1 to create an equal resistance across the entire landscape.

I correlated each resistance matrix with the matrix of pairwise F_{ST} values estimated in ARLEQUIN. I used a Mantel test in IBDWS version 3.23 with 10^4 randomizations to estimate the strength of association (R) between F_{ST} and each of the 82 IBR and 2 IBD matrices (Jensen *et al.* 2005). The model that produced the highest R value was presumed to be the best predictor of gene flow.

Results

Microsatellite Markers Evaluation

Two loci (Atex65 and AjeD75) deviated from Hardy-Weinberg equilibrium at more than four populations at an alpha level of 0.05. These two loci were therefore removed from further analyses. The remaining 9 loci were in HWE in at least 10 of the 13 tested groups and were retained for subsequent analysis (Table 1.7). These loci also showed no evidence of significant linkage disequilibrium after Bonferroni correction (Sokal 1995).

Delineation of Population Structure

The AMOVA analysis indicated that the variation between years was not significant relative to spatial variation so years were combined. The AMOVA revealed that -0.9% of total genetic variation occurred between breeding seasons, 5.65% of variation occurs among ponds within breeding season, and 2.26% of variation occurs among individuals within ponds. The majority of diversity (92.99%) occurred among individuals within ponds (Table 1.8). Variation breeding breeding seasons was found not to be greater than zero ($P = 0.7336$) while all other components had P values less than 0.05. Furthermore, in STRUCTURE results, I saw no consistent differences between cohorts with sites (not shown). I therefore pooled cohorts within ponds for subsequent population structure analysis.

The results from STRUCTURE analysis of 607 larval and metamorphic individuals (excluding individuals captured at silt fences between ponds) indicates a model of 17 discrete populations (K=17) had the highest average log-likelihood (Figure 1.3). However, this model was complex to interpret, created a number of non-geographically-meaningful subdivisions with ponds, appeared to overestimate K (Figure 1.4). Utilizing the Evanno *et al*. (2005) method of determining K based on the ΔK approach, a K of 3 would be the uppermost level of structure (Figure 1.5). However, exploratory examination of higher-K models indicated that a K=3 model (Figure 1.4) oversimplified populations structure and failed to recognize apparent genetic distinctions among the three flatwoods regions (see results below). Moreover, because within the K=3 model, admixture was prevalent and individuals did not cluster strongly into geographically meaningful groups, it did not make sense to conduct a subsequent, hierarchical STRUCTURE analysis within these groups to detect population sub-structure, as has been advocated by Evanno *et al.* (2005) and others. I therefore took a compromise approach and have also presented results

of a K= 5 model (Figure 1.4) that shows the primary distinction between the three flatwoods regions while preserving some of the within-pond complexity represented within the K=17 model. In particular, this K= 5 model indicates that pond SF and the East Bay flatwoods regions each are relatively genetically homogenous, whereas ponds in the Oglesby flatwoods are each comprised of a mosaic of up to four distinguishable genetic clusters, which are broadly shared across ponds within the region.

The second AMOVA analysis indicated that there was a greater component of variation between than within major flatwoods clusters (East Bay v. Oglesby v. pond SF). The AMOVA showed that 8.11% of total genetic variation occurs between the main flatwoods clusters while 3.16% of variation occurs among the ponds within the flatwoods. The majority of diversity occurred within individuals (88.73%). All variance components were found to be significantly greater than 0 ($P < 0.0001$ for each; Table 1.9).

Pairwise F_{ST} values among all sample sites indicated that there was significant variation between a majority of sites-pairs. Exceptions included 215 to 15, 32 to 33, 34 to 215, 32 to 34, 33 to 34, 34 to 49, 34 to 15, and 34 to 112. However, variation in sample size may have influenced these results. Six of these 8 non-significant comparisons involved Pond 34, which had relatively high F_{ST} values versus other ponds, but a small sample size of six individuals, which may have precluded significant test results. The two lowest values occurred between ponds 15 and 215 (F_{ST} = 0.004, P = 0.305) and between 4 and 53 (F_{ST} = 0.009, P = 0.006). Thus, significance tests aside, these two pond-pairs were the least genetically differentiated. Pairwise F_{ST} values ranged from 0.004 to 0.234 among between ponds (Table 1.10), with an average F_{ST} = 0.092. F_{ST} values showed significant positive relationship with pond spatial distance when pond SF was excluded (R = 0.7640, P = < 0.0001). However, when pond SF was included, the

relationship was still significant though the correlation coefficient decreased (R = 0.5910, P = < 0.0001). The traditional IBD using Euclidian distance was also performed separately for each flatwoods region. The Oglesby flatwoods by itself had a significant IBD relationship according to the Mantel test (R = 0.6370, P = 0.01), while the East Bay flatwoods showed no significant relationship between distance and gene flow (R = 0.4150, P = 0.226). Figure 1.6 illustrates all of the four above mentioned IBD configurations tested.

The site-based neighbor-joining tree shows a similar clustering of populations as the STRUCTURE analysis and agreed with the F_{ST} analysis regarding the genetic differentiation between ponds (Figure 1.7). The tree indicates a large division between ponds clustered in the Oglesby flatwoods and ponds clustered within East Bay flatwoods. The soccer field site (Pond SF) was indicated as being highly genetically distant from both clusters.

Dispersal between Ponds

Results from BAYESASS analyses showed that the mode of immigration rate per generation between ponds ranged from 0.002 to 0.273 (Table 1.11; Figure 1.8). The majority of between-pond migration rates were close to 0, with the exception of 5 to 53 at 0.047, 53 to 5 at 0.025, 212 to 4 at 0.013, and 53 to 4 at 0.273. Pond 15 was the only pond representing the East Bay region, and not surprisingly had immigration rates close to 0 from all ponds in Oglesby. The inferred high migration rate from pond 53 to pond 4 is consistent with the low F_{ST} value between these ponds (0.0086).

Estimation of Population Size and Assessment of Genetic Diversity

Estimates of mean effective population size generally were small, ranging from 20 to 60 individuals in most ponds (Table 1.12). In 3 of the 11 pond-by-cohort samples where N_e was estimable (i.e. $n \geq 10$), upper 95% confidence intervals included infinity, presumably due to small sample sizes in those ponds. Pond 53 in 2013-2014 also had a negative estimate of mean N_e, which can occur when sample size is small and/or the true N_e is large. However, the lower 95% confidence limit for this pond was 87 individuals, a relatively large value higher than the mean estimate for any other pond, suggesting that the true N_e was relatively large in this pond. Given the small sample sizes at some ponds and wide confidence intervals, N_e estimates should be interpreted more as indices of relative gene pool size than as absolute estimates of the number of breeders. These values still allow for a comparison between ponds and years. N_e was found to be similar across years, with little change except for pond 5 which showed a doubling in N_e estimate between years, which may have been influenced by variation in sample size between years. The minimum N_e was also compared to the pond size (in hectares). There was a significant positive relationship between N_e and pond area (R^2= 0.1027, P= <0.0001; Figure 1.9). Pond 53 was excluded from this analysis, because N_e was inestimable.

Most genetic diversity statistics, including mean number of alleles (A), allelic richness ($A_{R;}$ the mean number of alleles rarified based on the lowest sample size of n = 3), and expected heterozygosity (H_E) were found to be lowest in pond SF (3.44, 2.75, and 0.49, respectively; Table 1.13). Considering that pond SF is isolated from other ponds by urbanization, these lower estimates may be due to an increased effect of genetic drift on the population. The highest A_R and H_E estimates were found in ponds 33, 34, 53 and 4 (Table 1.13).

Landscape Influences on Gene Flow

In all, I tested 32 models featuring alternative landcover cost weighting schemes, 31 models featuring alternative elevation cost weighting schemes, 14 models featuring alternative slope cost weighting schemes and 4 models featuring combinations of the best-supported single-factor cost surfaces. Herein, I have presented results only from eight models per factor, which span the range of options tested and include best-supported models for each factor. All models were found to be positively correlated with genetic distance and had a statistically significant *P* value of less than 0.0001 (Table 1.14). Overall, landcover was the best predictor of F_{ST} between ponds (*R*= 0.8584). Woody wetlands, evergreen forest, scrub/shrub, and herbaceous landcovers were found to be the least resistant to gene flow while areas like deciduous forest, mixed forest, and all developed spaces were highly resistant to gene flow according to the model. The best elevation model was found to be slightly more informative than the null model (*R*= 0.6587) and slope was found to be less informative than the null (*R*= 0.6574). The combination of elevation and slope to landcover actually decreased the explanatory power of the model though only slightly (elevation *R*= 0.8166; slope *R*= 0.8463). I performed a traditional IBD using Euclidean distance as the base null including pond SF (*R*=0.3493) as well as excluding pond SF (*R*= 0.5840). CIRCUITSCAPE incorporates distance into the calculation of resistance between sites, so I used an IBD layer with all values set to 1 as the null model (*R*= 0.6583) for comparison of all models.

Discussion

Population Structure and Connectivity

Ponds clustered into three main genetically differentiated groups (Oglesby flatwoods, East Bay flatwoods, and pond SF). Based on the estimated levels of genetic differentiation and gene flow, I posit that the flatwoods regions function as metapopulations with occasional dispersal within, but probably not between them. Ponds within region were more genetically similar to each other than to those outside the region, based on results of STRUCTURE, the neighbor joining tree, and the AMOVA. Even though there are few barriers between these flatwoods regions, the sheer distance and lack of known active breeding ponds between the regions apparently are sufficient to preclude gene flow between the regions. These findings support an IBD model in which salamanders have higher migration between their immediately neighboring pond than those farther away. With the disappearance of ponds between these main clusters, the ability for connection between ponds has been greatly decreased. For example, though only 3-4 km from ponds in the East Bay flatwoods, Pond SF was just as genetically differentiated from these ponds as from ponds in the Oglesby flatwoods, much farther away. If you consider land cover, pond SF is almost entirely surrounded by urban development which could be preventing connectivity within the East Bay flatwoods region.

Even though the greatest observed genetic division was among pond SF and the two main regions, all but two pairs of ponds were clearly genetically differentiated (i.e. $F_{ST} > 0.01$), indicating that in most cases, each pond should be managed as though they are demographically independent. Even the two spatially closest ponds, 212 and 213, had relatively high F_{ST} value of 0.07, suggesting that RFS seldom exhibit gene flow between these ponds. From past studies, RFS have never been observed dispersing more than 1.7 km from their natal pond (Palis and

Hammerson 2008). Roughly half of ponds within the same region were within 1 km of each other, yet even these pond-pairs usually were genetically distinguishable, suggesting infrequent dispersal. Strong genetic differentiation ($F_{ST} > 0.05$) was found between some pairs of ponds that were within 0.6 km of each other. Migration rates estimated in BAYESASS corroborated evidence for limited gene flow among most ponds, with the exception of pond 4 which had an immigration rate of 0.27 from pond 53. The F_{ST} estimate between these ponds was also low (0.0086) indicating that there is gene flow occurring between these two ponds even though they are 0.7 km apart from each other. Observing the land cover that occurs between pond 53 and 4, there is woody wetland surrounded by evergreen forest that may act as a corridor between the ponds and funnel individuals from 53 into 4. Forest corridors have been shown to have a negative correlation with divergence in salamanders and help maintain habitat connectivity in fragmented environments (Gibbs 1998). Although the ponds on Eglin are genetically distinguishable, I hypothesize that the genetic drift occurring between ponds is likely due to anthropogenic effects of fire suppression and other management within the area, rather than long-term evolutionary isolation, and therefore that restoration of connection both via habitat management and other management methods are justified for RFS on Eglin.

The observed sub-structuring of local breeding populations was quite complex, with STRUCTURE indicating a *K* of 17 and multiple, genetically distinguishable genetic clusters within most Oglesby ponds, especially ponds 4 and 5. This division within ponds could be caused by several factors, including temporal differentiation due to consistently different arrival times at breeding ponds, spatial segregation of families among certain areas within ponds, or the influence of multiple founder events occurring in the past (Tennessen and Zamudio 2003). This brings about the idea that ponds could go through extinction and recolonization events frequently

but without high populations and limited connection between ponds, recolonization would be unable to occur. Unfortunately, we lack the data on arrival dates, precise spatial locations of breeding, or past founder events that we would need in order to parse these alternative explanations.

Effective Population Sizes of Subpopulations

All but one pond was estimated to have a relatively small effective population; where estimable, mean N_e ranged from 10 to 60 individuals. Mean N_e could not be estimated for Pond 53, but the lower 95% CL was substantially higher, at 87 individuals. N_e sets a lower limit for a viable population size and general guidelines propose that an $N_e > 500$ is needed over the long term to maintain adequate genetic diversity, while $N_e < 50$ is indicative of a population facing an imminent threat of inbreeding depression (Franklin 1980). Although small sample sizes precluded N_e estimation at some ponds and contributed to wide confidence intervals, nearly all sampled cohorts had N_e estimates below the threshold of 50 individuals. However, more recent reviews of N_e across various animal systems indicate that wild animal populations are smaller than once thought (Frankham 2009). A small N_e can occur from population bottlenecks, genetic isolation, low support for population size by the environment, reproductive skew, and sex-ratio asymmetry (Hartl and Clark 1997; Waples 2002; Wang *et al.* 2009; Wang *et al.* 2011).

My estimates for N_e are comparable to the N_e of other salamanders (Funk 1999; Jehle *et al.* 2002; Savage, 2010; Wang *et al.* 2011; Wang and Shaffer 2017) and other pond-breeding amphibians (Scribner *et al.* 1997; Driscol 1999; Rowe *et al.* 2004). Gill (1978) estimated an N_e of 25-185 for the red-spotted newt (*Notophalmus viridescens*). Long-toed salamanders were found to have an N_e of less than 100 according to Funk (1999). The N_e of the California tiger

salamander (*Ambystoma californiense*) was found to be 11-64 individuals per population by Wang (2011) and was found to be strongly correlated with pond size. Another study by Wang (2017) on *A. californiense* found that 10 breeding ponds had 8-43 effective breeders in 1995 and 6 ponds had 5-19 effective breeders in 2001. As in Wang's study, I found that the N_e varied by year but only slightly, with overlapping confidence intervals. This is not surprising since there is a relatively short time in between resampling year and the genetic differentiation stays consistent over long periods of time. Although the relationship of N_e to breeding pond size was positive, it was relatively weak ($R^2 = 0.1$) indicating that other factors than just pond size may more strongly affect breeding and recruitment.

Even though my N_e estimates are similar to other studies of salamanders, the high conservation status of RFS combined with the low number of extant breeding populations make the preservation of the known breeding ponds all the more important. Increasing N_e within the ponds is one way to protect genetic diversity especially with the population structuring seen in the previous section (Storfer *et al.* 2007; Charlesworth and Willis 2009)

Landscape Influences on Gene Flow

The landscape genetic analysis identified landcover type as the most important factor in regulating gene flow among ponds on Eglin Air Force Base. The presence of any urbanization, even that considered to be a low amount of urbanization, was found to be a hindrance to gene flow between metapopulations. Flatwoods salamanders migrate nocturnally, during or directly after rains associated with passing cold fronts (Palis 1997a). This is due to the fact that salamanders are prone to desiccation and the presence of water is needed for survival. Urban areas are poor at maintaining the moist environment needed and pose a formidable challenge to

dispersing salamanders (Peterman *et al.* 2014). Due to this, salamanders are less likely to perform exploratory movements and are less likely to travel through these environments. Connette and Semlitsch (2013) found that salamanders never moved more than 15.5 m over a three-hour period and surface activity is reduced when traversing unfavorable landcover (Connette and Semlitsch 2013; Peterman *et al.* 2014). This would mean that increased urbanization within the area could further reduce the amount of gene flow. Emergent herbaceous wetlands were also found to be a factor in the decrease of gene flow between populations. Flatwoods salamanders are found within mesic flatwoods and savannahs consisting of longleaf pine (*Pinus palustris*) and wiregrass (*Aristida stricta*) (Palis 1997b; Bevelhimer *et al.* 2008). These habitats are maintained by periodic summer fires that remove herbaceous growth, but due to the fire regime on Eglin, these prescribed burns do not occur at the frequency or timing needed to keep herbaceous growth down. This overgrowth makes movement through the landscape difficult for flatwoods salamanders whom move through areas predominantly consisting of wiregrass and would have a direct effect on the gene flow occurring on Eglin. The genetic drift occurring due to anthropogenic effects would imply that restoration efforts are justified to further the preservation of the populations on Eglin.

Elevation and slope were found to be poor predictors of gene flow. This could be due to the low amount of difference between elevations on the base, ranging only from 1-6 meters. Slope would seem like a good candidate for explaining gene flow, just from the energetic requirements needed to traverse areas with a high slope (Funk *et al.* 2005). However, with the lack of appreciable change in either elevation and slope on Eglin, these variables were not found to be any more informative than the simple unweighted distance (i.e. IBD model). In other studies, elevation and slope have been found to have varying effects on ambystomatid species.

Spear (2005) found that there was a significant positive relationship between genetic differentiation and elevation for tiger salamanders (*A. tigrinum*); while Zamudio (2007) found that elevation did not limit spotted salamander (*A. maculatum*) migration or dispersal. This is most likely due to the typical habitats in which these species reside. Tiger salamanders are restricted to grasslands and low foothills while spotted salamanders are found in piedmont and mountainous regions.

Results of this analysis suggest that RFS require pristine flatwoods habitat in order to traverse the landscape. Changes in land cover type, whether facilitated by natural or anthropogenic disturbances, have negative consequences on connectivity between ponds with urbanization having the most drastic effect. This is in line with other studies of the kind in other taxa including amphibians (Vos *et al.* 2001; Palo *et al.* 2004; Funk *et al.* 2005; Spear *et al.* 2005; Primmer *et al.* 2006; Clark, 2008; Peterman *et al.* 2014). On Eglin, proper management of the landscape is challenging, given that long leaf pine habitat requires summer fires to be maintained, which is difficult to do when close to urban areas. With this in mind, much of the habitat on Eglin faces overgrowth by woody vegetation not found in a typical long leaf pine environment. These overgrowth areas become unsuitable and reduce the connectivity between in the absence of ongoing, labor intensive management.

Recommendations for Conservation and Management

Flatwoods salamanders are a species in decline and are listed as endangered by the U.S. Fish and Wildlife Service as of February 10, 2009 (USFWS 2015). The protection and conservation of available flatwoods salamander habitats and the populations that live within them are a top priority for the continuation of the species. This means that increasing genetic

diversity both by increasing overall numbers of salamanders and thus increasing the N_e above 50 and increasing connectivity between breeding ponds are paramount. From my analysis on Eglin, flatwoods salamanders should continue to be monitored on the pond scale as this is the smallest manageable unit. Yearly monitoring of ponds allows for the ability to detect yearly recruitment of larva as well as to determine returning breeders to the pond. Maintaining a sufficiently large population size should be a driving goal in the conservation of the species on Eglin. While local population monitoring is a must, an ideal management strategy should also focus on the connectivity and gene flow between the ponds within the flatwoods. Connectivity between East Bay and Oglesby flatwoods is not currently likely, considering the distance and the lack of active breeding ponds between them. Restored connectivity could be achieved by both creating corridors between ponds that can be utilized by salamanders as well as by active translocation of individuals to ponds. Corridors are suggested to be effective as seen from the high immigration rates to Pond 4 from Pond 53 where wetland habitat is flanked by evergreen forest creating a path between the ponds. Within ponds, prescribed burning would increase the habitat for both adult and the larvae which would metamorphose within inundated wetlands. Headstarting of larvae in addition to habitat restoration would help to increase recruitment as the lack of additional individuals to the population is of concern. Overall, a better understanding of the basic biology of the species will help to further conservation efforts.

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Table 1.1 – Euclidean spatial distances between pairs of ponds on Eglin AFB (km).

	SF	15	215	32	33	34	112	49	5	53	4	212	213
SF	-												
15	3.06	-											
215	3.08	0.27	-										
32	3.19	0.14	0.25	-									
33	3.46	0.42	0.40	0.29	-								
34	3.95	0.93	0.87	0.79	0.50	-							
112	3.26	0.85	0.57	0.79	0.75	0.96	-						
49	15.48	12.60	12.49	12.47	12.18	11.68	12.23	-					
5	16.32	13.45	13.34	13.32	13.03	12.53	13.07	0.86	-				
53	16.29	13.40	13.29	13.26	12.97	12.47	13.03	0.83	0.36	-			
4	16.57	13.67	13.57	13.54	13.25	12.75	13.31	1.10	0.46	0.28	-		
212	16.82	13.90	13.81	13.77	13.48	12.97	13.56	1.46	0.94	0.68	0.48	-	
213	16.77	13.85	13.76	13.72	13.43	12.92	13.51	1.42	0.93	0.65	0.48	0.05	-

Table 1.2 – Microsatellite loci tested for utility in *Ambystoma bishopi*. Table includes original species developed for as well and the forward and reverse primer sequence, the motif, the size range found within the other species and the reference taken from.

Primer name (locus)	Species	Primer sequence (5' to 3') Forward	Primer sequence (5' to 3') Reverse	Motif	Amplicon size range (bp)	Reference (original)
AjeD108	Ambystoma jeffersonianum	CCTCTTGGGGTGTAAGTCTCTG	AAAGATGTGCCGTATAACTTGG	TAGA	105-180	Julian, 2003
AjeD162	Ambystoma jeffersonianum	AAATGTTCCAACCAGTCACAAC	GATTAAGCTAGAGGGCTTGTCC	TAGA	115-170	Julian, 2003
AjeD23	Ambystoma jeffersonianum	AAAACCTCTGGAGAAACATGAG	GAACACAGGCTACTAACAACAGG	TAGA	195-235	Julian, 2003
AjeD346	Ambystoma jeffersonianum	AGCAGGATTAGTGCTTAGATGC	TGGCAATGTTTACCTAAGAGAG	TAGA	160-195	Julian, 2003
AjeD422	Ambystoma jeffersonianum	CAAGGTGCTCAAGTTACTGTTC	CAAATTCTGTACCTGACTGCTG	TAGA	230-265	Julian, 2003
AcroB192	Ambystoma macrodactylum croceum	CACCAAGCTCTGAAGGCAAATC	ACTGGAGCGAGCTGTTAGCAAG	CCAT	113-133	Savage, 2009
AcroD037	Ambystoma macrodactylum croceum	CCGAGGACGTTGGTACTTTATAAGC	CTAGCGTCCTGTTTTTTGCTGTAA	TAGA	124-164	Savage, 2009
AcroD167	Ambystoma macrodactylum croceum	ATTGCCCAGATTGATCAGAGAGTC	GCCTACGGACTTTGGCACTTTAG	TCTA	102-114	Savage, 2009
AcroD176	Ambystoma macrodactylum croceum	AGTGGGATAAACACAGCACCATATC	GAGGGCTTTGGCACTTTATAAGC	TCTA	114-170	Savage, 2009
AcroD300	Ambystoma macrodactylum croceum	TGGTGGCATTACTGCTGACATGTAT	GCAATCAAGGACTCAAGATGGACTG	CTAT	192-228	Savage, 2009
AcroD330	Ambystoma macrodactylum croceum	GAAGCAGTTAGAGAAGATTGGAGAG	GGCACAATTTCATTTATTAAACAGG	GATA	136-176	Savage, 2009
AmaC40	Ambystoma maculatum	CATTTTCTTATTGCAGTTGTCG	ATTTAAACCTGGATTGCCTATG	TACA	155-175	Julian, 2003a
AmaD321	Ambystoma maculatum	GATGCCTTGAAACTTGTTCTTC	TGGTGCATCTATATTCCTCAAG	TATC	120-175	Julian, 2003a
AmaD367	Ambystoma maculatum	GTCTTCTCTCCACATGGTTTTG	TTCCTCTTAATGTTTTCCGTTG	TATC	165-220	Julian, 2003a
AmaD42	Ambystoma maculatum	GATGGAAAATCAATCAAGTGTG	TAACTAGCTGTCAATCGCTCTC	TAGA	125-160	Julian, 2003a
Atex65	Ambystoma texanum	TTCTGAGCTGTCCATGTTCATATGC	CGCTAGGAAGTCACATTTACTTTGTC	GATA	272-384	Williams, 2004
Atex74	Ambystoma texanum	TCAACGAAAGAGGTGTTGGGT	TCCAACGACAGCGGTATAAA	GACA	211-227	Williams, 2004
AjeD03	Ambystoma jeffersonianum	GACACCACTGGCACACATATAC	GAAGGGTGGTCAACAGAGAAC	TAGA	185-255	Julian, 2003
AjeD13	Ambystoma jeffersonianum	TTTAAACCTTAAGAGAAATCCCAG	CCATGTTGTCTGTCTTTGTGAG	TAGA	180-225	Julian, 2003
AjeD212	Ambystoma jeffersonianum	ACAAAAGTCGAAGGACTCCAC	AATGGTGCAGAATTTCATAAGG	TAGA	150-200	Julian, 2003
AjeD280	Ambystoma jeffersonianum	AATCTATCATGTGTTTGATCCG	AGAGCAAGACATAGAAAGCTGG	TAGA	240-265	Julian, 2003
AjeD283	Ambystoma jeffersonianum	TTGCACCCTTGGCAGATG	TGTAATGGGTCAGGCAATAATC	TAGA	120-160	Julian, 2003
AjeD294	Ambystoma jeffersonianum	GTTAGTCGAACTCCGGTTGAG	GTTTCTGTCCGTTGTTGTCTG	TAGA	230-280	Julian, 2003
AjeD314	Ambystoma jeffersonianum	ATAAGCAGGAGGAATTTACCAG	TTAAAGTCTGATTCAAGGCAAG	TAGA	105-255	Julian, 2003

AjeD326	Ambystoma jeffersonianum	AGCATCAAAGAGGATGAATGTC	ACAGGAGTTACATGCAGAGGTC	TAGA-CAGA	360-390	Julian, 2003
AjeD37	Ambystoma jeffersonianum	TATTGTTGCATGTAGGATTACC	CTTTAGGTCTTTCTTCCGCAC	TAGA	195-230	Julian, 2003
AjeD378	Ambystoma jeffersonianum	GGCAAACCATATTTTCCATAAC	AGAAACCTCTGGGTATTAAGGC	TAGA	210-290	Julian, 2003
AjeD448	Ambystoma jeffersonianum	CAGAATTTCCCACATCACTTTAG	AGGAACTGTCCATCATTGTTTC	TAGA-CAGA	125-260	Julian, 2003
AjeD46	Ambystoma jeffersonianum	CTCCTGCTATCGTCATCTTCTC	CTTCAGGACTACACTGGAAAGG	TAGA	290-420	Julian, 2003
AjeD75	Ambystoma jeffersonianum	ATGTCAGTGCAGCTATTTTGC	TTATATGTAGTGCCTGGATGCC	TAGA	145-190	Julian, 2003
AjeD84	Ambystoma jeffersonianum	CATGCATAGCATCCTGTGAG	ATATTTAACTGAGGCCTTTGGG	TAGA	140-350	Julian, 2003
AjeD94	Ambystoma jeffersonianum	ATATCCCATTCCATTGTTTCTG	ATGGACATTCACATGATCACC	TAGA	185-250	Julian, 2003
AcroD114	Ambystoma macrodactylum croceum	GGCTGAAGCCATTTCTTCTAAACAG	TGAGTCCTGAGGGCGAACAG	CTAT	116-132	Savage, 2009
AcroD190	Ambystoma macrodactylum croceum	TGTCAATGTGACGATGAACAGTACC	CGGACCAGCAGACAAATACAAGAC	CTAT	121-149	Savage, 2009
AcroD315	Ambystoma macrodactylum croceum	AGAACAAATAACAGTGAAAGAGAGC	AATACGTTTCTTTTGTTGTGAGC	TAGA	234-290	Savage, 2009
AcroD327	Ambystoma macrodactylum croceum	TTTGGCACAACTATATGTCTATCAAG	CCTAGAACAGAGAAGAAATAAGAATTAGG	TATC	162-202	Savage, 2009
AmmH123	Ambystoma macrodactylum macrodactylum	GGTTGCCTCCTGAGAACTTTATTTTC	ACAAACCCACTGACAACTTTGGAC	CTAT	110-118	Savage, 2009
AmmH136	Ambystoma macrodactylum macrodactylum	CCAAAATCGTGGGTTACTGTGTG	TGAGTGGCGCTATAGAAGAATTCAG	CTAT	120-160	Savage, 2009
Atex102	Ambystoma texanum	TTCAGGTGGATTCACAGTGC	CTGTGTTAGGGGGTTTCCTG	GATA	149-209	Williams, 2004
Atex133	Ambystoma texanum	CTTGAGGTTTGTGGTGCAAT	TATCGCCTTCCTGGCTCTTA	GATA	172-280	Williams, 2004
Atex141	Ambystoma texanum	GCTTCTTTTGCTTGCCTGTT	TTTCGCAATTGCTGATAAGG	GACA	151-159	Williams, 2004
Atex49	Ambystoma texanum	GAGGGGTGCTATATAAAAATCC	GTCTATAGTCTTTGCCTCAATC	GATA	103-175	Williams, 2004
Atex87	Ambystoma texanum	GGCGATTTTGCGCTATATAAAA	ATGATGCTTCAAACCAGAAC	GATA	135-219	Williams, 2004
Atex89	Ambystoma texanum	TAAAGCCCCTGTCCACAATC	TCAGTGCCTGGATACCCTTC	GATA	213-325	Williams, 2004
ATS10-7	Ambystoma tigrinum	GAGGCAGGATGATTTAGA	CTTGGCATTACTGATTAGG	GA	296-302	Mech, 2011
ATS14-3	Ambystoma tigrinum	GGGCACTGAAACGGAACACT	CCCCAAATGGCGTCCCT	CA	103-129	Mech, 2013
ATS4-20	Ambystoma tigrinum	TGTTTTGCCCTTATGTCG	GCCCAAATCCTAAAGAGTAAGT	CA	321-372	Mech, 2003
ATS4-25	Ambystoma tigrinum	ATAGGGGCCTCAAGTTAAG	GGCTACTAGATGGCGTTGT	CA	229-237	Mech, 2005
ATS5-7	Ambystoma tigrinum	GGGCTTGAATCATGTAGTGG	GGGAAGACTAGATGGCAATAAC	CA	240-282	Mech, 2007
ATS5-8	Ambystoma tigrinum	AGTCCCTCTCTATCTAATCTCG	ATTCTCCTGCCTGTATGTTT	CA	354-366	Mech, 2009
Abp01	Ambystoma bishopi	GACTGACACACGCTTCATGG	CCCTCAACTTTGCTCTTTCG	AG	264	Bohn, unpublished
Abp02	Ambystoma bishopi	GGAGATTCTGATGTTGTTGGTTC	ATTTGTCGTCCTTCCCTCAA	AAAG	289	Bohn, unpublished

Abp03	Ambystoma bishopi	GAGACACAATGGAAATGGCA	CATGTGCCCGGTAGAAAGAC	AATG	141	Bohn, unpublished
Abp04	Ambystoma bishopi	TTGCTCTACTTTGCCATGTGAT	CCTCACTGGCAACACCCTAT	AAAC	181	Bohn, unpublished
Abp05	Ambystoma bishopi	CTCAGGATACTGGAGGAGCG	ATATCATGTGACCCAACCCG	AG	311	Bohn, unpublished
Abp06	Ambystoma bishopi	TTGGGAACCTAGGTCACTGC	GGGAAGACAGAGCAGCAATC	AT	225	Bohn, unpublished
Abp07	Ambystoma bishopi	GGCTGGCTATGGACTGGAT	AGTAGGTTTGCCACAGCGTT	AC	300	Bohn, unpublished
Abp09	Ambystoma bishopi	CCTTTCTCTCTTCCTCTTCTGC	CTGGGTGGGCGATATAAGAA	AT	211	Bohn, unpublished
Abp11	Ambystoma bishopi	CGCCTTCTGCTGTTCTTTCT	TCGGACCGATACCAGGTAAA	AG	265	Bohn, unpublished
Abp15	Ambystoma bishopi	GAAATAGCGCTTAGGCTGTG	GCGTTTACCGCAAATCTCTC	ACAT	248	Bohn, unpublished
Abp16	Ambystoma bishopi	AGAGCTGGAACGAGGGTG	TTGCTCCTGTGAGTCTCTGC	AG	158	Bohn, unpublished
Abp17	Ambystoma bishopi	GTTGGTTTCCACTAGCCAGC	TGACAAAGTTACAGCGCCAG	AT	277	Bohn, unpublished
Abp18	Ambystoma bishopi	AAGGCTGTCCGTGATACACC	CTGCGTGGATCCTACTTTCC	AG	209	Bohn, unpublished
Abp19	Ambystoma bishopi	AAAGGGAGGGAACATGGAAA	ATCGGCAGATGAGCATTCTT	AG	242	Bohn, unpublished
Abp20	Ambystoma bishopi	AAATGGACATAGGCCTGCAT	AACTCATCAAACTGGCCCAC	AG	267	Bohn, unpublished
Abp21	Ambystoma bishopi	ATGCTCCAAAGGTGTCTCCC	ATGCATGACCAAGGTGTACG	AC	238	Bohn, unpublished
Abp22	Ambystoma bishopi	CCATTAGGTCGTTCCACCAC	TACGTGACGGCAACAGAAA	AG	250	Bohn, unpublished
Abp25	Ambystoma bishopi	GGGAACCAATGGTGCTAATG	TGCATCCCAGAAGGAATAGC	AAAT	195	Bohn, unpublished
Abp35	Ambystoma bishopi	AGGACCGTCATCAACGTCTC	TGCTCTGCCTTCCTCTTACC	AAG	262	Bohn, unpublished
Abp37	Ambystoma bishopi	CGTGGAAGAAGACGCCTAAG	CTAGACGACGCCGAAATGAT	AAG	273	Bohn, unpublished
Acg01	Ambystoma cingulatum	GCACATCACCAAGGAAGAGG	ATTCCTGCCACTGTCACTCC	AG	205	Bohn, unpublished
Acg02	Ambystoma cingulatum	CACCCAGCGTTAGAGGAAGT	ATGGAGAGGGAGATGGAAGG	AG	259	Bohn, unpublished
Acg03	Ambystoma cingulatum	GCGCTAGTATGCATGGTCTG	TAACGCTTCATGATTCAGGG	AC	241	Bohn, unpublished
Acg04	Ambystoma cingulatum	TTGTGAAAGACACTGAGGCG	ACGTATCGACCTCTAAGCGA	AC	203	Bohn, unpublished
Acg05	Ambystoma cingulatum	AAGACAGGTGATTGCATAGGG	AAGGTGGGCATGTGAGATTT	AG	114	Bohn, unpublished
Acg06	Ambystoma cingulatum	AACGGAGAGAGAACGGTGTG	GCTGTACGCTCCGGTATTGT	AG	112	Bohn, unpublished
Acg07	Ambystoma cingulatum	CTTAGCTCTGGTCACGGTCC	GCGGTTCCCAAGTCTAGAAA	AG	225	Bohn, unpublished

Acg08	Ambystoma cingulatum	TACAGCCAATTTGTTTGGCA	CAAACTCGTTTACCATTACCACC	AT	303	Bohn, unpublished
Acg09	Ambystoma cingulatum	AATGCATATATTTGGGCCGT	GCCAGAGAGGATTGGAAGAA	AAG	303	Bohn, unpublished
Acg10	Ambystoma cingulatum	TGTACGTTCACCTCTGTTGTCA	CGTACGGAGAGTGGGAGAGT	AT	172	Bohn, unpublished
Acg15	Ambystoma cingulatum	AGGAGTCCAGTGGAGGAAGG	CGGGACTGGCTAAAGGAAA	AG	143	Bohn, unpublished
Acg16	Ambystoma cingulatum	TGCATTTGTCCATGATGACTC	GATGGCTTTACCACTTGACCA	AC	288	Bohn, unpublished
Acg17	Ambystoma cingulatum	GGAAGGTTGCAGGTTTACCA	CGAAACAGGTTATGGCACCT	AT	286	Bohn, unpublished
Acg18	Ambystoma cingulatum	GTTTGGGCATACAGGGAATG	AGAATGCACTCACAGGGTCC	AAAT	233	Bohn, unpublished
Acg19	Ambystoma cingulatum	CCGGATTAGGTAAGTGGTGC	AAGGAGCCAAGGAGGAGAAA	AG	161	Bohn, unpublished
Acg22	Ambystoma cingulatum	AGTGAAACCGGTCCATAAGC	GTTGTTCAGTAGCAGCGCAC	AAAT	268	Bohn, unpublished
Acg26	Ambystoma cingulatum	GGATGGACCAGTTGGGAGTA	CCCTGCGCATTTGTACACTA	AAAC	301	Bohn, unpublished
Amb01	Ambystoma	AAACGCTGCTTGCGTTCTAT	TACATTTCCCAGAAGGCTCG	AG	276/287	Bohn, unpublished
Amb02	Ambystoma	TCTGGGTCTTCTCCAACAGG	GTTAACCATTCCCACCTCCA	AG	202/280	Bohn, unpublished
Amb05	Ambystoma	CCTCTCTCTTGCGCTCTCAT	ACTTACCCACAGCAGGGATG	AG	261/265	Bohn, unpublished
Amb06	Ambystoma	ATGGGACAGTGACGGGATAG	TGACTCAATGGTACTGCGGA	AC	294/296	Bohn, unpublished
Amb07	Ambystoma	AACCAGACCTGCTTCATGCT	CGGCTCGGTCTGTTAGAAGT	AT	138/136	Bohn, unpublished
Amb08	Ambystoma	CTCCACAGTTCAGGGTCCTC	TGCTTTCGGCACATAAACTG	AGG	318/307	Bohn, unpublished
Amb10	Ambystoma	AAGGAAAGACAGACGCGGTA	TCTACGTGCCGTTTACCTCC	AG	296/297	Bohn, unpublished
Amb14	Ambystoma	GGCTTTCTGTCCCTCTCAAA	AATGCCCTTCTAGCCTCGTT	AAG	242	Bohn, unpublished
Amb18	Ambystoma	AGGAACAGAGACGGAAGGGT	CTCTTATTTGGCTGCTTCCG	AG	238	Bohn, unpublished
Amb19	Ambystoma	CACCAGCACCCACTCAATC	CGGCAGGTGATGTGTTATGT	AC	171	Bohn, unpublished
Amb27	Ambystoma	ACAAGGACCATGCATTAGGG	TTGGTGGAGGAGGTCGTAAA	AG	306	Bohn, unpublished
Amb42	Ambystoma	TGCCAGGAATTTCTTAGTCCA	TGGTTCCGTGATGTCTACCTT	AG	215	Bohn, unpublished
Amb44	Ambystoma	TTCGAGAGAGCGTGAAGGAT	TGTGACAAACTTACTTGCCCTTT	AG	159	Bohn, unpublished

Table 1.3 – Locations of 13 ponds sampled on Eglin Air Force Base. The number (*n*) of individuals sampled and genotyped per site per sampling year is shown.

Pond	(*n*) 2013-2014	(*n*) 2015-2016	Total *n* per pond
SF	5	0	5
15	22	25	47
215	10	25	35
32	3	6	9
33	10	0	10
34	5	1	6
112	2	1	3
49	5	0	5
5	111	49	160
53	24	6	30
4	164	123	287
213	0	5	5
212	20	10	30

Table 1.4 – Examples of 8 models with different cost values used for land cover levels that were tested for affecting flatwoods salamander movement on Eglin Air Force Base.

Levels	Resistance values							
	$Cost_1$	$Cost_2$	$Cost_3$	$Cost_4$	$Cost_5$	$Cost_6$	$Cost_7$	$Cost_8$
Open Water	10	10	10	10	10	10	10	10
Developed, Open Space	8	10	10	10	10	10	10	10
Developed, Low Intensity	6	10	10	10	10	10	10	10
Developed, Medium Intensity	7	10	10	10	10	10	10	10
Developed, High Intensity	10	10	10	10	10	10	10	10
Barren Land	10	10	10	10	10	10	10	10
Deciduous Forest	5	5	1	10	10	10	10	10
Evergreen Forest	4	4	1	1	1	1	1	1
Mixed Forest	4	4	4	10	10	10	10	10
Scrub/Shrub	5	5	5	10	1	1	1	1
Herbaceous	3	3	3	3	3	3	1	1
Hay/Pasture	9	9	9	9	1	10	10	10
Cultivated Crops	9	9	9	9	9	1	10	10
Woody Wetlands	2	1	1	1	1	1	1	1
Emergent Herbaceous Wetlands	4	3	10	10	10	10	10	10
Road	9	9	9	9	9	9	9	9

Table 1.5 – Examples of 8 models with different cost values for varying elevation levels (m) that were tested for affecting flatwoods salamander movement on Eglin Air Force Base.

Levels	Resistance values							
	$Cost_1$	$Cost_2$	$Cost_3$	$Cost_4$	$Cost_5$	$Cost_6$	$Cost_7$	$Cost_8$
0-1	10	10	10	10	10	10	10	1
1.1-3	10	10	6	10	10	10	10	1
3.1-5	6	8	3	10	10	10	10	1
5.1-7	3	4	1	1	1	10	10	1
7.1-9	1	2	1	1	1	1	10	1
9.1-11	3	4	3	1	1	1	1	1
11.1-13	3	6	6	1	1	1	1	1
13.1-15	3	6	6	1	8	1	1	1
15.1-17	6	8	8	10	10	1	1	1
17.1-19	10	10	10	10	10	10	10	10
19.1-21	10	10	10	10	10	10	10	10
>21.1	10	10	10	10	10	10	10	10

Table 1.6 – Examples of 8 models with different cost values for varying slope levels (% change) that were tested for affecting flatwoods salamander movement on Eglin Air Force Base.

Levels	Resistance values							
	$Cost_1$	$Cost_2$	$Cost_3$	$Cost_4$	$Cost_5$	$Cost_6$	$Cost_7$	$Cost_8$
0-3	1	1	1	1	1	1	2	1
3.1-6	2	2	2	2	2	2	4	2
6.1-9	3	3	3	3	3	3	6	3
9.1-12	4	4	4	4	4	4	8	6
12.1-15	5	5	5	5	5	10	10	10
15.1-18	6	6	6	6	10	10	10	10
18.1-21	7	7	7	10	10	10	10	10
21.1-24	8	8	10	10	10	10	10	10
24.1-27	9	10	10	10	10	10	10	10
27.1-30	10	10	10	10	10	10	10	10
>30.1	10	10	10	10	10	10	10	10

Table 1.7 – Hardy-Weinberg equilibrium *P*-values by loci. (-) = loci was monomorphic (*) no test was run because the locus was already found to be unusable.

Pond	Year	*n*	Abp04	AcroD300	AjeD23	Atex65	AjeD75	AjeD162	AcroD330	AjeD314	AjeD37	AmaD367	AmmH136
SF	2013-2014	5	-	0.2366	1.0000	0.1454	0.2376	1.0000	1.0000	0.1514	0.3649	0.3030	1.0000
15	2013-2014	22	0.2175	0.9436	0.2702	0.6522	0.7851	0.2250	0.9605	0.1753	0.3930	0.2997	0.2131
15	2015-2016	25	1.0000	0.5214	0.2698	*	*	0.1857	0.2947	<0.0001	0.0225	0.0295	0.2033
215	2013-2014	10	1.0000	0.0387	0.2422	0.2174	0.3674	0.0963	0.2143	1.0000	0.9416	0.9679	0.8552
215	2015-2016	25	1.0000	0.0217	0.2333	*	*	0.0946	0.1483	1.0000	0.9684	0.9479	0.7920
32	2013-2014	3	1.0000	0.4689	1.0000	0.2003	0.0661	0.4640	1.0000	1.0000	0.4666	0.1997	1.0000
32	2015-2016	6	1.0000	0.7218	0.6353	*	*	0.5852	0.5878	-	0.2059	0.1966	1.0000
33	2013-2014	10	0.4790	0.8964	0.1484	0.1780	0.5064	0.8578	0.6627	0.2037	0.5067	0.1092	0.4649
34	2013-2014	5	0.4281	0.6972	0.6187	1.0000	0.1107	0.8476	0.2380	1.0000	0.0494	0.4889	0.5411
112	2013-2014	2	-	0.3335	0.3349	1.0000	0.3324	1.0000	1.0000	-	1.0000	1.0000	1.0000
49	2013-2014	5	-	0.7970	1.0000	0.6574	0.0494	1.0000	0.6174	1.0000	1.0000	0.2392	0.2379
5	2013-2014	111	1.0000	0.1841	1.0000	<0.0001	0.0002	0.0009	0.0117	0.0029	0.5832	0.2784	0.3122
5	2015-2016	49	1.0000	0.0008	0.9687	*	*	0.0029	0.1058	0.4397	0.0222	0.2297	<0.0001
53	2013-2014	24	1.0000	0.0654	1.0000	0.0122	0.0032	0.5998	0.6637	0.3259	0.4410	0.0020	0.3657
53	2015-2016	6	1.0000	1.0000	0.1430	*	*	1.0000	1.0000	1.0000	0.1425	0.3092	0.6595
4	2013-2014	164	1.0000	0.5709	0.0077	0.0132	0.0029	0.1299	0.1654	0.7220	0.7239	0.0738	0.8258
4	2015-2016	123	1.0000	0.0648	0.7833	*	*	0.0062	0.5038	0.9911	0.3104	0.0330	0.3859
213	2015-2016	5	1.0000	1.0000	0.4310	1.0000	*	0.0337	1.0000	0.1742	1.0000	0.1125	0.4917
212	2013-2014	20	0.5480	0.4836	0.0155	0.0020	0.4912	0.0784	0.1085	0.0362	0.0171	0.1160	0.1691
212	2015-2016	10	0.3072	0.8876	1.0000	*	*	0.0532	0.5208	0.7294	0.7506	0.1410	0.0413

Table 1.8 – AMOVA partition of microsatellite variation between breeding seasons with ponds that were sampled in 2013-2014 and 2015-2016 that had at least 5 individuals in each year. The statistical significance of each component scale was based on 10^4 permutations.

Source of Variation	Degrees of freedom	Molecular variance	Percentage of variation	*P*
Among Breeding Seasons	1	-0.31	-0.9	0.7336
Among Ponds within Breeding Season	14	0.19	5.65	0.0000
Among Individuals within Ponds	546	0.08	2.26	0.0044
Within Individuals	562	3.19	92.99	0.0000
Total	1123	3.43	100	

Table 1.9 – AMOVA partition of total microsatellite variation among three hierarchical scales. *P*-values were determined using 10^4 random permutations.

Source of Variation	Degrees of freedom	Molecular variance	Percentage of variation	*P*
Among Flatwoods	2	0.267	8.11	0.000
Among Ponds within Flatwoods	10	0.104	3.16	0.000
Within Individuals	1201	2.919	88.73	0.000
Total	1213	3.289	100	

Table 1.10 – Microsatellite genetic differentiation between pairs of ponds. Pairwise F_{ST} estimates are below the diagonal and the corresponding *P*-values (based on 10^4 permutations) are above the diagonal.

	SF	15	215	32	33	34	112	49	5	53	4	212	213
SF	-	<0.0001	<0.0001	0.0007	0.0004	0.0091	0.0192	0.0075	<0.0001	< 0.0001	<0.0001	<0.0001	0.0087
15	0.166	-	0.3052	0.0234	0.0065	0.0658	0.0119	<0.0001	<0.0001	< 0.0001	<0.0001	<0.0001	<0.0001
215	0.197	0.004	-	0.0430	0.0257	0.2166	0.0310	0.0002	<0.0001	< 0.0001	<0.0001	<0.0001	0.0002
32	0.167	0.025	0.025	-	0.0678	0.1444	0.0273	0.0055	<0.0001	<0.0001	<0.0001	<0.0001	0.0012
33	0.117	0.029	0.031	0.023	-	0.8141	0.0188	0.0022	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
34	0.141	0.031	0.012	0.021	0.021	-	0.1643	0.0791	<0.0001	0.0027	0.0007	0.0025	0.0241
112	0.234	0.078	0.057	0.053	0.079	0.037	-	0.0166	0.0080	0.0153	0.0140	0.0002	0.0405
49	0.181	0.132	0.121	0.078	0.111	0.063	0.108	-	0.0163	0.0175	0.0012	<0.0001	0.0467
5	0.170	0.117	0.114	0.079	0.116	0.088	0.061	0.032	-	<0.0001	<0.0001	<0.0001	<0.0001
53	0.158	0.113	0.100	0.083	0.106	0.065	0.069	0.047	0.026	-	0.0061	<0.0001	0.0047
4	0.181	0.112	0.089	0.090	0.112	0.070	0.064	0.062	0.032	0.009	-	<0.0001	0.0109
212	0.167	0.135	0.110	0.121	0.107	0.067	0.112	0.112	0.086	0.056	0.042	-	0.0007
213	0.216	0.163	0.136	0.152	0.160	0.091	0.138	0.074	0.074	0.058	0.041	0.075	-

Table 1.11 – Immigration rate estimates from BAYESASS for ponds with sample sizes of at least 30.

Posterior estimate of immigration rate		
Destination pond	Source pond	Mode (95% credible interval)
53	5	0.047 (0.008, 0.156)
53	4	0.003 (0.001, 0.044)
53	212	0.005 (0.001, 0.068)
53	15	0.003 (0.001, 0.047)
5	53	0.025 (0.005, 0.285)
5	4	0.002 (0.001, 0.035)
5	212	0.004 (0.001, 0.059)
5	15	0.003 (0.001, 0.045)
4	53	0.273 (0.176, 0.311)
4	5	0.005 (0.001, 0.074)
4	212	0.013 (0.001, 0.104)
4	15	0.002 (0.001, 0.035)
212	53	0.005 (0.001, 0.068)
212	5	0.002 (0.001, 0.040)
212	4	0.003 (0.001, 0.041)
212	15	0.002 (0.001, 0.038)
15	53	0.002 (0.001, 0.042)
15	5	0.002 (0.001, 0.039)
15	4	0.002 (0.001, 0.038)
15	212	0.002 (0.001, 0.039)

Table 1.12 – LDNe based estimates of the mean and 95% confidence limits (CL) of the effective population size (N_e) for each population with $n \geq 10$ sampled individuals. Where estimable, I have presented the harmonic mean N_e across the two years for a given pond, along with the lowest and highest CL from the sampled years. Negative mean N_e values indicate an N_e indistinguishable from infinity (INF).

Pond	Sample Year	Mean N_e	95% CL
4	2013-2014	22.7	(18.8-27.2)
	2015-2016	32	(19.9-52.7)
	Mean	26.6	(18.8-52.7)
5	2013-2014	20.0	(16-24.9)
	2015-2016	60.5	(30.2-229.4)
	Mean	30.1	(16-229.4)
15	2013-2014	22.0	(12.2-53.8)
	2015-2016	28.4	(15.3-79.8)
	Mean	24.8	(12.2-79.8)
33	2013-2014	23.6	(9-INF)
53	2013-2014	-611.1	(87.4-INF)
212	2013-2014	10.4	(5.9-19.4)
	2015-2016	15.6	(5.6-1840.2)
	Mean	12.5	(5.6-1840.2)
215	2013-2014	21.4	(7.2-INF)

Table 1.13 – Microsatellite genetic diversity statistics for breeding ponds of RFS, averaged across 9 loci (standard deviation in parentheses). Statistics include sample size (*n*), number of alleles per locus (*A*), allele richness standardized to a sample size of 3 individuals (A_R), observed heterozygosity (H_O), and expected heterozygosity (H_e).

Pond	*n*	*A*	A_R	H_O	H_E
SF	5	3.44 (1.42)	2.75 (1.03)	0.58 (0.19)	0.61 (0.27)
15	46	6.00 (2.65)	3.01 (0.75)	0.61 (0.20)	0.65 (0.24)
215	11	4.33 (1.58)	2.97 (0.69)	0.73 (0.17)	0.66 (0.23)
32	9	4.11 (1.05)	3.00 (0.77)	0.69 (0.29)	0.64 (0.28)
33	11	4.67 (1.32)	3.21 (0.55)	0.71 (0.15)	0.71 (0.23)
34	5	4.11 (0.93)	3.40 (0.61)	0.71 (0.21)	0.75 (0.24)
112	3	3.11 (1.36)	3.11 (1.36)	0.86 (0.16)	0.79 (0.28)
49	5	3.44 (1.42)	2.85 (0.96)	0.65 (0.24)	0.67 (0.25)
5	160	7.00 (2.69)	3.07 (0.95)	0.68 (0.25)	0.64 (0.30)
53	30	6.00 (2.40)	3.15 (0.93)	0.59 (0.21)	0.66 (0.29)
4	287	8.89 (4.62)	3.14 (0.81)	0.65 (0.16)	0.67 (0.26)
212	30	4.44 (1.59)	2.83 (0.65)	0.66 (0.14)	0.63 (0.23)
213	5	3.33 (0.71)	2.81 (0.60)	0.76 (0.26)	0.62 (0.25)

Table 1.14 – Results of Mantel tests with 10^4 iterations for the different landscape resistance value schemes and factor combinations, ordered from most- to least-strongly related to F_{ST}. All models had a *P*-values < 0.0001.

Model	R
$Landcover_8$	0.8584
$Landcover_7$	0.8578
$Landcover_5$	0.8471
$Landcover_8 + Slope_2$	0.8463
$Landcover_6$	0.8453
$Landcover_3$	0.8402
$Landcover_4$	0.8377
$Landcover_2$	0.8243
$Landcover_8 + Elevation_6$	0.8166
$Landcover_8 + Slope_2 + Elevation_6$	0.8002
$Landcover_1$	0.7731
$Elevation_6$	0.6587
$IBD_{EqualRes}$	0.6583
$Slope_1$	0.6574
$Slope_2$	0.6574
$Slope_3$	0.6574
$Slope_4$	0.6574
$Slope_5$	0.6574
$Slope_6$	0.6574
$Slope_7$	0.6572
$Slope_8$	0.6570
$Elevation_8$	0.6372
$Elevation_4$	0.6256
$Elevation_1$	0.6195
$IBD_{Euclidian}$	0.5910
$Elevation_2$	0.5756
$Elevation_7$	0.4852
$Elevation_3$	0.4238
$Elevation_5$	0.2100

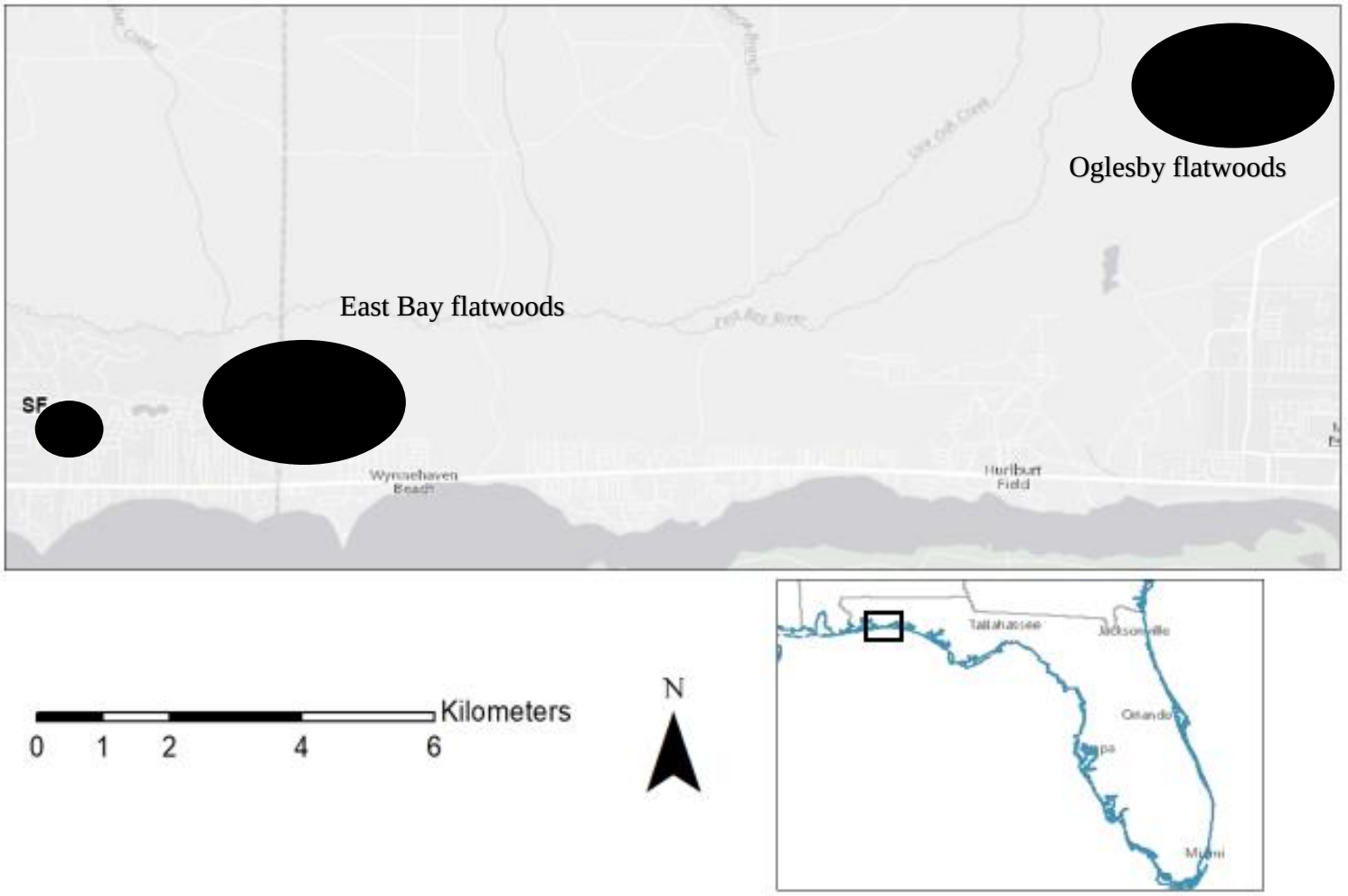

Figure 1.1 – Map depicting pond areas sampled for *Ambystoma bishopi* on Eglin Air Force Base. East Bay flatwoods includes ponds: 15, 32, 33, 34, 112, 215 and Oglesby flatwoods includes ponds: 4, 5, 49, 53, 212, 213.

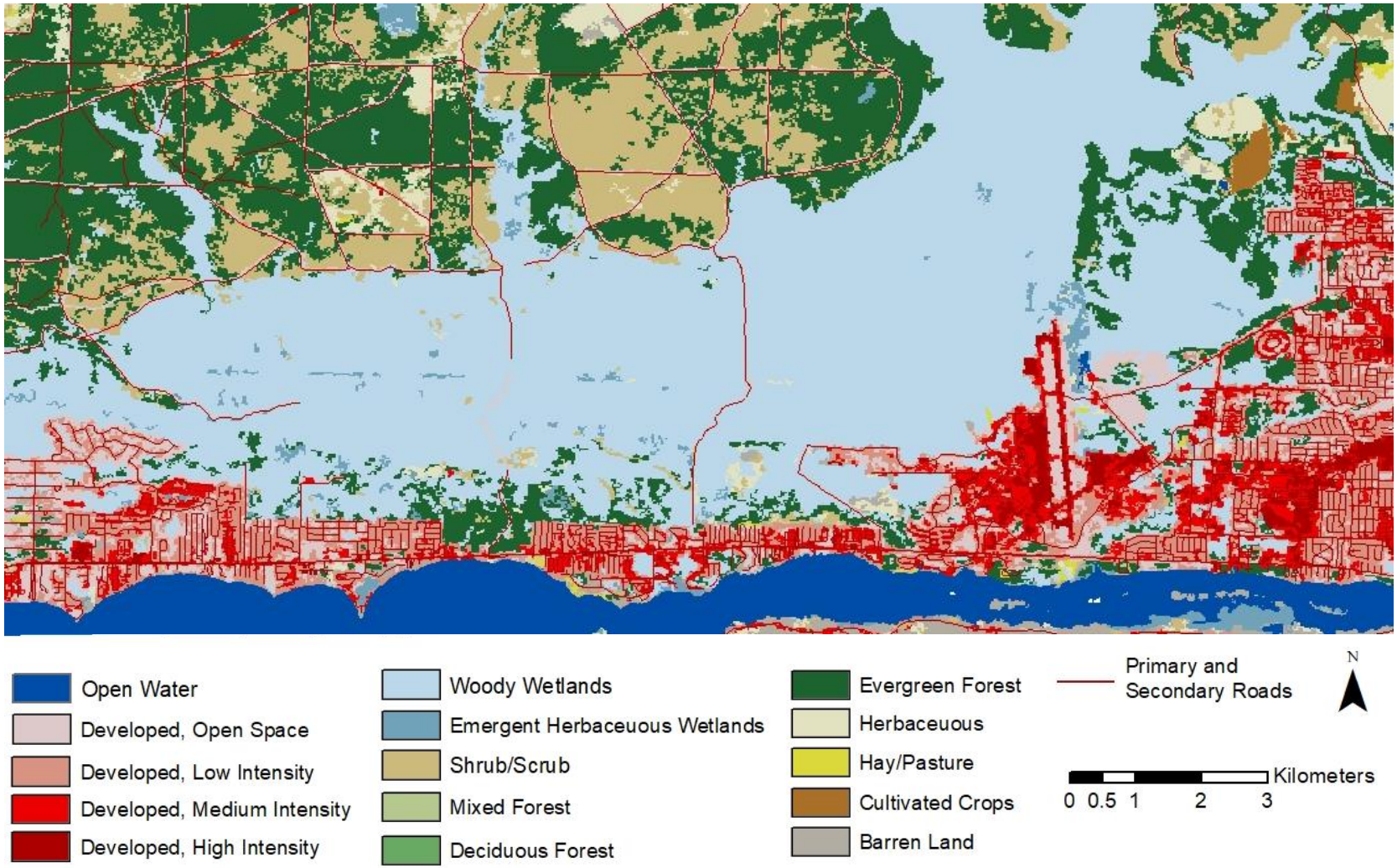

Figure 1.2 – Map depicting landcover types as well as roads found on Eglin Air Force Base.

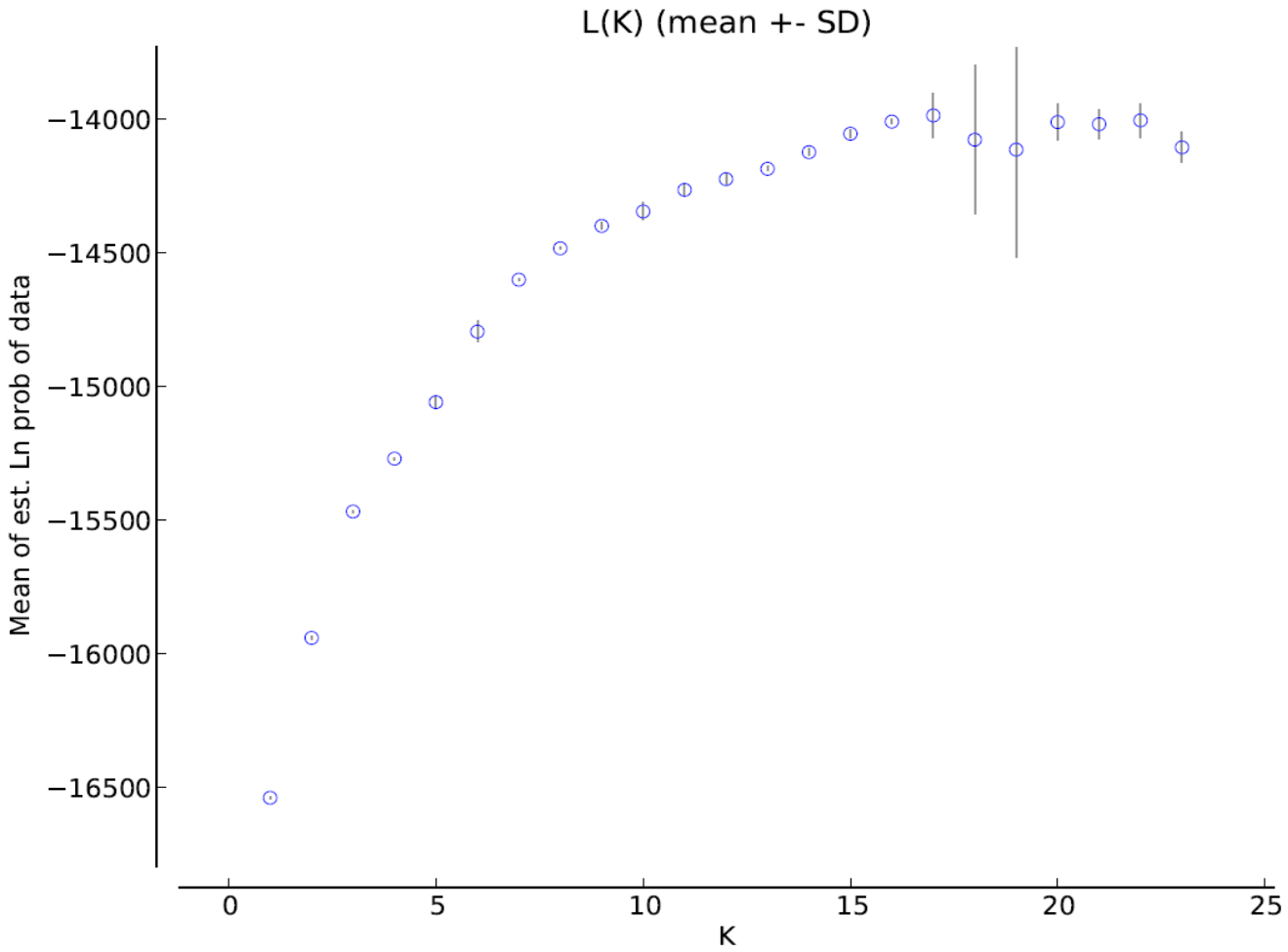

Figure 1.3 – Mean and standard deviation (error bars) log likelihood values of the 10 replicate models for each of 23 possible *K*-values used in STRUCTURE modeling.

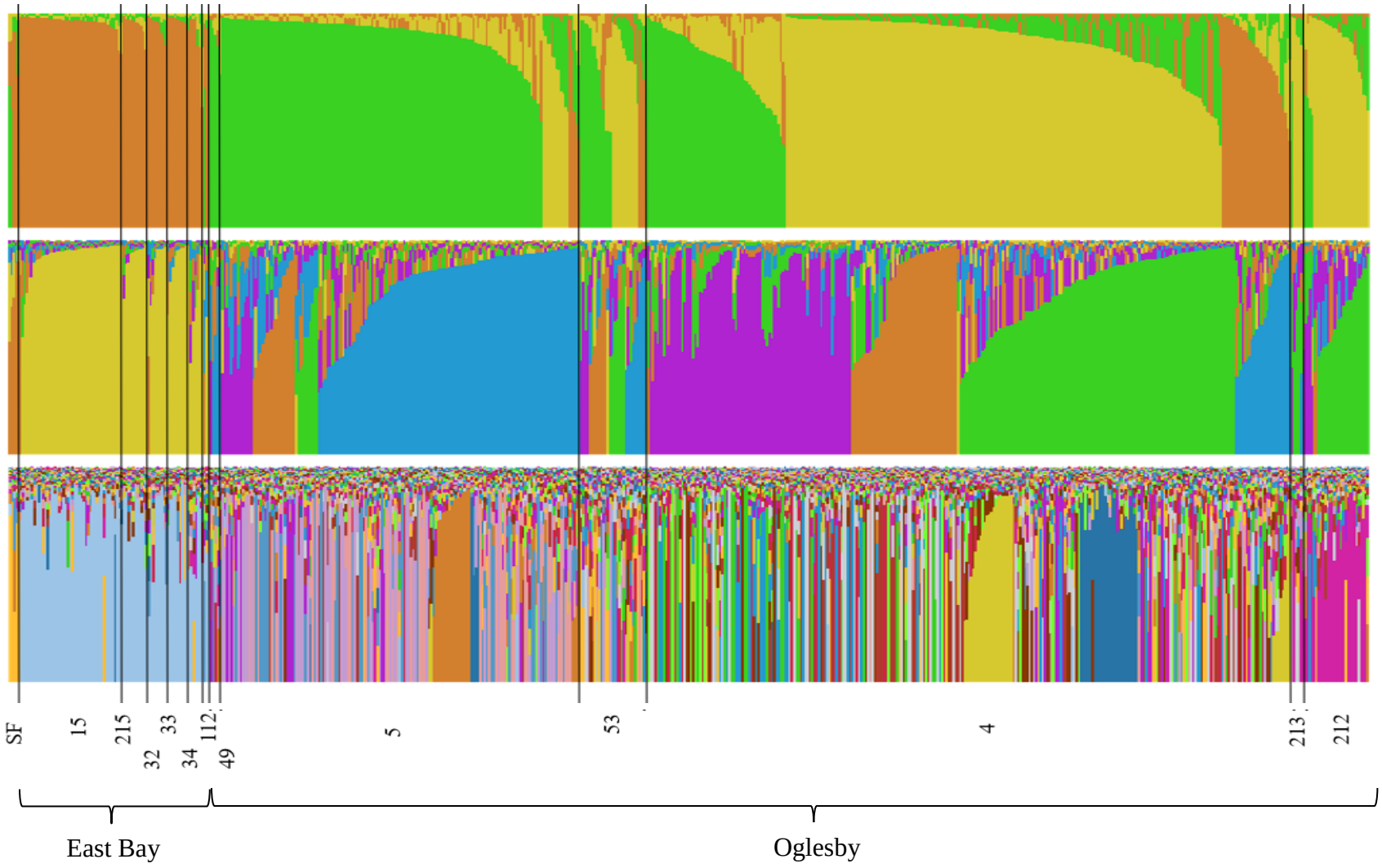

Figure 1.4 – Plot of the results from STRUCTURE models featuring *K*=3 (top panel), *K*=5 (middle panel), and *K*=17 (bottom panel). Pond names are located on the x axis with black bars denoting cut-off between ponds. Ponds are arranged West to East.

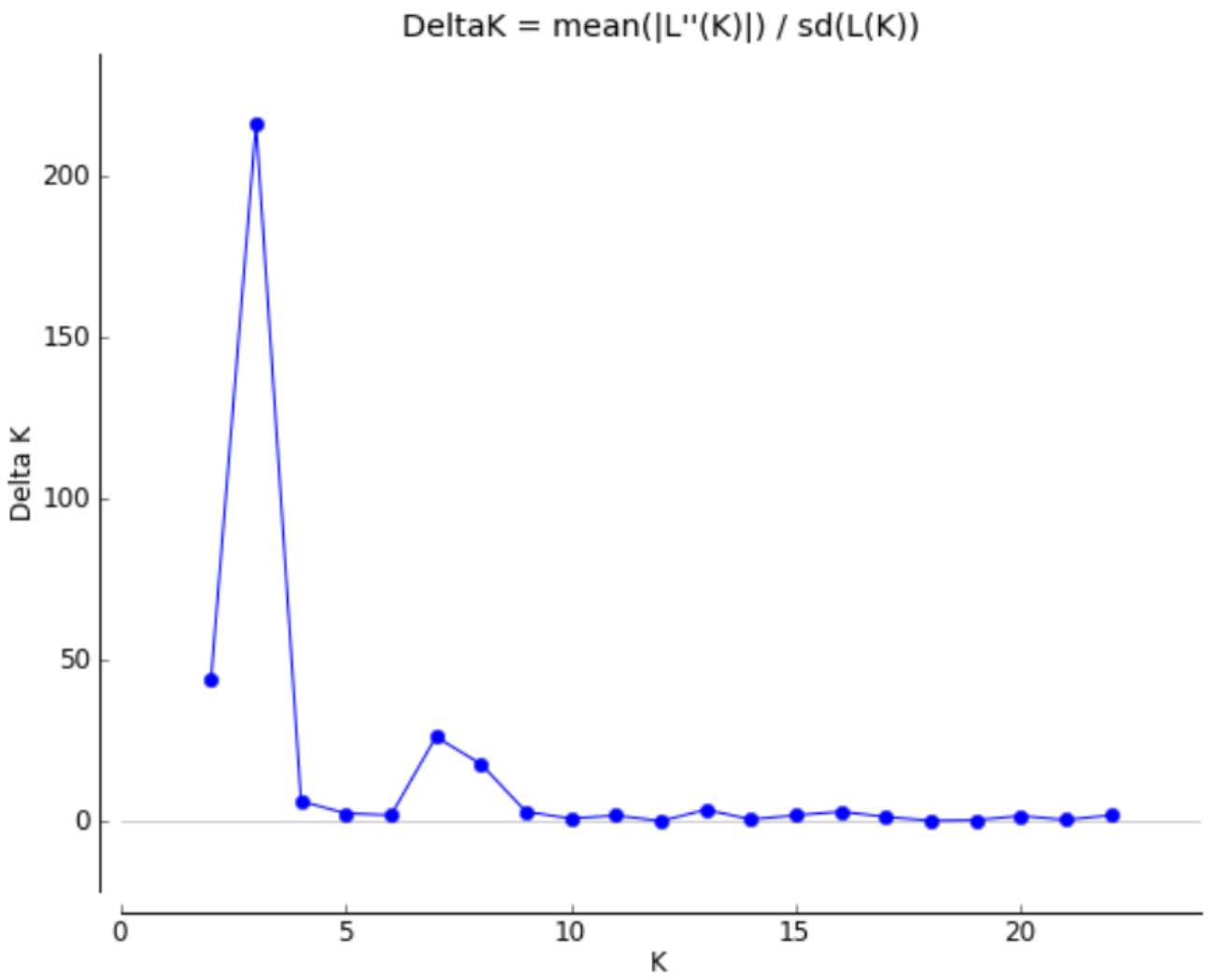

Figure 1.5 – Plot of Δ*K* based on the 2nd order rate of change of the likelihood distribution across 10 replicate models for each of the 23 possible *K* values in STRUCTURE.

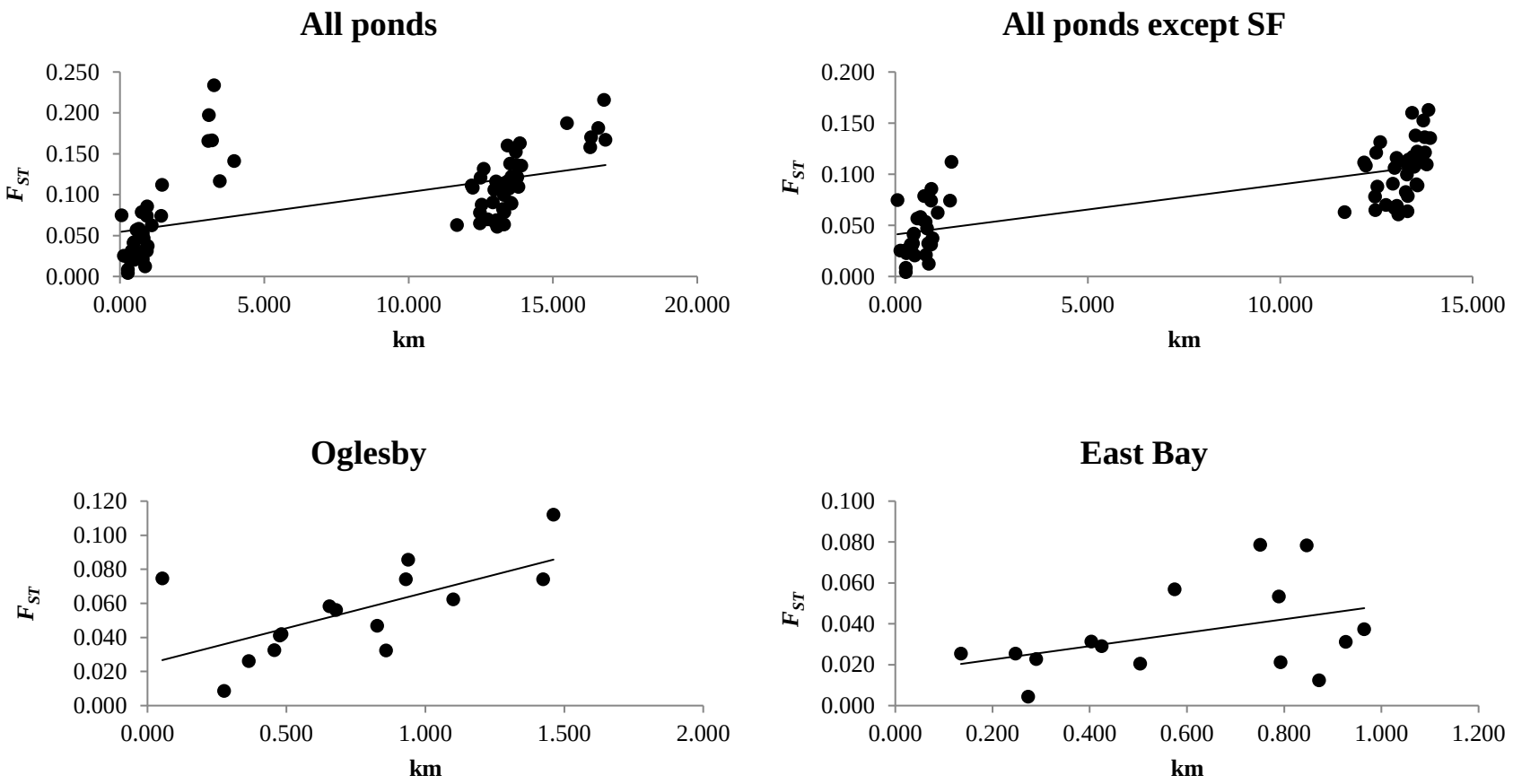

Figure 1.6 – Comparison of pairwise genetic distance (F_{ST}) to the Euclidian distance (km) using Mantel tests with 10^4 iterations. Graphs include all ponds (R = 0.5910, P = < 0.0001), all ponds excluding pond SF (R = 0.7640, P = < 0.0001), Oglesby flatwoods (R = 0.6370, P = 0.01), and East Bay flatwoods (R = 0.4150, P = 0.226).

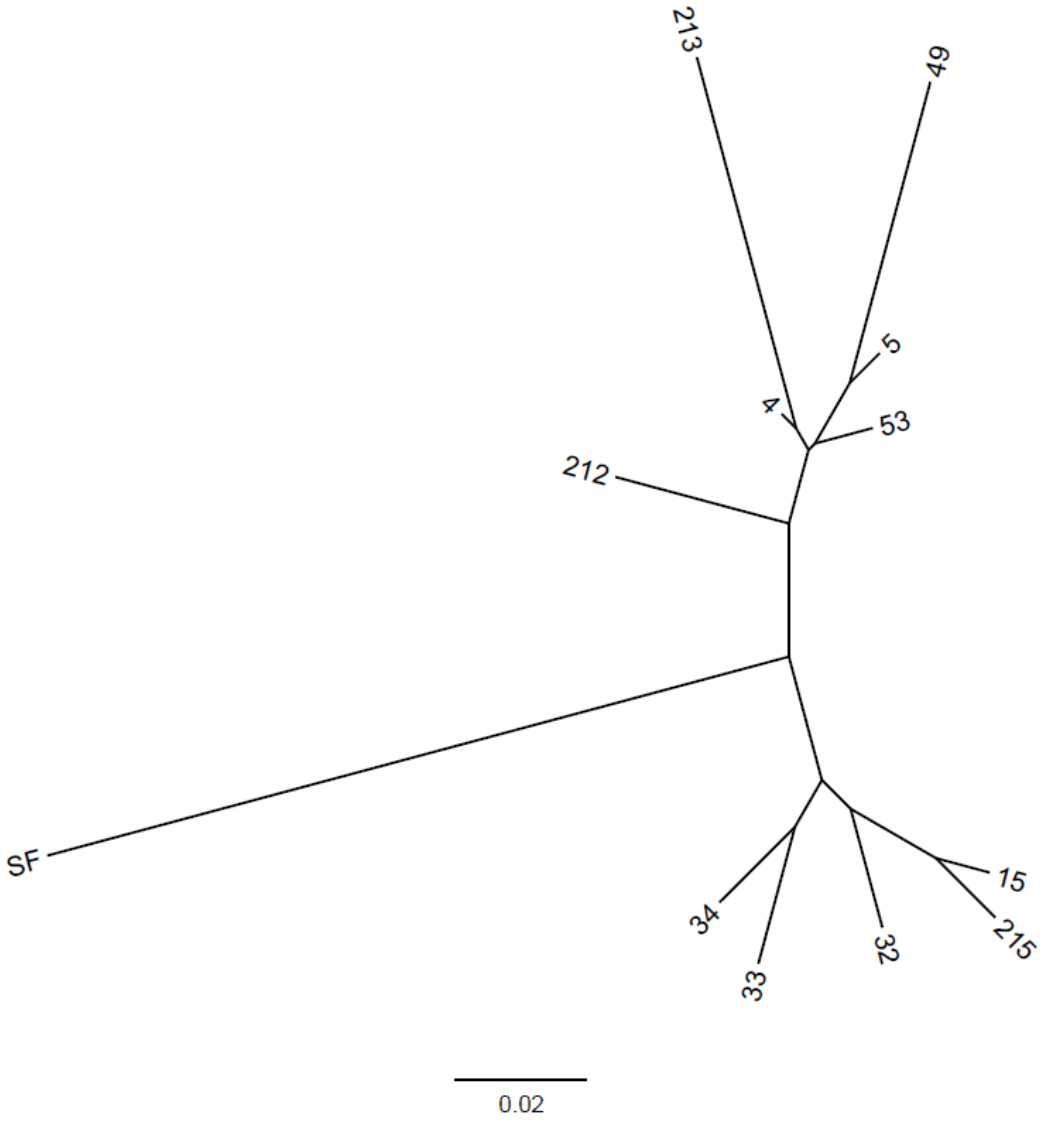

Figure 1.7 – Neighbor-joining tree based on a matrix of pairwise Nei's D_m values among all ponds with $n \geq 5$.

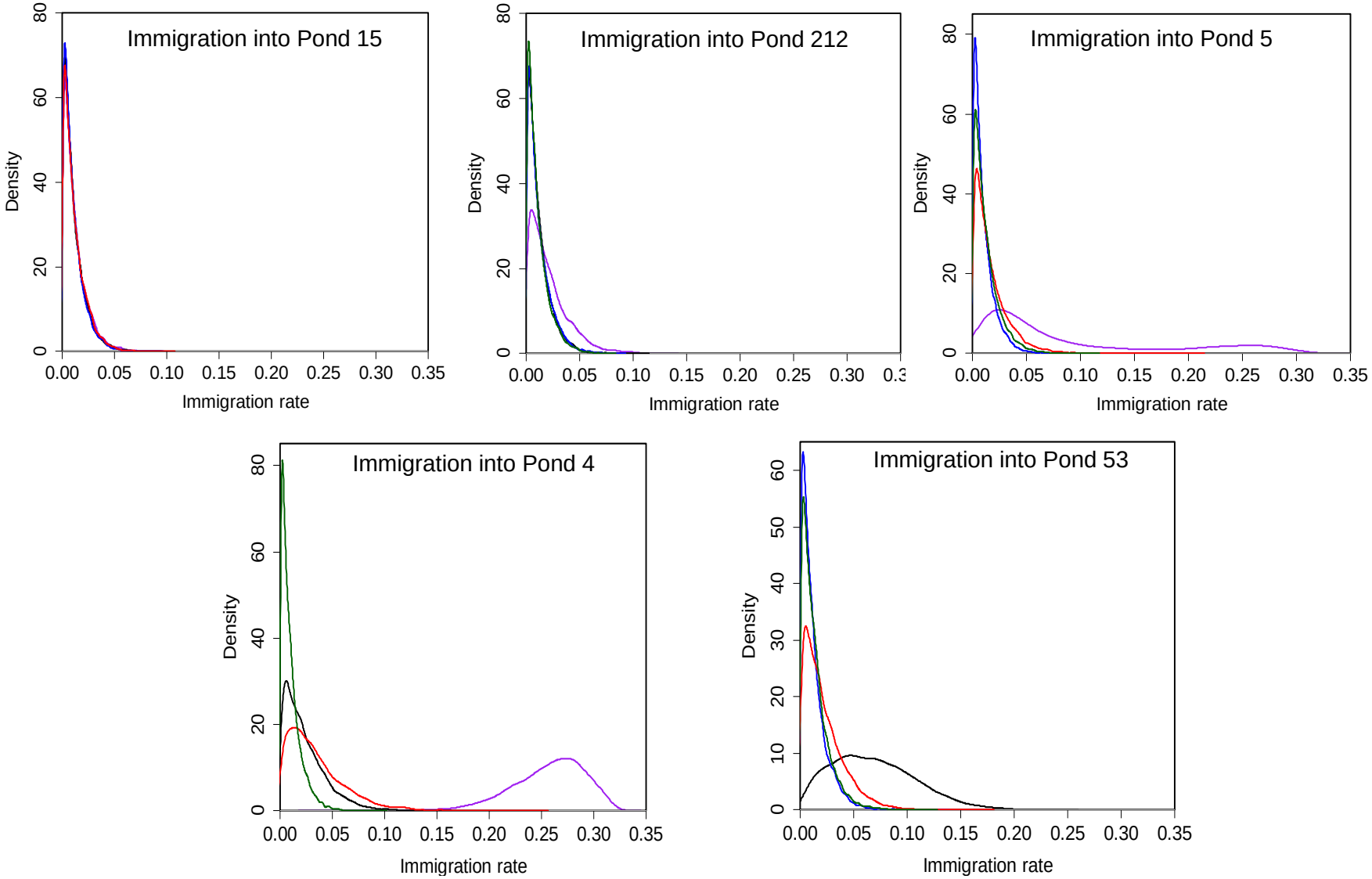

Figure 1.8 – Posterior densities of immigration rates into each pond (see panel titles) from each of four other ponds, as estimated by BAYESASS. Black = pond 5, Blue = pond 4, Red = pond 212, Green = pond 15, and Purple = pond 53.

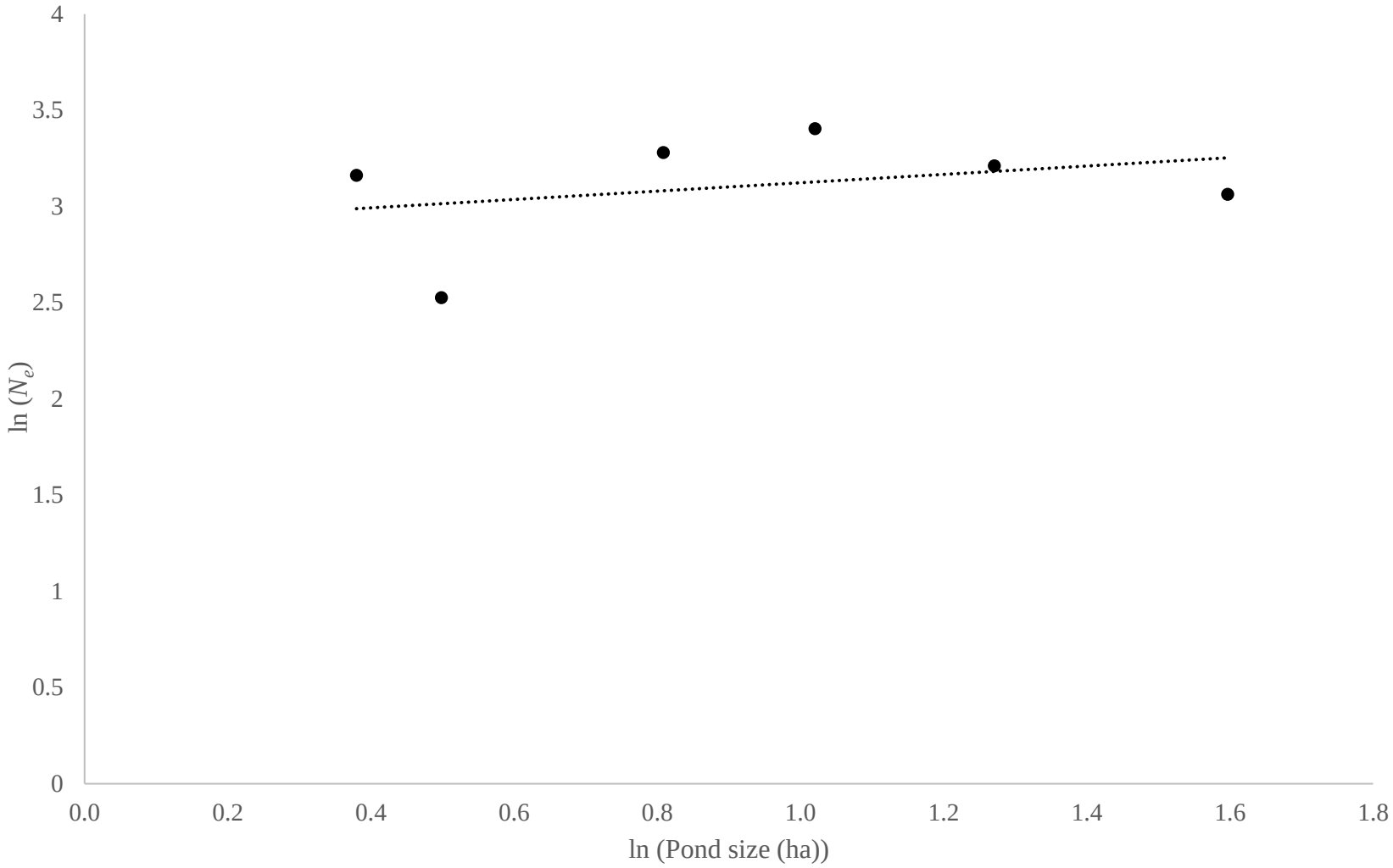

Figure 1.9 – The relationship between the natural log of N_e and the natural log of pond area. The linear regression trend (dotted) line was positive and significant (R^2=0.1027, P=<0.0001).

CHAPTER 2: BREEDING BIOLOGY OF RETICULATED FLATWOODS SALAMANDERS (*AMBYSTOMA BISHOPI*) ON EGLIN AIR FORCE BASE

Abstract

Relative to other ambystomatid salamanders, the breeding biology of the reticulated flatwoods salamander (RFS; *Ambystoma bishopi*), an endangered species endemic to pine flatwoods of the southeastern United States, is poorly known. I used a population genetic study of two breeding populations of RFS on Eglin Air Force Base, sampled across two breeding seasons, to better understand the mating system, distribution of reproductive success, and effective population size of the species. I analyzed variation at nine nuclear DNA microsatellite loci to reconstruct the wild pedigrees of sampled larvae, metamorphs, and adults. Polygamy was seen in both males and females, with members of both sexes producing offspring with up to nine partners. Only ~20% of sampled adults left observable offspring, and most full-sib and half-sib families consisted of < 3 and < 16 individuals, respectively. However, some families contributed disproportionately to cohorts, including large deduced full-sib and half-sib families of up to 11 and 56 individuals, respectively. Estimated effective population size ranged from 20 to 61 individuals across ponds, years, and methods, equating to a range of N_e/N ratios of from 0.16 to 0.70. This suggests that, although the pool of successful breeders at these ponds is typically small, it represents a relatively large proportion of adults that return to the pond. Based on my study, RFS utilize polygynandry to increase mating success, such that even though few individuals mate, those that do mate several times. Nonetheless, relatively small effective population sizes suggest that close monitoring, and potentially additional management of pond habitat and/or population size, would be prudent to ensure the persistence of the species.

Introduction

Mole salamanders (Order: Caudata, Family: Ambystomatidae) exhibit complex life-cycles, often requiring seasonal migration among distinct habitat types needed at different times over ontogeny. Adults are terrestrial, spending a majority of their lives in burrows and are seldom seen until the breeding season (Mitchell and Gibbons 2010). During the breeding season, individuals will migrate to breeding ponds from surrounding upland habitats and will only travel to breeding ponds during the correct environmental conditions, for example on warm rainy nights (Semlitsch and Bodie 1998; Kinkead and Otis 2007). The breeding ponds for Ambystomatidae can range in both size and hydroperiod, from highly ephemeral to more permanent (Wilbur 1980; Wilbur 1990; Semlitsch *et al.* 2015; Chandler *et al.* 2016). The breeding season for each species varies widely, with migration and egg laying occurring usually in the range of late fall to late spring. Time needed for larval metamorphosis is highly variable between species as well, with some species needing only a few weeks and others several months to develop into terrestrial juveniles. These juveniles then leave the breeding pond and are presumed to be the primary dispersers between populations (Gamble *et al.* 2007). Most newly metamorphosed individuals, however, stay close to the natal pond. Scott *et al.* (2013) found that 79% of marbled salamanders (*Ambystoma opacum*) remain within 90 meters of the breeding pond and only 2% move beyond 332 meters. Getting to suitable habitat can be difficult for juveniles no matter what the distance, with high mortality due to desiccation (Rothermel and Luhring 2005), predation (Rittenhouse *et al.* 2009), energy depletion (Scott *et al.* 2007) and density effects (Harper and Semlitsch 2007; Pittman and Semlitsch 2013). Rothermel and Semlitsch (2006) found that 83% of spotted salamanders (*Ambystoma maculatum*) do not survive 1 year after metamorphosis due to the causes listed above.

In order to increase probabilities of mating success and to counter this high larval mortality potential, ambystomatids have evolved a variety of complex reproductive behaviors. Many ambystomatid salamander species are aggregate breeders where both males and females congregate at their natal breeding site (Whiteley *et al.* 2014). This aggregation of both sexes increases male-male competition due to the higher ratio of males to receptive females (Emlen and Oring 1977). Males usually arrive to the breeding sites a few days before females (Bishop 1941; Hillis 1977) and males continue to arrive even after females are present. In several Ambystoma species, courtship between sexes is begun by mutual nudging, which triggers males to release spermatophores which are then picked up by females and eggs are fertilized internally (Gopurneko *et al.* 2007). On top of this, many species of salamanders have spermathecae (Sever 2003) and can fertilize their eggs with stored sperm which can create a potential for long-term sperm competition and cryptic female choice (Houck and Schwenk 1984; Tennessen and Zamudio 2003; Chandler and Zamudio 2008). Salamanders are the only vertebrates in which a cloacal sperm storage gland has evolved (Sever 1994). Marbled salamanders (*Ambystoma opacum*) oviposit their eggs in areas that will be flooded by rising water (Petranka 1998) and both have a polygynandrous mating system in which a female can mate with several males and vice versa. Males deposit spermatophores which females then pick up by squatting over them and removing the sperm caps with their cloacas (Arnold 1976). Sever *et al.* in 1995 found that marbled salamanders could store sperm from several males at one time, but sperm within the spermathecae degenerate within a month and no sperm persists more than 6 months. This would mean that offspring from a single female could be multi-paternal but only from males that were active in that breeding season.

Despite adaptations geared toward increasing fertilization at breeding ponds, biotic and abiotic habitat conditions at breeding ponds are highly dynamic over time, such that in any given breeding year, mating success may be skewed toward relatively few breeders (Funk *et al.* 1999; Myers and Zamudio 2004). For example, Gopurenko *et al.* (2007) found in a study of *Ambystoma texanum*, that of 32 males and 25 females sampled, only 17 males and 9 females produced offspring. A small N_e results in faster genetic drift, which causes population genetic diversity to decline. More closely related individuals end up mating with each other over time resulting in inbred offspring. The main consequence of inbreeding is homozygosis and the reduction of genetic diversity within the overall population (Charlesworth and Charlesworth 1987). Lost genetic diversity may constrain the potential to adapt to future environmental conditions (Conner and Hartl 2004). Components of reproductive fitness are depressed in inbreeding populations, and the risk of extinction is increased (Wright 1977; Thornhill 1993; Frankham 1995).

Our understanding of the reproductive biology and ecology of ambystomatids would be improved by additional studies that measure reproductive success and its variation within and among populations. For example, we might improve our basic knowledge of the mating habits and systems utilized and how this may relate to breeding behavior. Ambystomatids court females and then deposit spermatophores which the females then pick up. Spotted salamanders (*A. maculatum*) deposit many spermatophores, spend little time courting females, and have low success with each spermatophore. On the other hand, tiger salamanders (*A. tigrinum*), deposit few spermatophores, spend longer performing courtship, and have higher success with each spermatophore (Arnold 1976). This translates over into how different the paternity within egg masses are between species. In a study of tiger salamanders egg clutches, 44% were multiply

sired (Gopurenko *et al.* 2006) while in a study of spotted salamander egg clutches, more than 70% showed evidence of multiple paternity (Myers and Zamudio 2004).

With a better understanding of the reproductive biology and ecology of ambystomatids, we would improve our ability to effectively manage and conserve this relatively imperiled group. For example, reticulated flatwoods salamanders (*Ambystoma bishopi*) is one such species that is poorly understood and currently listed as endangered status (USFWS 2015). Potential RFS population management activities such as ensuring population growth and the preservation of genetic diversity, are hampered by poor information about their breeding biology and the family structuring that results from this.

The goal of this chapter is to better understand the reproductive biology and ecology of RFS on Eglin AFB, in particular by characterizing the mating system, distribution of reproductive success, and effective population size exhibited over two breeding seasons by populations occupying two focal ponds. I characterized these phenomena via genetic pedigree construction and other population genetic analyses based on variation measured at nine nuclear DNA microsatellite markers (described in Ch. 1). My specific objectives were to determine the breeding structure and success by answering the following questions:

1. What type of mating system does RFS exhibit on Eglin Air Force Base? Do one or both sexes show evidence for polygamy?
2. What proportion of the adults that come to the pond successfully produce offspring, and how does this vary between years and sexes?
3. Is there evidence of offspring returning as adults to their natal breeding pond to breed?
4. How does the effective population size (N_e) vary over time and space, and how does it compare to the population size (N) estimated over the same interval?

Methods

Sampling Collection

Tail clips from larval salamanders and toe clips from metamorph and adult salamanders were collected from 2 breeding ponds, ponds 4 and 5, in the 2013-2014 and 2015-2016 breeding seasons (Table 1). Personnel from Virginia Tech and from Eglin Air Force Base collected larval samples, as described in Chapter 1, and constructed drift fences that completely encompassed each wetland to collect adult and metamorphed individuals. Drift fences were set prior to the first heavy rainfall event in October and were left in place until mid- to late spring. Even though these drift fences encompassed the entire wetland, detection probabilities based on population size estimates from mark-recapture studies indicate a 70% chance of capturing an individual moving in and out of the pond (pers. comm. George Brooks). The drift fences used 60-cm high rolls of galvanized steel flashing that were buried 15-20 cm to reduce the chance of escape under the fence. Drift fences had funnel traps that were placed approximately 10 meters apart and were placed in pairs on either side of the fence. Funnel traps were 85 cm x 20 cm with 5 cm openings and were constructed using aluminum window screening. Traps were checked every evening to reduce mortality. More information on the methods and time periods for drift fence trapping can be found in Erwin *et al.* (2016).

Upon capture at the drift fences, data including morphometric, mass, and sex for adults via visual cues (gravidity, swollen cloaca) were taken. Tail clips from larvae and toe clips from metamorphed/adults (for mark-recapture purposes as well) of approximately 5-12mm in size, were taken using surgical scissors, cuticle trimmers, or a scalpel that had been sterilized by wiping with alcohol and then burning. Samples were then placed in a 1.5 mL microcentrifuge

tubes containing 95-100% ethanol. After returning from the field, samples were then kept in a -20°C freezer until extraction.

Laboratory Methods

Whole genomic DNA was extracted from tissue samples using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer protocols. Nine nuclear DNA microsatellite markers that were found suitable in tests I performed in chapter 1 were used in this study. PCR reaction mixes were 25 μL in total volume, and consisted of 12.5 μL GoTaq Mastermix (Promega, Madison, Wisconsin, USA), 1 μL forward primer, 1 μL reverse primer, 8.5 μL molecular-grade H_2O, and 2 μL of template DNA. I grouped these loci into three multiplexes for screening individuals as follows: Multiplex 1- AcroD300, Abp04, AjeD23, Atex65; Multiplex 2- AcroD330, AjeD75, AjeD162; and Multiplex 3- AjeD314, AjeD37, AmaD367, AmmH136. The PCR cycling conditions for multiplexes 1 and 2 were as follows: initial denaturation at 95°C (120 s), 35 cycles of denaturation at 95°C (30 s), annealing at 56°C (40 s), and extension at 72°C (90 s), followed by a final extension at 72°C (600 s). For multiplex 3, all conditions were the same except for the annealing step, which was set to 58°C.

Amplified PCR products were visualized on an ABI 3500 Genetic Analyzer with a Genescan 500HD LIZ dye standard (Applied Biosystems). Allele sizes were then independently scored by Dr. Roberts and me in GeneMapper (GeneMapper v4.0; Applied Biosystems). In case of disagreement the GeneMapper output was discussed until a consensus was reached.

Evaluation of Marker Power and Pedigree Construction

To evaluate the level of genetic resolution in the marker set, I used GenAlEx 6.5 (Peakall 2012) in order to determine the probability of identity (PI) and the probability of identity for full siblings (PI_{sibs}) for all individuals in each pond, with all ages and cohorts pooled. I also screened for larval and metamorph individuals in the same year that had the same genotype and found 2 individuals that were the same. For the further analyses I excluded the larval individual from simulations.

I then wanted to test my markers for their ability to delineate accurate pedigrees by determining the model sensitivity (the percentage of correctly matched pairs) and model specificity (the number of correctly unmatched pairs) of offspring-offspring and offspring-parent relationships. I created simulated datasets in which empirical adult genotypes were used to create simulated offspring genotypes. I randomly selected 26 males and 26 females genotyped from Pond 4 in 2015 to "mate" twice each with two different partners, each time producing 3 full-sib offspring nested within a half-sib family of 6 offspring. For these simulated datasets, I conservatively assumed a polygynandrous mating system based on other closely related ambystomatid species. Figure 2.1 shows an example of mating structure and how full-sibs and half-sibs are grouped. Each simulated offspring's genotype was created by randomly selecting one allele from each parent independently for each of the 9 loci. This simulated mating scheme (i.e. the "full" dataset) consisted of 156 full-sib pairs, 468 half-sib pairs, and 11,466 non-sib pairs.

In order to account for the likely situation that not all parents or offspring were sampled, I also created a dataset identical to the first, except that 6 males, 6 females, and 36 offspring were removed (i.e. not "sampled"). Even though the drift fences encompass the entire wetland, capture rate estimates indicate that approximately 30% of individuals may escape detection (pers. comm.

George Brooks). I represented this in the simulated data by removing one or both parents for some of the sets of offspring and by also removing some sets of offspring to represent unsuccessfully breeding adults and/or unsampled offspring. The resulting "reduced" dataset consisted of 120 full-sib pairs, 297 half-sib pairs, and 6,723 non-sib pairs.

I analytically deduced the family pedigrees of these two simulated datasets in COLONY 2.0 (Wang 2004; Jones and Wang 2010) which uses maximum likelihood to assign both sibship and parentage relationships. The model within COLONY 2.0 also can accommodate errors that would arise due to null alleles and other stochastic error (Jones *et al.* 2010). I assumed polygamous mating strategies for both males and females and assumed no inbreeding or clones. I used the long and medium run lengths and full-likelihood estimation with high precision, combining likelihoods over three independent replicate model runs. Full-likelihood methods take into account all the information from other individuals when constructing the pedigree instead of ignoring anything other than the focal pair as in pairwise methods. This can give a better understanding of the structure of the population as well as some insight into mate selection in the species. Both the "update allele frequency" and "sibship size" options were used. Sibship size prior was set to 0 since this variable is unknown in the population. For both mothers and fathers, I assumed a prior probability of 0.7 that any given offspring's actual father/mother was included in the dataset. This is based on detection probabilities at the drift fences (see above). Finally, I analyzed each simulated dataset using two different assumptions about genotyping error. Markers were given a genotyping error rate of either 0 or 0.05 per marker.

From this simulation-based analysis, I determined the optimal way to analyze my empirical data at ponds 4 and 5 from the 2013-2014 and 2015-2016 breeding seasons (Table 2.1). I included unknown-sex adults in both the potential-father and potential-mother input files.

Other input files were identical to those described above, except that based on simulation results (see Results), I conservatively assigned each marker an error rate of 0.

Estimation of Effective Population Size

In order to compare the size of populations between years as well as determine the potential strength of genetic drift, I estimated effective population size (N_e) for each pond at each breeding season. I estimated N_e separately for each cohort at each pond. First, I used the pedigree-based method implemented by COLONY. Within COLONY, N_e is estimated by taking a random sample of individuals from a population and using the frequencies of full and half sib dyads (Wang 2009). COLONY assumes that the individuals are taken at random from a single cohort of the population and unlike other methods, does not assume random mating (Wang and Santure 2009).

Second, I estimated N_e using the linkage disequilibrium approach, implemented in LDNe v1.31 (Waples and Do 2008). LDNe assumes that there is random mating, non-overlapping generations, and sampling of a single cohort. Due to the nature of reticulated flatwoods salamanders, the second of these assumptions is violated since once reaching sexual maturity, salamanders will return to the breeding pond for multiple breeding years. However, all populations should be similarly affected by this violation and thus the variation in N_e estimates should be relatively similar across all populations (Robinson and Moyer 2013). Thus, results should be interpreted as relative measures of gene-pool size that fall between N_e and the effective number of breeders (N_b). I used only the juvenile samples (larvae and metamorphs) for LDNe calculations, because adult samples comprised unknown mixtures of cohorts, which violates the assumptions of the estimation method. Rare alleles can have a large effect on estimating N_e using

the LDNe approach. Waples and Do (2010) suggest that the exclusion of alleles occurring at a frequency less than 2% yields the most accurate N_e so I excluded from calculations alleles that occurred at a frequency < 0.02. I also calculated 95% confidence intervals by jackknifing. Estimates of N_e for each cohort by pond sample were compared to adult population size (N) estimates made in these same breeding years based on a hierarchical Bayesian analysis of mark-recapture data collected at the drift fences (Brooks *et al.* in prep).

Results

Evaluation of Marker Power

Analyses indicated that my panel of 9 loci had sufficient resolution to accurately reconstruct wild pedigrees. First, probabilities of identity were low: PI for pond 4 was $6.4e^{-09}$ and PI_{sibs} was $6.2e^{-04}$, while PI for pond 5 was $5.6e^{-09}$ and PI_{sibs} was $6.8e^{-04}$ (Figure 2.2). Second, pedigree analysis of simulated data with known family pedigrees indicated that, at least under certain sets of assumptions, COLONY's algorithm had high accuracy (sensitivity and specificity). I found that models allowing for no error were highly accurate, even when some data were missing, but allowing for a 0.05 error rate dramatically reduced pedigree accuracy. The model assuming 0.05 error rate was far more conservative than the 0-error rate model in that fewer offspring-offspring and offspring-parent relationships were inferred (Table 2.2). However, even though these both did well identifying full-sib relationships and parent-offspring relationships, the program had some difficulty with identifying the more complex half-sib relationships. For the full dataset with 0.05 marker error, of the 557 half-sibs identified by COLONY 2.0, 198 (35%) of the observations were incorrect, with 85 of the incorrect assignments being actual full-sibs and 113 being actual non-sibs. The reduced dataset showed

similar results even with individuals removed to represent an incomplete sampling. For the some missing dataset with 0.05 marker error, of the 429 half-sibs observed, 202 (47%) of the observations were incorrect, with 60 of the incorrect assignments being actual full-sibs and 142 being non-sibs. Setting the error rate to 0 resulted in fewer incorrect calls. For the full dataset with 0 marker error, of the 574 total half-sibs identified by COLONY, 118 (21%) of the observations were incorrect, with 44 being actual full-sibs and 74 being non-sibs. For the reduced dataset with 0 error, of the 437 total half-sibs identified, 146 (36%) of the observations were incorrect with 48 of those being actual full-sibs and 98 being actual non-sibs. Many of these incorrect sib-ships were due to the fact that the program was unable to assign the offspring to its correct parent and thus assigned several to hypothetical parents resulting in incorrect relationships among offspring. Regardless of the settings used in COLONY 2.0, complete sampling of adults appeared to be important since when hypothetical parents are needed to be made to create complete pedigrees, COLONY will overestimate the number of adults and decrease family sizes resulting in incorrect relationships being formed.

Reconstruction of Wild Pedigrees

Based on simulation results, I analyzed the four empirical datasets (two ponds for two years) using COLONY models featuring a genotyping error rate of 0, long run length, and full-likelihood precision. Deduced pedigrees included both sampled adults that produced observed offspring and hypothetical adults that were created to account for deduced offspring. I found evidence for a polygynandrous mating system in that both females and males were identified to have multiple mating events. Multiple mating was equally common among females (46.7% of individuals overall) and males (46.3% overall). Although both sexes mated with a mode of one

mate, mating success was skewed, with some members of both sexes producing offspring with up to nine mates (Figure 2.3).

Most sampled adults (79% of both males and females) failed to leave deduced progeny, and those parents that did contributed unevenly to the new cohort. Although males and females most commonly were matched to only one offspring, two different males were matched to 13 offspring, and two females each were matched to over 20 offspring (Figure 2.4). Similarly, COLONY results showed that full-sib families were small, consisting of 1-3 individuals, but some contained as many as 11 individuals (Figure 2.5); likewise, half-sib family size was typically 1-16 individuals, but some cases up to 56 individuals (Figure 2.6). In general, patterns for mating structure were consistent across ponds and years. The number of offspring produced per parent was also similar regardless of pond or year.

Among individuals genotyped both as juveniles (in 2013-2014) and adults (in 2015-2016), I saw evidence for natal philopatry. I found that 10 juveniles (6 males, 4 females) from pond 4 and 2 juveniles (2 females) from pond 5 sampled in the 2013-2014 breeding season had genotypes matching adults identified in the 2015-2016 breeding season. Thus, these results indicate that both males and females had returned to the pond within two years.

Estimation of Effective Population Size

Estimated mean effective population sizes ranged among ponds and years from 20 to 61 individuals based on the LDNe method and from 27 to 58 individuals based on the COLONY method (Table 2.3). Ranges between the two estimates were similar, but COLONY estimated a higher N_e for three of the four ponds x years. For both methods, confidence intervals were

narrow, indicating a high precision of the estimates. Resulting N_e/N ratios were variable but relatively high, ranging from 0.16 to 0.70.

Discussion

Mating Strategy and Success

Based on its commonness across ponds and years, I posit that polygynandry is frequently utilized by flatwoods salamanders to increase reproductive opportunities. Pedigree analysis indicated that both sexes were involved in multiple mating events and that there was a slight skew for females having multiple mates compared to males, which is also seen in small-mouthed salamanders, *Ambystoma texanum* (Gopurenko 2007). Half-sib families were large, indicative of several mating events occurring, and several pedigrees showed multiple mates for both the males and females involved.

It was surprising that there were a large number of offspring whose parents had not been sampled, such that COLONY was forced to create hypothetical parents for these offspring. Several offspring for both ponds and years were only assigned one or no sampled parents, which could be caused by several possibilities. One is that the drift fences constructed around the ponds are not as effective as thought. Even though the drift fences surround the entire pond, estimates from Eglin suggest that there is a 70% chance of catching an individual as it moves in and out of the pond (pers. comm. George Brooks). Secondly, there is the possibility that some salamanders do not emigrate and stay within the breeding basin. Adult ambystomatid salamanders normally emigrate after the reproductive season and overwinter >50 m from the breeding pond (Semlitsch 1998; Regosin 2005; Gopurenko 2007), however there are some exceptions to this. *A. texanum* have had recorded post-mating migrations ranging from 0 m to 125 m with many adults found <

50 m from the breeding pond (Williams 1973). Yearlings, though not usually members of the breeding population, could still possibly be contributing to the effective population. Since small salamanders are more prone to desiccation (Spotila 1972; Semlitsch 1981) they may be confined to breeding ponds if a preceding dry summer prevented dispersal from the pond (Palis 1997). Finally, genotyping errors could have reduced the accuracy of pedigree assignments and caused some true parent-offspring pairs to be rejected. However, individuals that were sequenced multiple times showed error rates were low. Moreover, allowing for genotyping errors up to 5% did not increase the number of matches (data not shown). Whatever the case may be, the creation of hypothetical parents by COLONY likely overestimated the number of parents, as seen in the simulated dataset analyzed with missing adults. This would mean that family sizes would be fewer in number but larger in size than what I estimated, which would cause COLONY-based estimates of *Ne* to be upwardly biased. The fact that few of the sampled adults were assigned to offspring would support this as well. Within several other ambystomatids, mating success is skewed toward only a few individuals (Funk *et al.* 1999; Myers and Zamudio 2004) and my results support this for RFS as well.

Effective Population Sizes

Having adequate genetic diversity and the recruitment of offspring into the breeding population is crucial to having a sustainable population. In a 22-year study of the flatwoods salamander population in western Florida, Means *et al.* (1996) found that the population had decreased by almost 99%, nearly to extinction, due to habitat modification. The importance of keeping effective population sizes high is a key strategy for keeping both genetic diversity and potential recruitment up, especially in a species that displays philopatry. For pond 4, there was an

estimated increase in both the census (N) and effective population size (N_e) from the 2013-2014 breeding season to the 2015-2016 breeding season. Pond 5 also showed an increase N and N_e based on LDNe, whereas N_e based on COLONY was the same between breeding seasons. This is most likely due to the fact that in the 2015-2016 breeding season, no larvae were sampled from this pond. This would disproportionately affect COLONY's calculation of N_e since it is based on the construction of pedigrees and requires individuals from several families. N_e sets a lower limit for a viable population size and general guidelines propose that an $N_e > 500$ is needed to maintain adequate genetic diversity over the long term while $N_e < 50$ is indicative of a population facing the threat of inbreeding depression in the short term (Franklin 1980). Even with the increase in population size and effective breeders for the ponds, estimates are still low and close to or below the N_e indicative of inbreeding depression. Tracking the N_e over time is critical since N_e represents the number of individuals that are contributing to the propagation of the species to the next generation.

From the 2013-2014 breeding season, some offspring were found to return to their natal pond in the 2015-2016 breeding season. *A. bishopi* have been shown to home to and from breeding ponds to a particular terrestrial retreat (Palis 1997). This is especially concerning since even if population numbers for a pond were to increase, those individuals may be closely related. Moreover, if philopatry is strong, RFS may have limited potential to migrate to or recolonize other ponds. Results of Ch. 1 indicated that most breeding ponds are not connected by significant contemporary dispersal. Monitoring N_e gives insight into the genetic health of the population as a higher N_e would help to maintain genetic diversity within the population. The high N_e/N ratios indicate that a high proportion of the adult population are arriving at the ponds to breed. A majority of the adults seem to return to the breeding ponds during the breeding season in an

attempt to find a mate. Predictive models of N_e/N ratios usually predict a value of 0.25 to 0.75 (Nunney 1993). In a study of spotted salamanders (*A.maculatum*) in Tennessee, the N_e/N was found to be 0.1, though the census population size was found to be higher than typically found in ambystomatids (Armstrong 2012). The fact that my N_e/N ratios were between 0.16 to 0.70 are within a desirable range for population persistence. The N_e of a population is sensitive to variations in census size (Waples 2002) so my relatively high N_e/N ratios indicate that population had remained stable for at least a small period of time.

Conclusions and Direction for Future Study

The breeding biology of RFS on Eglin shows that even though much of the adult population arrive at the ponds to mate, few are successful in producing detectable offspring. Those that do find mates however are successful in that they have many mating events. This results in families that consist of several full and half siblings. The reconstruction of the wild pedigrees most likely would have shown greater family sizes had the adults from which offspring originated from could be identified. Though the N_e for the ponds indicated a cause for concern for the threat of inbreeding, compared to the census population estimates, the majority of adults are actively involved in the effective population.

Additional studies in the mating behavior of RFS would give insight into the factors that contribute to our results. Continued measurement of both N_e and N would allow for better understanding of the recruitment of adults to the population as well as how many of these adults integrate into the breeding population.

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Table 2.1 – Sample sizes for age and sex classes for ponds 4 and 5 from the breeding seasons of 2013-2014 and 2015-2016.

Pond	Breeding season	Known males	Known females	Unknown adult	Larval	Metamorph	Total
4	2013-2014	11	21	5	18	146	201
	2015-2016	28	43	6	31	92	200
5	2013-2014	6	14	3	51	60	134
	2015-2016	14	24	10	0	49	97

Table 2.2 – Simulation results from COLONY 2.0 of two datasets ("Full" and "Reduced") consisting of simulated families, analyzed assuming marker error rates of either 0 and 0.05. Model sensitivity (the percentage of correctly matched pairs) and model specificity (the number of correctly unmatched pairs) are reported for all possible relationships.

Dataset and assumed error rate	Relationship	True number of pairs	Sensitivity	Specificity
Full	Full-sib	156	155/156 (0.994)	11923/11934 (0.999)
0 error	Half-sib	468	456/468 (0.974)	11506/11622 (0.990)
	Paternity	156	153/156 (0.981)	3741/3744 (0.999)
	Maternity	156	151/156 (0.968)	3742/3744 (0.999)
Reduced	Full-sib	120	116/120 (0.967)	7013/7020 (0.999)
0 error	Half-sib	297	291/297 (0.980)	6789/6843 (0.992)
	Paternity	84	84/84 (1.00)	1833/1836 (0.998)
	Maternity	84	84/84 (1.00)	1833/1836 (0.998)
Full	Full-sib	156	88/156 (0.564)	11934/11934 (1.00)
0.05 error	Half-sib	468	359/468 (0.767)	11424/11622 (0.983)
	Paternity	156	137/156 (0.878)	3738/3744 (0.998)
	Maternity	156	126/156 (0.808)	3737/3744 (0.998)
Reduced	Full-sib	120	70/120 (0.583)	7020/7020 (1.00)
0.05 error	Half-sib	297	227/297 (0.764)	6641/6843 (0.970)
	Paternity	84	80/84 (0.952)	1831/1836 (0.997)
	Maternity	84	72/84 (0.857)	1831/1836 (0.997)

Table 2.3 – Effective population size (N_e) and total population size (N) estimates from two breeding ponds at two different breeding seasons on Eglin AFB. *n* (offspring) = number of offspring, LDNe N_e = effective population size calculated by LD method, COLONY N_e = effective population size calculated by COLONY, N = population size estimate based on mark-recapture study (see text). Values in parentheses are 95% confidence intervals.

Pond	Cohort	*n* (larvae)	*n* (metamorphs)	LDNe N_e	COLONY N_e	N	LDNe N_e/N	COLONY N_e/N
4	2013-2014	18	146	23 (19-27)	47 (32-70)	86 (75-97)	0.27	0.55
	2015-2016	31	92	32 (20-53)	58 (41-85)	195 (174-218)	0.16	0.30
5	2013-2014	51	60	20 (16-25)	28 (18-50)	40 (32-50)	0.50	0.70
	2015-2016	0	49	61 (30-229)	27 (17-47)	114 (90-146)	0.54	0.24

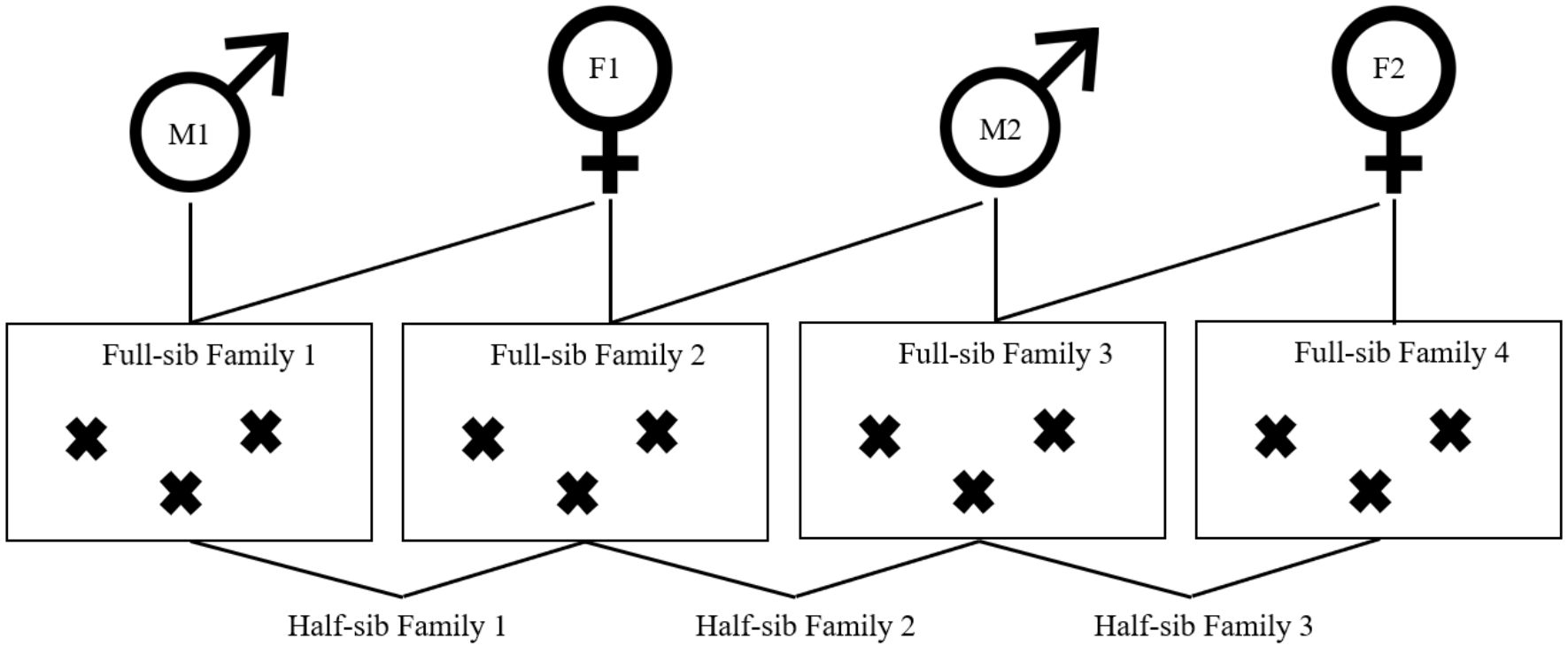

Figure 2.1 – Example pedigree showing of simulations for COLONY with males and females having two mating events each resulting in three offspring (represented by Xs).

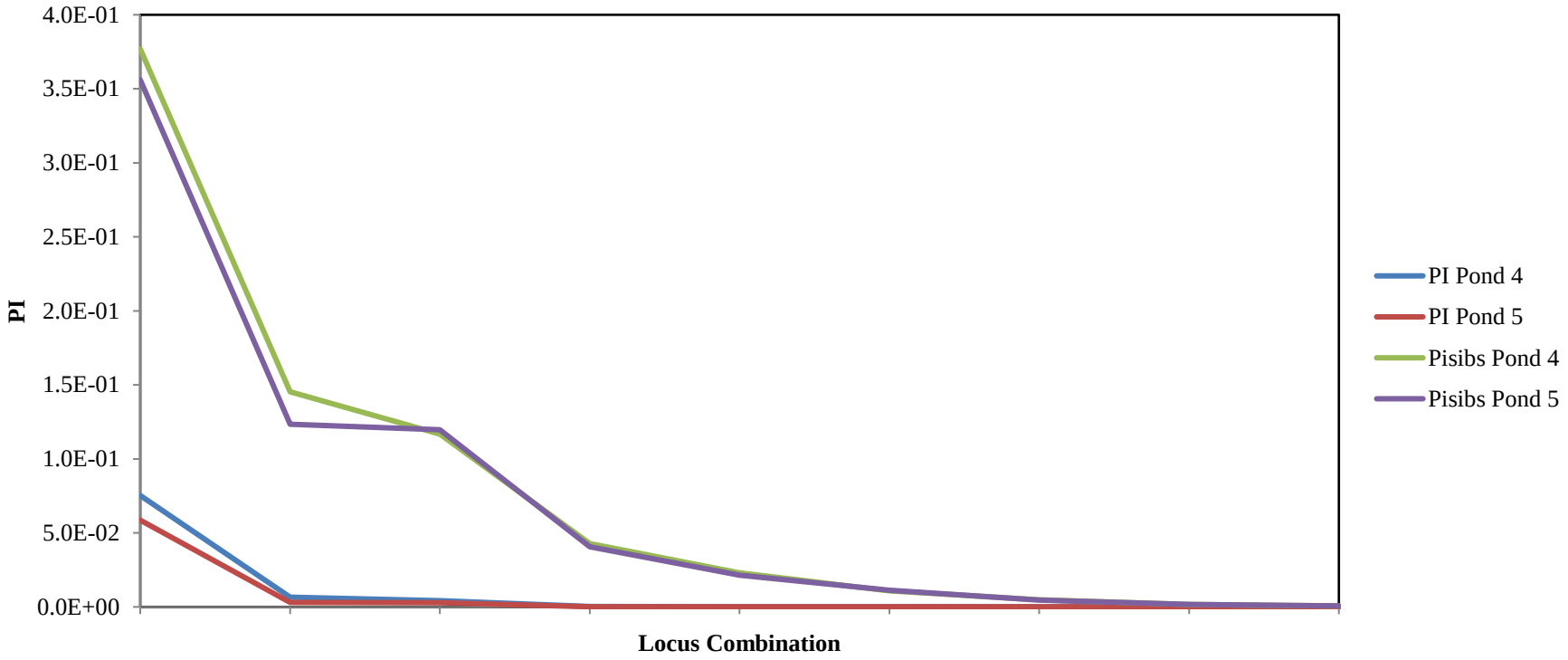

Figure 2.2 – Probability of identity as well as probability of identity for full siblings for ponds 4 and 5 across 9 nuclear DNA microsatellite markers.

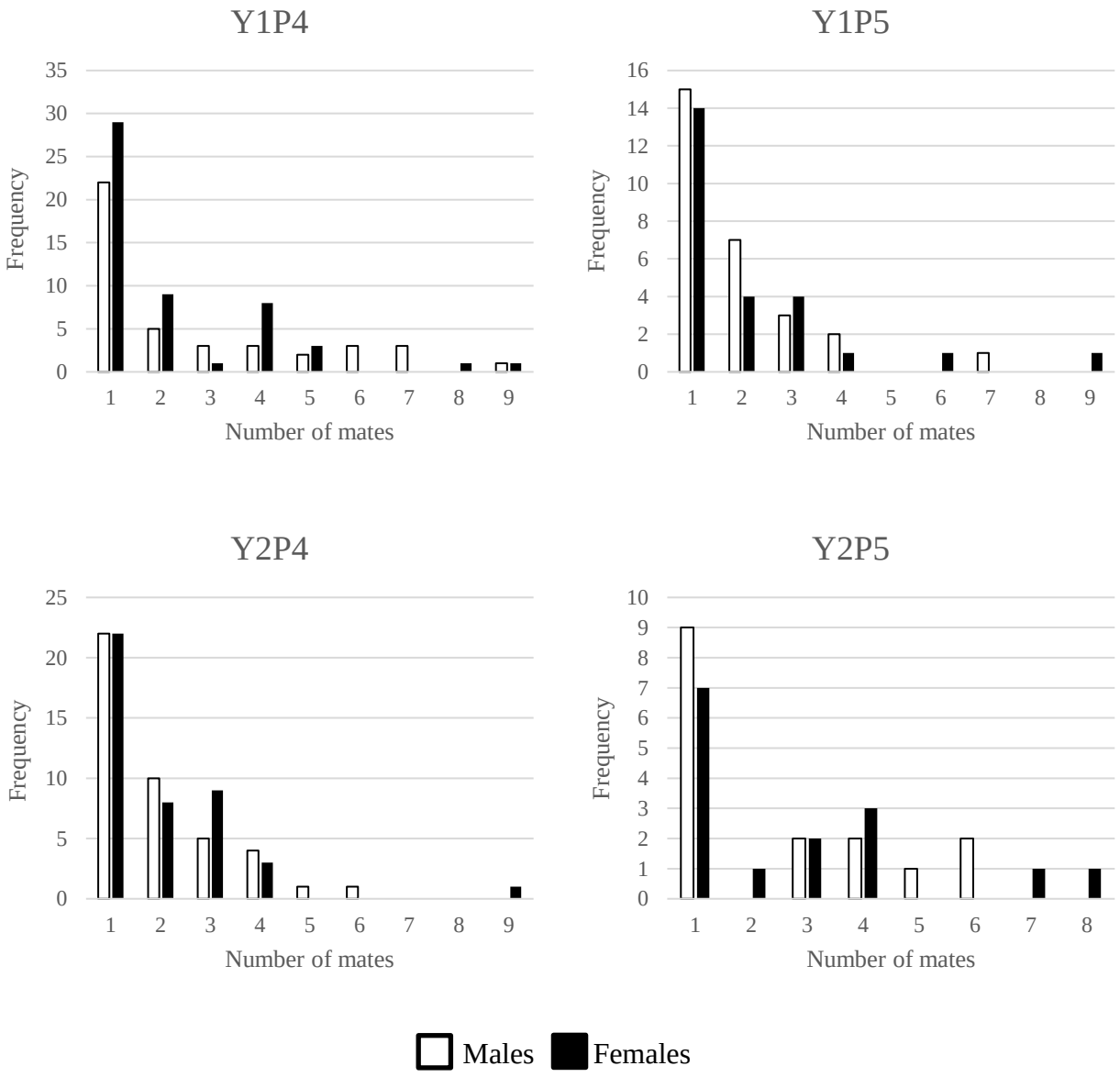

Figure 2.3 – Frequency distributions of mating success by sex, pond, and year. (Y1= 2013-2014; Y2= 2015-2016) and pond (P4= pond 4; P5= pond 5). An adult was considered to have mated successfully if it was assigned to at least one offspring by COLONY. This includes both sampled adults and hypothetical parents created by COLONY assigned to offspring.

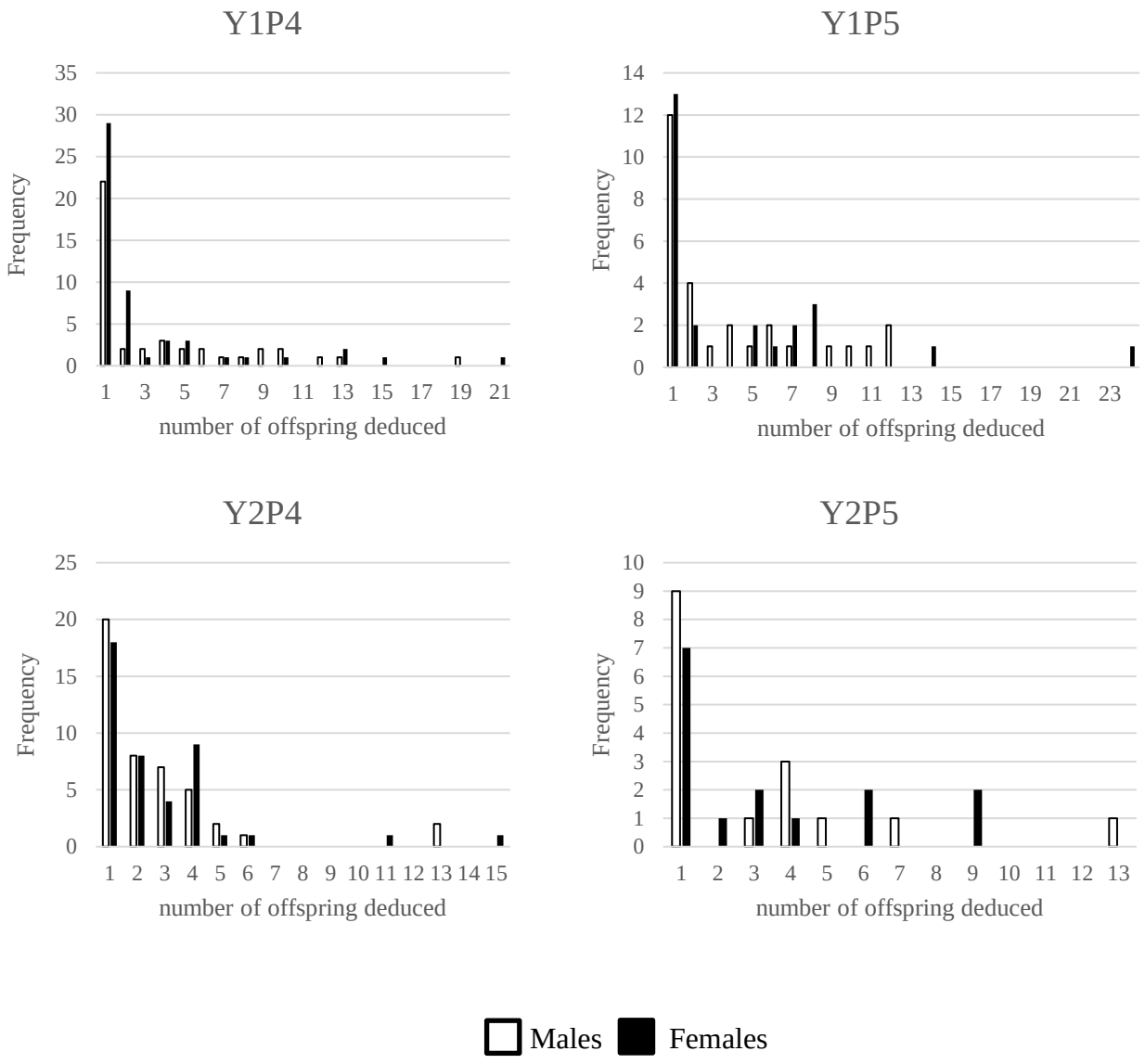

Figure 2.4 – Frequency distributions of reproductive success per individual by sex, pond, and year. (Y1= 2013-2014; Y2= 2015-2016) and pond (P4= pond 4; P5= pond 5). Only adults assigned to at least one offspring by COLONY were included. This includes both sampled adults and hypothetical parents created by COLONY assigned to offspring.

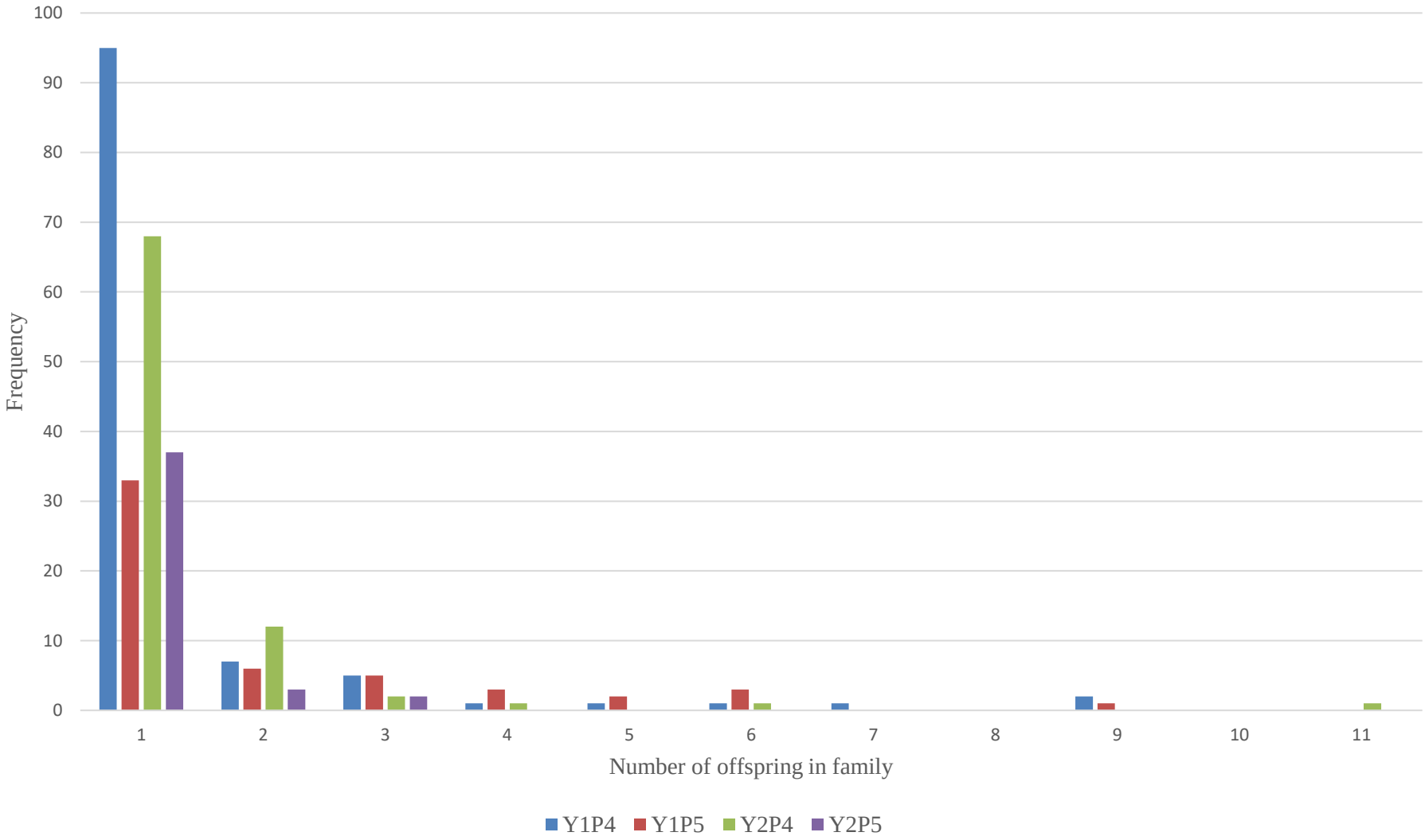

Figure 2.5 – Frequency distribution of deduced full-sib family sizes by year (Y1= 2013-2014; Y2= 2015-2016) and pond (P4= pond 4; P5= pond 5).

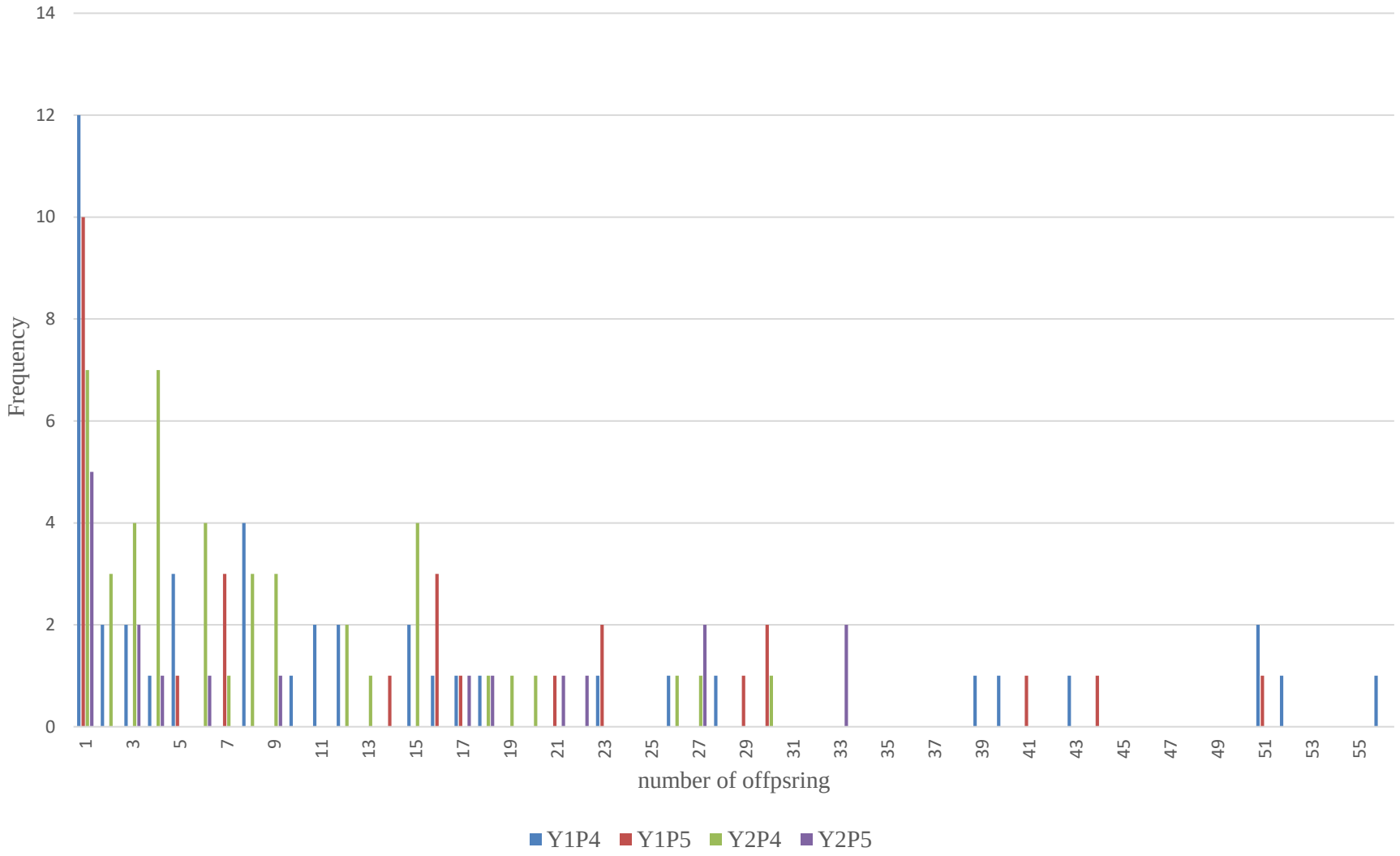

Figure 2.6 – Frequency of deduced half-sib family sizes by year (Y1= 2013-2014; Y2= 2015-2016) and pond (P4= pond 4; P5= pond 5).

GENERAL CONCLUSIONS

This study of reticulated flatwoods salamanders (RFS) was performed to increase the understanding of the population genetic structuring of an endangered species and the factors that contribute to this. The main goals of my thesis were 1) Determine the population structuring of RFS and the manageable units for species conservation, 2) Understand dispersal of RFS and factors that influence this, 3) Estimate the size and status of populations, 4) Recognize breeding biology and recruitment of RFS and how this affects population sizes, 5) Draw general conclusions on population declines and provide recommendations for future management.

Population Structuring of Reticulated Flatwoods Salamanders

Among ambystomatid species, population structuring varies from structuring at small spatial scales, as seen in tiger salamanders (*Ambystoma tigrinum melanostictum*) where gene flow is reduced at distances greater than 1 km (Spear *et al.* 2005), to no evidence of genetic differentiation across a large spatial area, as seen in the study system of spotted salamanders (*A. maculatum*) across a 2100-km area undertaken by Purrenhage *et al.* (2009). Prior to this study, a general idea of RFS population structuring was assumed based on prior knowledge of the species but an in-depth analysis of population structuring within RFS had not been performed. With the populations on Eglin, it was found that structuring could be placed into three different categories.

Groups of ponds that were clustered in the same flatwoods region <1 km apart were found to be more genetically similar than those outside of the region. These two flatwoods regions are separated by 11 km, much further than the 4.8 km at which Zamudio and Wieczorek (2007) found

that spotted salamanders (*A. maculatum*) begin to show genetic differentiation. Though there are potential breeding ponds that have been found between these regions on Eglin, no RFS have been found within these ponds for several years. These two flatwoods regions function as metapopulations with dispersal within but not between them and should be treated separately.

Within these clusters of ponds, the ponds themselves are genetically differentiated (i.e. $F_{ST} > 0.01$), indicating that in most cases, each pond should be considered a semi-independent population for management. Though these ponds are ≤ 1 km apart, genetic differentiation was still high with $F_{ST} > 0.05$ observed in ponds greater than 0.6 km apart. Migration rates between ponds showed evidence for low migration which is to be expected considering that several ambystomatid species display philopatry. For example, marbled salamanders (*Ambystoma opacum*) are known to return to their natal pools, with only 3.5–9% seeking new breeding areas (Scott 1994; Gamble *et al*. 2007). For the management purposes on Eglin, each pond should be considered as a management unit focused on keeping populations within the ponds stable and connectivity between ponds available for individuals that do disperse. This can be achieved by maintaining habitat and creating corridors between ponds for directed flow of dispersing individuals.

Finally, within ponds have shown evidence of sub-structuring as evident from STRUCTURE outputs showing a higher K than the number of ponds available. Some temporal aspect of pond arrival could explain this but further studies will be needed in order to better understand this system.

My findings suggest that RFS should be managed on a pond by pond basis while keeping connectivity available between ponds within the immediate vicinity. The use of translocations between ponds, either of adults or larval individuals, may be utilized to bolster gene flow between ponds.

Factors Influencing Dispersal

Reticulated flatwoods salamanders' ability to disperse between ponds on Eglin are affected both by the landscape and their biological tendency to return to their natal pond. Land cover type was found to have influence on the gene flow between ponds. The presence of any urbanization, even that considered to be a low amount of urbanization, was found to be a hindrance to gene flow between populations. Pond SF is genetically differentiated from both the East Bay and Oglesby flatwoods pond clusters and is almost completely surrounded by urbanization. Though there are only 3-4 km between pond SF and the East Bay flatwoods pond cluster, F_{ST} values indicated a higher amount of differentiation between the two than between East Bay and Oglesby which is separated by 11 km. Flatwoods salamanders migrate nocturnally, during or directly after rains associated with passing cold fronts (Palis 1997a). This is due to the fact that salamanders are prone to desiccation and the presence of water is needed for survival. Urban areas are poor at maintaining the moist environment needed and pose a formidable challenge to dispersing salamanders (Peterman *et al.* 2014). Due to this, RFS are less likely to travel through these environments. Emergent herbaceous wetlands were also found to be a factor in the decrease of gene flow between populations. Flatwoods salamanders are found within mesic flatwoods and savannahs consisting of longleaf pine (*Pinus palustris*) and wiregrass (*Aristida stricta*) (Palis 1997b; Bevelhimer *et al.* 2008). These habitats are maintained by periodic summer fires that remove herbaceous growth, but due to the fire regime on Eglin, these prescribed burns do not occur at the frequency or timing needed to keep herbaceous growth down. This overgrowth makes movement through the landscape difficult for flatwoods salamanders whom move through areas

predominantly consisting of wiregrass and would have a direct effect on the gene flow occurring on Eglin.

In addition to landscapes having an effect on connectivity, RFS's tendency to return to their natal pond for breeding decreases the amount of gene flow between ponds. Several ambystomatids, including *A. opacum*, *A. maculatum*, and *A. californiense*, display natal pond fidelity and *A. bishopi* is no exception to this (Portnoy 1990; Scott 1994; Trenham *et al.* 2001; Gamble *et al.* 2007; Semlitsch 2008). From this study, we found 12 larval individuals from the 2013-2014 breeding season that returned to their natal pond for the 2015-2016 breeding season. Inbreeding levels within ponds 4 and 5 were also found to be around 12% (indicative of individuals mating with their uncle/aunt, half-brother/half-sister, or grandfather/grandmother) which is evidence for the return of individuals to the same pond for several breeding seasons.

Size and Status of Breeding Ponds

Across Eglin, breeding pond effective population sizes ranged from 20 to 60 individuals which is in the threshold set by Franklin (1980) for concern of a population facing the threat of inbreeding depression. One pond however, pond 53, had mean N_e that could not be estimated for but the lower 95% CL was substantially higher, at 87 individuals. This pond also showed evidence of immigration into pond 4, which could indicate that when a pond reaches a high N_e, that dispersal from that pond will occur more frequently and that population will become a source population for surrounding ponds. Although my estimates of N_e should be considered as relative rather than absolute, smaller populations should be considered at a heightened risk. Though N_e was found to be low in a majority of the ponds, my findings were found to be comparable to other ambystomatid species. N_e of the California tiger salamander (*Ambystoma californiense*) was found

to be 11-64 individuals per population by Wang (2011) and was found to be strongly correlated with pond size. Another study by Wang (2017) on *A. californiense* found that 10 breeding ponds had 8-43 effective breeders in 1995 and 6 ponds had 5-19 effective breeders in 2001.

From the study of ponds 4 and 5 for the breeding seasons of 2013-2014 and 2015-2016, we did see an increase in both N_e and N, but with that came an increase of the inbreeding coefficient. This means that even though the population increased, many of those individuals were highly related to each other. Though it is important to keep population numbers up, keeping diversity within those populations is also vital for their health and longevity.

Breeding Biology and Recruitment

Between October and December, both species of flatwoods salamanders emerge from their burrows and migrate to deposit their eggs terrestrially in a moist microhabitat located in the basin of a dry breeding pond (Anderson and Williamson 1976, Gorman *et al.* 2014). The only other species of ambystomatid salamanders that lays its eggs terrestrially is the marbled salamander, *Ambystoma opacum* (Petranka 1998). By laying their eggs terrestrially instead of in an established pond, RFS have developed a breeding strategy to help better the odds for recruitment.

RFS show evidence for a polygynandrous mating system in which both males and females mate with several individuals during the breeding season. Polygynandry has also been seen in other ambystomatid species including the small-mouther salamander *A. texanum* (Gopurenko 2007). Of all the sampled adults for both ponds and both years, 21% of individuals were successful in producing at least one sampled offspring according to the reconstructed pedigrees. That being said, many of those that did produce young were successful in that they had many offspring, 24 was the highest seen for one individual. From the two years sampled, there was evidence that there

was recruitment of individuals from both the increase of N_e and N from the 2013-2014 breeding season to the 2015-2016 breeding season.

Management Recommendations

In all, continued management and monitoring of breeding ponds on Eglin will aid in the viability of populations. Management at the pond level is advised since these are the smallest manageable unit. Habitat between the ponds within regions as well as the ponds themselves should be maintained to allow connectivity of dispersing individuals as well as to increase recruitment of individuals. This means that both urbanization and overgrowth of fire intolerant and non-native plants need to be reduced in order to allow for ease of movement between ponds. If translocations of individuals do occur to counteract inbreeding depression, it is suggested that translocations only occur between ponds within the same region as those outside of the region would not occur naturally. Headstarting of larval individuals may be useful in increasing recruitment as a majority of mortality occurs during this period. Further studies in pond arrival times of individuals as well as basic biology of adults would give insight into further management of the species.

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