

ENVIRONMENTAL INFLUENCES ON MOSQUITOFISH REPRODUCTION

By

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To my parents, Margaret and Peter

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Reproduction in all organisms is regulated by a wide range of environmental factors. In fishes, these include (among other factors): temperature, photoperiod, nutrition, and social interactions. In the past 50 years, an additional factor has emerged: anthropogenic pollution. Many widely used contaminants (PCBs, pesticides, fertilizers, plasticizers) are now distributed throughout our environment, particularly in aquatic systems. Several of these have been shown to disturb normal development, growth, and reproduction of vertebrates through disruptive interactions with the endocrine system.

In this extended seasonal study, we evaluated reproductive parameters in adult *Gambusia holbrooki* captured from two central Florida lakes: Lake Apopka (with a documented history of organochlorine contamination) and Lake Woodruff Wildlife Refuge (reference site). Relative to the Lake Woodruff population, males and females from Lake Apopka exhibited increased hepatosomatic indices; females also exhibited altered estradiol patterns and an unexpected increase in fecundity. Males from Lake

Apopka exhibited increased testicular size, but decreased sperm counts and sperm viability in some months, particularly at the end of the reproductive season.

In a second suite of studies, we assessed reproduction, at one point during the breeding season, among adult *Gambusia*, captured from eight Florida springs with varying concentrations of nitrate. Nitrate contamination of Florida springs is a growing concern. In fact, nitrate concentrations in some springs exceed the EPA-established concentration limit for drinking water (10 mg/L NO₃-N). Nitrate exposure is associated with altered development, reduced steroidogenesis, and diminished reproductive success in a number of species. In male mosquitofish exposed to elevated nitrate concentrations (4 to 5 mg/L NO₃-N), we observed increased gonadosomatic index, reduced 11-ketotestosterone concentrations, and reduced sperm counts. Females from springs with elevated nitrate concentrations exhibited reduced embryo dry weights and a decreased rate of reproductive activity, based on presence or absence of vitellogenic oocytes.

Taken together, our results suggest that long-term exposure to environmental contaminants, specifically organochlorines and nitrate, is associated with altered reproductive outcomes. In addition, the temporal nature of our research greatly expands and integrates our knowledge of *Gambusia* reproduction in terms of seasonal variation and basic life history.

CHAPTER 1
OVERVIEW OF *Gambusia* BIOLOGY, STUDY SITES, AND CONTAMINANTS OF
INTEREST

Study Overview

Aquatic environments are inherently variable and often exhibit pronounced temporal changes that influence the biology and ecology of their resident flora and fauna. Many changes are natural in that they are non-anthropogenic. Some are also repetitively cyclical and represent phenomena to which the animals in the system are adapted. Mosquitofish, for example, become reproductively active in the spring, when water temperatures exceed 20 to 22°C, and they end activity in the fall when daylength shortens to less than 12 h (Chapters 1 and 2 of our study; Fraile et al., 1994; Koya and Kamiya, 2000). It is likely that this seasonal pattern evolved around practical considerations of prey abundance, mate readiness, and larval survivorship; factors that affect fitness, and that generally vary with seasonal environmental change (Winemiller, 1993).

In addition to the natural factors that regulate reproduction, aquatic animals are increasingly subject to “un-natural” regulation by anthropogenic endocrine-disrupting contaminants (EDCs). Widespread use of pesticides, fertilizers, plastics, and other industrial chemicals has increased exponentially over the past 50 years (Danielopol et al., 2003). The effect is so dramatic that epidemiologists can track the related rise in human reproductive disorders that are causally linked to contaminant exposure (Carlsen et al., 1992; Skakkebaek et al., 1998).

Contaminant-induced reproductive changes in the human population probably occur after similar changes in wildlife. This is because most wildlife species are smaller than humans and potentially sensitive to lower doses. Those with shorter generation times could be more susceptible to cumulative, cross-generational changes. In addition, some species possess a degree of phenotypic plasticity that makes them more likely to exhibit overt symptoms. For example, in a wide variety of fishes, sexual differentiation is highly labile, and exposure of differentiating fishes to estrogens (regardless of genotypic sex) can produce morphologically female monocultures (Piferrer, 2001). Therefore, studies of wild animals are useful in risk assessment and should be used to guide environmental policy to improve conservation, ecological sustainability, and human health and welfare.

Our study investigated temporal patterns of reproduction in wild adult *Gambusia holbrooki* (eastern mosquitofish) captured from Lake Woodruff and Lake Apopka in central Florida. These lakes were sampled because other biological data on fishes (Gallagher et al., 2001; Toft et al., 2003) and alligators (Guillette et al., 2000) from these lakes suggest that they provide a comparative system in which to study the impact of EDCs on reproductive variables. In addition to sampling these lakes, we conducted a single-month reproductive study of *Gambusia holbrooki* captured from eight artesian springs, located along the Suwannee and Santa Fe Rivers in north Florida. These springs represent (at present) a gradient of nitrate contamination and provided a natural experimental opportunity to assess the potential relationship between nitrate concentration and variation in mosquitofish reproduction. The remainder of this chapter details mosquitofish life history, clarifies why I chose to work with mosquitofish, and

presents an overview of reproductive endocrinology in fishes. This is followed by descriptions of the two lakes and eight springs sampled in our study, along with a review of the effects of pesticides and nitrate on fish reproduction. Finally, I present the hypotheses that guided our study.

Gambusia Life History

Taxonomy and Distribution of *Gambusia holbrooki*

Within the genus *Gambusia* (family Poeciliidae), there are approximately 45 species with a native range that extends from New Jersey, west to Iowa and the Mississippi River drainage; and south to the Gulf coast, the Caribbean, and Mexico. From Mexico, the native range continues south through Central America, to Columbia (reviewed by Parenti and Rauchenberger, 1989). Of these 45 species, *Gambusia holbrooki* (eastern mosquitofish) and *Gambusia affinis* (western mosquitofish) are the most widely studied. In addition to their native ranges in the southern United States, these two species have been introduced for biological control of mosquito larvae and are now found on all of the continents except Antarctica (reviewed by Courtenay and Meffe, 1989). Thus, their functional range is substantially larger than their native range.

The focus of our study is *Gambusia holbrooki*, which is distinguished from its western sister species (*Gambusia affinis*), by a thin geographic and biological line, across which hybridization does occur (Walters and Freeman, 2000). Morphologically, the two species are differentiated by fin ray counts. *Gambusia holbrooki* possess 8 dorsal and 11 anal fin rays, whereas *G. affinis* have 7 dorsal and 10 anal fin rays (Walters and Freeman, 2000).

In their native range, *G. affinis* are found west of Mobile Bay in Alabama; whereas *G. holbrooki* occur to the east, and the zone of sympatry lies within the Mobile River

Basin (Wooten et al., 1988). Successful interspecific hybridization is possible between *G. holbrooki* females and *G. affinis* males, while the alternative hybridization of *G. affinis* females and *G. holbrooki* males does not result in viable offspring (Black and Howell, 1979). This one-way incompatibility is probably caused by species differences in the sex chromosomes (Black and Howell, 1979). *Gambusia holbrooki* do not exhibit heteromorphic sex chromosomes in either sex, although inheritance patterns of male-linked melanism suggest an XY system of sex determination (Angus, 1989; Black and Howell, 1979). Conversely, among *G. affinis*, females possess heteromorphic sex chromosomes and males possess homomorphic sex chromosomes indicative of ZW sex determination (Black and Howell, 1979). According to Scribner and Avise (1994), in areas where the two species co-exist, *G. holbrooki* will quickly (within 4 generations) out-compete *G. affinis* in terms of mitochondrial and nuclear allele frequency, an outcome that is likely to be related to the one-way chromosomal incompatibility described above. Scribner et al. (1999) concluded that offspring from *G. holbrooki* females also have some selective advantage. Because *G. holbrooki* and *G. affinis* do hybridize where they co-occur, they were originally considered two subspecies of *Gambusia affinis* (thus *G. affinis holbrooki* and *G. affinis affinis*). For the reasons above, the two are now considered separate species (Wooten et al., 1988), but pre-1988 literature often refers to *Gambusia affinis*, without indicating the subspecies.

Habitat and Diet

Gambusia holbrooki are small, sexually dimorphic, viviparous omnivores that inhabit fresh and brackish waterways ranging from ephemeral ponds and ditches, to rice fields; to streams, ponds, rivers, lakes, springs, estuaries, and marshes (Daniels and Felley, 1992; McKinsey and Chapman, 1998; Porte et al., 1992; Toft et al., 2003; Vargas

and de Sostoa, 1996). Adult sizes range from 13 to 32 mm for males and 17 to 63 mm for females (Chapters 2 to 6 of our study; Vargas and de Sostoa, 1996). Although they will eat algae and detritus, mosquitofish prefer animal prey, including insects, anuran eggs, crustaceans, rotifers, *Daphnia*, and round worms (Meffe and Snelson, 1989). The life expectancy of mosquitofish is generally no more than 2 years (Vargas and de Sostoa, 1996). Although females mature about 20 days later than males (Koya et al., 2003), they typically grow faster, achieve a larger size, and outlive males (Vargas and de Sostoa, 1996).

Female Reproductive Cycle

Mosquitofish are reproductively active in the spring, summer, and fall in Florida (our study), Japan (Koya et al., 1998; Koya and Iwase, 2004), and Spain (Vargas and de Sostoa, 1996); or all year round in Hawaii (Haynes and Cashner, 1995). Female *Gambusia* mature about 110 days after they are born (Koya et al., 2003), and produce sequential, synchronized broods throughout their breeding season: as one brood nears parturition, the next cohort of oocytes are accumulating yolk (Koya and Kamiya, 2000). Females store sperm and thus can produce multiple broods, even in isolation from males, assuming they have mated once before (Hubbs, 1999). This feature contributes to their success as founder species. Fertilization of oocytes occurs after yolk accumulation, when eggs exceed 1.7 mm in diameter (Koya et al., 2000). Yolked oocytes become atretic if they do not reach maturity with their cohort (Koya et al., 2000). Litter size ranges from 1 to 245 precocious offspring, depending on female size and time of year (Chapters 1 and 4 of our study; Vargas and de Sostoa, 1996). Gestation (which takes place within the single, fused ovary) lasts 22 to 39 days, depending on temperature and photoperiod (reviewed by Koya et al., 1998). After birth, the larvae live independently of the parents.

Koya and Kamiya (2000) showed experimentally that (in *Gambusia affinis* collected from an irrigation canal in central Japan) ovarian recrudescence begins with the rise in springtime temperature, regardless of daylength. In that population, they found that vitellogenesis occurred at 14°C and pregnancy at 18°C. In the fall, vitellogenesis ended when daylength was less than 12.5 h, regardless of temperature. The final brood finished its development at a temperature-dependent rate and ovarian regression occurred after final parturition.

Mosquitofish are typically classified as lecithotrophs because they produce large yolky eggs (Haynes, 1995). However, lecithotrophy implies that embryo nutrition is limited to the yolk placed in the oocyte before fertilization. By definition, lecithotrophs lose weight during gestation because of respiratory losses. However, several studies, including ours, show that embryos gain in diameter and wet and dry weight during gestation (Chapters 1 and 4 of our study; Vargas and de Sostoa, 1996). This process is supported by appropriate changes in liver size that are presumably related to vitellogenesis (Chapters 1 and 4 of our study). Furthermore, (using *Gambusia geiseri*) Marsh-Matthews et al. (2001) showed that maternal transfer of tritiated leucine to embryos was measurable within 2 hours of injection. Their findings suggest that (in addition to yolk provisioning) mosquitofish provide directly for their embryos during gestation via matrotrophy.

Male Reproductive Cycle

Mature poeciliid males are readily identified by their gonopodium (a grooved bony modification of the anal fin used to transfer spermatozeugmata to the genital opening of the female). The gonopodium forms during puberty by elongation of anal fin lepidotrichia and fusion of pterygiophores 3, 4, and 5, with a species-specific

arrangement of hooks at the tip (Rosa-Molinar et al., 1994). This is in contrast with females, which have a fan-shaped anal fin without fusion of the pterygiophores.

The structure and mechanical control of the gonopodium are supported by the internal skeleton, which (like the anal fin) becomes masculinized in response to androgens during puberty (Ogino et al., 2004; Rosa-Molinar et al., 1994, 1996). Specifically, in males (but not females), the hemal spine at vertebra 13 is resorbed; and the hemal spines at vertebrae 14, 15, and 16 thicken, elongate, and swing anteriorly (swing caudally in females). Spines 14, 15, and 16 are connected by interosseal ligaments (poorly developed in females) to the fused proximal pterygiophores of the anal fin. Therefore, as the hemal spines bend forward, they pull the anal fin in an anterior direction. This movement aligns the gonopodium with the male's urogenital opening so that sperm can be delivered via the gonopodium during mating. The movement also places the anal fin and its appendicular support at the fish's center of gravity (Rosa-Molinar et al., 1996). This improves fine-motor control of the gonopodium, which the male must abduct across his body with enough precision to insert the hooked tip briefly into the female. The entire copulatory event takes less than a second and is performed while both fish are in motion (Rosa-Molinar et al., 1996). Lastly, the mature male skeleton provides support for the large muscle used to maneuver the gonopodium (Rosa-Molinar et al., 1996).

Males copulate with females frequently, making attempts even before they are fully mature (Bisazza et al., 1996). Males possess mature spermatozoa about 90 days after birth (Koya et al., 2003), although some variation among individuals or populations is likely. In mosquitofish, the testes are fused into a single, round, white-colored organ that

is located centrally in the abdomen, dorsal to the origin of the gonopodium (Fraile et al., 1992). A single vas deferens connects the gonopodium to the efferent ducts that coalesce within the central lumen of each testis (Fraile et al., 1992).

The outer wall of the testis is lined with spermatogonia (Fraile et al., 1992). In spring through fall, spermatogonia proliferate in successive waves of mitosis, forming nests (cysts) of primary spermatocytes bounded by Sertoli cells (Fraile et al., 1992). In a process that takes approximately 30 days, spermatocytes within a single cyst undergo synchronized meiosis and differentiation to produce spermatids and ultimately tailed spermatozoa (Fraile et al., 1992; Koya and Iwase, 2004). As the cysts mature, they move from the periphery of the testis to the center, where they are released to the efferent sperm ducts as spherical aggregates of sperm (spermatozeugmata), with tails in the center and heads on the periphery (Fraile et al., 1992). At this point, Sertoli cells no longer surround the spermatozeugmata; instead, they hypertrophy and become part of the efferent duct tubule (Fraile et al., 1992). The tubules secrete a gelatinous matrix that holds the spherical structure of the spermatozeugma together until it reaches the oviduct of a female (reviewed by Meffe and Snelson, 1989). As winter approaches, production of new spermatocytes ceases. Through the winter, stored cysts of mature spermatozoa occupy most of the testicular volume, and will be used during early spring copulation, which occurs before the first wave of spring spermatogenesis is complete (Koya and Iwase, 2004).

Like most teleosts, male mosquitofish exhibit seasonal variation in sperm production that is regulated by changes in temperature and photoperiod (Fraile et al., 1994). However, factors that initiate mosquitofish spermatogenesis are not yet fully

understood. Males that have recently entered testicular quiescence cannot be stimulated to produce new sperm by increasing temperature or photoperiod (Fraile et al., 1993). However, fish captured at the end of their quiescent period will proliferate spermatogonia, even if temperatures remain low and days are short (Fraile et al., 1994). Increasing ambient temperatures are required for differentiation of spermatogonia to spermatocytes, and photoperiod must lengthen for spermatocytes to enter meiosis (Fraile et al., 1994; De Miguel et al., 1994).

***Gambusia holbrooki* as a Sentinel Species**

I chose this prolific species for their small size, wide availability, rapid time to maturity (about 3 months)(Koya et al., 2003), and viviparity. In addition, endocrine-disrupting contaminants (EDCs) that impact reproduction are often lipophilic and therefore bioaccumulate in the fatty tissues of exposed animals (Porte et al., 1992). Mosquitofish are intermediate in the food web, and are thus likely to bioaccumulate contaminants absorbed by their prey. Maternal provisioning of the egg, and later the embryo (in viviparous species like *Gambusia*) involves transfer of stored lipids from the mother to the offspring (Meffe and Snelson, 1993). For individuals with high body loads of accumulated contaminants, this provisioning process can result in an exceptionally high dose of contaminants (relative to ambient concentrations) to the offspring during the period of sexual differentiation (Harding et al., 1997).

Developmental exposure to EDCs can permanently alter the organization of the reproductive system (Guillette et al., 1995). This could result in significant reproductive problems at the population level. According to Nakamura (1978) and Sone et al. (2005), *Gambusia affinis* larvae are sexually differentiated by the time they are born. Although in another study, Koya et al. (2003) report that, at two days before birth, all *Gambusia*

affinis embryos are female, with oocytes present in their gonads. At birth, some of the offspring had developed testes instead. Thus, Koya et al. (2003) suggest that *G. affinis* exhibit embryonic protogyny; however, after birth, these fish are gonochoristic.

In addition to certain convenient aspects of their biology (noted above), I chose to study *Gambusia* because they are already the subject of a few endocrine disruption studies. For example, abnormal vitellogenin production occurs in males after exposure to estrogenic compounds (Tolar et al., 2001). Angus et al. (2002) observed enlarged testes and livers among male *Gambusia* inhabiting water contaminated with treated sewage effluent. Note that the presence of excreted ethynylestradiol, derived from birth control pills gives sewage effluent an estrogenic character (Schultz et al., 2003). Dreze et al. (2000) observed skewed sex ratios that favored females, and/or delayed sexual development in male *Gambusia* exposed to estrogenic 4-nonylphenol. Orlando et al. (2002) showed increased ovarian and brain aromatase (converts androgens to estrogens) activity among adult female mosquitofish caught from the Fenholloway River in Florida. The Fenholloway River is polluted with paper mill effluent, which in other studies has been shown to masculinize the anal fins of female mosquitofish (Bortone and Cody, 1999; Howell et al., 1980; Parks et al., 2001). This last example of masculinized anal fins is one of the more famous early cases of endocrine disruption in fishes and is described in greater detail below.

In 1978, masculinized female mosquitofish, *Gambusia affinis holbrooki*, were discovered in Elevenmile Creek (Escambia County, Florida), downstream from a paper mill (Howell et al., 1980). The females possessed partially to fully formed gonopodia, complete with the hooks, spines, and fusion of fin rays 3, 4, and 5. There was no

evidence of gonadal masculinization: masculinized females possessed only ovarian tissue. In addition, Howell et al. (1980) observed the masculinized sexual behavior of these females, who pursued normal and masculinized females while swinging and thrusting their gonopodia. Immature males (12 to 13 mm standard length) from the same collection sites (downstream from the paper mill) displayed precocious gonopodial development and more aggressive courtship behavior compared to normal males (Howell et al., 1980).

Howell et al. (1980) hypothesized that the masculinizing effect of paper-mill effluent was due to androgenic chemicals in the effluent. This hypothesis is supported by Turner (1941) and Angus et al. (2001), who induced gonopodial development in immature female *Gambusia affinis* with ethynyl testosterone and 11-ketotestosterone, respectively. Androstenedione, a precursor to testosterone, was later identified in Fenholloway water (Durhan et al., 2002; Jenkins et al., 2001). However, after running *in vitro* tests of androgenic activity, Durhan et al. (2002) concluded that the isolated androstenedione was not responsible for the androgenic character of Fenholloway water. Howell et al. (1980) made the interesting observation that female *Gambusia* are genetically capable of gonopodial development, but the potential remains dormant in the normal absence of male androgen levels. This suggestion makes sense in light of the sexual bipotentiality of mosquitofish embryos described above.

Overview of Reproductive Endocrinology in Fishes

Although gonadal activity is often the focus of teleostean fish reproduction studies, reproduction really begins in the hypothalamus. In response to environmental stimuli such as photoperiod, the hypothalamus secretes gonadotropin releasing hormone (GnRH), which causes the anterior pituitary to release two gonadotropic hormones

(GTH) (Norris, 1997). These are sometimes called GTH-I, similar to mammalian follicle-stimulating hormone (FSH); and GTH-II, similar to mammalian luteinizing hormone (LH). FSH stimulates spermatogenesis and oogenesis; LH causes final gamete maturation and ovulation or sperm release (Norris, 1997). In both ovaries and testes, LH affects gametes by stimulating the synthesis of progesterone-derived 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) (Kobayashi et al., 1993; 2002).

In addition to gamete production, LH and FSH also stimulate gonadal steroidogenesis in teleosts (Norris, 1997; Schulz and Miura, 2002). The three steroids relevant to our study are estradiol-17 β , testosterone, and 11-ketotestosterone (11-KT). Although all three hormones occur in both sexes, we focused on estradiol in females and testosterone and 11-KT in males. In females, estradiol promotes sexual maturation, gonadal growth, hepatic vitellogenesis (yolk precursor production), and oogenesis (Kobayashi et al., 2002; Norris, 1997). In males, testosterone promotes sexual maturation, development of secondary sex characters, spermatogenesis (particularly toward the end), sperm quality, spawning, and sexual behavior (Norris, 1997, Ogino et al., 2004; Toft et al., 2003, Wu et al., 2003). It is likely that 11-KT also participates in these processes; it is best known for its ability to induce spermatogonial proliferation, which usually is not accomplished by testosterone (Schulz and Miura, 2002).

Lakes

For the studies described in Chapters 1 through 3, we sampled fish from Lake Apopka and Lake Woodruff in central Florida. These lakes differ in terms of their ecology and contaminant loads; some of the main differences are highlighted below. We selected these lakes because they are geographically close, and thus subject to the same photoperiod. Furthermore, our lab has previously published detailed contaminant data

coupled with comparative reproduction studies of alligators from these two lakes (Guillette et al., 1999; Guillette et al., 2000). These earlier findings stimulated the hypotheses tested in our study.

Lake Apopka

Lake Apopka is impacted by a nearby (1 mile away) EPA-designated Superfund site. Superfund status indicates that the area is contaminated with uncontrolled hazardous waste and poses a recognized risk to the environment or human health. Main pollutants in Lake Apopka include polychlorinated biphenyls (PCBs) and several organochlorine pesticides, including Dicofol, DDT and its metabolites p,p'-DDD and p,p'-DDE, Dieldrin, Endrin, Mirex, Methoxychlor, Chlordane, Toxaphene, and trans-Nonachlor (Guillette et al., 2000). These chemicals were washed into the lake from agricultural lands, or the Superfund site, where a Dicofol (15% DDT) spill occurred in 1980. The chemicals listed here have been identified and measured in both alligator eggs and alligator serum taken from animals living in Lake Apopka (Guillette et al., 2000; Heinz et al., 1991). In addition, elevated concentrations of several of these compounds have been measured in the tissues of mosquitofish (U.S. Fish and Wildlife Service, unpubl. data) and brown bullheads (Gallagher et al., 2001) from Lake Apopka.

In mosquitofish, contaminant concentrations are around 0.17 mg/kg for DDT, 9.2 mg/kg for Toxaphene, 1.1 mg/kg for p,p'-DDD and 0.54 mg/kg for trans-Nonachlor (Greg Masson, U.S. Fish and Wildlife Service, pers. comm.). Alligator egg dosing studies of 0.1 to 10 mg/kg of DDE, DDD, or trans-Nonachlor have caused alterations in sex determination, endocrine function, secondary sex characteristics and/or gonadal anatomy (Crain, 1997; Matter et al., 1998; Rooney, 1998). Compared to cohorts from Lake Woodruff, female alligators from Lake Apopka exhibit above-normal plasma

estradiol concentrations and abnormal ovarian morphology (with large numbers of polyovular follicles and polynuclear oocytes). Male alligators have lower-than-normal plasma testosterone concentrations, poorly organized testes, and abnormally small phalli (Guillette et al., 1994). In addition, alligator eggs from Lake Apopka exhibit low hatchability; and neonates show increased rates of mortality, poor motor coordination, changes in metabolism (particularly in liver and steroidogenic enzymes), and altered gene expression (reviewed in Guillette et al., 2000; Guillette and Gunderson, 2001). Given these previous studies, it is reasonable to expect that *Gambusia* in Lake Apopka are subject to reproductive alterations in association with contaminant exposure.

Lake Woodruff

Lake Woodruff is a National Wildlife Refuge. While not contaminant-free, alligators captured from Lake Woodruff (relative to Lake Apopka) have fewer chemicals in their body tissues; and those chemicals occur at lower concentrations (Guillette et al., 1999). For this reason, our laboratory has traditionally used Lake Woodruff as a reference site. In addition to contaminant concentrations, other water-quality measures also distinguish the two lakes. Based on our data (in conjunction with average data for 2000 to 2003, taken from EPA's STORET public-access database, <http://www.epa.gov/storet/dbtop.html>), Lake Woodruff has a different seasonal temperature profile that favors cooler temperatures in fall and spring, lower nitrogen and phosphorus concentrations, greater water clarity (Secchi depth), and lower turbidity and total suspended solids relative to Lake Apopka. In evaluating the results of our study, we considered these ecological differences, and also considered lake-associated variation in contaminant concentrations.

Florida Springs and Nitrate

Overview

Over the past 40 years, concentrations of nitrate ($\text{NO}_3\text{-N}$) in several of Florida's artesian springs have increased from less than 0.1 mg/L to more than 5 mg/L (Katz, 2004). The highest measured concentration was 38 mg/L $\text{NO}_3\text{-N}$ in a small spring along the Suwannee River in northern Florida (Katz et al., 1999). This is almost four times the EPA drinking-water standard of 10 mg/L $\text{NO}_3\text{-N}$. Most of the nitrate comes from inorganic fertilizers applied to land, ultimately leaching through the ground to recharge Florida's aquifer (Katz, 2004). A potentially important ecological problem often caused by increased nitrates is eutrophication, which can increase algal and plant growth. The excessive flora can cause fluctuations in aquatic light levels and dissolved oxygen concentrations, thus affecting survival and diversity of aquatic organisms and overall community structure (Attayde and Hansson, 1999; Capriulo et al., 2002; Irfanullah and Moss, 2004).

Apart from the negative ecological effects of eutrophication, nitrate can also directly harm animals living in affected aquatic systems. These effects range from gross toxicity to subtle, but equally alarming, changes in physiology and development (Guillette and Edwards, 2005). For example, mortality of the larvae of cutthroat trout, Chinook salmon, and rainbow trout occurs at $\text{NO}_3\text{-N}$ concentrations ranging from 2.3 to 7.6 mg/L (Kincheloe et al., 1979). Survival of chorus frog and leopard frog tadpoles decreased significantly after exposure to 10 mg/L $\text{NO}_3\text{-N}$ (Hecnar, 1995). This concentration is considered the upper limit for safety in drinking water (EPA, 1996). On the other hand, the 96 h median lethal concentration (LC50) for fathead minnow larvae is 1,341 mg/L $\text{NO}_3\text{-N}$; while it is 462 mg/L $\text{NO}_3\text{-N}$ for adult *Daphnia magna* (an

invertebrate) (Scott and Crunkilton, 2000). These examples show that sensitivity to nitrate varies greatly among species, and often depends on the stage of development at the time of exposure.

The best-known human health effect of nitrate is methemoglobinemia (blue-baby syndrome) (Gatseva et al., 1996; Scott and Crunkilton, 2000). This condition occurs when nitrate interacts with hemoglobin in the blood, causing it to crystallize. A similar condition, called brown blood disease, occurs in fishes exposed to high nitrite levels. Tissue hypoxia and cyanosis result because the crystallized hemoglobin cannot function as an oxygen carrier. Risks associated with methemoglobinemia are the reason maximum nitrate concentration in drinking water is regulated at 10 mg/L NO₃-N (EPA, 1996). In addition to methemoglobinemia, nitrate and nitrite have been implicated in mild hepatic degeneration in rats (Gatseva et al., 1999); reduced steroidogenesis in rats (Panesar, 1999; Panesar and Chan, 2000), frogs (Barbeau, 2004), and alligators (Guillette and Edwards, 2005); decreased human sperm motility; and increased human sperm mortality (Rosselli et al., 1995).

Nitrogen Cycling, *In Vivo* Nitrogen Metabolism, and Effect of Nitrate on Steroidogenesis

Nitrogen is naturally cycled in terrestrial and aquatic ecosystems. For example, ammonia excreted by fishes is converted to nitrite (NO₂) by aerobic nitrifying bacteria (*Nitrosomonas* sp.), and then oxidized to more stable nitrate (NO₃) by *Nitrobacter* bacteria. Nitrate is assimilated by plants as a nutrient, or can be converted back to nitrite and then atmospheric nitrogen (N₂) by anaerobic denitrifying bacteria. In anoxic environments, or when the nitrifying or denitrifying activity of bacterial populations is

overwhelmed (as can result from overfeeding in aquaculture systems), nitrite levels can spike, placing fish populations at immediate risk for brown blood disease.

In vivo, conversions between nitrate and nitrite also occur. Nitrate and nitrite enter the bodies of freshwater animals by crossing the gill epithelia and accumulating in extracellular fluid (Jensen, 1995). In crustaceans, nitrate and nitrite are transported against the concentration gradient by substituting for chloride in the bicarbonate-chloride exchange mechanism that normally participates in the osmoregulatory and respiratory functions of the gill (Jensen, 1995; Lee and Pritchard, 1985). In crayfish, nitrate uptake is pH dependent with uptake increasing as water pH declines (Jensen, 1995). *In vivo*, NO_3 can be converted to nitrite (NO_2), and then nitric oxide (NO) (Panesar and Chan, 2000; Samouilov et al., 1998). Nitrite is converted to NO in various ways, including endogenous nitrite reductase (NR), nitric oxide synthase (NOS), various non-NOS enzymatic and non-enzymatic mechanisms, and low pH (Cadenas et al., 2000; Doblender and Lackner, 1996; Kozlov et al., 1999; Lepore, 2000; Meyer, 1995; Nohl et al., 2001; Panesar and Chan, 2000; Samouilov et al., 1998; Stuehr and Marletta, 1985; Vanin et al., 1993; Weitzberg and Lundberg, 1998; Zweier et al., 1999). Nitric oxide is a gas that diffuses through tissues, playing diverse roles in vasodilation, cell-to-cell signaling, neurotransmission, and immunity. One specific action of NO is the inhibition of steroid hormone synthesis (DelPunta et al., 1996; Kostic et al., 1998; Panesar, 1999; Panesar and Chan, 2000; Vanvoorhis et al., 1994; Weitzberg and Lundberg, 1998).

In normal steroidogenesis, free cholesterol is taken into the mitochondria and converted to progesterone (the precursor of testosterone, 11-ketotestosterone, and estradiol). This involves steroidogenic acute regulatory protein (StAR) and cytochrome

P450 enzymes such as P450-sidechain cleavage enzyme (SCC) and 3 β -hydroxysteroid dehydrogenase (3 β HSD). Nitric oxide inhibits these enzymes, with the result that steroid hormone production is reduced (Panesar and Chan, 2000). This has serious implications for reproduction and development, since both directly depend on appropriate hormone levels.

Effects of Nitrates on Sperm Motility and Viability

Effects of nitrate or nitric oxide (NO) on sperm motility and viability have been investigated only recently, and results are conflicting. Certainly, if steroidogenesis is inhibited by nitrate, nitrite, or NO, then spermatogenesis could be affected since both androgens and estrogens are required for spermatogenesis (Cochran, 1992; Hess et al., 1997; Miura et al., 1991; Vizziano et al., 1996). Additionally, NOS and NO are associated with activation of eggs and sperm (acrosome reaction) (Kuo et al., 2000; Revelli et al., 2001). Rosselli et al. (1995) found that human sperm incubated with NO have decreased motility and increased mortality. The percentage of immotile and dead sperm also correlated positively with nitrite-nitrate levels in the seminal plasma of the sperm donors (Rosselli et al., 1995). Furthermore, bulls exposed orally to nitrates (100 to 250 g/day/animal) showed reduced sperm motility, increased sperm abnormalities, and degenerative lesions in the spermatocyte and spermatid germ layers of the testis (Zrally et al., 1997).

Summary of Nitrate's Effects on Reproduction

Evidence in the literature shows that nitrate can affect sperm quality and the synthesis of sex steroid hormones, with possible deleterious down-stream effects on other

reproductive variables, which rely on appropriate hormone concentrations. Hypotheses based on this evidence are investigated in the 4th and 5th chapters of our study.

Review of Endocrine Disruption in Fishes

Table 1-1 shows known effects of endocrine disruption in fishes. To date, more data are available regarding effects on males than on females. Although numerous studies of nitrate-mediated endocrine disruption are published for mammals, this area is understudied in fishes.

Hypotheses and Goals

Based on the review presented here, we hypothesized that adult female and male *Gambusia holbrooki*, captured from sites with high organochlorine concentrations (Lake Apopka) or high nitrate (springs), would exhibit altered reproductive parameters compared with reference or low nitrate sites. Based on previous studies (Table 1-1), we expected to observe increased hepatosomatic index, reduced gonadosomatic index, altered steroid hormone profiles (estradiol in females; testosterone and 11-ketotestosterone in males), reduced embryo number, decreased embryo weights; poor sperm quality; and diminished gonopodial length among fish from contaminated sites. In addition to our assessment of possible endocrine disruption in the sampled populations, we expected to observe seasonal variation in reproduction related to changes in temperature and photoperiod.

Overall, our goal was to understand the potential for endocrine disruption in our study systems in context of the seasonal reproductive cycle and some aspects of ecological variation. We measured variables that are informative in terms of basic reproductive biology of *Gambusia*, are likely targets of endocrine disruption (Table 1-1: note parameters highlighted in gray), and that, if disrupted, could affect fitness. Field

studies such as mine are useful because they measure reproductive characteristics in the wild, under natural conditions that are impossible to replicate in the lab. Thus, they can be used to generate hypotheses regarding any observed variation in reproduction, and suggest causal mechanisms. However, it is understood that, without complementary experimental studies that test field-generated hypotheses, field studies are correlative: they suggest, but do not show cause and effect.

Class of Endocrine Disruptors [†]	Sample Compounds	Observed Alterations in Reproduction Caused By or Associated With Endocrine Disruptor Exposure	References
Estrogen	Estradiol ^{cghrstx} 4-Tert-pentylphenol ^{fgh} 4-Tert-octylphenol ^{sx} Octylphenol ^l P-nonylphenol ^a 4-Nonylphenol ^{de} Nonylphenol ^w Endosulfan ^w Kepone ^b DDD ^{byz} Bisphenol A ⁱ PCBs ^{iyz} Treated sewage effluent ^{klmnop} Chlordane ^{qyz} Chronic hypoxia (1 ± 0.2 mg/L) ^v Toxaphene ^{vz} DDE ^{vz} Organochlorine mixture ^{vz*}	<p>↑Plasma vitellogenin^{ghlmnps} ♀♂</p> <p>↑Hepatosomatic index^{lt} ♂</p> <p>↑Ovotestes/intersex^{aghkmnqs} ♂</p> <p>↑Oviduct formation^{gfk} ♂</p> <p>↓Delayed puberty/persistent immature testes^d ♂</p> <p>↓Gonadosomatic index^{tux} ♂</p> <p>↔Sertoli cell structure^t ♂</p> <p>↓Gonadal development^v ♀♂</p> <p>↓Diameter of seminiferous tubules^h ♂</p> <p>↑Atrophy of germinal epithelium^h ♂</p> <p>↓Primordial germ cell number^{fg} ♂</p> <p>↔PCG distribution in developing gonad^w</p> <p>↑Malformed germ cellsⁿ (intersex fish)</p> <p>↓Delayed spermatogenesis^{ghn} ♂</p> <p>↑Loss of spermatogenic cysts^h ♂</p> <p>↓Sperm Counts^{iknz} ♂</p> <p>↓Milt volume^{knt} ♂ (intersex fishes)</p> <p>↓Sperm motility^{kv} ♂ (intersex fishes)</p> <p>↑Occluded reproductive ducts^{nt} ♂ (intersex fishes)</p> <p>↔Delayed gonopodial development^{cd} ♂</p> <p>↓Genital papilla length^r ♂</p> <p>↓Adult coloration^x ♂</p> <p>↓Courtship behavior^c ♂</p> <p>↓Fertilization success^{kv} ♀♂ (intersex fishes)</p> <p>↓Embryonic/larval survival^{ejuv} ♀♂</p> <p>↓Oocyte maturation^b ♀</p> <p>↑Oocyte atresiaⁿ ♀</p> <p>↓Embryo growth^s ♀♂</p> <p>↓Ca⁺⁺, amino acid availability to fetuses during gestation^s</p> <p>↓Delayed hatching^{iv} ♀♂</p> <p>↑E₂ binding in liver^s ♀</p> <p>↓Serum E₂, T^v ♀</p> <p>↑Plasma T, 11-KT^y ♀</p> <p>↔ Plasma E₂, Tⁿ (intersex fish)</p> <p>↑Serum E₂^{vy} ♂</p> <p>↓Serum T, 11-KT^{puv} ♂</p> <p>↑17, 20-DHP^u ♂</p>	<p>^aBarnhoorn et al., 2004</p> <p>^bDas and Thomas, 1999</p> <p>^cDoyle and Lim, 2002</p> <p>^dDreze et al., 2000</p> <p>^eFairchild et al., 1999</p> <p>^fGimeno et al., 1997</p> <p>^gGimeno et al., 1998a</p> <p>^hGimeno et al., 1998b</p> <p>ⁱHaubruge et al., 2000</p> <p>^jNakayama et al., 2005</p> <p>^kJobling et al., 2002b</p> <p>^lLye et al., 1997</p> <p>^mRodgers-Gray et al., 2001</p> <p>ⁿJobling et al., 2002a</p> <p>^oBatty and Lim, 1999</p> <p>^pFolmar et al., 1996</p> <p>^qHarshbarger et al., 2000</p> <p>^rKirby et al., 2003</p> <p>^sRasmussen et al., 2002</p> <p>^tRasmussen and Korsgaard, 2004</p> <p>^uSchultz et al., 2003</p> <p>^vWu et al., 2003</p> <p>^wWilley and Krone, 2001</p> <p>^xToft and Baatrup, 2001</p> <p>^yGallagher et al., 2001</p> <p>^zToft et al., 2003</p>

Table 1-1 Continued

Class of Endocrine Disruptors [†]	Sample Compounds	Observed Alterations in Reproduction Caused By or Associated With Endocrine Disruptor Exposure	References
Anti-Androgen	Vinclozolin ^{abc} DDE ^{abde} Flutamide ^{ab}	<ul style="list-style-type: none"> ↓Sperm count^{abce} ♂ ↓Fertilization success^c ♂ ↓Adult coloration^{abc} ♂ ↓GSI^a ♂ ↓Courtship behavior^{abc} ♂ ↓Delayed maturation^b ♂ ↓Gonopodial development^b ♂ ↑Serum E₂^d ♂ ↑Plasma T, 11-KT^d ♀ 	<ul style="list-style-type: none"> ^aBaattrup and Junge, 2001 ^bBayley et al., 2002 ^cBayley et al., 2003 ^dGallagher et al., 2001 ^eToft et al., 2003
Androgen	11-ketotestosterone ^b Paper mill effluent ^{acdef} Methyl-testosterone ^{fg}	<ul style="list-style-type: none"> ↑Gonopodial development^{abcdf} ♀ Male biased sex ratio^e ↑Male coloration^f ♀ ↓Number of reproductive females^f ♀ ↑Intersex^g ♀♂ 	<ul style="list-style-type: none"> ^aHowell et al., 1980 ^bAngus et al., 2001 ^cBortone and Cody, 1999 ^dJenkins et al., 2001 ^eLarsson and Forlin, 2002 ^fLarsson et al., 2002 ^gHahlbeck et al., 2004
Aromatase ^{**} Inhibition	Tributyltin ^{abcd}	<ul style="list-style-type: none"> Male-biased sex ratio^d ♀♂ ↓Sperm counts^a ♂ ↑Sperm lacking flagella^d ♂ ↓ATP content of sperm^b ♂ ↓Lactate dehydrogenase activity in sperm^b ♂ ↓Sperm motility^{bd} ♂ ↓Fertilization success^c ♀♂ ↓Hatchability^c ♀♂ ↓Embryo survivorship^c ♀♂ 	<ul style="list-style-type: none"> ^aHaubruge et al., 2000 ^bRurangwa et al., 2002 ^cNakayama et al., 2005 ^dMcAllister and Kime, 2003
Other	Landfill leachate ^{ab}	<ul style="list-style-type: none"> Male-biased sex ratio^{ab} ↓GSI^{ab} ♀ ↓Brain aromatase activity^a ♀ ↓Plasma T, E₂^a ♀ ↓Delayed vitellogenesis^b ♀ 	<ul style="list-style-type: none"> ^aNoaksson et al., 2001 ^bNoaksson et al., 2004
	Nitrate ^c	<ul style="list-style-type: none"> ↓Spawning^c ♂ ↓Egg number^c ♀ ↓Fertilization rate^c ♀♂ ↓Delayed hatching time^c ♀♂ ↓Hatching rate of the eggs^c ♀♂ 	^c Shimura et al., 2002

Table 1-1 Continued

<p>[†]Endocrine disruptor classification is general and non-exclusive. Several chemicals operate through a number of mechanisms that vary with context. For example, DDE has been called an estrogen, an anti-estrogen, and an anti-androgen. Classifications presented here depend on the papers cited.</p> <p>*Organochlorine mixture – refers to the chemical mixture detected in the plasma of juvenile alligators from Lake Apopka. The mix includes PCBs, DDE, DDD, mirex, endrin, dieldrin, trans-nonachlor, and oxychlorodane. These chemicals cause male to female sex reversal of reptile embryos (reviewed by Guillette et al., 2000)</p> <p>**Aromatase catalyzes the conversion of testosterone to estradiol, and androstenedione to estrone (Johnson and Everitt, 1995).</p>
<p><i>Italicized descriptors</i> refer to papers on <i>Gambusia</i> species. Superscripts match sample compounds, observed effects, and author citations. Highlighted descriptors are related to hypotheses tested in our study. ♀ = female. ♂ = male. (Intersex fish(es)) = the sex of the fish(es) in the cited study was an abnormal mix of female and male. ↑ = increase. ↓ = decrease or inhibition. ↔ = altered. 11-KT = 11-ketotestosterone. ATP = adenosine triphosphate. DDD, DDE = metabolites of the insecticide DDT. GSI = gonadosomatic index. E₂ = estradiol. HSI = hepatosomatic index. PCBs = polychlorinated biphenyls. PGC = primordial germ cell. T = testosterone. T₃ = tri-iodothyronine. 17, 20-DHP = 17α-20β-dihydroxy-progesterone.</p>

CHAPTER 2
TEMPORAL REPRODUCTIVE PATTERNS FOR FEMALE MOSQUITOFISH
CAPTURED FROM TWO FLORIDA LAKES

Introduction

In the field of endocrine disruption, Howell et al. (1980) first recognized mosquitofish as a model species when they reported that females living downstream from paper mills exhibited masculinized anal fin development. Since then, a handful of other studies have been published (i.e., Dreze et al., 2000; Porte et al., 1992; Toft et al., 2003; Toft and Guillette, 2005), suggesting that mosquitofish are a valuable model.

In part, the value of mosquitofish (*Gambusia holbrooki* and their western sister, *Gambusia affinis*) as sentinel species is associated with their ubiquitous global distribution, which has resulted from their widespread use as a biological control agent for mosquitoes (Courtenay and Meffe, 1989). This practice continues today (based on a May 2005 internet search of government pest control programs), despite the fact that most countries with introduced populations have found them to be ineffective, undesirable, or both (Courtenay and Meffe, 1989). The “undesirability” is due to *Gambusia*'s extraordinary ability to adapt and proliferate in new environments (Courtenay and Meffe, 1989). Introduced *Gambusia* can have broad negative impacts on the biota of aquatic ecosystems. They harass or out-compete native fishes, and eat a wide variety of aquatic invertebrates, as well as the eggs, larvae, or adults of many anuran and fish species, including food and sport fishes (reviewed by Arthington and Lloyd, 1989). Outside their native range, these activities disturb habitats, cause economic losses, and,

ironically, can eliminate other native mosquito predators, which are often more efficacious (Courtenay and Meffe, 1989; Danielson, 1968).

Mosquitofish are successful because they breed continuously during the reproductive season, producing several sequential and slightly overlapping broods of 1 to 245 precocious offspring at approximately 22-39 day intervals, depending on environmental temperature (reviewed by Koya et al., 1998). A second reason for their success is their ability to tolerate poor water quality, high levels of pollution, and ongoing habitat disturbance by humans (Courtenay and Meffe, 1989). In addition, their generalist diets (which expose mosquitofish to a variety of potential contaminant sources) and intermediate position in the food web suggest that they bioaccumulate high concentrations of lipophilic contaminants (like PCBs) relative to their environment. This aspect of their biology is useful in biomonitoring programs that assess biological effects of anthropogenic contaminants. For example, Porte et al. (1992) detected seasonal variation in PCBs and organophosphate (OP) pesticides in *Gambusia* muscle tissue that reflected seasonal inputs of these chemicals to the Ebro Delta in Spain, an area that is heavily used for rice farming. In that study, the authors noted that PCB concentrations were low in females sampled bi-monthly during the April – November breeding season, but increased significantly in December and February, when females are reproductively quiescent. Given that mosquitofish produce large (1.5 to 2 mm (Vargas and de Sostoa, 1996)), yolky oocytes; this indirect evidence suggests that female mosquitofish can lighten their body contaminant loads by offloading contaminants as they lose fat reserves to yolk production. Moreover, during gestation, female *Gambusia* have been shown to transfer radiolabeled leucine and 4-nonylphenol (a xenoestrogen) to their offspring

following maternal exposure (Marsh-Matthews et al., 2001; Thibaut et al., 2002). In other fish species, when females bioaccumulate contaminants, they typically pass those contaminants to their offspring in doses that are concentrated, relative to ambient concentrations (Harding et al., 1997; Nakayama et al., 2005). This can affect maternal fitness by impacting offspring survival, development, or fertility (Black et al., 1998; Nakayama et al., 2005; Rasmussen et al., 2002; Saiki and Ogle, 1995).

Given the global potential of *Gambusia* as model organisms for examining the effects of environmental contaminants on reproduction, coupled with their ecological and economic impacts, as described above, our study was undertaken to learn more about seasonal/temporal patterns of reproduction among female *Gambusia holbrooki* in their native range. We collected mosquitofish from two lakes in central Florida: Lake Apopka is eutrophic, with a 50-year history of agricultural, municipal, and industrial contamination, whereas Lake Woodruff National Wildlife Refuge is oligotrophic, is less impacted by human activities, and served as a reference site for a number of related studies (e.g. Guillette et al., 2000). Mosquitofish exhibit great genetic diversity coupled with a potential for phenotypic plasticity in life history characters (Downhower et al., 2000; Haynes and Cashner, 1995; Meffe et al., 1995; Stockwell and Vinyard, 2000). These features contribute to the success of *Gambusia* as an introduced species (Greene and Brown, 1991). Therefore, the second goal of our study was to assess reproductive variation between the two lake populations in context of lake-associated environmental differences.

Methods

Field and Tissue Collections

Between March 2001 and June 2002, 16 monthly collections of adult female *Gambusia holbrooki* were made from Lake Apopka (north shore, near Beauclair Canal) and Lake Woodruff Wildlife Refuge (northwest shore, Spring Garden Lake) in central Florida, USA. Fish of mature size (> 1.7 cm, based on our experience) were captured using a 3-mm mesh dip net. Fish maturity was verified in the laboratory from the presence of clearly differentiated white or yolked oocytes in the ovary (Haynes, 1995). Fish were considered reproductively quiescent (mature, but not pregnant) at stage 0, when no yolk was visible in the oocytes (Fig. 2-1). Each lake collection took 1 day, plus additional days to process fish. Thus monthly collections from the two lakes were made on different days (average of 4 days apart).

Each monthly sample, ranging from 39 – 60 fish per lake, was divided into two subsets. The first subset was used to obtain ovarian and hepatic weight, embryo number, and embryo stage. These fish ($n = 23 - 31$ per month, per lake) were held live in aerated coolers filled with lake water, fed flake fish food ad libitum, and processed within 1-2 days of capture. Before necropsy, fish were over-anesthetized in room-temperature water containing 0.1% MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma #A5040). The ovary (0.1 to 322 mg) and liver (0.1 to 31.5 mg) were removed and weighed to the nearest 0.1 mg. Embryos (stage 3 and greater) were separated from the ovarian stroma, counted, and staged according to Haynes (1995) (Fig. 2-1). Embryos were staged at half stages (e.g., 4.5) if they were transitional between two stages. Unfertilized oocytes that were younger than stage 3 were staged, but not counted. Note that, within a brood, embryos are generally synchronized in their development. Average

embryo wet weight for each brood was calculated as ovarian weight divided by embryo number. Note that this value is exaggerated slightly for larvae just before birth (stage 9 – 10) because, at that time, oocytes for the next brood have started to accumulate yolk, thus adding weight to the ovary that is not related to the counted embryos.

The second subset of captured fish was used for measurement of muscle estradiol concentrations. These fish ($n = 7 - 12$ per month, per lake; mean = 10) were frozen immediately at capture and held on ice while in the field. Upon return to the laboratory, the caudal fin was removed, and all caudal peduncle tissue posterior to the gonad was cut from the fish, weighed (average = 71 mg), and frozen (-80°C). Estradiol was measured on lipid extracts of these peduncle tissues, as described below. Because the caudal peduncle is primarily muscle, we will refer to it as muscle for the remainder of the chapter. Heppell and Sullivan (2000) showed that seasonal patterns of muscle and plasma estradiol concentrations were comparable in female gag grouper, although actual concentrations in muscle (measured as pg/g) were an order of magnitude lower than plasma concentrations (measured as ng/ml).

For all fish (both subsets), standard length (SL) was measured to the nearest 0.01 cm from the snout tip to the caudal peduncle using calipers. Fish were blotted dry and weighed with an electronic balance to the nearest milligram.

Water temperature, pH, and conductivity data were obtained at the time and location where fish were sampled, using a handheld *Ultrameter* (Model 6P, Myron L Company, Carlsbad, CA). To describe additional water quality parameters for the two lakes, we referred to STORET, a public-access EPA database of environmental measurements (<http://www.epa.gov/storet/dbtop.html>). We gathered data from 2000 –

2003 on nitrogen and phosphorus concentrations, secchi depth, turbidity, and total suspended solids. It should be noted that the usefulness of this database is limited by the relatively few sampling dates recorded each year: Lake Apopka was sampled 5 times in 2000, and once in each of 2001-2003; Lake Woodruff was sampled 6 times in 2000, twice in 2001, once in 2002, and 3 times in 2003. Our lake comparison based on STORET data reflects the average values for measurements made in 2000 – 2003 and thus provides a helpful, but generalized view of water quality in each lake.

Muscle Estradiol Measurements

For measurement of muscle concentrations of 17- β -estradiol, frozen peduncle tissues were thawed in glass tubes, on ice, and homogenized in 1 ml 65mM borate buffer (pH 8.0). Homogenate was extracted twice with 5 ml diethyl ether. For each extraction, ether and homogenate were mixed together for two minutes using a multi-tube vortex mixer. For the first extraction, tubes were allowed to settle for three minutes to separate phases. For the second extraction, phases were separated by centrifugation for two minutes. The aqueous phase was frozen in a methanol bath chilled to -25°C with dry ice. The ether from both extractions was combined in a second glass tube and evaporated under dry forced air.

Hormone concentrations were determined using validated enzyme immunoassay (EIA) kits (Cat No. 582251) purchased from Cayman Chemical Company (Ann Arbor, Michigan). Dry extract was reconstituted in 500 μ l EIA buffer so that samples would fall within the range of the standard curve. EIAs were run as recommended by Cayman with an 18 h refrigerated incubation to increase sensitivity. Data were quantified against a standard curve linearized using a logit transformation of B/Bo (bound sample/maximum

bound). Duplicate or triplicate interassay variance (IAV) samples at two dilutions were included with each plate. The coefficient of variance among all plates, averaged for the two dilutions was 22.6%. To normalize sample estradiol concentrations across assays, we multiplied by a correction factor derived from the relationship between individual plate IAV values and the mean IAV values for all plates.

Statistics

Estradiol data points lying more than three standard deviations from the mean for all samples ($n = 9$, 3%) were excluded from the data set. Using a correlation matrix, data were screened for correlations among standard length (SL), body weight, ovarian and hepatic weight, embryo number, embryo wet weight, and embryo stage. We also looked for correlations between maternal body size and muscle estradiol concentrations.

Apparent correlations were visualized using linear regression following \log_{10} transformation of the variables. Embryo number was positively related to maternal SL ($r^2 = 0.44$, $p < 0.0001$). Likewise, hepatic weight was positively related to maternal body weight ($r^2 = 0.57$, $p < 0.0001$). Therefore, mean embryo number and hepatic weight were adjusted for body size (using ANCOVA) when appropriate.

We used ANOVA or ANCOVA to compare mean response variable values between consecutive months within a lake and to compare mean response variable values between lakes in each month. Changes in embryo wet-weight and adjusted hepatic weight during gestation were similarly compared between stages within a lake, and between lakes at each stage. Correlation, regression, and ANOVA analyses were completed using Statview 5.0. ANCOVA analyses and calculations of adjusted means were completed using SPSS 13.0. Results were considered significant at $p \leq 0.05$.

Gonadosomatic Index

Gonadosomatic index (GSI) is a traditional measurement of fish ovarian weight relative to body size. Mean GSI is often used to indicate temporal changes in female (or male) reproductive status. However, in viviparous mosquitofish, GSI is not an accurate measure, particularly in a seasonal study that invokes mean GSI values to describe reproductive trends. This is because females are not reproductively synchronized as a group: in any given month during the reproductive season, females exhibit all stages of embryonic development (Fig. 2-2) (although within a female, the embryos are synchronized). Furthermore, ovarian weight is determined in part by embryo number, which varies with maternal body size. For these reasons, we do not consider GSI a valid measurement for female mosquitofish, particularly when evaluated as an average value, and thus have not included it as a response variable.

Results

Environmental Differences between Lakes

Water temperature, which exhibited a seasonal pattern, was similar between the two lakes, except at the end of fall and beginning of spring, when the temperature of Lake Apopka was about 5°C warmer (Fig. 2-3). Mean conductivity, which differed slightly between lakes, ranged from 430 to 766 μS (mean = 591 μS) in Lake Apopka and from 700 to 1800 μS (mean = 1231 μS) in Lake Woodruff. However, this difference is unlikely to be biologically relevant to osmotic balance (1800 μS < 4% seawater). Lake pH fluctuated monthly at both sites, ranging from 6.78 to 8.73 (mean = 7.62) in Lake Apopka and 6.47 to 8.47 (mean = 7.22) in Lake Woodruff. Fluctuations in pH did not follow a predictable pattern in either lake, and thus we could not detect any particular effect of pH on the measured variables relevant to our study. Additional descriptive

information on lake water quality is shown in Table 2-1. On average (2000 to 2003), Lake Woodruff has lower nitrogen and phosphorus concentrations than the more eutrophic Lake Apopka. Lake Woodruff also has greater water clarity (Secchi depth) and lower turbidity and total suspended solids. Our visual observations of water clarity in the two lakes corroborate these data.

Temporal and Lake-Associated Variation in Response Variables Related to Reproduction

Body size

In general, the adult female mosquitofish collected from Lake Apopka were bigger than those from Lake Woodruff (Fig. 2-4). Mean body size of captured females from both lakes was higher at the beginning of the reproductive season, and lower at the end of the year. This change could reflect fall recruitment of newly matured fish born earlier in the year (which, although mature, will not finish growing until the next spring). We observed that 1.7 cm was the smallest size for mature females from either lake, suggesting that this is the minimum size for female reproductive activity. However, all fish at this size need not be mature, as we did observe immature fish that were up to 2.0 cm in standard length.

Temporal changes in reproductive activity

Females collected from both lakes were reproductively active ($\geq 90\%$ pregnant) during the spring summer and fall, and quiescent for at least two months during the winter (Figs. 2-2 and 2-5). The timing of spring recrudescence was different between the two sample populations, with females from Lake Woodruff being delayed by 2 to 3 months (Fig. 2-5). In addition, spring reproductive recrudescence was more synchronized in females from Lake Apopka compared to those from Lake Woodruff,

which were more variable in their timing of reproductive onset (Fig. 2-5). This difference in timing is probably due to spring temperature differences between the lakes (Fig. 2-3), as Lake Apopka warms up more rapidly in the spring. The observed pattern suggests that temperature, rather than photoperiod, is the cue responsible for spring onset of reproductive activity in mosquitofish.

Embryo number, size, and stage of development

In sample populations from both lakes, embryo number was significantly ($p < 0.0001$) and positively related to female standard length (SL), although the strength and slope of this relationship was different between females from the two lakes (Apopka: $r^2 = 0.54$; Woodruff: $r^2 = 0.14$; ANCOVA indicated a significant interaction between lake and SL, $p < 0.0005$) (Fig. 2-6). In Lake Apopka, large females produced many more offspring than small females, a trend that was not as strong among females from Lake Woodruff (Fig. 2-6). On average, females from Lake Apopka produced 12.5 embryos per brood, whereas females from Lake Woodruff produced only 5.1 embryos per brood. Mean litter size (adjusted for maternal standard length) was typically larger among females from Lake Apopka, except at the beginning and end of the reproductive season, when litter sizes were similar in the two lakes (Fig. 2-5).

Because litter sizes were larger among females from Lake Apopka, it is reasonable to hypothesize that a tradeoff between embryo number and embryo size exists such that Apopka embryos might be smaller than Woodruff embryos. This, however, was not the case. Although all embryos gained wet weight as they developed, there was no lake-associated difference in embryo wet-weight at birth, based on ANOVA (Fig. 2-7). We did observe lake related differences in embryo wet weight between stages 9 – 10,

although this could be an artifact of the method of wet weight calculation, as explained above in Methods.

Hepatosomatic index

We observed that mean hepatic weight (adjusted for female body weight) was dependent on embryonic stage, an association that is probably related to vitellogenesis. For maximum sample size, we combined fish from both lakes and noted that adjusted hepatic weight increased from stage 0 to stage 2.5, declined gradually through stage 6 – 8, and rose again between stages 8 and 10.5 (Fig. 2-8A). Note that females in transition between broods do not exhibit stage 0 (reproductive quiescence, no yolked oocytes present) as broods overlap slightly (Fig. 2-1, see photographic panel for stage 2). When HSI data were split between lakes, we observed a similar gestation-related pattern in HSI among females from both lakes, although adjusted hepatic weight was often higher among females from Lake Apopka (Fig. 2-8B).

Estradiol

Temporal variation in female muscle estradiol (E_2) concentrations was evident and often differed between the two lake populations (Fig. 2-9). Females from Lake Apopka exhibited a significant decline in E_2 from September through December, followed by three significant peaks in February, April, and June. Females from Lake Woodruff also exhibited significant fluctuations in E_2 throughout the year, but the amplitude of the fluctuation was less than for females from Lake Apopka. In addition, females from Lake Woodruff exhibited a fall peak in muscle E_2 that was significantly higher than E_2 concentrations in Apopka cohorts.

Discussion

Female mosquitofish in central Florida begin reproductive activity in spring when water temperatures exceed 22°C, and end activity in fall when daylength shortens to between 12 and 11 h, after which females no longer produce new broods, but presumably complete gestation of broods already in progress. Interestingly the influence of temperature in spring overrides short photoperiods, because we observed reproductive females in the late January collection from Lake Apopka, when daylength was 10.5 h, but water temperature reached 27°C. Similarly, decreasing photoperiod in the fall overrides temperature, because fewer than 25% of Apopka females were pregnant in late October (daylength = 11 h), even though daytime water temperatures could still exceed 25°C. These observations are similar to those of Koya and Kamiya (2000), who observed that, in a Japanese population of *Gambusia affinis*, spring vitellogenesis occurred when temperature exceeded 14°C, and pregnancy proceeded when temperature exceeded 18°C. In the same study, Koya and Kamiya (2000) reported that sexually active females ceased vitellogenesis when photoperiod shortened to 12.5 h.

Previously published data, and data presented here, indicate that Lake Apopka is more eutrophic in terms of nitrogen and phosphorus content and contains more estrogenic or antiandrogenic endocrine-disrupting compounds when compared to Lake Woodruff (EPA STORET database, <http://www.epa.gov/storet/dbtop.html>; Guillette et al., 1999). Moreover, Lake Apopka has lower water clarity as described by Secchi depth, turbidity, and total suspended solids. Exposure to estrogenic chemicals is often related to reduced fecundity. For example, dosing of female medaka or fathead minnows with estradiol or bisphenol A (weakly estrogenic) has been shown to increase vitellogenesis and, at higher

doses, reduce or abolish egg production (Palace et al., 2002; Patyna et al., 1999; Scholz, and Gutzeit, 2000; Sohoni et al., 2001). Likewise, estradiol exposure arrests embryo development in zebra *Danios* (Kime and Nash, 1999). In addition, reduced water clarity in Lake Apopka might be expected to reduce reproductive output of females because of the lower light availability, as shown experimentally for *G. affinis* by Hubbs (1999).

Despite these data, which would predict lower fecundity among Apopka females relative to females from Lake Woodruff, we found the opposite. In our study, females from Lake Apopka were significantly larger and more fecund, even when fecundity is adjusted for maternal body size. Moreover, their increase in fecundity was not accompanied by a decrease in embryo size, suggesting that Apopka females genuinely have greater reproductive output compared to Woodruff females. The larger size of Apopka females suggests that they grow faster and/or live longer than females from Lake Woodruff. This may be due to higher primary productivity in Lake Apopka, driven by increased nutrient loads, which results in greater food availability with respect to mosquitofish. In a field study of Poeciliid growth rates, Grether et al. (2001) reported that guppy females and juveniles grew faster in rainforest streams with higher primary productivity.

As with fecundity, adjusted hepatic weight was higher among Apopka females relative to females from Lake Woodruff. It is likely that fecundity and hepatic weight are causally related, but unclear in which direction. For example, with their higher fecundity, Apopka females probably require increased yolk production. In mosquitofish, yolk is derived, in part, from vitellogenin produced in the liver in response to circulating estrogens (Tolar et al., 2001). Thus, the increased need for vitellogenesis could explain the increased hepatic weight observed among Apopka fish. Alternatively, the estrogenic

contaminants that characterize Lake Apopka (Guillette et al., 1999) could stimulate vitellogenesis (Palace et al., 2002; Sohoni et al., 2001). In an unexpected twist, contaminant-stimulated vitellogenesis could actually permit the increased fecundity of Apopka females. However, offspring survivorship remains to be tested.

In the first half of 2002, we noted a significant rise in muscle estradiol concentrations among females from Lake Apopka compared to females from Lake Woodruff. Gallagher et al. (2001) reported estradiol concentrations for female bullheads caught in January and July from Lake Apopka and Lake Woodruff, but they observed no lake-associated differences. However, their limited number of collection dates could explain their non-detection of changes in endogenous estradiol. In other studies of female fishes captured from sites affected by estrogenic pollution, like Lake Apopka, increased estradiol concentrations have been reported. This is true, for example, of female walleye, captured from water contaminated with estrogenic treated sewage effluent (Folmar et al., 2001).

Conclusions and additional hypotheses. Data presented here indicate that female mosquitofish in central Florida exhibit a well-defined reproductive cycle. In addition, we observed lake-associated variation in hepatosomatic index and muscle estradiol concentrations that suggest a subtle level of estrogenic endocrine disruption among Apopka females, consistent with other studies. However, in conjunction with these observations, we also report that females from Lake Apopka exhibit greater reproductive output in terms of embryo number, compared to females from Lake Woodruff. Although relative survivorship of larval and juvenile fish remains to be established, our present data

suggest that chemical pollution in Lake Apopka might not disrupt resident mosquitofish at the population level.

In comparison to other species, mosquitofish are recognized for their ability to adapt to variable or polluted environments (Courtenay and Meffe, 1989). This ability is marked by increased heterozygosity and overall genetic diversity among exposed individuals (Downhower et al., 2000; Stockwell and Vinyard, 2000; Theodorakis and Shugart, 1997). Genetic diversity is supported by *Gambusia*'s mating system, in which females often mate with more than one male, resulting in broods characterized by multiple paternity (Zane et al., 1999). Greene and Brown (1991) observed that larger female *Gambusia affinis* were more likely than small females to mate with multiple males. They also observed that multiply inseminated females were more heterozygous than singly mated females, and, that the offspring of multiply inseminated females exhibited greater genetic diversity. Females from Lake Apopka are larger than females from Lake Woodruff, and it would be interesting to know if their degrees of heterozygosity and genetic diversity were also greater. If that was the case, *Gambusia* could provide an informative model for the evolution of characters that are adaptive in polluted environments.

Water Parameter	Apopka*	Woodruff*
Phosphorus as P (mg/L)	0.11 ± 0.03	0.07 ± 0.01
Nitrite (NO ₂) + Nitrate (NO ₃) as N (mg/L)	1.15 ± 0.71	0.05 ± 0.02
Nitrogen, Kjeldahl (sum of free ammonia and organic nitrogen) (mg/L)	2.57 ± 0.42	1.27 ± 0.06
Secchi disk depth (m)	0.39 ± 0.09	0.76 ± 0.06
Turbidity (NTU)	12.24 ± 2.66	3.27 ± 0.69
Total suspended solids (TSS) (mg/L)	50.50 ± 7.12	8.53 ± 3.45
*Mean value for lake taken from sampling dates in 2000 to 2003; data retrieved from EPA's public-access STORET database (http://www.epa.gov/storet/dbtop.html).		

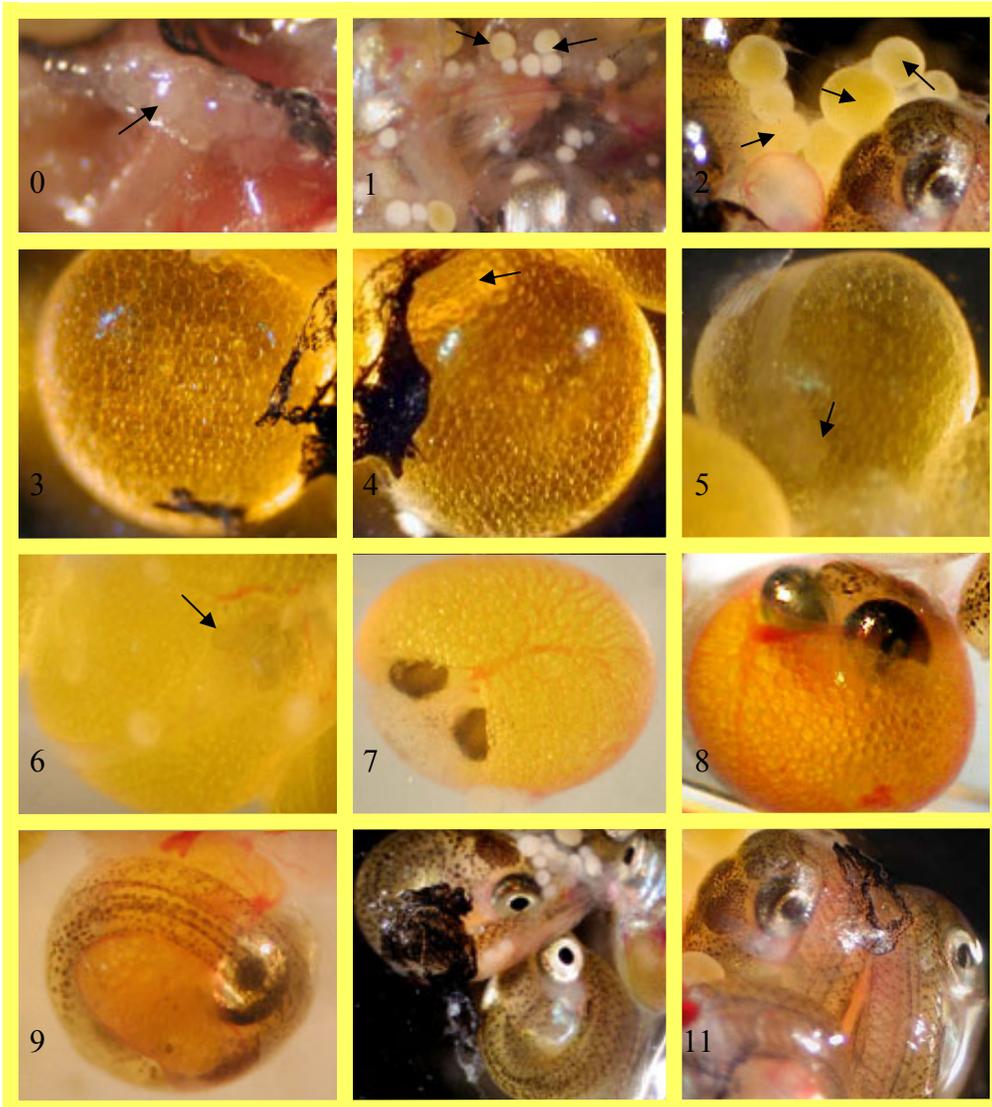


Figure 2-1. Stages of embryonic development for eastern mosquitofish (*Gambusia holbrooki*). Stage 0 consists of small, white oocytes (black arrows), indicating a mature, but reproductively quiescent ovary. Stages 1 and 2 indicate progressive yolking of oocytes (black arrows). Note that broods overlap. Stage 3 is the first stage at which all yolked oocytes are similar in size. Stage 4 is distinguished by presence of the blastodisk (black arrow). Stage 5 embryos have elongated, and optic discs (black arrow) are visible but unpigmented. Stage 6 embryos exhibit pigmented optic discs (black arrow). Stage 7 embryos are enlarged, have some skin pigmentation and advanced, but incomplete eye development. Stage 8 embryos exhibit fully formed and pigmented eyes, pigmented skin, but tail does not overlap head. Stage 9 embryos retain a large yolk sac but tail overlaps head. Stage 10 embryos have absorbed most their yolk sacs and often survive if removed from the ovary. Stage 11 embryos have absorbed their yolk sacs and are ready for birth. Staging is based on Haynes (1995).

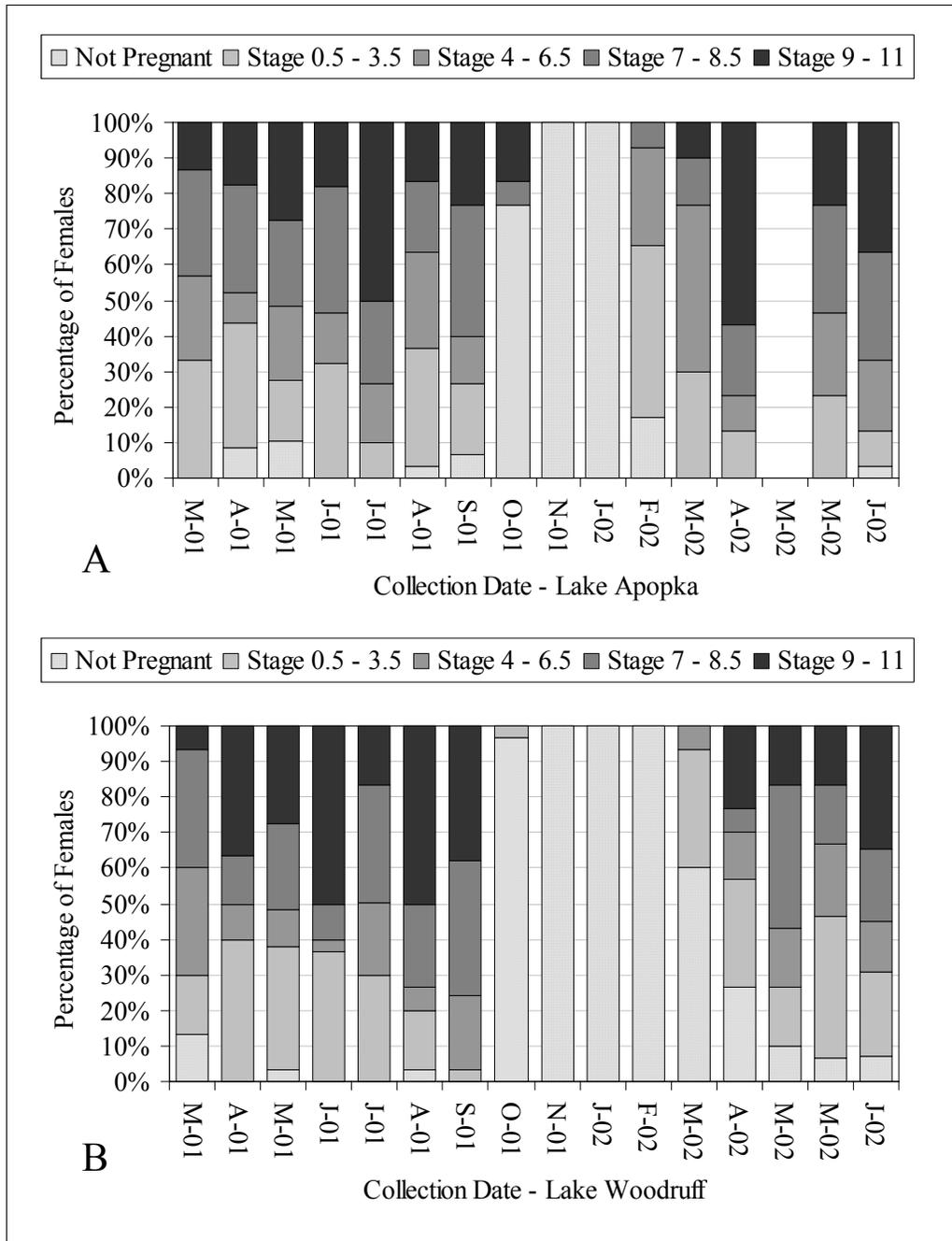


Figure 2-2. Percentage of female mosquitofish with broods at the indicated stages of embryonic development. A) Females from Lake Apopka. B) Females from Lake Woodruff. Embryos are developmentally synchronized within a brood, but, as this graph shows, they are not synchronized across broods. Females presenting as “not pregnant” were mature, but reproductively quiescent. Note that the first May 2002 collection is missing from Lake Apopka: drought conditions on the lake prevented boat access for two weeks.

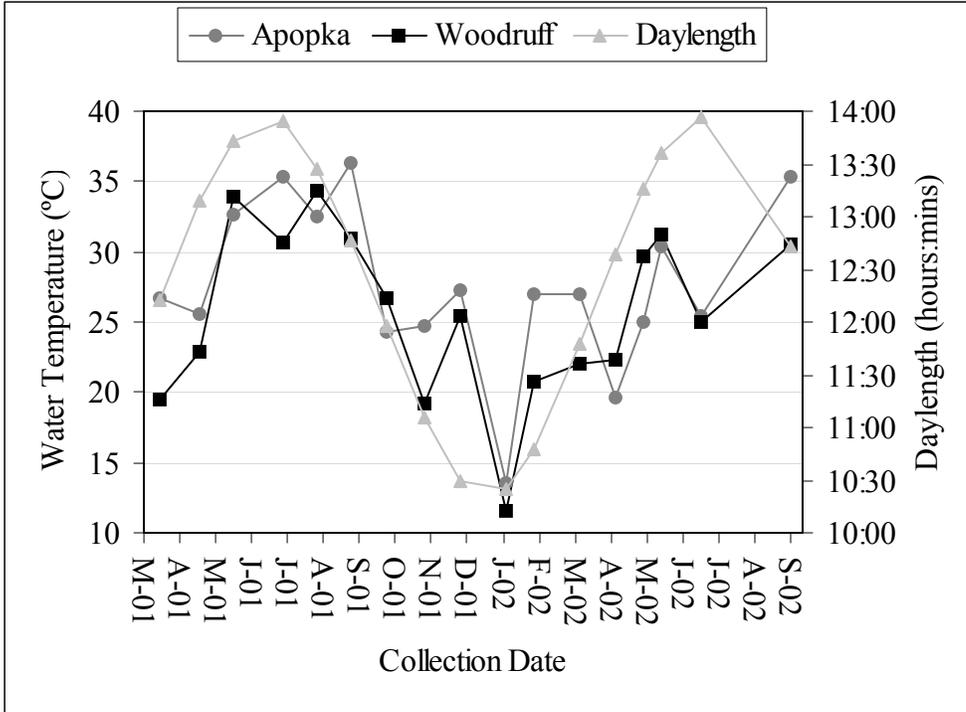


Figure 2-3. Seasonal changes in water temperature for Lake Apopka and Lake Woodruff, shown with ambient photoperiod for each collection date

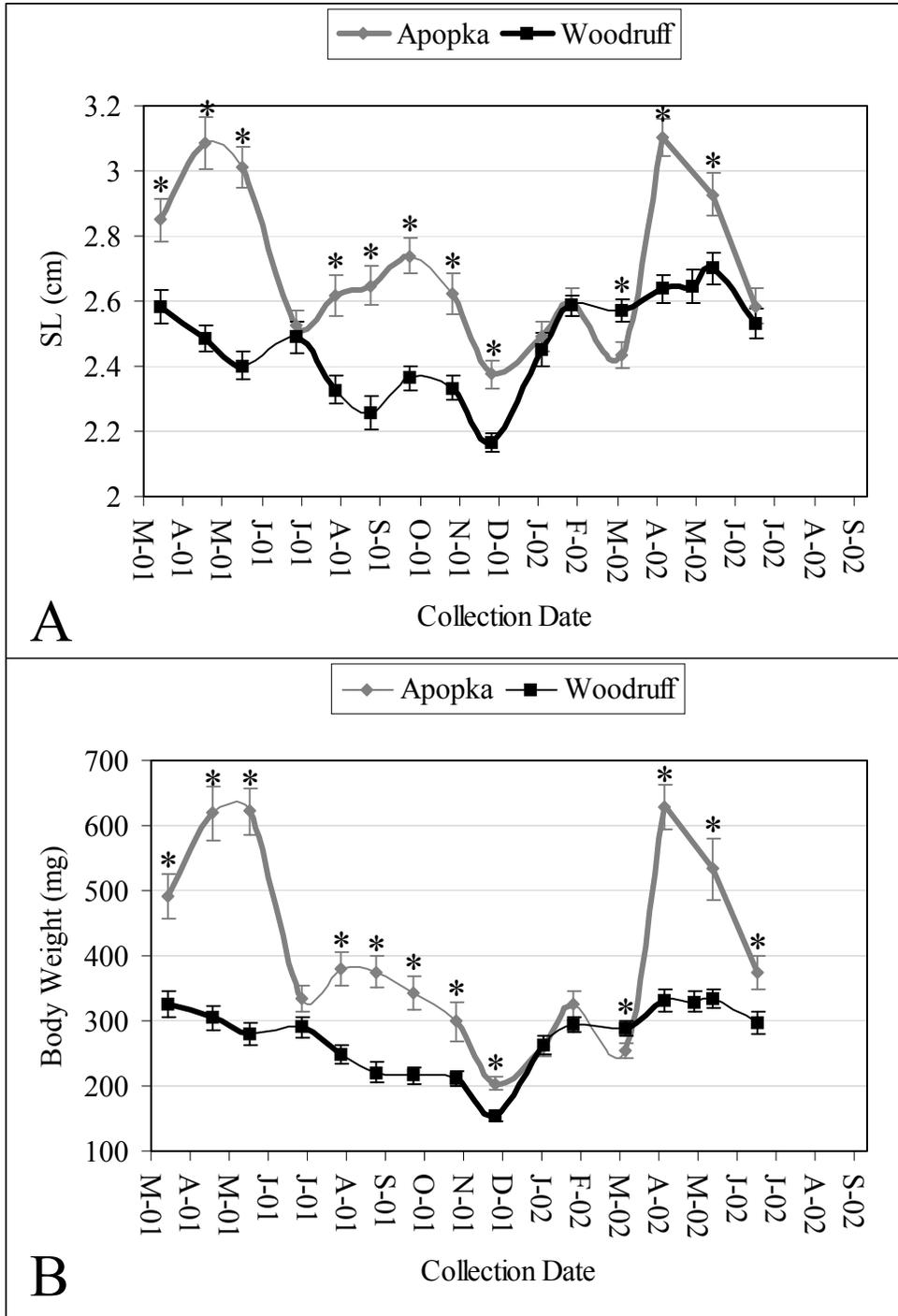


Figure 2-4. Temporal variation in body size of adult female mosquitofish from Lake Apopka and Lake Woodruff. A) Standard length (SL). B) Body weight. Graphs show means ± 1 SE. *Months in which the mean body size of fish from the two lakes are significantly different (ANOVA, $p \leq 0.05$). A heavier line between data points indicates a significant temporal change within a lake (ANOVA, $p \leq 0.05$).

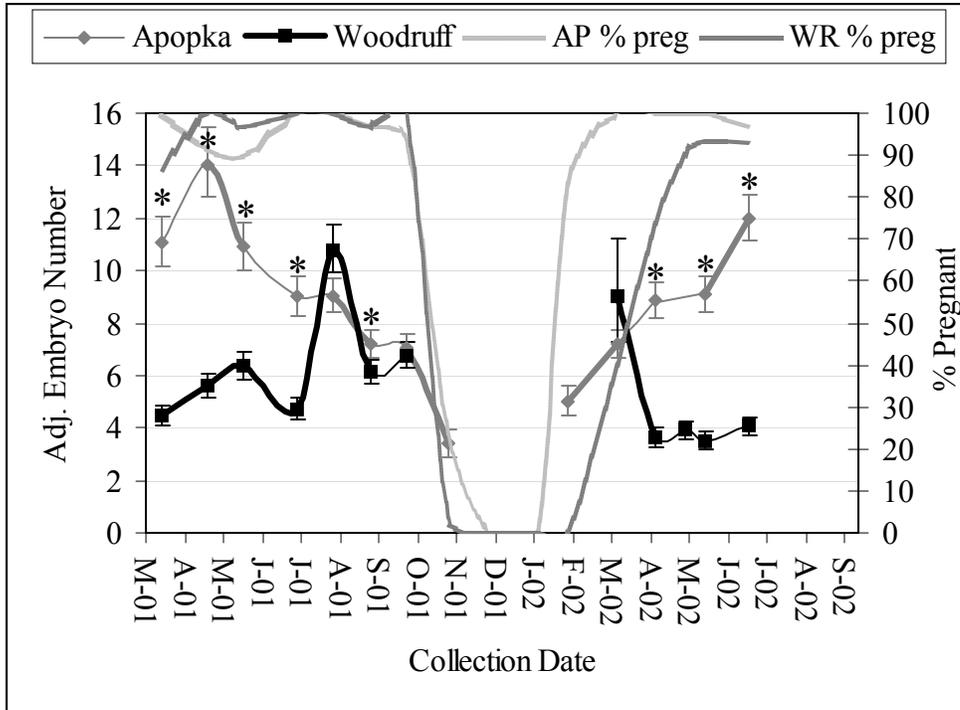


Figure 2-5. The right hand y-axis shows the percentage of sampled females from each lake that were pregnant (contained yolked oocytes) at each collection date. The left hand y-axis quantifies temporal variation in mean embryo number (litter size), adjusted for standard length, of adult female mosquitofish from Lake Apopka and Lake Woodruff. Graph shows means \pm 1 SE. *Months in which the mean adjusted embryo number of fish from the two lakes are significantly different (ANCOVA, $p \leq 0.05$). A heavier line between data points indicates a significant seasonal change in embryo number within a lake (ANCOVA, $p \leq 0.05$).

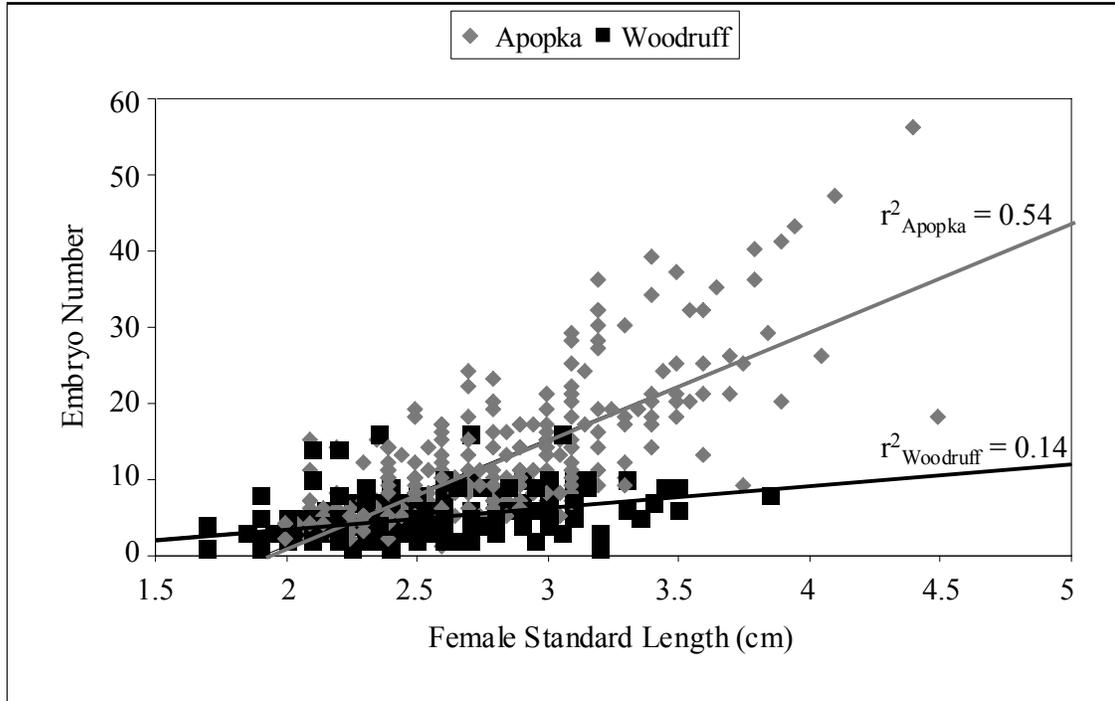


Figure 2-6. Embryo number (litter size) observed for female mosquitofish of different standard lengths. Data for fish from Lake Apopka and Lake Woodruff are shown separately. Regression is significant for both populations ($p < 0.0001$).

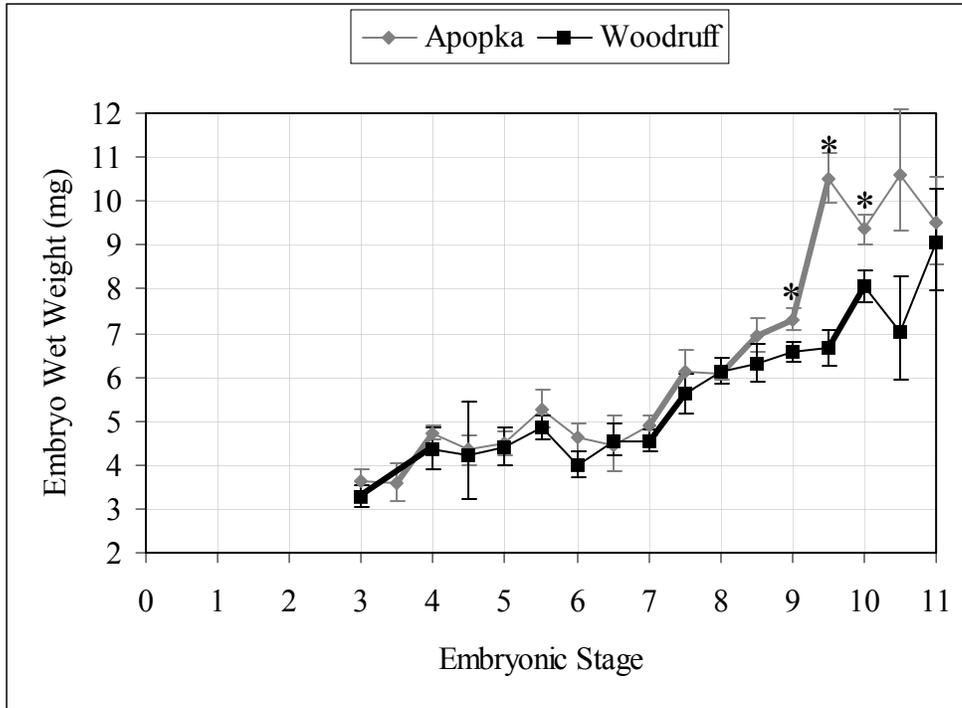


Figure 2-7. Mosquitofish embryonic wet weight at different developmental stages. Data for fish from Lake Apopka and Lake Woodruff are shown separately. Graph shows means ± 1 SE. *Stages at which the mean embryo wet weight was significantly different between females from the two lakes (ANOVA, $p \leq 0.05$). A heavier line between data points indicates significant stage-to-stage variation within a lake (ANOVA, $p \leq 0.05$).

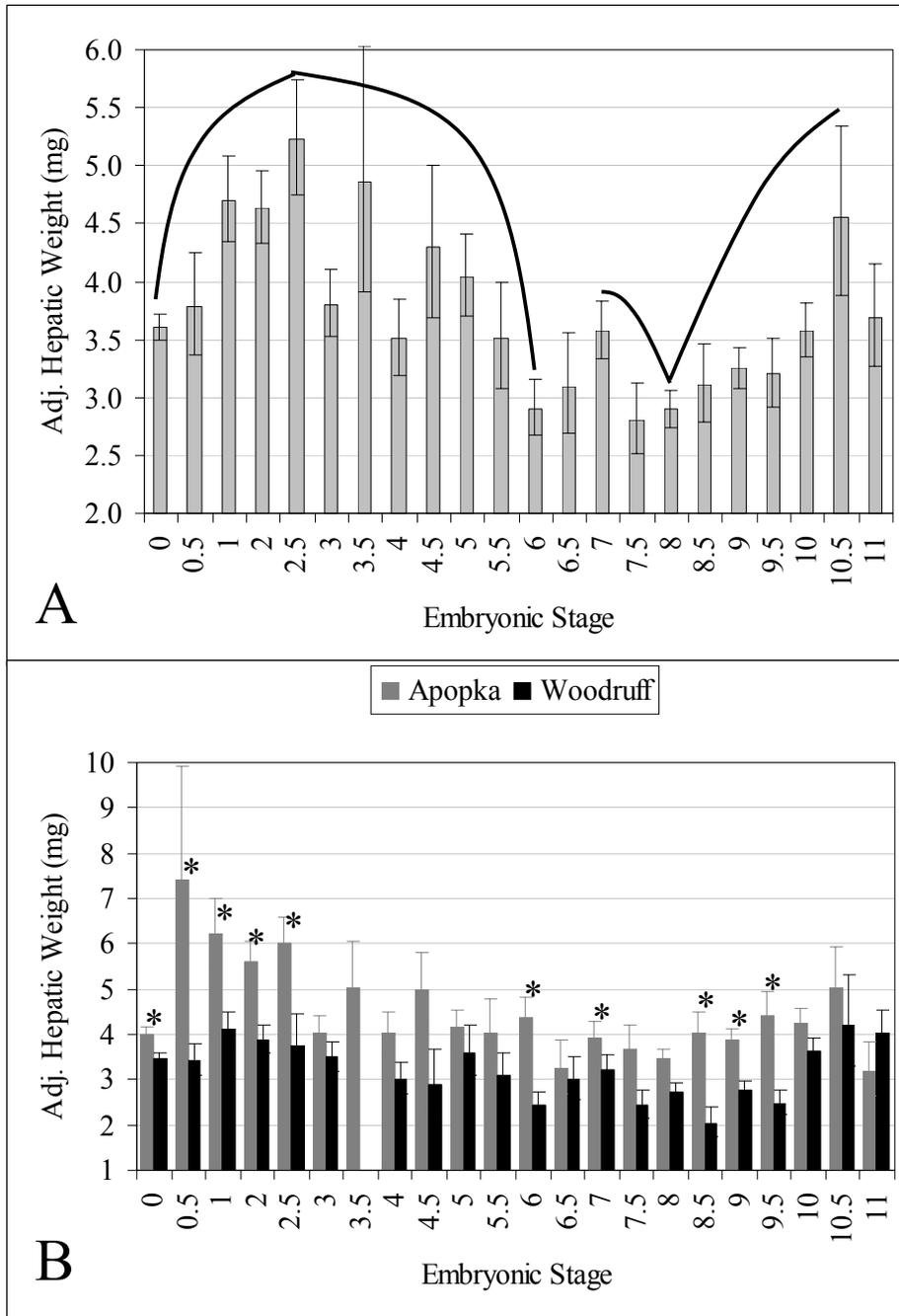


Figure 2-8. Mean hepatic weight (± 1 SE), adjusted for female body weight of mosquitofish with embryos at different stages. A) Fish from Lake Woodruff and Lake Apopka combined. B) Fish from Lake Woodruff and Lake Apopka shown separately. Curved lines in (A) indicate significant changes in hepatic weight across stages (ANCOVA, $p \leq 0.05$). Similar significant trends were observed in (B) (not shown for graph clarity). *Indicates that adjusted mean hepatic weight at a given stage of embryonic development was different between lake populations (ANCOVA, $p \leq 0.05$).

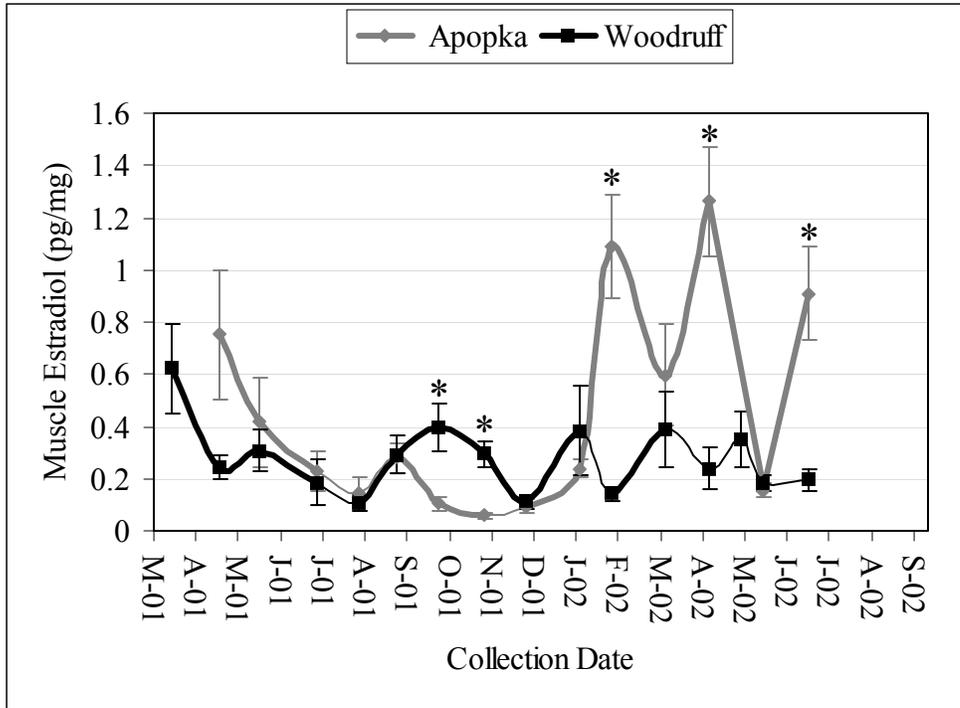


Figure 2-9. Temporal variation in muscle estradiol concentrations of adult female mosquitofish from Lake Apopka and Lake Woodruff. Graph shows means \pm 1 SE. *Months in which the mean estradiol concentrations of fish from the two lakes are significantly different (ANOVA, $p \leq 0.05$). A heavier line between data points indicates a significant seasonal change within a lake (ANOVA, $p \leq 0.05$).

CHAPTER 3
SEASONAL SPERM QUALITY IN MALE *Gambusia holbrooki* (EASTERN
MOSQUITOFISH) COLLECTED FROM TWO FLORIDA LAKES

Introduction

Sperm count and sperm viability are important measures of male fertility. In several human populations, sperm counts have decreased over the past 50 years, an observation that is related to increased subfertility and infertility among men (Carlson et al., 1992; Swan et al., 2000; Jensen et al., 2002). The underlying causes of reduced human sperm counts remain controversial. However, a variety of studies in non-human animals, particularly fishes, suggest a link with environmental contaminants that have been shown to disrupt endocrine function and/or reproductive development (Gray, 1998; Toft and Guillette, 2005; Jobling et al., 2002). For example, in sexually developing and adult guppies, decreases in spermatogenesis and stripped sperm counts have been observed after exposure to vinclozolin (fungicide) or p,p'-DDE (DDT metabolite) (Baatrup and Junge, 2001; Bayley et al., 2002). Similar observations have been reported in swordtails exposed to nonylphenol (plasticizer) (Kwak et al., 2001); goldfish or zebrafish treated with estradiol or ethynylestradiol (associated with sewage effluent), respectively (Schoenfuss et al., 2002; Van den Belt et al., 2002); and in adult Japanese medaka exposed to 4-tert-octylphenol (estrogen mimic) (Gronen et al., 1999).

In addition to these experimental studies, field studies have shown similar effects among wild English flounder and roach captured from waterways contaminated with treated sewage effluent (Lye et al., 1998; Jobling et al., 2002b). Toft et al. (2003)

previously reported reduced sperm counts among male mosquitofish collected from Lake Apopka in Florida (USA). Over the three months during which sampling occurred, males from Lake Apopka exhibited an average of 47% fewer stripped sperm cells per mg testis compared with cohorts captured from Lake Woodruff (reference lake). Unlike Lake Woodruff, Lake Apopka has a history of contamination by p,p'-DDE and other endocrine-disrupting contaminants with estrogenic or antiandrogenic activity (Guillette et al., 1999). In a follow-up study, Toft and Guillette (2005) observed reduced sperm counts among reference mosquitofish exposed for 1 month to water from Lake Apopka.

The annual spermatogenic cycle of mosquitofish has previously been described for populations sampled in León Province, Spain, and central Japan (Fraile et al., 1992; Koya and Iwase, 2004). Based on these reports, the cycle involves a continuous period of spermatogenesis (spring through fall), followed by a shorter period of winter quiescence. In mosquitofish, the testes are fused into a single, round, white-colored organ that is located centrally in the abdomen, dorsal to the origin of the gonopodium (Fraile et al., 1992). The grooved gonopodium is used by the male to transfer spermatozeugmata to the genital opening of the female. This structure forms during puberty by fusion and modification of the anal fin rays under stimulation by endogenous androgen (Angus et al., 2001; Ogino et al., 2004). A single vas deferens connects the gonopodium to the efferent ducts that coalesce from within the central lumen of each testis (Fraile et al., 1992). The outer wall of the testis is lined with spermatogonia (Fraile et al., 1992). In spring through fall, spermatogonia proliferate in successive waves of mitosis, forming nests (cysts) of primary spermatocytes bounded by Sertoli cells (Fraile et al., 1992). In a process that takes approximately 30 days, spermatocytes within a single cyst undergo

synchronized meiosis and differentiation to produce spermatids and ultimately tailed spermatozoa (Fraile et al., 1992; Koya and Iwase, 2004). As the cysts mature, they move from the periphery of the testis to the center, where they are released to the efferent sperm ducts as spherical aggregates of sperm (spermatozeugmata), with tails in the center and heads on the periphery (Fraile et al., 1992). At this point, Sertoli cells no longer surround the spermatozeugmata, but rather, they hypertrophy and become part of the efferent duct tubule (Fraile et al., 1992). The tubules secrete a gelatinous matrix that holds the spherical structure of the spermatozeugma together until it reaches the oviduct of a female (reviewed by Constantz, 1989). As winter approaches, production of new spermatocytes ceases. Through the winter, stored cysts of mature spermatozoa occupy most of the testicular volume, and will be used during early spring copulation, which occurs before the first wave of spring spermatogenesis is complete (Koya and Iwase, 2004).

Like most teleosts, mosquitofish exhibit seasonal variation in sperm production that is regulated by changes in temperature and photoperiod (Fraile et al., 1994). However, factors that initiate mosquitofish spermatogenesis are not yet fully understood. Males that have recently entered testicular quiescence cannot be stimulated to produce new sperm by increasing temperature or photoperiod (Fraile et al., 1993). However, fish captured at the end of their quiescent period will exhibit proliferation of spermatogonia, even if temperatures remain low and days are short (Fraile et al., 1994). Increasing ambient temperatures are required for differentiation of spermatogonia to spermatocytes, and photoperiod must lengthen in order for spermatocytes to enter meiosis (Fraile et al., 1994; De Miguel et al., 1994).

We conducted our study from May 2001 to September 2002 to extend our understanding of seasonal sperm production in mosquitofish populations from Lakes Apopka and Woodruff in central Florida. In addition, we tested our hypothesis that low sperm counts per mg testis, previously reported for fish from Lake Apopka, are related to reduced sperm counts per spermatozeugma. The resulting data will help identify possible mechanisms by which sperm count can be affected among males from Lake Apopka. In addition to sperm counts, we measured seasonal changes in sperm viability among fish from both lakes.

Methods

Field Collections

Between May 2001 and September 2002, 15 monthly collections of adult male *Gambusia holbrooki* were made from Lake Apopka (north shore, near Beauclair Canal) and Lake Woodruff Wildlife Refuge (northwest shore, Spring Garden Lake) in central Florida, USA. Mature fish (identified by their hooked gonopodium) were captured using a 3-mm mesh dip net. Average monthly sample size for the sperm study was 15 fish per lake, with a range of 5 to 21. Concurrently, we captured additional fish for testicular histology ($n = 3$ to 10 per month, per lake). Water temperature, pH, and conductivity data were obtained at the time and location where fish were sampled, using a handheld *Ultrameter* (Model 6P, Myron L Company, Carlsbad, CA). Fish were held live in aerated coolers filled with lake water, fed flake fish food ad libitum, and processed 1 to 2 days after capture. Fish were over-anesthetized immediately before sperm collection in room-temperature 0.1% MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma #A5040). Standard length (SL) was measured to the nearest 0.01 cm from the snout tip to the caudal peduncle using calipers. Gonopodium length was measured using an ocular

micrometer mounted on a dissecting microscope. Fish were blotted dry and weighed with an electronic balance to the nearest milligram (range was 56 to 463 mg).

Testicular Histology

Fish captured for testicular histology were anaesthetized in 0.1% MS222. Testes were removed and fixed in Smith's fixative (aqueous solution with 0.2% potassium dichromate, 12% acetic acid, and 47% neutral buffered formalin) for 24 h, followed by storage in 75% ethanol. Testes were dehydrated overnight, by transferring tissues to progressively drier ethanol solutions, infused with and embedded in paraffin, and sectioned at 10 μ m. Mounted sections were stained using a trichrome procedure with Harris' hematoxylin, fast green, and Biebrich scarlet orange. Testes were considered reproductively active when spermatocytes and/or spermatids (in addition to spermatogonia and spermatozoa), were present, and quiescent when only spermatozoa and spermatogonia were present (Fraile et al., 1992; Koya and Iwase, 2004) (Fig. 3-1). The stages of spermatogenesis are shown in Figure 3-1.

Sperm Collection

Each fish was rinsed in distilled water and placed on its side, in a small petri dish containing enough 150 mM KCl to barely cover the fish. With the gonopodium abducted, spermatozeugmata (szm) were stripped from the fish by gently pressing down on the abdomen anterior to the gonopodium and sweeping caudally, using the smooth, rounded end of large forceps with the two tips taped together (Fig. 3-2A). Typically, hundreds of variably sized szm are ejected from the fish after 1-2 sweeps (Figs. 3-2B, C, D). The fish was removed from the dish, and duplicate or triplicate sub-samples of a known number of szm (average = 23) were randomly drawn up in 300 μ l KCl using a micropipette. This collection step must be done rapidly because the KCl activates the

spermatozoa, causing them to disperse from the szm in a matter of minutes (Figs. 3-2E, F). We collected and counted only intact szm. The KCl containing the sperm was placed in a 12 x 75mm polystyrene culture tube and kept on ice until the samples were counted (up to 3.5 h). Based on validation tests, sperm maintained on ice remain viable for at least 4 h (0 to 5% change in the number of live sperm in 9 replicate samples measured repeatedly over 5 h).

Sperm Staining

All sperm samples were stained at the same time using the Live/Dead Sperm Viability Kit available from Molecular Probes, Inc. (Cat. #L-7011). Samples containing 300 μ l sperm were first stained with 15 μ l fluorescent SYBR Green, diluted 500 fold from kit stock. Samples were vortexed and incubated on ice for 10 minutes, after which 1.5 μ l propidium iodide (PI) stock were added. Samples were again vortexed and incubated on ice for at least 10 minutes more. SYBR Green and PI are nucleic acid stains that differentially stain live sperm green and dead sperm orange, respectively. Sperm are characterized as dead if their cell membrane is compromised, allowing the rapid entry of propidium iodide. To protect fluorescent stains from light, tubes and stains were handled under low light conditions and tubes were incubated in a covered cooler containing ice.

Sperm Counts and Viability

To obtain absolute sperm numbers (expressed as sperm number per spermatozeugma (szm)), we needed to quantify the volume of sample processed by the flow cytometer. This volume was measured indirectly by adding a known number of fluorescent particles (average of 23,000 particles per 300 μ l sperm sample) to each tube and then counting the number of particles processed with the sperm sample. We used AccuCount 5.2 micron fluorescent particles (Spherotech Inc., Cat. #ACFP-50-5) because

they were similar in size to the sperm, and their red fluorescence spectra allowed them to be distinguished from SYBR Green and PI. Particles were suspended in a small buffer volume (average = 21 μ l) that was added to individual samples. Sperm samples were vortexed immediately before counting to ensure homogeneous suspension of the particles.

Flow cytometric analysis was performed on a FACSort flow cytometer (BD Biosciences, San Jose, CA). This instrument uses an argon-ion laser emitting 15 mW of 488 nm light to illuminate the cells. Data for 10,000 to 20,000 particles were collected and analyzed using CellQuest 3.3 software (BD Biosciences). Forward and side light scatter measurements and green (530 +/- 15nm), orange (585 +/- 21 nm), and red (> 650 nm) fluorescence measurements were collected for each sample. The instrument threshold was set on forward light scatter. Sperm cells were identified using a gate on the forward vs. side light scatter dot plot (Fig. 3-3). The contents of this gate were displayed in a second plot that gated green (SYBR Green) and orange (PI) fluorescing particles separately (Fig. 3-3). Cells emitting only green fluorescence were counted as live, and cells emitting any orange fluorescence (indicating a breach in the cell membrane) were counted as dead cells. Calibration particle counts were quantified from their peak on a separate red-fluorescence histogram (Fig. 3-3). Our methods are similar to those used for Nile tilapia (Segovia et al., 2000)

Calculations

Absolute sperm count per spermatozeugma was calculated using the formula: $(L+D)(B)/(b)(S)$, where L = number of live sperm counted; D = number of dead sperm counted; B = number of calibration particles added per sample; b = number of calibration particles counted; and S = number of spermatozeugmata originally added to the tube.

Sperm viability was measured as the percentage of live sperm: $L/(L+D)$. Live sperm count per szm was calculated based on this percentage.

Statistics

Data points lying more than three standard deviations from the mean value for sperm count or percentage of live sperm were excluded from the data set (applied to 6 fish or 1.5% of the total sample). Combined data were screened for correlations among SL, body weight, gonopodium length (adjusted for SL), sperm count per szm, percentage of live sperm, and live sperm count per szm using a correlation matrix. Gonopodium length was adjusted for SL (using ANCOVA) because the two were significantly correlated ($r^2 = 0.70$; $p < 0.0001$). Relationships between mean sperm parameters and water temperature, pH, and conductivity measured each month were assessed using linear regression. Differences in sperm data between lakes in any given month were tested using ANOVA. Monthly variation in sperm data within each lake was tested using Fisher's PLSD post-hoc test for ANOVA, with capture date as the independent variable.

Results

Sperm count per spermatzeugma (szm), percent live sperm, and live sperm count per szm did not correlate with SL, body weight, or adjusted gonopodium length ($r^2 < 0.01$). There also was no correlation between sperm count per szm and percent live sperm ($r^2 < 0.0001$). In addition, sperm count and viability were not significantly related to monthly water temperature, pH, or conductivity ($r^2 < 0.14$; $p > 0.06$), although the lowest p -value obtained ($p = 0.06$) suggests a seasonal effect of water temperature on percent live sperm (Figs. 3-4 and 3-5). Figure 3-4 illustrates seasonal change in water temperature and photoperiod during the study. In addition, 2002 was characterized by significant drought, particularly through the summer, and the water level of Lake Apopka

was more affected than that of Lake Woodruff in the areas where *Gambusia* were captured.

Sperm Viability

In 2001, percent live sperm remained stable at about 85% (with a brief drop to 75% noted in July) from May through the beginning of December among males from Lake Woodruff (reference lake) (Fig. 3-5). From December through mid-March 2002, mean viability dropped to 45%, followed by steady recovery to previous levels through mid-May. Among Woodruff fish, viability was maintained from May through the last collection in early September (Fig. 3-5). Based on testicular histology obtained from cohorts captured at the same time, male *Gambusia* from Lake Woodruff reduced production of new spermatocytes in early October, with reinitiation observed in early January. Through the winter, the fish stored spermatozoa, which progressively lost viability until gonadal recrudescence in the spring (Fig. 3-5).

Overall, sperm viability (percent live) among fish from Lake Apopka followed a similar trend to those from Lake Woodruff, with spring recrudescence occurring at a similar time (Fig. 3-5). However, the winter decline in viability started 1 month earlier (November) among males from Lake Apopka (Fig. 3-5). In addition, from June through September 2002, there was a significant decline in sperm viability (percent viability = 50%) among fish from Lake Apopka relative to cohorts from Lake Woodruff. This decline was not expected based on summer data from 2001 (Fig. 3-5). Testicular histology revealed a decrease in spermatogenesis in September, but not in June. Note that no collections were made between June and September.

Sperm Counts

During the collection period, mean sperm counts varied from 3400 – 7200 sperm per spermatozeugma (szm) among male mosquitofish collected from the two lakes (Fig. 3-6). In late August 2001, males from Lake Woodruff exhibited significantly higher sperm counts per spermatozeugma (szm) relative to males from Lake Apopka, although the opposite trend was observed 1 year later, in early September 2002 (Fig. 3-6).

Woodruff males also had higher sperm counts in late January 2002 due to a third seasonal peak in sperm count that was not observed among fish from Lake Apopka (Fig. 3-6). The other two seasonal peaks observed among males from both lakes occurred in July and October-November 2001 (Fig. 3-6). Note that this second seasonal peak in sperm counts occurred 1 month earlier among Apopka males, compared to Woodruff males (late October versus late November 2001) (Fig. 3-6).

Although cyclic variation in sperm counts was observed during 2001, sperm counts apparently remained constant from mid March through September 2002 (Fig. 3-6). However, no samples were collected between mid June and September 2002, when the first seasonal peak should occur, as predicted by data from 2001. Therefore the apparent lack of periodicity observed in 2002 may be an artifact of sampling date. In addition, drought conditions in 2002 may account for additional variation between the two sampling years.

As a measure of fertility, live sperm count per szm is the most relevant of the sperm measures presented here (Fig. 3-7). For both lakes, live sperm count (2000 to 5800 live sperm per szm) followed the same seasonal pattern described above for total sperm counts (Fig. 3-7). However, in late August 2001 and throughout the winter, males from Lake Woodruff exhibited higher live sperm counts relative to cohorts from Lake Apopka

(Fig. 3-7). We observed the same effect in September 2002. At no time did live sperm counts from Apopka males exceed those observed among males from Lake Woodruff.

Discussion

Male mosquitofish from both Lake Woodruff and Lake Apopka exhibited cyclic spermatogenesis, with the number of sperm per spermatozeugma rising and falling two to three times per year. In addition, sperm viability followed a distinct seasonal pattern, particularly among fish from Lake Woodruff, with viability maintained at 75 to 95% during the breeding season, when sperm are continuously released and replenished, but dropping to as low as 45% during winter quiescence, when stored sperm presumably degrade over time. We observed that spring testicular recrudescence was related to increasing temperature followed by increasing photoperiod. This observation is similar to previous reports of mosquitofish described by Fraile et al. (1994) and De Miguel et al. (1994).

Seasonal Variation in Sperm Counts

In our previous study (Toft et al., 2003), we observed that mosquitofish from Lake Apopka exhibited an average of 47% fewer stripped sperm cells per mg testis compared with cohorts captured from Lake Woodruff. In our study, we report sperm counts as sperm number per spermatozeugma. Based on our study, the lake-associated difference in total ejaculated sperm number observed by Toft et al. (2003) is probably not due to reduced sperm counts per spermatozeugma, at least not during most of the year (late January 2002 was an exception). However, the fact that we observed temporal variation in the number of sperm per spermatozeugma is an interesting finding in itself, because it suggests that the rates of spermatogonial mitosis or apoptosis vary on a seasonal basis. Our finding adds to previous mosquitofish studies, which describe monthly variation in

the number of cysts, or volume of testis, devoted to a given stage of sperm cell development (Fraile et al., 1992; Koya and Iwase, 2004). These authors attributed most of the variation to seasonal changes in copulatory behavior, and differences in the time required for each stage of sperm cell development. Copulatory behavior could also regulate the number of sperm per spermatozeugma. For example, since most mosquitofish broods exhibit multiple paternity (Zane et al., 1999), suggesting that sperm competition affects individual mosquitofish fitness (Evans et al., 2003), it is possible that males increase the number of sperm per spermatozeugma when competition is high. To our knowledge, this hypothesis has not been tested in *Gambusia*. Evans et al. (2003) found that male *Gambusia* housed with 3 females and another male for eight days (high risk of sperm competition) mated more often and used more sperm when placed with a novel female, relative to males housed with females alone for eight days (low risk of sperm competition). In this system, the housing regime (eight days with or without other males) did not affect the number of stripped sperm retrieved from treated males that were not mated to novel females. However, the authors did not test the effects of mating frequency on sperm production rate. In addition, eight days is too short a time frame to test effects on spermatogenesis, which typically requires 30 days in *Gambusia* for a single sperm cycle (Koya and Iwase, 2004).

As mentioned above, temporal variation in sperm count per spermatozeugma could be due to changing rates of spermatogonial mitosis. Spermatogonia undergo mitosis for two reasons. The first is to maintain a population of spermatogonial stem cells; the second is to produce nests (called cysts in *Gambusia*) of primary spermatocytes that undergo meiosis (reviewed by Miura and Miura, 2003). Some of the factors that regulate

spermatogonial mitosis have been determined for Japanese eels and were recently reviewed by Miura and Miura (2003). Briefly, spermatogonial stem cell production is regulated by estradiol, which, in eels, stimulates the expression of a protein called “eel spermatogenesis related substance” (eSRS34). Interestingly, estradiol and eSRS34 stimulation result only in renewal of germ cells; they do not promote spermatocyte proliferation and meiosis. For these events to occur, the testis must synthesize 11-ketotestosterone (11-KT), which it does in response to gonadotropins released from the pituitary. 11-KT works in conjunction with IGF-1 and activin B, both secreted by Sertoli cells, to promote spermatogonial proliferation and meiosis. Additional regulation of any step in these pathways would affect spermatogonial mitosis and possibly explain temporal changes in sperm counts per spermatocyte. For example, environmental cues, such as temperature and female reproductive pheromones, have been shown to stimulate gonadotropin release in male goldfish (Kobayashi et al., 2002).

Lake-Associated Variation in Sperm Counts and Quality

In the months when lake related variation was observed (August to September 2001, November 2001 to February 2002, June to September 2002), male mosquitofish from Lake Woodruff exhibited significantly higher live and/or total sperm counts per spermatocyte and/or greater viability relative to males from Lake Apopka. An exception occurred in early September 2002, when males from Lake Apopka exhibited higher total sperm counts, but their live sperm counts were still significantly lower than males from Lake Woodruff.

The differences in sperm quality between the two lake populations are due to three main observations. First, Woodruff males exhibited three seasonal peaks in sperm count, in July, late November, and late January, relative to two seasonal peaks in July and

October among fish from Lake Apopka. Second, while fish from both lakes exhibited decreased sperm viability during the winter, the period of low viability began 1 month earlier (and was therefore 1 month longer) among fish from Lake Apopka. Third, in June through September 2002, sperm viability among fish from Lake Apopka was significantly reduced relative to cohorts from Lake Woodruff, and to patterns established by both populations in 2001. The unpredicted drop may be related to the drought conditions, which severely affected Lake Apopka's water levels in summer 2002, particularly in the shallow periphery where mosquitofish are found. Low water levels, especially in combination with warm temperatures and the eutrophic conditions of Lake Apopka are likely to be associated with reduced dissolved oxygen concentrations (unfortunately, daily data on oxygen concentrations are not available for the study period). In carp, chronic hypoxia has been shown to reduce serum concentrations of testosterone and estradiol, impede gonadal development, and reduce spawning success, sperm motility, and fertilization rate (Wu et al., 2003).

Interestingly, the January peak in sperm count observed among Woodruff males coincides with the time of declining winter sperm viability. This January peak may be adaptive in that it dilutes the impact of lower viability, allowing males to maximize their live sperm counts well into January or even February, when some females might be entering spring ovarian recrudescence (Chapter 2). If this is the case, then the lack of a January peak in sperm counts among males from Lake Apopka could be deleterious. Alternatively, the January strategy among males from Lake Woodruff may be a plastic and possibly costly response that is cued to female reproductive activity. Females from Lake Woodruff, collected at the same time as males in our study, produced vitellogenic

oocytes 6 to 7 weeks later in the spring, compared to females from Lake Apopka. Therefore the third peak in sperm count observed among Woodruff males may be a response to delayed recruitment among female cohorts. Koya and Iwase (2004) observed similar synchrony between the sexes.

In addition to these social factors, the higher concentrations of estrogenic or anti-androgenic contaminants in Lake Apopka (Guillette et al., 1999) could explain the reduced sperm quality observed among Apopka mosquitofish in some months. This hypothesis is supported by the fact that elevated concentrations of p,p'-DDE (antiandrogenic) and toxaphene (estrogenic) have been measured in the body tissues of mosquitofish collected from Lake Apopka (US Fish and Wildlife Service, unpubl. data). Estrogenic and antiandrogenic molecules, including 4-tert-pentylphenol, 4-tert-octylphenol, nonylphenol, p,p'-DDE, bisphenol A, and estradiol have been shown to cause reduction of primordial germ cell numbers or spermatogenic cysts, progressive disappearance of spermatozoa and spermatogenic cysts, degeneration of sperm cells, or inhibition of spermatogenesis in male carp, guppies, and platyfish (Gimeno et al., 1998a, b; Kinnberg et al., 2000; Kinnberg and Toft, 2003). Additionally, vinclozolin, a fungicide with anti-androgenic activity has been shown to reduce testis cord number, increase germ cell apoptosis, and reduce sperm motility among rats exposed during embryonic development (Uzumcu et al., 2004).

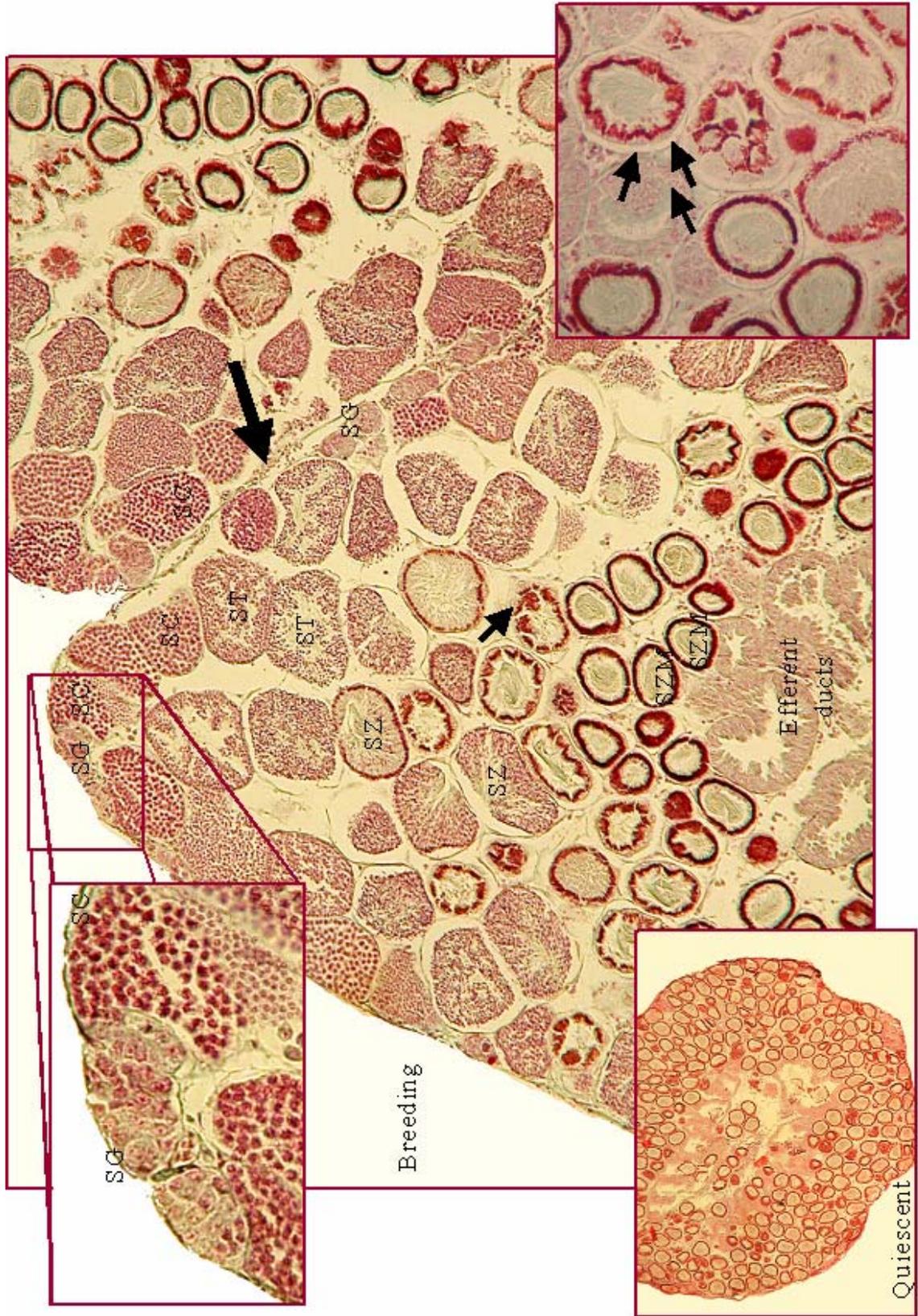
One additional hypothesis is that mosquitofish are more sensitive to endocrine disruption in some months, particularly if the amplitude of endogenous signals is already low. For example, alligators exhibit seasonal variation in testicular response to gonadotropin (Edwards et al., 2004), suggesting that dosage of components in a signaling

pathway is important and seasonally variable. Presence of endocrine disruptors may attenuate the efficacy of a signaling system, and this may be especially disruptive at either the end or beginning of the breeding season, when signal concentrations may not be optimized. This hypothesis could explain the lack of a third January peak in sperm count per spermatozuogma and the early drop in winter sperm viability observed among fish from Lake Apopka.

Conclusions

Overall, we observed temporal variation in both sperm counts per spermatozuogma and sperm viability. Mosquitofish from Lake Apopka exhibited reduced total sperm counts, live sperm counts, and sperm viability at several points during the 15 months of collections. Taken together with previous studies from our laboratory, in which we reported reduced total sperm counts per mg testis among mosquitofish captured from Lake Apopka and among reference mosquitofish exposed to water from Lake Apopka for 1 month (Toft et al., 2003; Toft and Guillette, 2005), we conclude that chemical components in Lake Apopka are a likely, but not sole, cause of reduced sperm quality among resident mosquitofish. The underlying causes of reduced sperm quality could include increased apoptosis or degeneration of germ cells and sperm cells, reduced mitosis of germ cells leading to germ cell renewal (estradiol mediated), and reduced efficacy of signaling pathways associated with spermatogenesis, particularly during periods of transition between breeding and testicular quiescence.

Figure 3-1. Testicular histology of *Gambusia holbrooki*, showing breeding (main picture) and quiescent (inset, lower left) states. SC = spermatocytes, SG = spermatogonia, ST = spermatids, SZ = spermatozoa, SZM = spermatozeugmata. Inset on lower right shows Sertoli cells (small arrows) surrounding spermatozeugmata and lining efferent ducts. Sertoli cells enclose all spermatogenic cysts, but are most visible around spermatozoa because they hypertrophy immediately before releasing spermatozeugma into an efferent duct. Large arrow indicates the line along which the testes fused during development.



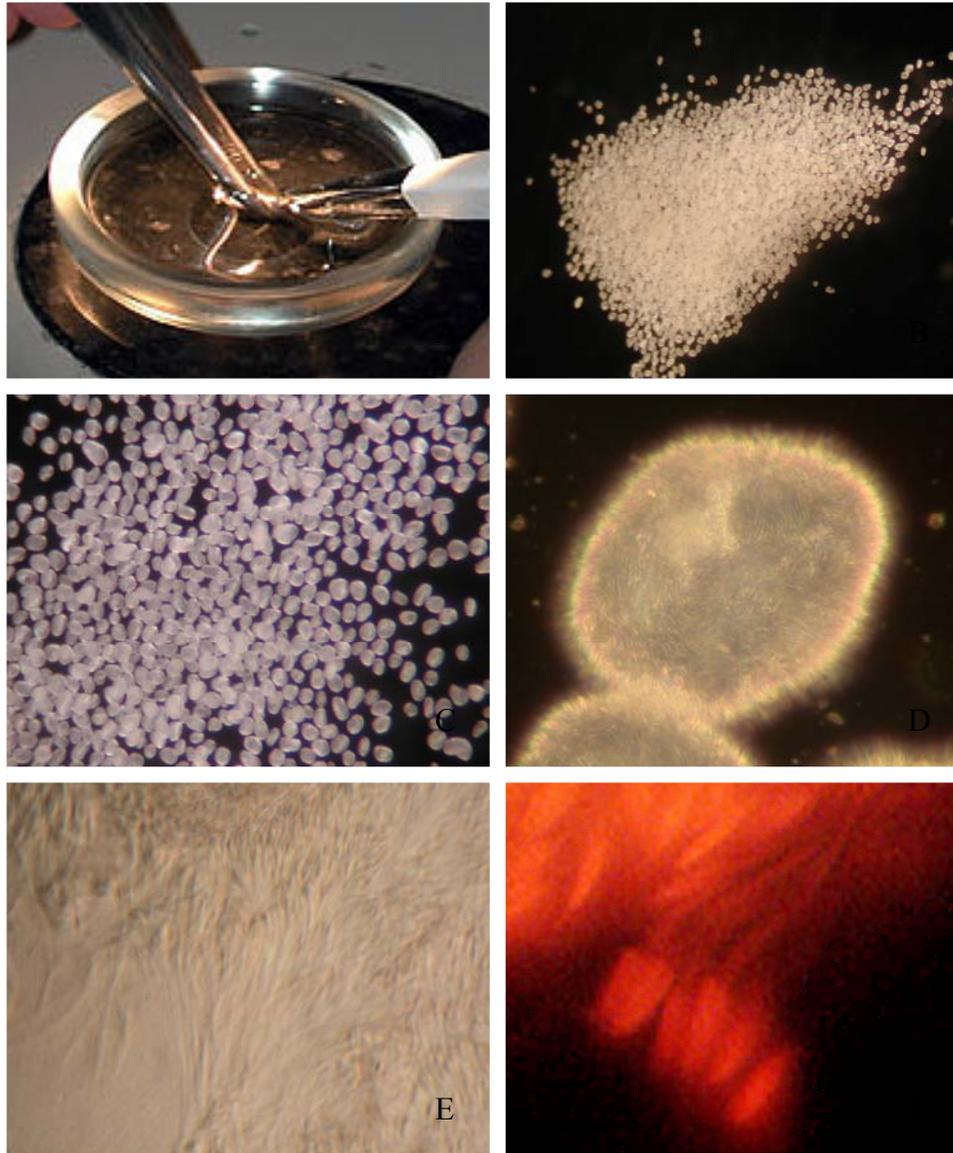


Figure 3-2. Sperm methods for *Gambusia holbrooki*. Spermatozeugmata (szm) are stripped from an anaesthetized male mosquitofish by gently pressing down on the abdomen anterior to the gonopodium and sweeping caudally, using the smooth, rounded end of large forceps with the two tips taped together. A) The fish is held in place by a second pair of forceps. B) Hundreds to thousands (number is highly variable) of szm are retrieved from a single fish. C) Szm vary in shape and size. D) Each szm contains a few thousand spermatozoa, arranged with heads toward the outside of the szm, and tails in the center. E) Once szm are removed from the male, they disperse in a few minutes from the gelatinous matrix that holds them together. F) *Gambusia* sperm exhibit an elongated head and single flagellum.

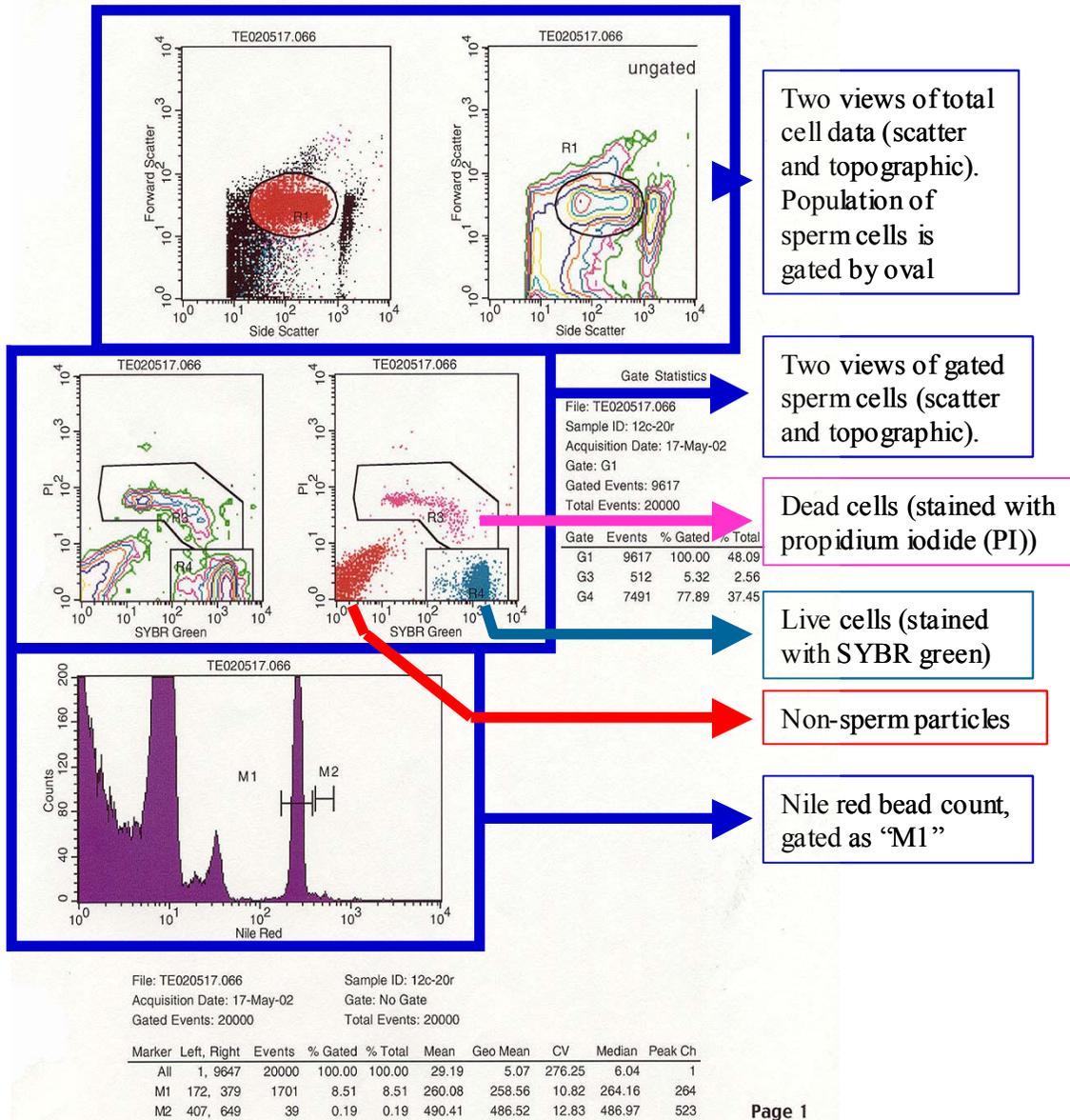


Figure 3-3. *Gambusia* sperm counts flow cytometry printout. The top panel shows two views of the total sample, gated within the oval. The middle panel shows two views of three particle populations (dead sperm cells, live sperm cells, and non-sperm particles) that are distinguished by their respective wavelength of florescence. The last panel indicates the gated bead count, used to calibrate the volume of sample taken up by the flow cytometer.

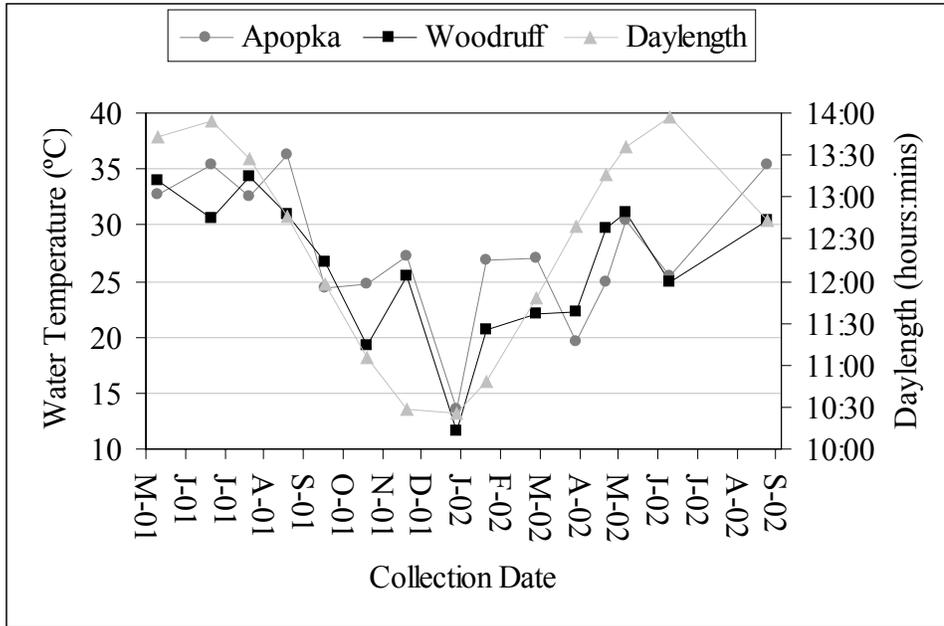


Figure 3-4. Water temperature and daylength data for the collection period. Daylength data source: Astronomical Applications Dept., U. S. Naval Observatory, Washington, D.C. 20392-5420. This graph duplicates Figure 2-2, but is presented again here because it is highly relevant to the interpretation of data in this chapter.

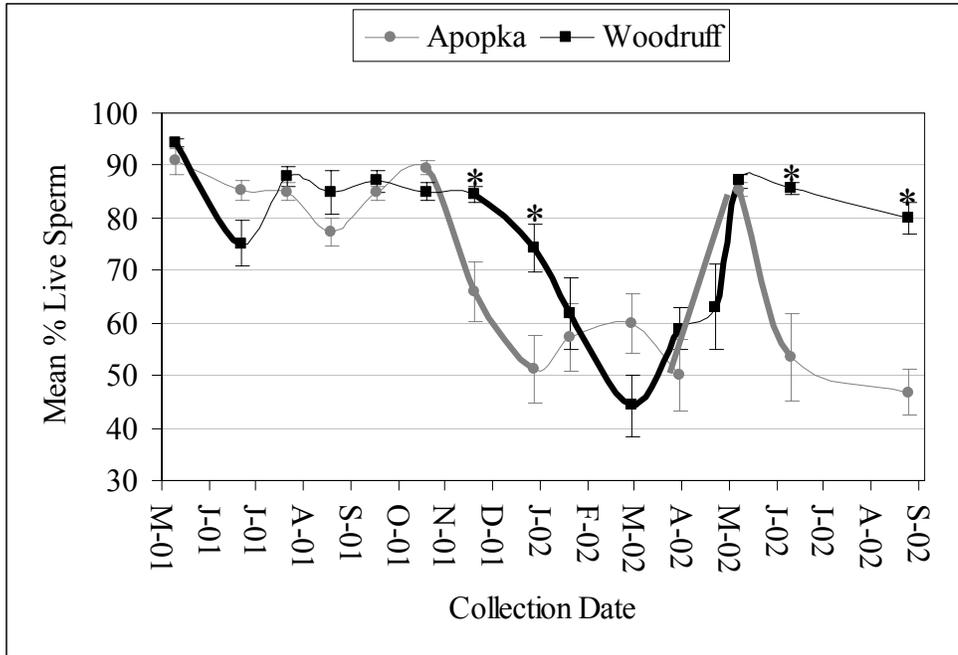


Figure 3-5. Mean percent live sperm (± 1 SE) observed among adult male *Gambusia holbrooki* collected from two lakes in central Florida. Collections were made between May 2001 and September 2002. Significant variation over time within a lake is indicated by bolded trend lines (ANOVA, $p \leq 0.05$). *Indicates a significant lake effect within a given month (ANOVA, $p \leq 0.05$).

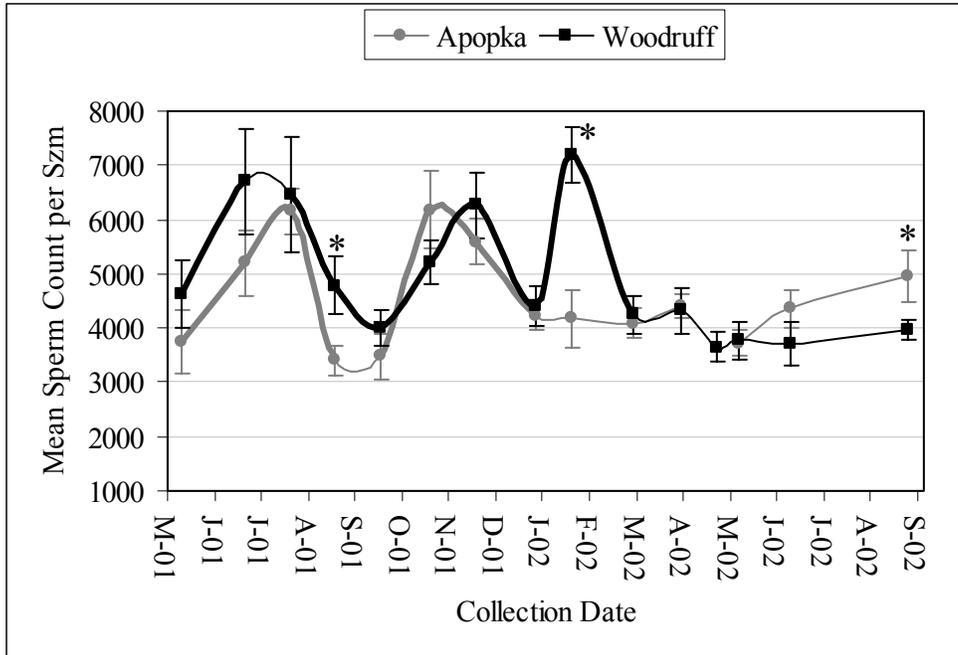


Figure 3-6. Mean sperm count per spermatozeugma (szm) (± 1 SE) observed among adult male *Gambusia holbrooki* collected from two lakes in central Florida. Collections were made between May 2001 and September 2002. Significant variation over time within a lake is indicated by bolded trend lines (ANOVA, $p \leq 0.05$). *Indicates a significant lake effect within a given month (ANOVA, $p \leq 0.05$). Note that no collections were made between June and September 2002, which may explain the lack of periodicity otherwise predicted by data from 2001.

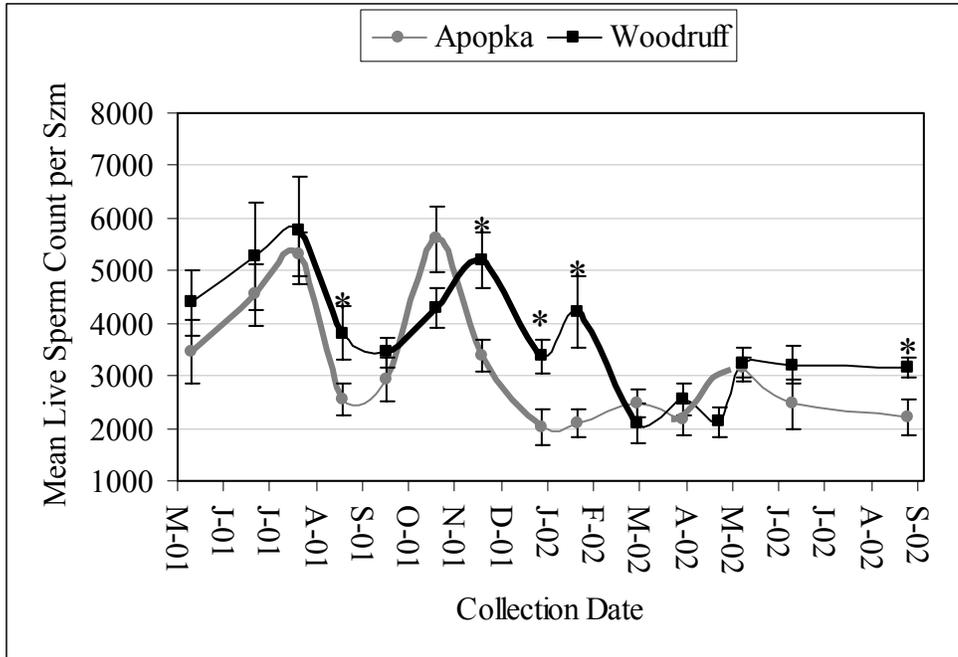


Figure 3-7. Mean live sperm count per spermatozeugmatum (szm) (± 1 SE) observed among adult male *Gambusia holbrooki* collected from two lakes in central Florida. Collections were made between May 2001 and September 2002. Significant variation over time within a lake is indicated by bolded trend lines (ANOVA, $p \leq 0.05$). *Indicates a significant lake effect within a given month (ANOVA, $p \leq 0.05$). Note that no collections were made between June and September 2002, which may explain the lack of periodicity otherwise predicted by data from 2001.

CHAPTER 4
SEASONAL VARIATION IN BODY SIZE, MUSCLE ANDROGEN
CONCENTRATIONS, AND TESTICULAR AND HEPATIC WEIGHTS AMONG
MALE MOSQUITOFISH FROM TWO LAKES IN CENTRAL FLORIDA

Introduction

Fish reproduction is regulated by a wide variety of abiotic and biotic environmental factors. These include temperature and photoperiod (Fraile et al., 1994), nutrition (Cech et al., 1992), and behavioral interactions between the sexes and among individuals of the same sex (Bisazza et al., 2001; McPeck, 1992). In addition to these natural factors, most aquatic systems are now impacted by anthropogenic contaminants, which also have the potential to disrupt reproductive function of fishes (Baatrup and Junge, 2001; Jobling et al., 2002a).

Many of the chemicals that now pollute aquatic systems have been shown to affect male fish reproduction by changing steroid hormone action or metabolism. For example, estradiol and estrogenic chemicals like nonylphenol, octylphenol, pentylphenol, and the chemical mixture found in treated sewage effluent, are associated with delayed puberty, persistently immature testes, or ovotestes, a condition in which males develop ovarian tissue within their testes (Barnhoorn et al., 2004; Dreze et al., 2000; Gimeno et al., 1998a; Toft and Baatrup, 2001). Ovotestes are also associated with abnormal presence of an oviduct and reduced semen quality in terms of volume, morphology, and fertilization success (Jobling et al., 2002b).

In a previously published study, conducted in conjunction with the first three months of this project, we reported reduced sperm counts, slightly shorter gonopodia,

higher whole-body testosterone concentrations, and increased testicular and hepatic weights among adult male mosquitofish from Lake Apopka in central Florida (Toft et al., 2003). The Lake Apopka population was compared to those from a nearby reference lake, Lake Woodruff. Unlike Lake Woodruff, Lake Apopka has a history of chemical and nutrient contamination from agricultural, industrial, and municipal sources (Guillette et al., 2000). Previously, the contamination in Lake Apopka has been characterized as estrogenic and anti-androgenic, based on the chemicals measured in the serum and eggs of resident alligators and body tissues of resident mosquitofish (Guillette et al., 1999; Guillette et al., 2000; Heinz et al., 1991; U.S. Fish and Wildlife Service, unpubl. data). Measured contaminants include several pesticides, like dieldrin, endrin, mirex, oxychlorane, *trans*-nonachlor, DDT, p,p'-DDE and DDD (DDT metabolites), and toxaphene (Guillette et al., 1999; Heinz et al., 1991; U.S. Fish and Wildlife Service, unpubl. data). These chemicals have been experimentally shown to alter steroid and thyroid hormone synthesis and degradation, cause male to female sex reversal of alligators and red-eared slider turtles, and agonize or antagonize steroid receptor binding and activity (for reviews, see Akingbemi and Hardy, 2001; Guillette et al., 2000; Guillette and Gunderson, 2001; Willingham and Crews, 2000).

In a follow-up to the mosquitofish study cited above, Toft and Guillette (2005) observed reduced sperm counts and altered sexual behavior among mosquitofish after a one-month exposure to water from Lake Apopka versus water from two reference sites. In that study, fish originated from one of the reference sites. In another paper, Gallagher et al. (2001) reported elevated levels of plasma estrogens among male bullhead catfish from Lake Apopka as compared with male bullheads from Lake Woodruff. These studies

expanded upon the more established Apopka-Woodruff alligator literature. That is, relative to alligators from Lake Woodruff, alligators from Lake Apopka exhibit poor hatching success (Woodward et al., 1993), changes in gonadal morphology (Guillette et al., 1994), altered hepatic enzyme expression and activity (Gunderson et al., 2001), and reduced plasma testosterone and phallus size in males (Guillette et al., 1999). The gonadal and steroid abnormalities are observable at hatching suggesting that changes occur during development (Guillette et al., 1995). Given the observed relationship between environmental contaminants and altered reproductive variables among fishes and alligators, we designed our study to further examine possible impacts of contaminants on mosquitofish reproduction. Here, we focus on male mosquitofish (*Gambusia holbrooki*), captured monthly for 17 months from the Apopka-Woodruff model system. The seasonal component of our study is intended to place any observed, lake-associated alterations in reproduction in context of the seasonal cycle and “normal” environmental variation like temperature or photoperiod.

Methods

Field Collections

Between March 2001 and September 2002, 17 monthly collections of adult male *Gambusia holbrooki* were made from Lake Apopka (north shore, near Beauclair Canal) and Lake Woodruff Wildlife Refuge (northwest shore, Spring Garden Lake) in central Florida, USA. Fish were captured using a 3-mm mesh dip net and those with a well-developed and hooked gonopodium were considered mature (Angus et al., 2001). Each lake collection took 1 day, plus additional days to process fish. Thus monthly collections from the two lakes were made on different days (average of 4 days apart). For 12 of the 17 months, Lake Woodruff was sampled first.

Water temperature, pH, and conductivity data were obtained at the time and location where fish were sampled, using a handheld *Ultrameter* (Model 6P, Myron L Company, Carlsbad, CA). Each monthly sample, ranging from 37 to 67 fish per lake, was divided into three subsets. The first subset was used to obtain testicular and hepatic weight. These fish (n = 6 to 21 per month, per lake; mean = 18) were held live in aerated coolers filled with lake water, fed flake fish food ad libitum, and processed within 1 to 2 days of capture. Before necropsy, fish were over-anesthetized in room-temperature 0.1% MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma #A5040). The testis (0.4 to 9.9 mg) and liver (0.1 to 8.0 mg) were removed and weighed to the nearest 0.1 mg.

The second subset was used for the measurement of androgen (testosterone and 11-ketotestosterone) concentrations in the caudal peduncle tissues. Since the peduncle is primarily muscle, we will refer to it as muscle for the remainder of the chapter. Androgen concentrations were measured in muscle rather than plasma because *Gambusia* are too small to yield an adequate amount of plasma. These fish (n = 6 to 12 per month, per lake; mean = 10) were frozen immediately at capture and held on ice while in the field. Upon return to the laboratory, the caudal fin was removed, and all peduncle tissue posterior to the gonad was cut from the fish, weighed (average = 60 mg), and frozen at -80°C. Androgens were measured on lipid extracts of these peduncle tissues (referred to as “muscle androgens”), as described below.

The third subset was used for sperm analysis, as reported in Chapter 3. For all fish, standard length (SL) was measured to the nearest 0.01 cm from the snout tip to the caudal peduncle using calipers. Fish were blotted dry and weighed with an electronic balance to

the nearest milligram. Gonopodium length was measured to the nearest 0.05 mm, using an ocular micrometer mounted on a dissecting microscope. In mosquitofish, the gonopodium is a grooved structure, formed by the fusion of several anal fin rays, and used to transfer sperm to the female, as fertilization is internal in this viviparous species (Batty and Lim, 1999). We have included measurement of the gonopodium because fin ray fusion and elongation are androgen dependent, and the development of this secondary sex character can be perturbed by exposure to antiandrogens such as p,p'-DDE (present in Lake Apopka) and flutamide (Bayley et al., 2002; Ogino et al., 2004).

Muscle Androgen Measurements

For measurement of muscle concentrations of testosterone and 11-ketotestosterone, frozen muscle (peduncle) tissues were thawed in glass tubes, on ice, and homogenized in 750 μ l 65 mM borate buffer (pH 8.0). Homogenate was extracted twice with 5 ml diethyl ether. For each extraction, ether and homogenate were mixed together for two minutes using a multi-tube vortex mixer. For the first extraction, tubes were allowed to settle for three minutes to separate phases. For the second extraction, phases were separated by centrifugation for two minutes. The aqueous phase was frozen in a methanol bath chilled to -25°C with dry ice. The ether from both extractions was combined in a second glass tube and evaporated under dry forced air.

Hormone concentrations were determined using validated enzyme immunoassay (EIA) kits (Cat No. 582701 (T); 582751 (11-KT) purchased from Cayman Chemical Company (Ann Arbor, Michigan). Dry extract was reconstituted in 600-800 μ L EIA buffer so that samples would fall within the range of the standard curve. EIAs were run as recommended by Cayman with an 18 h refrigerated incubation to increase sensitivity.

Data were quantified against a standard curve linearized using a logit transformation of B/Bo (bound sample/maximum bound). Duplicate interassay variance (IAV) samples at two dilutions were included with each plate. The coefficient of variance among all plates, averaged for the two dilutions was 18.5% for T, and 11.7% for 11-KT. To normalize sample androgen concentrations across assays, we multiplied by a correction factor derived from the relationship between individual plate IAV values and the mean IAV values for all plates.

Statistics

Androgen data points lying more than three standard deviations from the mean ($n = 3$ for T assays (1%); 7 for 11-KT assays (2%)) were excluded from the data set. Data were screened for correlations between standard length (SL) or body weight, and gonopodium length, testicular and hepatic weight, and androgen concentrations using a correlation matrix. Apparent correlations were visualized using linear regression. For variables that scaled significantly with body size, body size was entered as a covariate when ANCOVAs were performed.

Monthly means for each lake were compared using ANOVA or ANCOVA. To improve homogeneity of variance, all variables (with the exception of SL and body weight, when analyzed alone) were \log_{10} transformed. Androgen concentrations were $\log_{10}(y+1)$ transformed to avoid negative log values (Zar, 1999). *A priori* pairwise LSD comparisons were made between lakes for each month and between consecutive months within each lake. This latter analysis was used to describe seasonal variation within a lake. Correlation, regression, and ANOVA analyses were completed using Statview 5.0. ANCOVA analyses and calculations of adjusted means were completed using SPSS 13.0.

Results

Abiotic Factors

Seasonal variability in water temperature (12°C in January to about 35°C during May to August) was similar between the two lakes, with the exception that Lake Apopka exhibited an early warming period (about 7°C warmer) between February and March of both years (Fig. 4-1). Photoperiod varied seasonally from 10.5 to 14 h (Fig. 4-1). Water pH varied from 6.5 to 8.5, with a mean of 7.2 in Lake Woodruff, and from 6.8 to 8.7, with a mean of 7.5 in Lake Apopka. Conductivity was generally higher in Lake Woodruff, ranging from 700 to 1807 $\mu\text{S}/\text{cm}$, with a mean of 1196 $\mu\text{S}/\text{cm}$ (peaks occurred in March/April both years). Conductivity in Lake Apopka ranged from 433 to 765 $\mu\text{S}/\text{cm}$, with a mean of 583 $\mu\text{S}/\text{cm}$.

Body Size

Body weight and standard length (SL) were significantly related ($r^2 = 0.74$; $p < 0.0001$). In 2001, males from Lake Apopka were significantly larger in six out of nine months, in terms of SL (Fig. 4-2) and body weight (Fig. 4-3), than males from Lake Woodruff. In the first half of 2002, fish from both lakes were similar in SL and weight (Figs. 4-2 and 4-3), but in late summer, fish from Lake Woodruff were longer and heavier (Figs. 4-2 and 4-3). As a general pattern, average male body size appears to decline during the breeding season, beginning sometime in March to May, and reaching a seasonal low in September to October, quickly rising again by October or November. In 2001, the spring decline among males from Lake Apopka was delayed by 2 months compared to males from Lake Woodruff (Figs. 4-2 and 4-3). However, in 2002, mean body size of males from both lakes began to decline at about the same time.

Gonopodium Length

Gonopodium length was positively related to SL ($r^2 = 0.67$, $p < 0.0001$). In some months, in both 2001 and 2002, we observed a lake-associated difference in adjusted gonopodium length (Fig. 4-4). However, there was no pattern with regards to which lake had fish with longer or shorter gonopodia. In addition, the maximum lake-associated difference in adjusted gonopodium length was about 0.25 mm (Fig. 4-4). Seasonally, adjusted gonopodium length appeared to fluctuate greatly, particularly during the winter months (Fig. 4-4). This is likely due to variation in the body size of sampled males.

Androgens

Testosterone concentrations were negatively correlated with SL ($r^2 = 0.12$; $p < 0.0001$). Seasonally, there was substantial lake-associated variation in androgen concentrations (Fig. 4-5). For 11-KT, peak seasonal concentrations represent an increase of 2 to 10 fold (Woodruff), or 2 to 5 fold (Apopka) relative to seasonal lows (Fig. 4-5A). For adjusted testosterone, peak seasonal concentrations represent an increase of 2 to 5 fold (Woodruff), or 4 to 6 fold (Apopka) relative to seasonal lows (Fig. 4-5B). Males from Lake Woodruff exhibited two annual peaks of 11-KT, one in March-April-May and one in September-October (Fig. 4-5A). This second peak occurred in 2001, but was not apparent by September 2002 when the last samples were collected. The annual low in 11-KT concentrations occurred in January among fish from Lake Woodruff. Like males from Lake Woodruff, a spring peak in 11-KT among males from Lake Apopka occurred between March and May (Fig. 4-5A). However, males from Lake Apopka did not exhibit a significant rise in 11-KT in September, although they did exhibit a small peak in June 2002, a peak that was not observed in 2001 (Fig. 4-5A). Finally, males from Lake

Apopka exhibited an additional peak in 11-KT in January, the time that coincided with the lowest annual 11-KT concentrations among males from Lake Woodruff (Fig. 4-5A).

Like muscle 11-KT concentrations, muscle testosterone (T) concentrations varied both seasonally and between lakes. In 2002, the March-April peak in T coincided with the spring peak in 11-KT among fish from both lakes (Fig. 4-5B). However, this pattern was not observed in 2001. Likewise, fish from both lakes exhibited high T concentrations in September-October 2001 (Fig. 4-5B). As with 11-KT concentrations, this pattern had not yet developed by September 2002. Fish from Lake Woodruff exhibited a third peak in T in December just before the annual low in January-February (Fig. 4-5B). This peak was not observed among fish from Lake Apopka. However, fish from Lake Apopka exhibited a third rise in muscle T concentrations in June (2002 only), which coincided with the June 2002 rise in muscle 11-KT concentrations (Fig. 4-5B). This June 2002 peak in T was not observed among fish from Lake Woodruff.

Testicular Weight

Testicular and hepatic weights were positively related to body weight ($r^2 = 0.35$, $p < 0.0001$; $r^2 = 0.46$, $p < 0.0001$, respectively). Except during November 2001 and March 2002, adjusted testicular weights were significantly higher among males from Lake Apopka compared to males from Lake Woodruff (Fig. 4-6). The difference in actual adjusted testicular weight in most months is substantial. For example, the greatest lake-associated difference was observed at the end of April 2001, when adjusted testicular weight among fish from Lake Apopka was 145% greater than that for fish from Lake Woodruff (Fig. 4-6). Among fish from Lake Woodruff, testicular weight peaked in late March, early August (2001), and late October (2001) (Fig. 4-6). The March peak was consistent between 2001 and 2002 and coincided with the beginning of the spring period

of elevated androgen (both T and 11-KT) concentrations (Figs. 4-5 and 4-6). Likewise, the early August peak precedes the elevated androgen concentrations observed in late August-September, and the late October peak in testicular size precedes elevated testosterone concentrations observed in late November. No testicular measurements were made in August or October 2002, so a year-to-year comparison of this variable is not available.

As observed among fish from Lake Woodruff, males from Lake Apopka exhibited similar seasonality in testicular size; with increased size often preceding observed elevations in androgen concentrations (Figs. 4-5 and 4-6). In 2001 and 2002, testicular size peaked in April, coinciding with elevated 11-KT concentrations during both years. Similarly, testicular weights were increased in early August, preceding high testosterone concentrations in late September. It should be noted that testicular size data are missing for fish from Lake Apopka in late August. The testosterone data suggest that testicular weight remained high throughout August and early September among these fish. Finally, testicular size peaked in late January, possibly explaining the high 11-KT concentrations also observed at that time. This late January peak in testicular weight also precedes elevated concentrations of both 11-KT and T in March.

Hepatic Weight

The most notable lake associated differences in adjusted hepatic weight occurred in May – June 2001 and 2002, when mean hepatic weight was significantly greater among fish from Lake Apopka (Fig. 4-7). Temporal fluctuation occurred throughout the study period among fish from both lakes, although the patterns on each lake are dissimilar and frequently oppose each other (Fig. 4-7). Fish from Lake Woodruff exhibited temporal peaks in early August 2001, November 2001, and March/late April 2002. Each peak in

hepatic weight was followed by a seasonal low, suggesting a pulsatile pattern. Fish from Lake Apopka exhibited seasonal peaks in June 2001 and 2002, and in late January 2002. Except in March 2002, hepatic weights among fish from Lake Apopka remain elevated year-round with respect to seasonal lows observed among fish from Lake Woodruff (Fig. 4-7).

Discussion

Body Size

Size at maturity among male mosquitofish is highly variable, being affected by photoperiod, mating strategy, female preference, and sex ratio (Bisazza et al., 1996; Zulian et al., 1993). Although females appear to prefer larger males, small males generally have a mating advantage, possibly because they can approach females without detection (Bisazza et al., 2001). Small size reduces mating success only when the sex ratio is male-biased, as is often the case at the end of the reproductive season (Bisazza and Marin, 1995; Zulian et al., 1995). Male body size is not a strongly heritable trait. Zulian et al. (1993) report that, regardless of paternal size, length at maturity is greater for males raised in groups, and this larger body size sometimes occurs in conjunction with delayed maturation. In the same study, they report that mosquitofish reared under short photoperiods (9 h of light) matured earlier and at a smaller size. Taken together, these studies predict that male body size should be small at the beginning of the breeding season, and larger at the end. Our data did not follow this pattern. Instead, we observed a general decline in body size among sampled fish during the breeding season, until October, when females become reproductively quiescent (Chapter 2). Although we did not collect sex ratio data, our body size data suggest that, in Lake Apopka and Lake Woodruff, the sex ratio does not become male biased as the breeding season progresses.

Declining body size during the breeding season could reflect increasing recruitment of small males that were born in the same year that they were sampled. The increase in body size during reproductive quiescence probably reflects continued winter growth by the established male population.

Although the sampled males from both lakes exhibited declining body size during the breeding season in 2001, we noted that the spring decline among males from Lake Apopka was delayed by 2 months compared to males from Lake Woodruff.

Interestingly, females from Lake Apopka become reproductively active in late January, about 1.5 months earlier than those from Lake Woodruff (Chapter 2). If the Apopka breeding season begins early, and males mature rapidly at a small size because photoperiod is short and the sex ratio is female biased (as suggested by the studies described above), then we would expect mean male body size to decline earlier in the breeding season rather than later. The observed delay in body size decline suggests that maturation was delayed among males from Lake Apopka, relative to those from Lake Woodruff, at least in 2001. Possible causes for delayed maturation include an overall higher density of males year-round (Zulian et al., 1993) or exposure to an antiandrogen such as p,p'-DDE (Bayley et al., 2002). DDE makes up most of the contaminant load measured in the serum of alligators from Lake Apopka (Guillette et al., 1999).

Furthermore, elevated concentrations of p,p'-DDE have been measured in mosquitofish from Lake Apopka (5300 mg/kg) (US Fish and Wildlife Service, unpubl. data). Although delayed maturation among males from Lake Apopka is a likely explanation for differences in body size between the two lake populations in 2001, the effect did not persist in 2002.

Gonopodium Length

Lake associated differences in adjusted gonopodial length were maximized at about 0.25 mm or 4%. However, fish with shorter (or longer) gonopodia did not always come from the same lake. We reported similarly low variation in gonopodial length in a previous paper (Toft et al., 2003). To our knowledge, no data are available to indicate if this degree of variation is large enough to confer any functional changes.

Androgens

Both 11-ketotestosterone (11-KT) and testosterone (T) exhibited temporal variation. We previously reported whole-body testosterone concentrations of 1000 – 1600 pg/g for male mosquitofish captured from Lake Apopka and Lake Woodruff in March – May 2001 (Toft et al., 2003). Muscle testosterone concentrations reported here for the same period are about 150 pg/g for fish from both lakes. These data suggest that testosterone concentrations in the testis and possibly the brain, which are included in the whole-body measurement, are higher than circulating testosterone concentrations, as measured in the muscle. Our measured concentrations of muscle 11-KT were similar to muscle 11-KT concentrations measured in male gag grouper (Heppell and Sullivan, 2000).

In African catfish and Japanese eels, 11-KT, rather than T, is implicated in the stimulation of spermatogenesis (Cavaco et al., 1998; Miura and Miura, 2001). Based on sperm count and viability data (Chapter 3), male mosquitofish exhibit active spermatogenesis by May, after a period of winter quiescence. Our data suggest that 11-KT, which rises in March-April, is associated with spermatogenic activation among fish from both lakes. A small peak in 11-KT in September among fish from Lake Woodruff, but not Lake Apopka may explain why males from Lake Woodruff have more viable

sperm later in the year (Chapter 3). Interestingly, the January peak in 11-KT among fish from Lake Apopka was not associated with any observable effects on spermatogenesis, even up to three months later (Chapter 3). It is likely that during winter quiescence, testes are not responsive to androgen stimulation in terms of spermatogenesis, thus the reason for the January 11-KT peak among fish from Lake Apopka is not clear.

In 2002, males from Lake Apopka exhibited a large rise in muscle 11-KT concentrations in April, preceding an expected rise in sperm viability in May (Chapter 3). However, viability dropped substantially and unexpectedly in June, in association with a large rise in muscle T concentrations. In African catfish, the stimulatory effect of 11-KT on spermatogenesis can be blocked by co-treatment with T (Cavaco et al., 2001). Although we cannot explain the June rise in muscle T concentrations, which did not occur in 2001, nor among males from Lake Woodruff, the June rise in muscle T concentrations may explain the concomitant decrease in sperm viability observed among fish from Lake Apopka in 2002 (Chapter 3).

Testicular Weight

Among males from both lakes, temporal variation in testicular size was often positively related to changes in muscle androgen concentrations. The period of increased testicular size, implying increased testicular activity, ranged from March to November, followed by a period of winter quiescence. This observation is supported by our data on sperm quantity and viability (Chapter 3). It is also similar to previously published observations examining *Gambusia* reproduction in Japan, although the reproductive season for the Japanese populations was shorter (Koya and Iwase, 2004). Although the pattern among fish from the two lakes was similar, testicular weights were significantly higher among males from Lake Apopka compared to males from Lake Woodruff. On the

other hand, this did not translate into substantially higher androgen concentrations or sperm counts (Chapter 3) among Apopka fish. This observation suggests that testes of males from Lake Apopka are hypertrophied, a pathological state that may be necessary to maintain androgen homeostasis. That is, per unit mass, androgen synthesis by testicular tissues is reduced among males from Lake Apopka, but androgen concentrations are maintained at normal levels by the compensatory growth of Leydig cells. Histology data associated with this project will be evaluated in a future study.

Leydig cell hyperplasia and hypertrophy can be induced by exposure to both androgen receptor antagonists (i.e., flutamide, p,p'-DDE, or the fungicide Vinclozolin) and estrogen agonists (diethylstilbestrol (DES) or 17 β -estradiol) (Du Mond et al., 2001; Mylchreest et al., 2002; O'Connor et al., 2002; Rai and Haider, 1991). Both DDE (antiandrogenic) and toxaphene (estrogenic) occur in elevated concentrations in tissue samples from mosquitofish collected from Lake Apopka (US Fish and Wildlife Service, unpubl. data). In our study, the estrogenic nature of Apopka contaminants may be more relevant in terms of testicular enlargement since a study of the effects of antiandrogens on the gonadosomatic index of guppies yielded no effect (Bayley et al., 2002). Some risk assessment studies suggest that hormonally induced Leydig cell proliferation is related to development of Leydig cell adenoma and possibly carcinoma (Clegg et al., 1997).

Hepatic Weight

As with testicular weight, adjusted hepatic weight was generally higher among mosquitofish from Lake Apopka compared to Lake Woodruff, particularly between May and September. Increased hepatic weight can be associated with long or short-term exposure to toxicants, as is the case for male rodents exposed to di-n-butylphthalate (Wine et al., 1997), p,p'-DDE (Kang et al., 2004), or toxaphene (Hedli et al., 1998); or

fed contaminated salmon from the Great Lakes (Arnold et al., 1998). Increased hepatic weight can also be due to induction of vitellogenesis, which can occur in juvenile or male fishes, including mosquitofish, exposed to estrogenic substances (Tolar et al., 2001; Verslycke et al., 2002).

Summary

The study presented here illustrates temporal variation in body size, androgen concentrations, and testicular and hepatic weights in two populations of male mosquitofish inhabiting subtropical lakes in central Florida, USA. Males are reproductively active from March (temperature $\geq 20^{\circ}\text{C}$; daylength ≥ 11.5 h) through November (temperature = $20\text{-}25^{\circ}\text{C}$; daylength ≤ 11 h). Induction of reproductive activity appears to be controlled by a combination of daylength and temperature since, in central Japan (Koya and Iwase, 2004), male mosquitofish reproduction begins when the temperature is approximately 14°C and daylength is 13 h. Our data suggest that 11-KT, rather than testosterone, is responsible for induction of spermatogenesis, as reported in other fish species (Cavaco et al., 1998; Miura and Miura, 2001).

Given previous studies of alligators by our laboratory, we hypothesized that mosquitofish captured from Lake Apopka would exhibit altered reproductive characters in comparison to a reference population. We observed delayed maturation among males from Lake Apopka and enlarged testicular and hepatic size during most of the year. As discussed above, these altered characters could be indicative of exposure to anti-androgenic or estrogenic contaminants, such as those found in Lake Apopka.

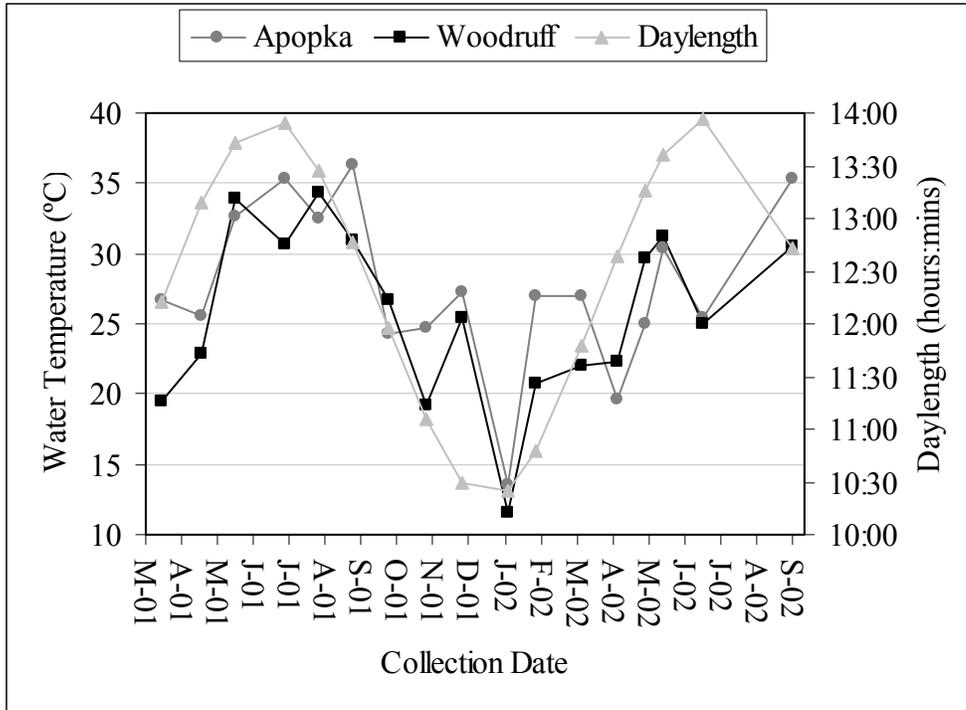


Figure 4-1. Water temperature for Lake Apopka and Lake Woodruff, shown with ambient photoperiod for each collection date. This graph duplicates Figures 2-2 and 3-4, but is presented again here because it is highly relevant to the interpretation of data in this chapter.

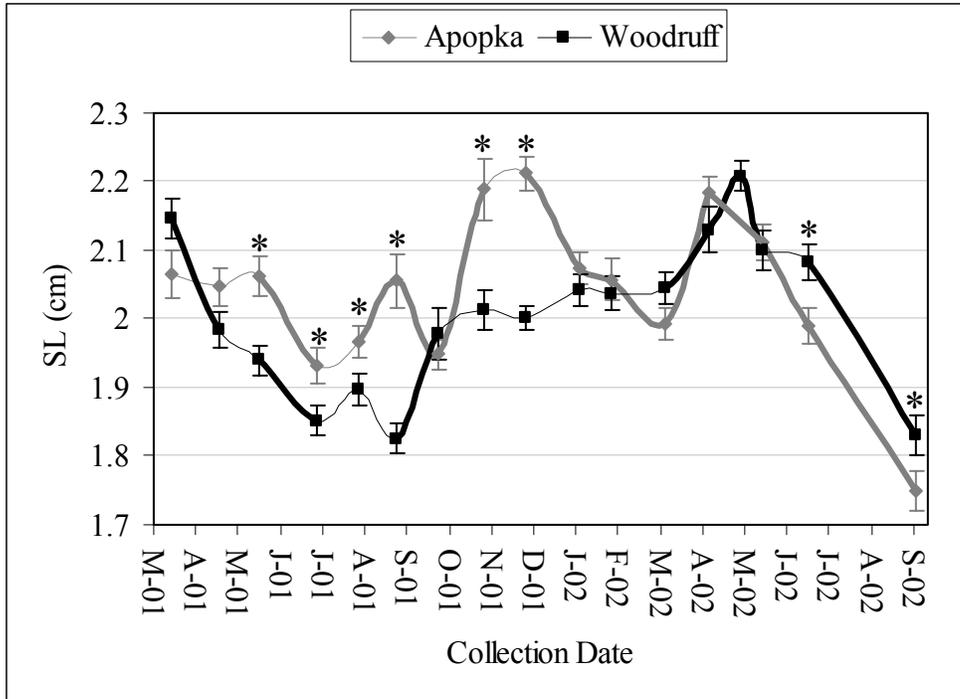


Figure 4-2. Mean standard length (SL) (± 1 SE) of adult male mosquitofish from Lake Apopka and Lake Woodruff. *Months in which the mean SL of fish from the two lakes are significantly different (ANOVA, $p \leq 0.05$). A heavier line between data points indicates significant month-to-month variation within a lake (ANOVA, $p \leq 0.05$). Slashed bars (//) indicate that time between consecutive collections exceeds 1 month and that the indicated trend is extrapolated.

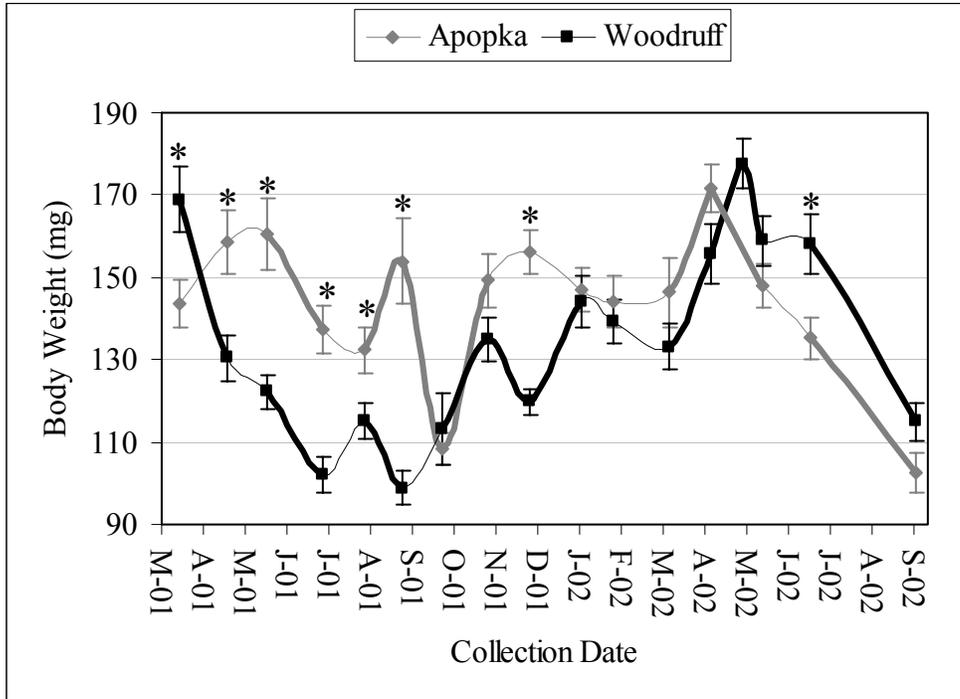


Figure 4-3.

Body weight of adult male mosquitofish from Lake Apopka and Lake Woodruff. Graph shows means ± 1 SE. *Months in which the mean body weights of fish from the two lakes are significantly different (ANOVA, $p \leq 0.05$). A heavier line between data points indicates significant month-to-month variation within a lake (ANOVA, $p \leq 0.05$). Slashed bars (/) indicate that time between consecutive collections exceeds 1 month and that the indicated trend is extrapolated.

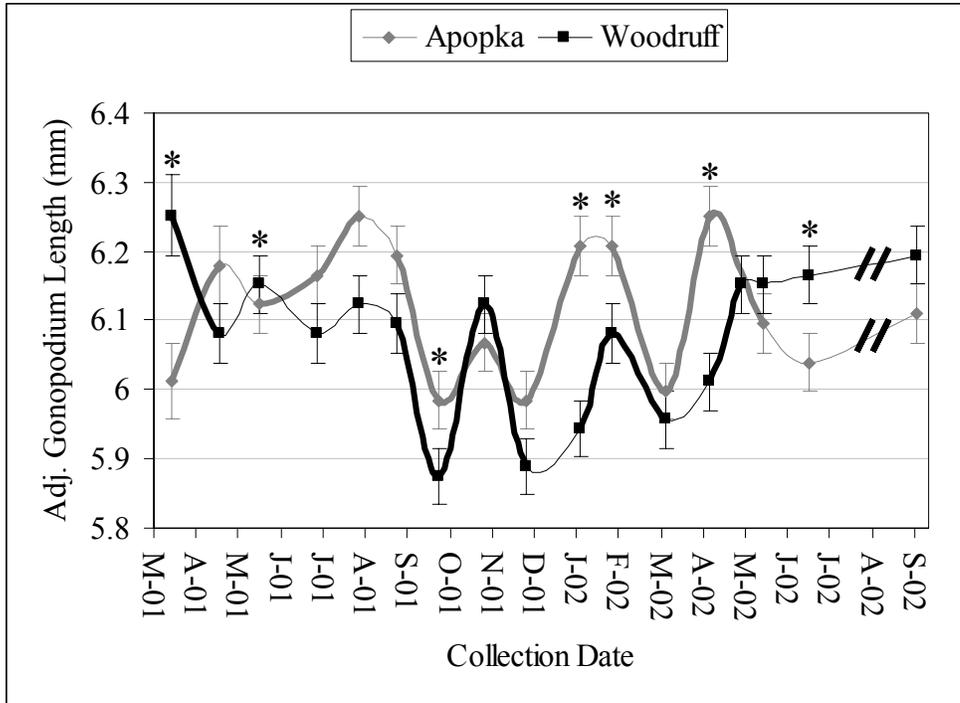


Figure 4-4. Mean gonopodium length (± 1 SE), adjusted for standard length, of adult male mosquitofish from Lake Apopka and Lake Woodruff. *Months in which the mean adjusted gonopodium lengths of fish from the two lakes are significantly different (ANCOVA, $p \leq 0.05$). A heavier line between data points indicates significant month-to-month variation within a lake (ANCOVA, $p \leq 0.05$). Slashed bars (//) indicate that time between consecutive collections exceeds 1 month and that the indicated trend is extrapolated.

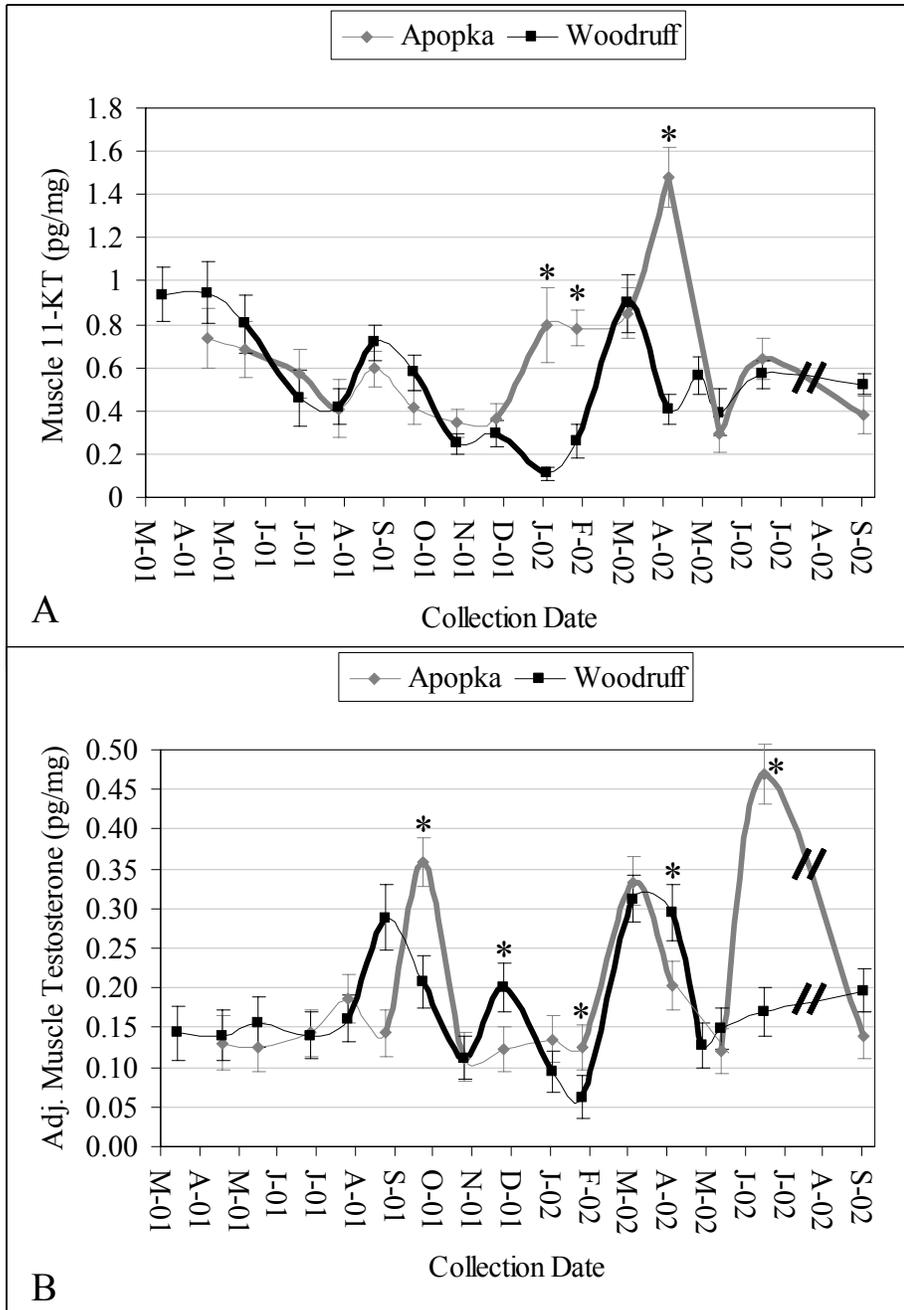


Figure 4-5. Mean muscle androgen concentrations (± 1 SE) for adult male mosquitofish from Lake Apopka and Lake Woodruff. A) 11-Ketotestosterone (11-KT). B) Testosterone (T). T concentrations are adjusted for male standard length. *Months in which the mean androgen concentrations of fish from the two lakes are significantly different (AN(C)OVA, $p \leq 0.05$). A heavier line between data points indicates significant month-to-month variation within a lake (AN(C)OVA, $p \leq 0.05$). Slashed bars (//) show that time between collections exceeded 1 month and that the indicated trend is extrapolated.

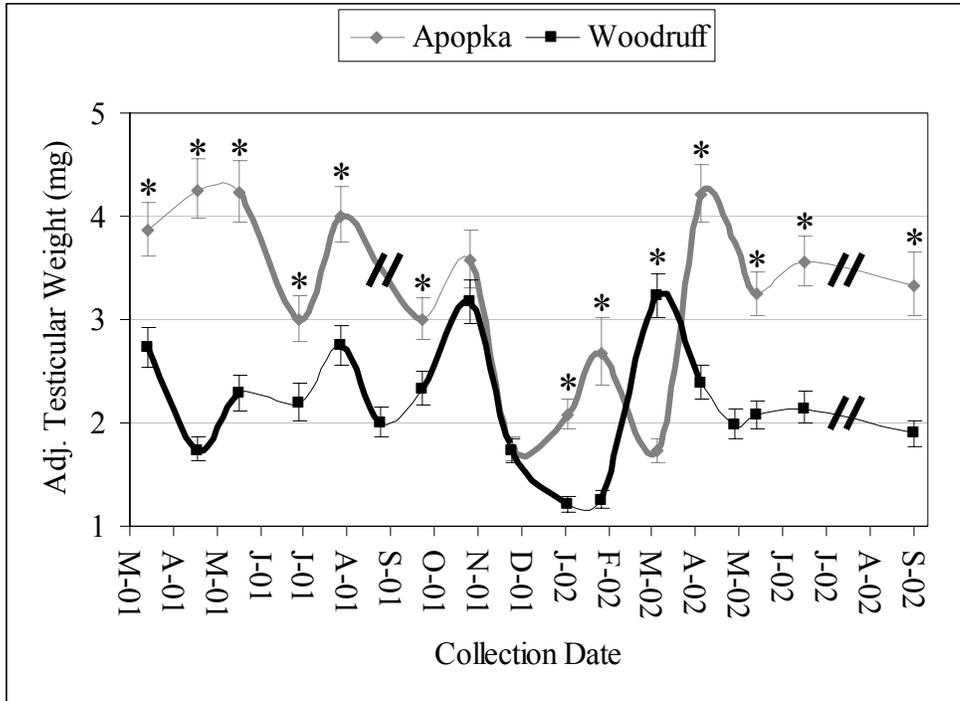


Figure 4-6. Testicular weight, adjusted for body weight, of adult male mosquitofish from Lake Apopka and Lake Woodruff. Graph shows means ± 1 SE. *Months in which the mean adjusted testicular weights of fish from the two lakes are significantly different (ANCOVA, $p \leq 0.05$). A heavier line between data points indicates significant month-to-month variation within a lake (ANCOVA, $p \leq 0.05$). Slashed bars (//) indicate that time between consecutive collections exceeds 1 month and that the indicated trend is extrapolated.

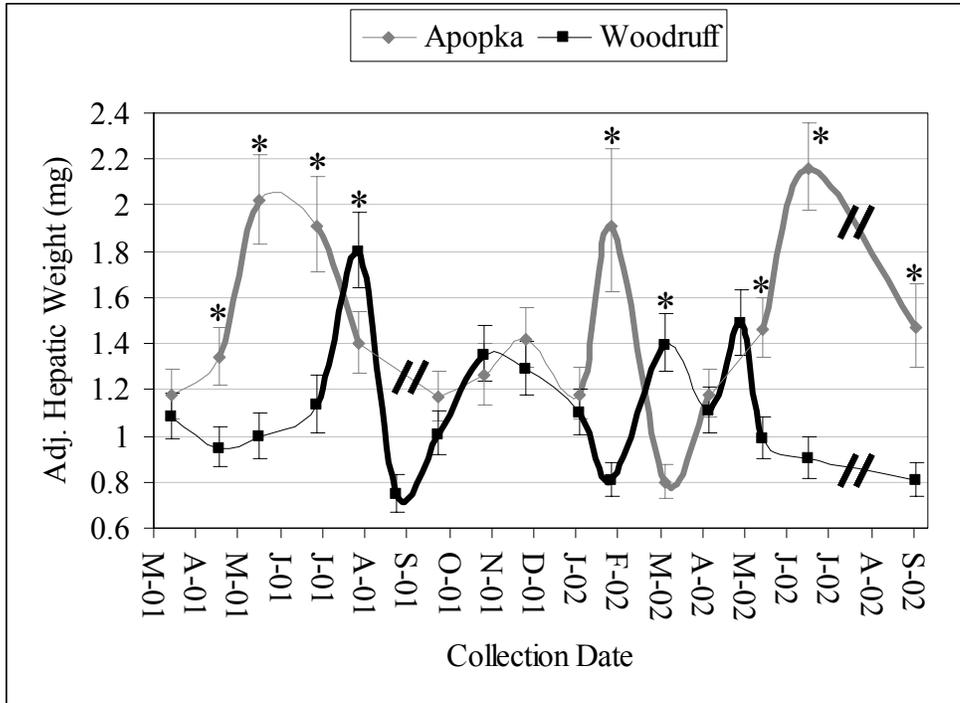


Figure 4-7. Hepatic weights, adjusted for body weight, of adult male mosquitofish from Lake Apopka and Lake Woodruff. Graph shows means \pm 1 SE. *Months in which the mean adjusted hepatic weights of fish from the two lakes are significantly different (ANCOVA, $p \leq 0.05$). A heavier line between data points indicates significant month-to-month variation within a lake (ANCOVA, $p \leq 0.05$). Slashed bars (//) indicate that time between consecutive collections exceeds 1 month and that the indicated trend is extrapolated.

CHAPTER 5
WATER QUALITY INFLUENCES REPRODUCTION IN FEMALE MOSQUITOFISH
(*Gambusia holbrooki*) FROM EIGHT FLORIDA SPRINGS¹

Introduction

Freshwater nitrate contamination is a growing international concern. While the drinking water standard is 10 mg/L NO₃-N in the United States and 11.3 mg/L NO₃-N in Europe (European Council 1998; US EPA 1996), natural water bodies can exceed 100 mg/L nitrate (reviewed by Rouse et al. 1999). In Iowa, a statewide well-water survey reported that 18% of rural drinking water wells were contaminated with nitrate concentrations that exceeded 10 mg/L NO₃-N (Kross et al. 1993). Nitrate usually enters surface and ground water in runoff from point and non-point sources, including fields, golf courses, private gardens, livestock feedlots, and sewage treatment facilities (Berndt et al. 1998; Katz et al. 1999). Under normal circumstances, nitrogen is naturally cycled by bacterial and plant communities in aquatic ecosystems. However, if these organisms are limited (e.g., low light, low phosphorus) and unable to remediate excess nitrate concentrations, nitrate can accumulate. Elevated aquatic nitrate potentially affects reproduction and survival of exposed animals by directly influencing their physiology (Guillette and Edwards, 2005).

Aquatic animals are exposed to nitrate primarily through ingestion or epithelial absorption across gills or skin (Onken et al. 2003). In crabs, nitrate can cross the gills,

¹**Disclaimer:** The views expressed herein are those of the author and do not necessarily reflect the views of the Florida Department of Environmental Protection, which provided funding for this project.

sometimes against a concentration gradient, by substituting for chloride in the chloride-bicarbonate exchange mechanism that normally regulates the osmotic and respiratory functions of the gill (Lee and Pritchard 1985; Onken et al. 2003). The ability of the gill epithelium of freshwater fishes to accumulate Cl^- suggests that nitrate can also accumulate, as shown in shrimp (Cheng and Chen 2002). Thus, like chloride, the circulating nitrate concentration could exceed that of the surrounding water.

Evidence suggests that sensitivity to nitrate is species specific. Kincheloe et al. (1979) reported larval mortality of Chinook salmon, rainbow trout, and cutthroat trout at concentrations as low as 2.3 to 7.6 mg/L $\text{NO}_3\text{-N}$. The 96 h median lethal concentration (LC50) for fathead minnow larvae is 1,341 mg/L $\text{NO}_3\text{-N}$ (Scott and Crunkilton 2000), and the lethal dose for adult and juvenile medaka is 100 mg/L $\text{NO}_3\text{-N}$ (Shimura et al. 2002). A range of sublethal effects of nitrate has also been reported. For example, Greenlee et al. (2004) observed increased apoptosis and reduced cell number in cultured pre-implantation mouse embryos exposed to 1 mg/L ammonium nitrate. In an accumulated nitrate test, in which nitrate built up over the course the experiment, Shimura et al. (2002) observed delayed hatching time and reduced fertilization and hatching rates of eggs produced by adult medaka exposed for two months to a maximum of 75 mg/L $\text{NO}_3\text{-N}$. In that test, the offspring also exhibited reduced juvenile growth rates. At 50 mg/L $\text{NO}_3\text{-N}$, Shimura et al. (2002) observed reduced spawning and fecundity (measured as egg number) among adult medaka exposed to nitrate as juveniles. Several authors have suggested that nitrate can impact reproduction in vertebrates through effects on steroid hormone balance or impacts on nitric oxide regulation (DelPunta 1996; Panesar and Chan 2000; Vanvoorhis et al. 1994).

Nitrate metabolism has not been studied in detail in fish. However, in mammals, nitrate can be converted *in vivo* by reversible reactions to nitrite and then nitric oxide (NO) by a number of suggested mechanisms (Kozlov et al. 1999; Lepore 2000; Panesar and Chan 2000; Samouilov et al. 1998; Weitzberg and Lundberg 1998). Nitric oxide is a gas that diffuses through tissues, playing diverse roles in vasodilation, cell-to-cell signaling, neurotransmission, and immunity. The mammalian ovarian cycle and ovulation are regulated, in part, by interactions among gonadotropins, progesterone, estradiol, and NO (Al-Hijji et al. 2001; Rupnow et al. 2001; Vanvoorhis et al. 1994; Yamagata et al. 2002). In a broad sense, NO appears to inhibit steroid hormone synthesis by inhibiting several steroidogenic enzymes or other major factors in this pathway. These include steroidogenic acute regulatory protein (StAR), and the enzymes P450-sidechain cleavage (P450_{SCC}), 3 β -hydroxysteroid dehydrogenase (3 β HSD), and aromatase (DelPunta et al. 1996; Panesar and Chan 2000; Stocco and Guillette, unpubl. data; Vanvoorhis et al. 1994; Weitzberg and Lundberg 1998; Yamagata et al. 2002).

Given the observed and hypothesized effects of nitrate on vertebrate reproduction and growth, we investigated the relationships between nitrate and several reproductive variables in wild female mosquitofish captured from eight Florida springs with varying concentrations of nitrate. We also considered the potential influence of four other environmental parameters, including temperature, pH, conductivity, and dissolved oxygen.

The Florida springs present an excellent system for our study because they vary in nitrate concentration; yet appear similar in many other respects, such as relatively constant year-round temperature, pH, conductivity, and clarity. In addition, unlike most

surface water sites, spring water arises from ground water sources. This suggests that water quality of spring water is more stable over time, compared to other surface waters. Specifically, water quality and chemistry of spring water primarily reflects the composition of the underground aquifer rock with which it comes in contact during its time underground (residence time) (Scott et al., 2004). Residence times range from several days to thousands of years, depending on the geology and flow rate of the spring (reviewed in Scott et al., 2004). Our study depends on water data taken only at the time of our fish collections. Thus we cannot describe temporal variation in water quality. However, given the underground source of spring water, it is likely that our measured values are representative of spring conditions over the short term (weeks to months) preceding our study. This statement is supported by other data we collected during 2003 (unpublished) and the emerging database on spring water quality initiated by Florida's Suwannee River Water Management District (available online at http://www.srwmd.state.fl.us/water+data/surfacewater+quality/search+surfacewater+quality+data.asp?county_code=F001&Submit=GO).

Methods

Field Collections and Water Quality

Between May 18 and June 7, 2003, adult female *Gambusia holbrooki* (eastern mosquitofish) were collected using 3-mm mesh dip nets or seines from eight Florida springs with varying degrees of nitrate contamination. The sampled springs are located along the Santa Fe and Suwannee Rivers in north-central Florida. Fish were selected if they were mature. This was judged by size in the field and confirmed during necropsy based on presence of differentiated follicles. Mature fish from the sampled springs exhibited a standard length ≥ 2 cm.

As fish were captured, they were randomly parsed into one of two groups. Fish placed in the group for estradiol analysis (n = 13 to 17 per spring) were immediately chilled on ice. Fish used for necropsy (n = 30 per spring) were taken live to the laboratory, using aerated coolers of water taken from the capture site. Fish in the necropsy group were dissected within 1 day of capture to examine ovarian and hepatic weight, embryo number, and embryo dry and wet weight.

Water quality data were obtained at the time and location where fish were captured. Water temperature, pH, and conductivity were measured using a handheld *Ultrameter* (Model 6P, Myron L Company, Carlsbad, CA). Dissolved oxygen was measured using an YSI oxygen probe (Model 550A). In addition, water samples were filtered through a 1-micron glass fiber filter (Millipore Cat. No. AP4004700), chilled on ice, and stored at -20°C until they were analyzed for nitrate using an auto-analyzer (Bran+Luebbe Technicon II with colorimeter). This method uses a copper-cadmium column to reduce nitrate to nitrite, which then reacts to form a colored solution that can be assessed colorimetrically. Therefore, nitrate concentrations are reported as ppm (mg/L) nitrogen in the forms of nitrate and nitrite combined (NO₃-N).

Body Size and Dissections

Adult standard length (SL) was measured from the snout tip to the caudal peduncle using calipers. Fish were blotted dry and weighed with an electronic balance to the nearest milligram. Ovaries and livers were removed and weighed to the nearest 0.1 mg. Ovarian wet weight ranged from 1.6 to 874.2 mg, ovarian dry weight ranged from 0.3 to 200.3 mg, and hepatic weight ranged from 1.6 to 94.8 mg. Mature females were considered reproductive if their ovaries contained at least 1 vitellogenic (yellow rather than white) oocyte. To assess fecundity, we determined the developmental stage of the

embryos (based on Haynes 1995), and counted embryos that were stage 3 or older. Counted embryos were dried in an oven for 24 h at 40 °C. In *Gambusia*, embryos develop within the ovary in synchronized waves and account for most of the ovarian weight. Therefore, mean embryo weight, both wet and dry, for each female was calculated by dividing the total wet and dry weight of a brood by the embryo number (Meffe and Snelson, 1993). For stage 11 embryos (just before birth), wet weights are slightly exaggerated by the presence of yolked ovarian follicles under development as part of the subsequent brood.

Estradiol Concentration

Estradiol-17 β concentrations were measured on extracts of mosquitofish tissue using enzyme immuno-assay (EIA) kits (Cat No. 582251) purchased from Cayman Chemical Company (Ann Arbor, Michigan), and validated in our lab for this purpose. All body tissue posterior to the gonad and anal fin was collected from each fish, and the fresh wet weight obtained after the caudal fin was removed. This tissue is primarily muscle, and will be referred to as muscle for the remainder of the chapter. Tissue was stored at -80°C until it was thawed on ice, homogenized in 1 ml 65mM borate buffer (pH 8.0), and extracted twice with 5 ml diethyl ether. For each extraction, the ether and homogenate were mixed for two minutes using a multi-tube vortex mixer. For the first extraction, tubes were allowed to settle for three minutes to separate phases. For the second extraction, phases were separated by centrifugation for two minutes. After phase separation, the aqueous portion was frozen in a methanol bath chilled to -25°C with dry ice. The lipophilic layers from both extractions were combined in a new tube, and the ether was evaporated under dry forced air. Dry extract was reconstituted in up to 4 ml

EIA buffer and diluted as necessary (up to 1:100) so that samples would fall within the range of the standard curve. EIAs were run as recommended by Cayman with an 18 h refrigerated incubation to increase sensitivity. Data were quantified against a standard curve that was linearized using a logit transformation of B/Bo (bound sample/maximum bound).

Statistics

At the beginning of our analysis, we intended to evaluate relationships between water quality factors, such as nitrate, and various measured reproductive variables. However, as we progressed through the analysis, it became clear that several response variables were interrelated and that these relationships needed to be described before we could examine the influence of water quality on reproduction.

Relationships among reproductive variables

To discover how different reproductive variables related to each other, we combined the study populations and constructed a correlation matrix based on data from individual fish. Estradiol concentrations were not included in the matrix because they were measured on a separate subset of fish (separate subsets were used to avoid altered sex steroid concentrations due to capture stress). To improve linearity, all data (except embryo stage) were \log_{10} -transformed. After the correlation analysis, colinear pair-wise combinations of reproductive variables were visualized using simple regression. Ovarian weight and embryo number were strongly related to more than one other response variable (Table 5-1). Therefore, for these two variables, we used forward stepwise regression to rank the relative importance of each regressor.

Relationships among water quality parameters and reproductive variables

To determine which environmental parameters were important predictors of the measured reproductive variables, we used forward stepwise regression. The five water parameters (NO₃-N, temperature, conductivity, dissolved oxygen, pH), expressed as a mean for each spring, were entered as independent variables, and their collective statistical influence was evaluated for each dependent variable, also expressed as a mean or adjusted mean. Adjusted means were calculated using ANCOVA following log-log transformation. Dependent variables included body size (SL, weight, and condition (expressed as mean (log₁₀ weight) adjusted for (log₁₀ SL)), estradiol concentration (log₁₀ (E₂+1)), embryo weight (wet and dry), number of non-reproductive, mature females captured (out of 30 total from each spring), hepatic weight adjusted for body weight, and embryo number adjusted for standard length². Possible colinearities between pairs of independent variables were assessed using a correlation matrix. No significant colinearities were detected ($r^2 < 0.41$, $p > 0.09$ for all pairwise correlations). When more than one independent variable entered into the stepwise model, we calculated partial correlation coefficients using a partial correlation matrix of the dependent and relevant independent variables.

At the conclusion of the stepwise analysis, we visualized the effects of single independent variables (water parameters) on individual response variables using simple linear regression. We observed that temperature was an important predictor for several

² Results from the correlation/simple regression analyses indicated that embryo number correlated positively with both standard length ($r^2 = 0.64$; $p < 0.0001$) and maternal body weight ($r^2 = 0.74$; $p < 0.0001$). Compared to body weight, standard length is a more appropriate covariate because it is independent of the response variable (embryo number).

reproductive variables. However, for all these variables, particularly condition, the significant influence of temperature was driven by a lower temperature at Ruth Spring. The temperature of Ruth Spring was 0.9 to 1.8°C less than the other seven sites. Given that this is a seemingly small difference, we repeated the stepwise analysis after excluding temperature as an independent variable.

In addition to the above stepwise analysis, $\log_{10}(E_2+1)$ -transformed estradiol concentrations were also compared among fish from the different springs using ANOVA. Adjusted means were calculated and compared using an ANCOVA model in SPSS, version 12.0. All other analyses were performed using Statview 5.0, and results were considered significant at $\alpha = 0.05$.

Outliers

During the analysis, we omitted three measured estradiol values (2.5%) that were more than three standard deviations from the mean for all fish in the study. One female from Ruth Spring was omitted because she exhibited unusually high fecundity compared to the mean for all females in the study (245 versus an average of 27 embryos *in ovario*).

Results

Relationships among Reproductive Variables

Standard length and female body mass (\log_{10} - \log_{10} transformed) were highly correlated ($r^2 = 0.95$) (Table 5-1). In addition, hepatic weight correlated positively with maternal body weight ($r^2 = 0.62$) and embryo number correlated positively with standard length ($r^2 = 0.64$) (Table 5-1). Adjusted hepatic weight was influenced by stage of embryonic development, being highest during the period of yolk deposition to the embryos (stages 0.5 – 2.5) and then dropping for the remainder of gestation (Fig. 5-1A). Likewise, ovarian weight and embryo number were also influenced by other factors

(Table 5-1). *Gambusia* embryos develop inside the maternal ovary and, according to our data, consistently gain wet weight during the course of gestation (as stage increases) (Fig. 5-1B). Embryo dry weight also increases at the beginning of gestation, but stabilizes between stages 4.5 – 8, and then decreases as offspring approach parturition (Fig. 5-1C). There appears to be a tradeoff between embryo number and embryo dry weight (but not wet weight) such that a female may have many smaller embryos or fewer large ones (Table 5-1). The outcome of this tradeoff is influenced by maternal body weight, as larger females generally produce more offspring, and those offspring exhibit increased wet weights in a manner that may be stage-dependent (Table 5-1). Therefore, the relationship between ovarian weight and body mass, traditionally expressed as the gonadosomatic index (GSI), is complicated by gestational wet weight gain and embryo number, which in turn is influenced by maternal body mass and embryo dry weight (Table 5-1). Since females in any given population are not synchronized with regard to gestational stage, it could be misleading to compare populations using GSI as a singular measure of reproductive health or fecundity (as is a common practice in the piscine literature) without knowing the gestational stage or degree of tradeoff between embryo size and number.

Water Quality

Table 5-2 shows the collection sites and provides abiotic water data. Ranges across the eight springs for each water parameter were as follows: temperature: 21.4 to 23.2 °C; pH: 7.02 to 7.35; conductivity: 347 to 479 μ S; dissolved oxygen: 0.39 to 5.22 mg/L; and NO₃-N: 0.22 to 5.06 mg/L.

Relationships between Water Quality and Reproduction

Detailed results of the stepwise regression analyses are shown in Table 5-3. The direction of individual interactions is indicated by the partial correlation coefficients. With all five water parameters included, nitrate significantly predicted the number of non-reproductive females sampled from the springs (Fig. 5-2). Temperature exhibited a negative relationship with condition and adjusted embryo number (Fig. 5-3), and a positive relationship with embryo dry weight; embryo dry weight was also related negatively to nitrate (Table 5-3). However, for these three variables, the influence of temperature appears to be driven by the Ruth Spring data point, which is cooler than the other sites by less than 2 °C. If Ruth Spring is excluded from the model, the negative relationship between temperature and adjusted embryo number becomes marginal ($r^2 = 0.53$; $p = 0.06$), and the relationships between temperature and condition or embryo dry weight are lost ($p > 0.3$).

With temperature excluded from the list of potential independent variables in the stepwise model, we found that nitrate still played a significant role in predicting embryo dry weight (negative relationship, Fig. 5-4). We checked our data to be sure that this negative association between nitrate and embryo dry weight could not be explained by differences in stage among embryos from different springs (Fig. 5-1C).

Dissolved oxygen was the only variable to enter into the stepwise model for predicting adjusted hepatic weight (Fig. 5-5). Again, this association between oxygen concentration and hepatic weight could not be explained by differences in developmental stage of the embryos (Fig. 5-1A). Based on our data, embryo wet weight and maternal estradiol concentrations were not influenced by the water quality parameters we

measured (Table 5-3 and Fig. 5-6). In addition, we did not observe significant differences in muscle estradiol concentrations among springs ($p = 0.15$).

Discussion

At the outset of our study, we hypothesized that low concentrations of environmental nitrate (1 – 5 mg/L NO₃-N) would be related to changes in reproduction and growth of mosquitofish captured from Florida springs. Our data indicate a significant association between increasing nitrate and reduced embryo dry weight. We also observed a strong relationship between increased nitrate and reduced reproductive activity among mature females. In addition to these findings regarding nitrate, we observed that many of the measured reproductive variables were interrelated. In addition, variation in *Gambusia* body size and embryo number and dry weight were related to temperature, and hepatic weight was related to dissolved oxygen concentration.

We hypothesize that the observed negative relationship between nitrate and embryo dry weight is due to nitrate-induced alterations in thyroid function. Environmentally relevant concentrations of nitrate have been shown to reduce thyroid function, feeding behavior, and growth rate in a variety of vertebrates such as sharks, amphibians, and mammals (Allen et al. 1996; Crow et al. 1998; Jahreis et al. 1991; Schuyttema and Nebeker 1999; Zaki et al. 2004; Zraly et al. 1997). Nitrate exposure has been associated with goiter and reductions in plasma thyroxine (T₄), plasma tri-iodothyronine (T₃), iodine availability, iodine uptake, hypothalamic concentrations of growth hormone releasing factor, and plasma concentrations of somatomedin-C and IGF-1, which are part of the growth hormone axis (Crow et al. 1998; Jahreis et al. 1991; Kursa et al. 2000; Simon et al. 2000; Zraly et al. 1997). The importance of thyroid function during development and

growth suggests that embryos, fetuses, and juveniles could be more susceptible than adults to the disruptive effects of nitrate exposure.

In addition to the observed relationship between low embryonic growth and nitrate, we noted that the number of reproductive females captured during sampling was negatively related to nitrate concentration. That is, as nitrate levels went up, fewer reproductive females (less than 54% in Fanning Spring) were captured relative to the total number of sexually mature females caught. In our other studies of *Gambusia* collected from Florida lakes, we typically observe that 95% of mature females are reproductively active (Edwards et al. unpubl. data). Since *Gambusia* incorporate yolk into oocytes before fertilization (Koya et al. 2000), our observation does not imply disrupted fertilization. Rather, it suggests that nitrate, or its metabolites (nitrite, nitric oxide) may influence some aspect of vitellogenesis or vitellogenin sequestering during oogenesis. Vitellogenesis occurs in the liver, and is stimulated by estrogens (Tolar et al. 2001). If estrogens are decreased by nitrate or its metabolites, then vitellogenesis could be similarly decreased. Yamagata et al. (2002) demonstrated that *in vitro* steroidogenesis by rat granulosa cells could be decreased by exposure to a nitric oxide donor. We did not find a relationship between estradiol concentration in the body tissue and nitrate concentrations in the springs. Nor did we observe a relationship between estradiol and frequency of reproductive females. However, it is possible that nitrate may alter the action of estradiol in the liver. Alternatively, the reduced frequency of reproductively active females could be due to a delayed onset of seasonal reproductive activity among some females in the population. Both hypotheses require further testing.

Hypoxia in the springs was related to increased adjusted hepatic weight, and explained 84% of the variance in this variable. In cultured mammalian hepatocytes and whole animals, hypoxia and related acidosis stimulate sodium accumulation in liver cells, which causes cell swelling. If this swelling is not excessive, the cell membrane will remain intact and the cell will avoid necrosis (Carini et al. 1999). In addition, necrosis is also avoided if hepatocytes are preconditioned by early but intermittent exposure to hypoxia (Carini et al. 2001), as may be the case for wild *Gambusia*. We informally screened hepatic histology for several male fish captured with the females in our study and did not observe necrotic cells in fish from low or high oxygen sites.

Matrotrophy

We have noted in the literature that *Gambusia* are classified as lecithotrophs (Constantz, 1989). However, our data suggest that *Gambusia* are matrotrophic, at least during the first two thirds of development (through stage 8), when both embryo dry weight and wet weight are stable or rising (Figs. 5-1B and C). Meffe and Snelson (1993) observed a similar increase in *Gambusia* embryo dry weight during gestation. In our study, hepatic weight (adjusted for body weight) was highest during the period of yolk deposition to the embryos (stages 0.5 – 2.5) (Fig. 5-1A) and then dropped for the remainder of gestation. This suggests that vitellogenesis is greatest at the beginning of gestation (Koya et al. 2000). Although vitellogenesis apparently drops by stage 3, embryos gain or maintain dry weight through to stage 8. Between stages 8 and 11, the yolk sac diminishes rapidly and some dry weight is lost (Fig. 5-1C). Based on these observations, we suggest that *Gambusia* exhibit some direct, matrotrophic support of embryo growth during the first two thirds of development, and rely on egg yolk reserves for the completion of gestation. This observation of matrotrophy in *Gambusia* is

supported by other recent evidence of maternal nutrient transfer (Marsh-Matthews et al. 2001). The gain in wet weight before birth (a 4-fold increase on average) could be adaptive in that larger larvae often exhibit better survivorship (Hare and Cowen 1997).

Conclusion

Our data suggest that growth and reproductive parameters in *Gambusia* are highly interrelated and subject to influence from a variety of environmental factors, including nitrate, temperature, and dissolved oxygen. In particular, nitrate exposure is related to reduced dry weight of developing *Gambusia* embryos during gestation and reduced rate of reproductive activity among mature females. These findings, along with those of other studies cited here, suggest that nitrate may act as an endocrine disruptor, possibly affecting signaling patterns associated with the thyroid, liver, and gonad. The mechanisms associated with these alterations require extensive study, as nitrate contamination of aquatic ecosystems is a global concern.

Response Variable	Correlated with	<i>r</i> (indicates direction of relationship)	<i>r</i> ²	<i>p</i> -value	
Body weight	Standard Length	0.98	0.95	< 0.0001	
Hepatic weight	Body weight	0.79	0.62	< 0.0001	
Embryo dry weight	Embryo number	-0.32	0.10	< 0.0001	
Embryo wet weight	Stage**	0.81	0.66	< 0.0001	
Embryo wet weight	Maternal body weight**	0.46	0.21	< 0.0001	
Response Variable	Step	<i>r</i> ²	<i>p</i> -value	Other Reproductive Variables	Partial <i>r</i>
Ovary weight	1	0.86	< 0.0001	Body weight	0.93
	2	0.91	< 0.0001	Body weight Stage	0.92 0.57
	3	0.97	< 0.0001	Body weight Stage Embryo number	0.69 0.82 0.82
Embryo number	1	0.64	< 0.0001	Standard Length	0.80
	2	0.77	< 0.0001	Standard Length Embryo dry weight	0.86 -0.61

*Analyses involving embryo number or data derived from embryo number (embryo weight) do not include females with embryos younger than stage 3 (Haynes, 1995) because such embryos are variable in size and difficult to count.

** Both stage and maternal body weight are significantly correlated with embryo wet weight. However, if both are included in a stepwise regression model for embryo wet weight, only stage enters the model.

Spring	Location (GPS)	Water Temperature (°C)	pH	Conductivity (uS)	Dissolved O ₂ (mg/L)	NO ₃ -N (mg/L)
Blue	N 29°49'49.2"; W 082°40'56.6"	23.2	7.27	346.5	5.22	1.51
Fanning	N 29°35'15.0"; W 082°56'08.0"	22.60 \pm 0.06	7.09 \pm 0.01	470.9 \pm 3.6	1.89 \pm 0.20	4.03 \pm 0.41
Hart	N 29°40'30.4"; W 082°57'05.0"	22.35 \pm 0.25	7.10 \pm 0.01	402.1 \pm 0.9	0.39 \pm 0.07	0.81 \pm 0.04
Lily	N 29°49'48.6"; W 082°39'37.7"	22.3	7.19	425.1	0.84	0.32
Manatee	N 29°29'20.6"; W 082°58'40.0"	22.85 \pm 0.25	7.16	479.1 \pm 0.6	1.94 \pm 0.14	1.26 \pm 0.16
Peacock	N 30°07'18.0"; W 083°07'57.0"	22.50 \pm 0.44	7.35 \pm 0.07	362.2 \pm 1.6	2.02 \pm 0.27	1.69 \pm 0.15
Poe	N 29°49'33.0"; W 082°38'58.0"	22.40 \pm 0.06	7.19 \pm 0.01	415.1 \pm 0.2	0.59 \pm 0.28	0.22 \pm 0.01
Ruth	N 29°59'44.0"; W 082°58'38.0"	21.37 \pm 0.50	7.02 \pm 0.07	404.2 \pm 4.3	1.17 \pm 0.44	5.06 \pm 0.61

Table 5-3. Relationships of water quality parameters and response variables measured in adult female *Gambusia holbrooki* collected from eight Florida springs.

Response Variable	Step	r^2	p -value	Water Parameter	Partial r
Standard length (SL)	0				
Body weight	0				
Condition	1	0.56	0.03	Temperature	-0.75
Adjusted hepatic weight	1	0.85	0.001	Dissolved O ₂	-0.92
Adjusted embryo number	1	0.76	0.005	Temperature	-0.87
Mean embryo dry weight	1	0.68	0.012	Temperature	0.82
	2	0.83	0.01	Temperature NO ₃ -N	0.78 -0.69
Mean embryo wet weight	0				
Estradiol	0				
Number of non-reproductive females	1	0.57	0.03	NO ₃ -N	0.75
Temperature removed from the analysis*					
Condition	0				
Adjusted hepatic weight	1	0.85	0.001	Dissolved O ₂	-0.92
Adjusted embryo number	0				
Mean embryo dry weight	1	0.56	0.03	NO ₃ -N	-0.75
Number of non-reproductive females	1	0.57	0.03	NO ₃ -N	0.75

*With the exception of the robust relationship between temperature and embryo number, the significant influence of temperature is largely driven by a lower temperature at Ruth Spring, which is 0.9-1.8°C less than the other seven sites. Given that this is a seemingly small difference, we repeated the stepwise analysis after excluding temperature as an independent variable.

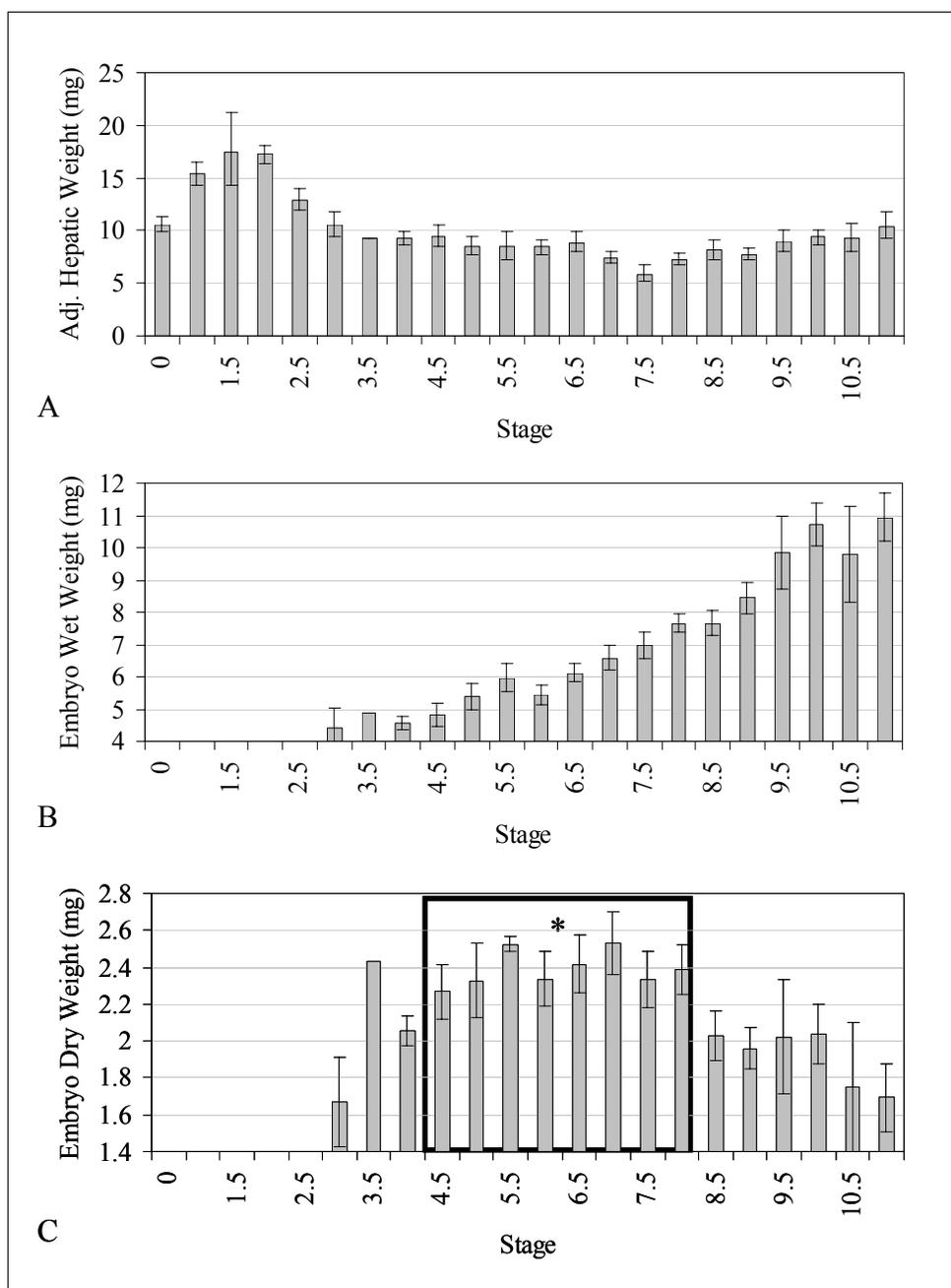


Figure 5-1. A) Mean maternal hepatic weight, adjusted for body weight; B) embryo wet weight; and C) embryo dry weight plotted by embryonic stage (stages based on Haynes, 1995). Data at stage 3.5 were limited to a single female. Embryo weights represent the sum of the embryo and yolk sac. We did not obtain oocyte/embryo weight data at stages younger than 3 because those oocytes are small and highly variable in size. *The collective mean dry weight of embryos between stages 4.5 – 8 was significantly greater than the collective mean dry weights of embryos either younger or older (ANOVA, $p = 0.0004$).

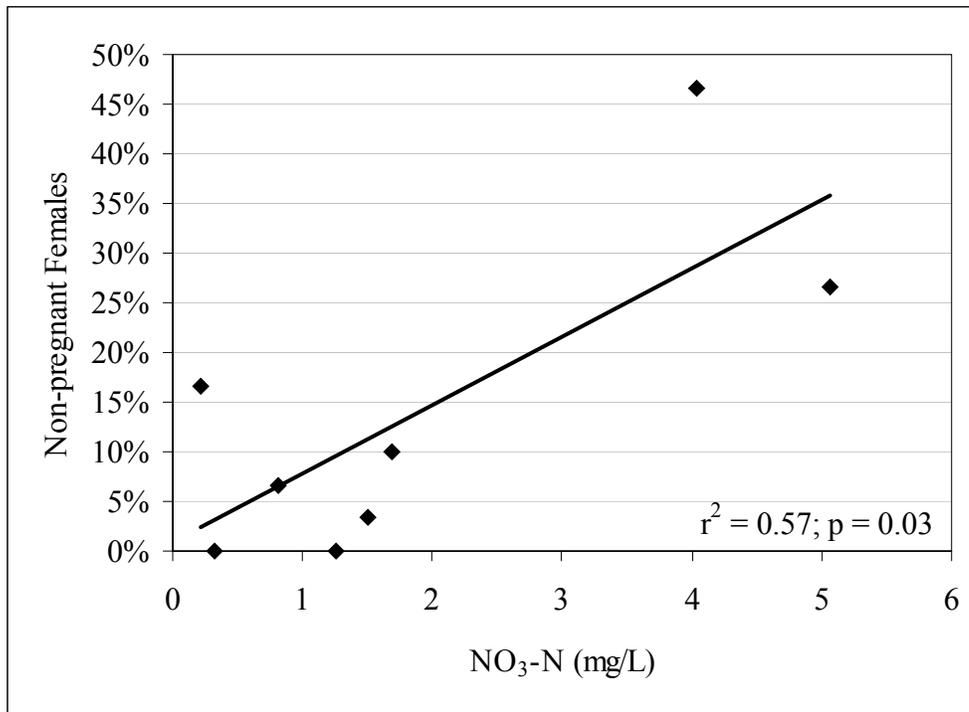


Figure 5-2. Percentage of non-reproductive, mature females sampled from Florida springs with varying nitrate concentrations. Total samplings from each spring consisted of 30 mature females.

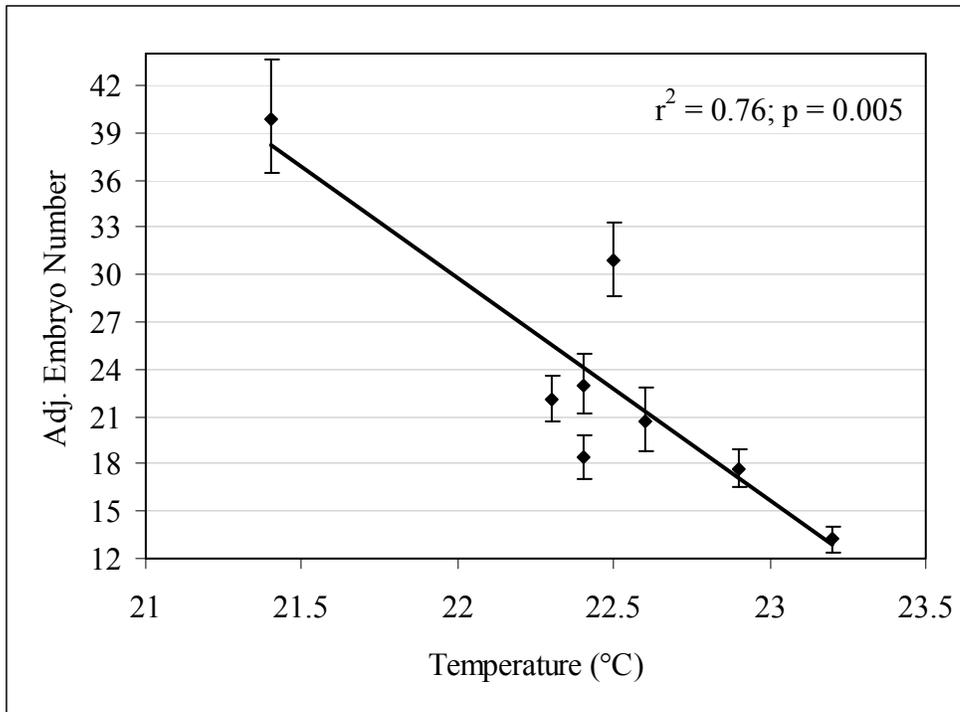


Figure 5-3. Mean embryo number, adjusted for maternal body weight for females captured in Florida springs with varying mean temperatures. Graph shows means \pm 1 SE.

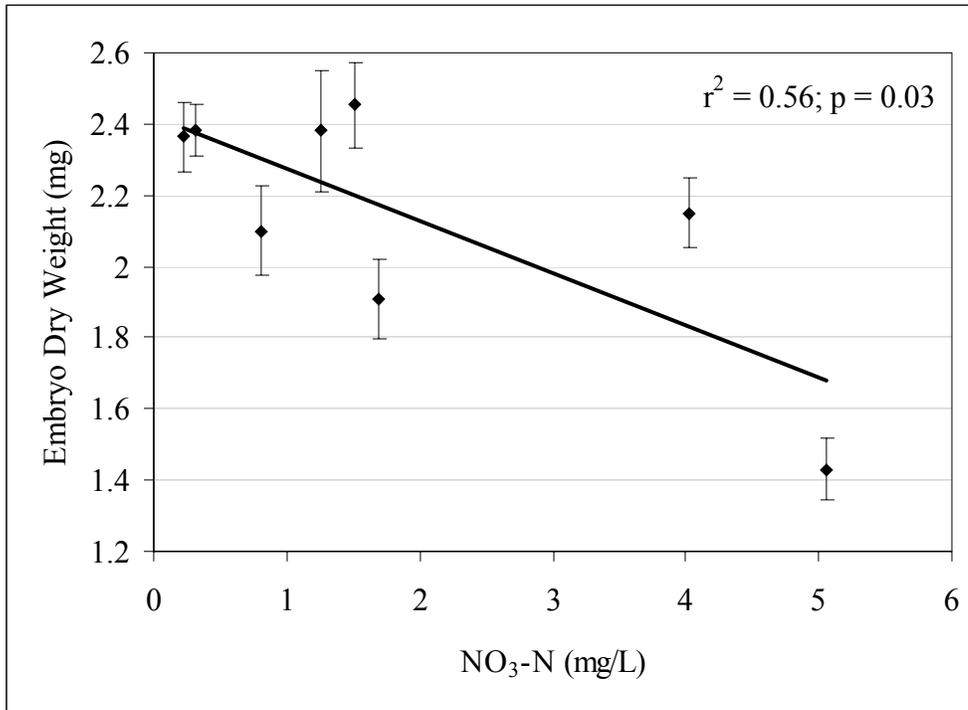


Figure 5-4. Embryo dry weight (mg) for embryos taken from females captured in Florida springs with varying concentrations of nitrate. Graph shows means \pm 1 SE.

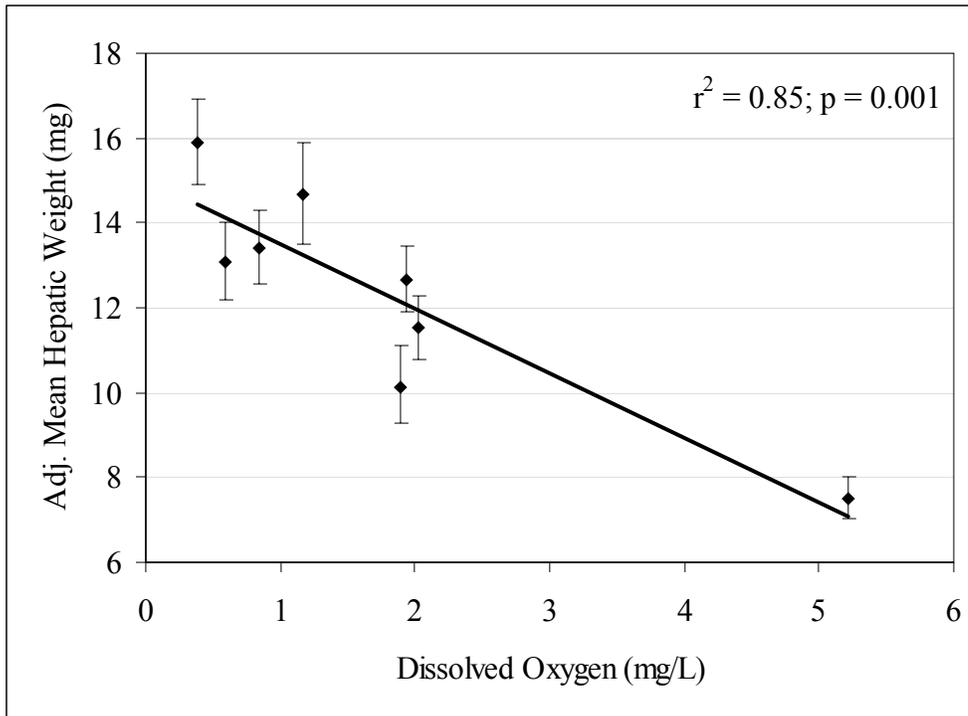


Figure 5-5. Mean hepatic weight, adjusted for body weight, for females captured in Florida springs with varying dissolved oxygen concentrations. Graph shows adjusted means \pm 1 SE.

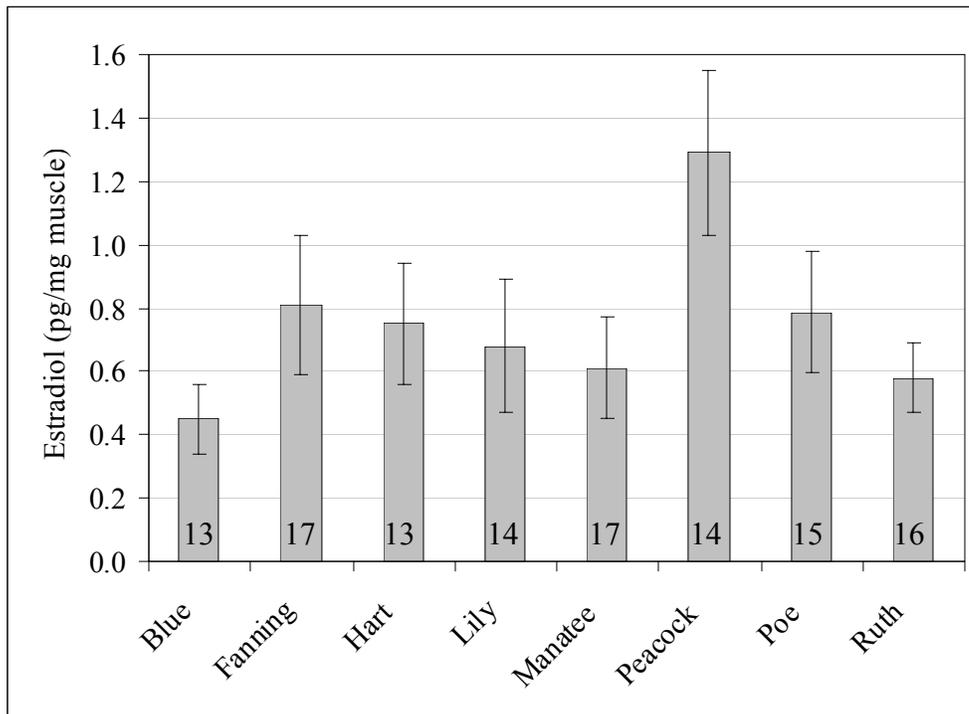


Figure 5-6. Muscle estradiol concentrations for females from each spring. Graph shows means \pm 1 SE. Means are not statistically different (ANOVA, $p = 0.15$). Numbers at base of data columns indicate sample size.

CHAPTER 6
WATER QUALITY INFLUENCES REPRODUCTION IN MALE MOSQUITOFISH
(*Gambusia holbrooki*) FROM EIGHT FLORIDA SPRINGS³

Introduction

In the past 30 years, nitrate concentrations in many Florida springs and coastal waters have increased significantly, due largely to anthropogenic sources such as fertilizer application and discharge of treated sewage (Barile, 2004; Katz, 2004; Katz et al., 2004). Nitrate concentrations in Florida springs vary from a presumed background concentration of 1 mg/L NO₃-N to 38 mg/L NO₃-N (Katz et al., 1999). This latter value is almost 4-fold higher than the current U.S. drinking water standard for nitrate (10 mg/L NO₃-N) (US EPA, 1996). Florida springs are not the only sites of groundwater nitrate contamination. According to the Minnesota Pollution Control Agency (2000), 13.7% of sampled wells exceed 10 mg/L NO₃-N. In Pennsylvania's Susquehanna River Basin, 45% of sampled wells in agricultural areas exceed 10 mg/L NO₃-N (Lindsey et al., 1997), and in Iowa, a statewide well-water survey reported that 18% of rural drinking water wells were contaminated with nitrate concentrations that exceeded 10 mg/L NO₃-N (Kross et al. 1993). Clearly, drinking water is at risk for nitrate contamination, and consequently, health risks associated with nitrate exposure must be determined.

A number of recent vertebrate studies report that nitrate exposure reduces reproductive hormone concentrations, fertilization rates, and semen quality, suggesting a direct, negative effect on reproductive function (DeLPunta et al., 1996; Kostic et al., 1998;

³**Disclaimer:** The views expressed herein are those of the author and do not necessarily reflect the views of the Florida Department of Environmental Protection, which provided funding for this project.

Panesar and Chan, 2000; Rosselli et al., 1995; Shimura et al., 2002; Zrally et al., 1997; for review, see Guillette and Edwards, 2005). Nitrate can elicit its effects through *in vivo* conversion to nitrite and then nitric oxide (NO), a potent signaling molecule that can reduce androgen concentrations and sperm motility, maturation, and viability through a variety of mechanisms (DelPunta et al., 1996; Kostic et al., 1998; Panesar and Chan, 2000; Ratnasooriya and Dharmasiri, 2001; Rosselli et al., 1995). Nitric oxide has been shown to inhibit several steroidogenic enzymes, including steroidogenic acute regulatory protein (StAR) and cytochrome P450 enzymes such as P450-sidechain cleavage (SCC) and 3 β -hydroxysteroid dehydrogenase (3 β HSD) (DelPunta et al., 1996; Panesar and Chan, 2000). The effects of nitrate on spermatogenesis could either be indirect through alterations of steroidogenic enzyme action or expression, or direct, via the influence of nitric oxide on germ cell or sperm cell apoptosis. Nitric oxide is implicated in both normal and abnormal regulation of apoptosis of spermatogonia, spermatocytes, and spermatids (Lue et al., 2003; Di Meglio et al., 2004; El-Gohary et al., 1999; Zini et al., 1996).

Given these observed effects of nitrate or its metabolite, NO, on reproduction, we hypothesized that adult male mosquitofish (*Gambusia holbrooki*), captured from springs contaminated with nitrate would exhibit altered reproduction relative to those from less contaminated springs. Our evaluation of reproductive health was based on adult body size, gonopodium length, gonado- and hepato-somatic indices, androgen concentrations (testosterone and 11-ketotestosterone), total and live sperm counts, and sperm viability. In addition to nitrate, we measured water temperature, pH, conductivity, and dissolved oxygen. In mosquitofish, the gonopodium is a grooved, bony extension of the anal fin

that elongates during puberty in response to testosterone, and is used for copulation (Rosa-Molinar et al., 1994). Male mosquitofish package their sperm in spherical sperm packets called spermatzeugmata. Since the spermatzeugma, which facilitates transfer of sperm to the female, is a functional and quantifiable reproductive unit, we measured the number of sperm per spermatzeugma.

Methods

Field Collections and Water Quality

Between May 18 and June 3, 2003, adult male *Gambusia holbrooki* (eastern mosquitofish) were collected from eight artesian springs located along the Suwannee and Santa Fe Rivers in north-central Florida (Table 6-1). Fish were captured using a 3-mm mesh dipnet or seine. The eight springs were selected for two reasons. First, they all had mosquitofish populations large enough for sampling, and second, they represent a gradient of nitrate concentrations, which, at the time of sampling ranged from 0.2 to 5.1 mg/L NO₃-N.

Fish maturity was assessed in the field by looking for a well-developed and hooked gonopodium (Angus et al., 2001). Sampled fish from each spring were divided into three subsets. Sample sizes for each response variable are given in Table 6-2. One subset was immediately chilled on ice for androgen (testosterone (T) and 11-ketotestosterone (11-KT)) analysis. The other two subsets were transported live to the laboratory in aerated coolers containing water from the capture site. Within 2 days of capture, these fish were anesthetized in 0.1% MS-222 (3-aminobenzoic acid ethyl ester methanesulfonate salt, Sigma #A5040), and processed for testicular and hepatic weights from one subset, or sperm counts and sperm viability from the second subset. Testicular weight and sperm

data were not taken from the same fish as the process of stripping sperm is likely to distort the gonad, and the removal of sperm affects testicular weight.

On the day of the collection, between noon and 3 pm, water quality data were obtained at the spring boil and the fish collection site(s) within the spring basin. If fish were captured at multiple locations within the spring basin, the associated water quality measurements were averaged (standard errors given in Table 6-1). Water temperature, conductivity, and pH were measured using a handheld Ultrameter (Model 6P, Myron L Company, Carlsbad, CA). Dissolved oxygen was measured using an YSI oxygen probe (Model 550A). Nitrate concentrations were measured as follows. Water samples (50 ml) were filtered through a 47-mm glass fiber filter (0.7 μm nominal pore size), chilled on ice, and stored in clean glass containers at -20°C until they were analyzed in duplicate using an auto-analyzer (Bran+Luebbe Technicon II with colorimeter). This method uses a copper-cadmium column to reduce nitrate to nitrite, which then reacts to form a colored solution that can be assessed colorimetrically. Therefore, nitrate concentrations are reported as ppm (mg/L) nitrogen in the forms of nitrate and nitrite combined ($\text{NO}_3\text{-N}$). All instruments were calibrated using standard solutions before each sampling event. Ranges across the eight springs for each water parameter were as follows: temperature: 21.4 to 23.2 $^{\circ}\text{C}$; pH: 7.02 to 7.35; conductivity: 347 to 479 μS ; dissolved oxygen: 0.59 to 5.22 mg/L; and $\text{NO}_3\text{-N}$: 0.22 to 5.06 mg/L (Table 6-1).

Body Size and Dissections

Standard length (SL), body weight, and gonopodium length were measured on all fish. SL was measured from the snout tip to the caudal peduncle using calipers. Fish were blotted dry and weighed with an electronic balance to the nearest milligram (range

was 68 to 615 mg). Gonopodium length was measured to the nearest 0.01 cm, using an ocular micrometer mounted on a dissecting microscope. For GSI and HSI, testes (1.0 to 13.6 mg) and livers (0.1 to 5.6 mg) were removed and weighed to the nearest 0.1 mg.

Muscle Androgen Measurements

Testosterone (T) and 11-ketotestosterone (11-KT) were measured on extracts of mosquitofish caudal peduncle tissue (referred to as “muscle androgens” for the remainder of the chapter). Upon return to the laboratory, all peduncle tissue posterior to the gonopodium and gonad was collected from each fish iced in the field. After removal of the caudal fin, the caudal peduncle was weighed (average = 60 mg) and stored at -80°C. For extraction, peduncle tissues were homogenized (Polytron homogenizer, Kinematica AG, Littau, Switzerland) in 750 µl of 65 mM borate buffer (pH 8.0), and extracted twice with 5 ml diethyl ether, mixed with the homogenate for 2 minutes using a multi-tube vortex mixer. For the first extraction, tubes were allowed to settle for three minutes to separate phases. For the second extraction, phases were separated by centrifugation for 2 minutes. The aqueous phase was frozen in a methanol bath cooled to -25°C with dry ice. The ether from both extractions was combined in a second tube and evaporated under dry forced air.

Hormone concentrations were determined using validated enzyme immunoassay (EIA) kits (Cat No. 582701 (T); 582751 (11-KT), Cayman Chemical, Ann Arbor, Michigan). Dry extract was reconstituted in 1000 to 1500µl EIA buffer so that samples would fall within the range of the standard curve. EIAs were run as recommended by Cayman with an 18 h refrigerated incubation to increase sensitivity. Data were quantified against a standard curve linearized using a logit transformation of B/Bo (bound

sample/maximum bound). Duplicate interassay variance (IAV) samples at 2 dilutions were included with each plate. The coefficient of variance among all plates, averaged for the 2 dilutions was 10.0% for T, and 12.3% for 11-KT. To normalize sample androgen concentrations across assays, we multiplied by a correction factor derived from the relationship between individual plate IAV values and the mean IAV values for all plates.

Sperm Counts and Sperm Viability

Males were over-anesthetized immediately before sperm collection in room temperature MS-222. Each fish was rinsed in distilled water and placed on its side, with gonopodium abducted, in a petri dish containing enough 150 mM KCl to cover the fish. Spermatozeugmata were stripped from the fish by gently pressing down on the abdomen, anterior to the gonopodium, and sweeping caudally using the smooth, rounded end of a pair of large forceps with the two tips taped together. Triplicate samples of a known number of spermatozeugmata (20 to 40) were drawn up in 300 μ l KCl using a micropipette, and placed in tubes on ice until the sample was counted using a flow cytometer. The method described below was previously characterized for *Gambusia* sperm (Chapter 3). Based on validation tests, sperm are stable for 3 to 4 h.

Just before counting, the sperm samples were stained with 15 μ l florescent SYBR green dye, incubated on ice for 20 to 30 minutes, and then counter-stained with 1.5 μ l florescent propidium iodide (PI) for 10 minutes. These stains are available in a Live/Dead Sperm Viability Kit (#L-7011, Molecular Probes, Inc.). Before staining, the purchased SYBR green was diluted 500 fold in DMSO. SYBR Green and PI are nucleic acid stains that differentially stain live sperm green and dead sperm orange, respectively.

Sperm are characterized as dead if their cell membrane is compromised, allowing the rapid entry of PI.

To obtain absolute sperm numbers (expressed as sperm number per spermatozeugma), the flow rate of the flow cytometer was calibrated by adding a known number of Nile-red fluorescent beads to each sperm sample (20,000 beads suspended in 20 μ l buffer per 300 μ l sperm sample). Pre-counted fluorescent beads (SPHERO AccuCount fluorescent particles, 5.2 μ m, Cat. No. ACFP-50-5) were purchased from Spherotech (Libertyville, Illinois). Beads were added to sperm and vortexed immediately before counting.

Flow cytometric analysis for 20,000 particles was performed on a FACSort flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using CellQuest 3.3 software (BD Biosciences). Forward and side light scatter measurements and green (530 \pm 15 nm), orange (585 \pm 21 nm), and red ($>$ 650 nm) fluorescence measurements were collected for each sample. The instrument threshold was set on forward light scatter. Sperm cells were identified using a gate on the forward vs. side light scatter dot plot. The contents of this gate were displayed in a second plot that gates green (SYBR Green) and orange (PI) fluorescing particles separately. Cells emitting only green fluorescence were counted as live, and cells emitting any orange fluorescence (indicating a breach in the cell membrane) were counted as dead cells. Nile-red bead counts were quantified from their peak on a red-fluorescence histogram.

Absolute sperm count per spermatozeugmatum (szm) was calculated as follows:
 $\text{sperm/szm} = [(L+D)(B)]/[b(S)]$, where L = number of live sperm counted; D = number of dead sperm counted; B = number of beads added per sample; b = number of beads

counted; and S = number of spermatozeugmata originally added to the tube. Sperm viability, measured as the percentage of live sperm, was calculated as % live = $[L/(L+D)]*100$.

Statistical Analysis

Relationships among reproductive and morphometric variables

To examine if different reproductive and morphometric response variables were related, we combined the spring populations and constructed a correlation matrix based on data from all individual fish. The three subsets of response variables (GSI and HSI; androgens; and sperm data) were evaluated as separate studies since different fish were used in each subset. To improve linearity and homogeneity of variance, \log_{10} -transformation was used for body, hepatic, and testicular weights, and androgen data ($\log_{10}(y+1)$) (Zar, 1999). Percentage of live sperm was arcsine-square-root transformed (Zar, 1999). During the androgen analyses, we omitted four fish (3%) with measured androgen values that were more than three standard deviations from the mean. After the correlation analysis, colinear pair-wise combinations of response variables were visualized using simple regression.

Correlation and regression results indicated that standard length and body mass were significantly related ($r^2 = 0.92$). Similarly, testicular ($r^2 = 0.68$) and hepatic weights ($r^2 = 0.55$) scaled positively with body weight, and gonopodium length scaled with SL ($r^2 = 0.75$). Therefore, in subsequent step-wise regression analyses (described below), mean values for testicular and hepatic weights were adjusted for body weight, gonopodium length was adjusted for SL, and condition was calculated as mean body weight adjusted for SL, after \log_{10} transformation of both variable and covariate.

Relationships among water quality parameters and reproductive variables

Measured variables among fish from the eight springs were compared using ANOVA or ANCOVA (when a covariate was appropriate). We observed spring related differences in all response variables ($p < 0.02$) except muscle testosterone concentration ($p = 0.07$) and percentage of live sperm ($p = 0.32$). For response variables that differed, we identified which of the water quality parameters were important predictors of the variables using forward stepwise regression. The five water parameters ($\text{NO}_3\text{-N}$, temperature, conductivity, dissolved oxygen, pH), expressed as a mean for the fish collection sites at each spring, were entered as independent variables, and their collective statistical influence was evaluated for each dependent variable, also expressed as a mean or adjusted mean. Dependent variables included SL, weight, condition, adjusted gonopodium length, GSI (adjusted mean testicular weight), HSI (adjusted mean hepatic weight), muscle 11-KT concentrations, and total and live sperm counts per spermatozeugma.

When more than one independent variable entered into the stepwise model, we calculated partial correlation coefficients using a partial correlation matrix of the dependent and relevant independent variables. Possible colinearities between pairs of independent variables were assessed using a correlation matrix. No significant colinearities were detected ($r^2 < 0.41$, $p > 0.09$ for all pairwise correlations). We did, however, note that the two springs with the highest nitrate concentrations had the lowest pH readings, an observation that we address in our interpretation of the results. At the conclusion of the stepwise analysis, we visualized the effects of single water parameters on response variables (means or adjusted means for each spring) using simple linear regression.

Temperature appeared to be an important predictor for several reproductive variables. However, this effect was driven by the lower temperature at Ruth Spring. The measured temperature of Ruth Spring at the time of the fish collection was 0.9 to 1.8°C less than the other seven sites. Given that this small difference may be within the error of natural variation, we repeated the stepwise analysis without including temperature as an independent variable. Likewise, we observed that water pH was predictive of muscle 11-KT concentration and HSI. However, because the sites with lowest pH had the highest nitrate, and because we have hypothesized that nitrate can impact the variables we have measured, we also compared muscle 11-KT concentrations and HSI between fish from the high nitrate sites (Fanning and Ruth) and fish from all other “low” nitrate sites using ANOVA. This component of our investigation is of interest because water pH can affect gill permeability and nitrate uptake (Jensen, 1995).

Adjusted means were calculated using an ANCOVA model in SPSS, version 13.0. All other analyses were performed using Statview 5.0, and results were considered significant at $\alpha = 0.05$.

Results

Water Quality

There were no significant colinearities among pairs of water quality variables ($r^2 < 0.41, p > 0.09$). However, the springs with the highest nitrate concentrations also exhibited the lowest pH values. The relationship between these two variables for the eight springs is better described by a curve than a line, which is why colinearity was not detected (Fig. 6-1).

Relationships among Reproductive and Morphometric Variables

Standard length and male body mass were highly correlated ($r^2 = 0.88$; $p < 0.0001$). Likewise, hepatic and testicular weights were positively correlated with body weight ($r^2 = 0.57$, $p < 0.0001$ and $r^2 = 0.68$, $p < 0.0001$, respectively). Gonopodium length was significantly related to SL ($r^2 = 0.75$; $p < 0.0001$). We did not observe a significant correlation between muscle testosterone and 11-ketotestosterone concentrations. The ratios of T to 11-KT in individual fish were both above and below 1.0, suggesting that the two androgens are regulated independently. Finally, neither androgens, nor the T to 11-KT ratio, nor sperm parameters were strongly related to body size. Total sperm counts and percentage of live sperm were unrelated. Sample sizes for all variables are given in Table 6-2.

Relationships among Water Quality Parameters and Reproductive Variables

We observed significant spring-related differences in all response variables ($p < 0.02$, based on ANOVA/ANCOVA) except muscle testosterone concentration ($p = 0.07$) and percent live sperm ($p = 0.32$), which averaged 86.5%. The low p -value associated with variation in muscle testosterone variation among fish from the different springs is driven by higher T concentrations in fish from Peacock spring (Fig. 6-2A). Mean testosterone concentrations were generally higher than 11-KT concentrations (Fig. 6-2A, B)

Results of the stepwise regression analyses are shown in Table 6-3. The direction of individual interactions is indicated by the partial correlation coefficients. Although SL, body weight, and condition differed significantly among fish from the different springs, the differences were not explained by any of the measured water parameters, based on regression analyses. Nonetheless, fish pooled from the two high nitrate sites (4-

5.1 mg/L NO₃-N) were significantly smaller (in terms of SL and weight) than fish pooled from the six lower nitrate sites (0.2 – 1.7 mg/L NO₃-N) ($p < 0.0001$).

With all five water parameters included, adjusted mean gonopodium length was positively related to nitrate concentration ($r^2 = 0.67$; $p = 0.01$) (Figure 6-3A). GSI and HSI were negatively related to temperature ($r^2 = 0.73$, $p = 0.007$ and $r^2 = 0.75$, $p = 0.005$, respectively), whereas total and live sperm counts per spermatozeugma were positively related to temperature ($r^2 = 0.61$, $p = 0.02$ and 0.53 , $p = 0.04$, respectively) (Fig. 6-4A, B, C, D). However, if Ruth spring, which had the lowest temperature, is removed from the analysis, the regression relationships between these four variables and temperature become nonsignificant.

Muscle 11-KT concentrations were positively related to pH ($r^2 = 0.56$; $p = 0.03$) (Fig. 6-5). After controlling for the effects of pH in the stepwise regression, 11-KT was also negatively related to dissolved oxygen concentrations ($r^2 = 0.79$; $p = 0.003$). As with testosterone, fish from Peacock spring exhibited the highest muscle concentrations of 11-KT (Fig. 6-2B). However, unlike the analysis of testosterone, if fish from Peacock are removed from the ANOVA, significant spring related variation in muscle 11-KT is still detected ($p = 0.03$). Furthermore, muscle 11-KT concentrations were significantly lower among males from high nitrate springs compared to males from low nitrate springs ($p < 0.001$, with or without Peacock fish included in the analysis) (Fig. 6-6).

With temperature excluded from the list of potential independent variables in the stepwise model, we found that nitrate played a more significant role in predicting GSI (positive relationship, $r^2 = 0.63$; $p = 0.02$) (Fig. 6-3B) and total sperm count per spermatozeugma (negative relationship, $r^2 = 0.50$; $p = 0.05$) (Fig. 6-3C). Despite the

total sperm count results, we noted that nitrate did not predict the number of live sperm per spermatozeugma when temperature was removed from the model. This is because the percentage of live sperm among fish from the two highest nitrate springs (Ruth and Fanning) averaged 88%, compared to 85% among fish from the six low nitrate springs. The change was not significantly different between the two groups ($p = 0.06$), but accounts for some discrepancy between total versus live sperm counts. Functionally, live sperm counts are more relevant.

Discussion

Relationships between Nitrate and Reproduction

As predicted by our hypothesis, nitrate concentration in the springs was predictive of reproductive variation among resident male mosquitofish. Fish from high nitrate springs exhibited a suite of symptoms that included testicular hypertrophy, decreased muscle concentrations of 11-KT, and lower total sperm counts per spermatozeugma. Muscle 11-KT concentrations were also positively related to water pH. Because springs with low pH had the highest nitrate, we conclude that the interaction between pH and nitrate is important, although little data are available to explain the interaction. One possible mechanism, based on data from crabs, is as follows: nitrate and nitrite enter the body of a freshwater fish by crossing the gill epithelia and accumulating in extracellular fluid; these ions are transported against a concentration gradient by substituting for chloride in the bicarbonate-chloride exchange mechanism that normally participates in the osmoregulatory and respiratory functions of the gill (Doblender and Lackner, 1996; Jensen, 1996; Lee and Pritchard, 1985; Panesar, 1999); because chloride cells in the gill also participate in acid-base regulation (Hirose et al., 2003), their function is likely to be affected by pH. In crayfish, nitrate uptake is pH dependent, with uptake increasing as

water pH declines (Jensen, 1995). Thus, as suggested by our data, the physiological influence of nitrate may be magnified by lower water pH.

Gonopodium Length

In addition to the above variables, fish from high nitrate springs possessed longer gonopodia (adjusted for standard length). It is not clear whether this significant association results from direct or indirect effects of nitrate on mosquitofish physiology or if the association is accidental. In *Gambusia*, gonopodium length appears to be highly plastic and affected by a number of selective pressures. For example, Langerhans et al. (2005) observed that female *G. affinis* and *G. hubbsi* preferred males with longer gonopodia, whereas males with shorter gonopodia survive better when predators are present. This is because a longer gonopodium hinders the burst-swimming behavior used to evade predators (Langerhans et al., 2005). However, Jennions and Kelly (2002) observed that male *Brachyrhaphis episcopi* (Poeciliidae) exhibited longer gonopodia in the presence of predators. The authors of both these studies suggested that differences in food availability (potentially nitrate dependent) or flow rates (which vary among the springs) might also explain variation in gonopodial length.

Testicular Hypertrophy and Muscle 11-KT Concentrations

Testicular hypertrophy could be a compensatory response to reduced steroidogenesis (11-KT in our study) (Mylchreest et al., 2002). As explained previously, nitrate can be converted *in vivo* to nitrite and then nitric oxide, especially under changing pH conditions, and nitric oxide has been shown to reduce testicular steroidogenesis (Panesar and Chan, 2000). When target tissues detect that circulating androgens are low, either because they are reduced, or because the androgen receptors are blocked or down-regulated, gonadotropins are released from the pituitary. In male rats treated with

antiandrogens, the gonadotropin release is followed by compensatory hypertrophy and/or hyperplasia of Leydig cells (O'Connor et al., 2002). This Leydig cell increase could account for the increased testicular size associated with nitrate exposure. The observed decreases in 11-KT could also, in part, explain the lower total sperm counts exhibited by males from high nitrate springs, since 11-KT is required for successful spermatogenesis in teleosts (Miura and Miura, 2003). In African catfish and Japanese eels, 11-KT is the primary androgen involved in spermatogenesis, but its concentration in relation to that of testosterone is also relevant, as both androgens provide feedback to the pituitary (Cavaco et al., 2001; Schulz and Miura, 2002).

Spermatogenesis

In addition to possible indirect negative effects on spermatogenesis through reductions in synthesis of 11-KT, the influence of nitrate on spermatogenesis may also be direct. In *Gambusia*, multiple spermatogonia are grouped in cysts, surrounded by Sertoli cells (Fraile et al., 1992). At the onset of spermatogenesis, these germ cells undergo mitosis, forming hundreds of spermatocytes, which then complete meiosis to form a few thousand spermatids (Koya and Iwase, 2004). After spermatozoa are formed, the cyst is released to the sperm duct as a spermatozeugma (Koya and Iwase, 2004). Therefore, sperm count per spermatozeugma, which we measured in our study, depends on the rates of cell division and the incidence of apoptosis. If nitrate is converted *in vivo* to nitrite and then nitric oxide (Modin et al., 2001; Panesar and Chan, 2000; Weitzberg and Lundberg, 1998), it has the potential to reduce sperm numbers per spermatozeugma by increasing apoptosis of sperm cells at various stages of development (germ cells, spermatocytes, and spermatids), as has been observed in mammals (Di Meglio et al., 2004; El-Gohary et al., 1999; Lue et al., 2003; Zini et al., 1996). This would explain the significant, negative

relationship we observed between nitrate and total sperm counts per spermatozeugma. Although we observed a negative relationship between nitrate and total sperm count, we did not observe spring-related differences in the percentage of live sperm, suggesting that, if increased apoptosis is responsible for lower total sperm counts among fish from high nitrate springs, then it occurs mostly at the level of the germ cell, or degenerate sperm cells must be cleared from the spermatozeugmata before they are released to the sperm duct.

Conclusions

Though correlative, our findings indicate that environmental exposure to nitrate is related to changes in several reproductive variables among adult male mosquitofish. Future experimental studies are needed to establish cause and effect. These studies should focus on the processes of steroidogenesis and spermatogenesis in relation to nitrate concentrations as low as 4 mg/L NO₃-N. In addition, studies are needed to investigate possible interactions between nitrate uptake and pH, given the widespread and well-documented occurrences of fresh water acidification (Dunson et al., 1992).

Table 6-1: Florida collection sites for male *Gambusia holbrooki*. Parameter values (± 1 SE) were obtained at the time and location(s) of the fish collection.

Spring	Location (GPS)	Water Temperature (°C)	pH	Conductivity (uS)	Dissolved O ₂ (mg/L)	NO ₃ -N (mg/L)
Blue	N 29°49'49.2"; W 082°40'56.6"	23.2	7.27	346.5	5.22	1.51
Fanning	N 29°35'15.0"; W 082°56'08.0"	22.60 \pm 0.06	7.09 \pm 0.01	470.9 \pm 3.6	1.89 \pm 0.20	4.03 \pm 0.41
Hart	N 29°40'30.4"; W 082°57'05.0"	22.35 \pm 0.25	7.10 \pm 0.01	402.1 \pm 0.9	0.39 \pm 0.07	0.81 \pm 0.04
Lily	N 29°49'48.6"; W 082°39'37.7"	22.3	7.19	425.1	0.84	0.32
Manatee	N 29°29'20.6"; W 082°58'40.0"	22.85 \pm 0.25	7.16	479.1 \pm 0.6	1.94 \pm 0.14	1.26 \pm 0.16
Peacock	N 30°07'18.0"; W 083°07'57.0"	22.50 \pm 0.44	7.35 \pm 0.07	362.2 \pm 1.6	2.02 \pm 0.27	1.69 \pm 0.15
Poe	N 29°49'33.0"; W 082°38'58.0"	22.40 \pm 0.06	7.19 \pm 0.01	415.1 \pm 0.2	0.59 \pm 0.28	0.22 \pm 0.01
Ruth	N 29°59'44.0"; W 082°58'38.0"	21.37 \pm 0.50	7.02 \pm 0.07	404.2 \pm 4.3	1.17 \pm 0.44	5.06 \pm 0.61

Table 6-2. Male Mosquitofish Sample Sizes

Spring	SL, body weight	Gonopodium length	GSI	HSI	Muscle 11-KT	Muscle T	Total sperm per szm	Viable Sperm (% live)	Live sperm count
Blue	35	34	19	20	19	19	15	14	14
Fanning	50	49	20	20	15	15	15	15	15
Hart	39	38	20	20	19	19	16	16	16
Lily	34	34	20	19	20	20	14	13	13
Manatee	41	41	20	20	18	18	15	15	15
Peacock	51	51	18	18	13	13	15	12	12
Poe	55	55	20	20	14	15	18	19	18
Ruth	51	51	20	20	14	14	14	15	14

SL = Standard Length

GSI = Gonadosomatic Index

HSI = Hepatosomatic Index

11-KT = 11-ketotestosterone

T = testosterone

Szm = spermatozeugma

Table 6-3. Significant relationships among water quality parameters from fish-collection sites and response variables measured in adult male *Gambusia holbrooki* collected from eight Florida springs.

Response Variable	Step	r^2	p -value	Water Parameter	Partial r
Standard length	0				
Body weight	0				
Condition	0				
GSI (log ₁₀ testicular weight adjusted for log ₁₀ body weight)	1	0.73	0.007	Temperature	-0.85
	2	0.91	0.003	Temperature NO ₃ -N	-0.87 0.81
HSI (log ₁₀ hepatic weight adjusted for log ₁₀ body weight)	1	0.75	0.005	Temperature	-0.87
Gonopodium length (adjusted) for SL)	1	0.67	0.01	NO ₃ -N	0.82
Sperm count per szm	1	0.61	0.02	Temperature	0.78
Live sperm count per szm	1	0.53	0.04	Temperature	0.73
Muscle 11-ketotestosterone	1	0.56	0.03	pH	0.75
	2	0.91	0.003	pH Dissolved oxygen	0.95 -0.89
Temperature removed from the analysis*					
GSI (log ₁₀ testicular weight adjusted for log ₁₀ body weight)	1	0.63	0.02	NO ₃ -N	0.79
Sperm count per szm	1	0.50	0.05	NO ₃ -N	-0.71
HSI (log ₁₀ hepatic weight adjusted for log ₁₀ body weight)	0				
Live sperm count per szm	0				
*The significant influence of temperature on GSI, HSI, and sperm count data are largely driven by a lower temperature at Ruth Spring, just 0.9-1.8°C less than the other seven sites. Given that this small difference may be within the error of natural variation, we repeated the stepwise analysis without temperature as an independent variable.					

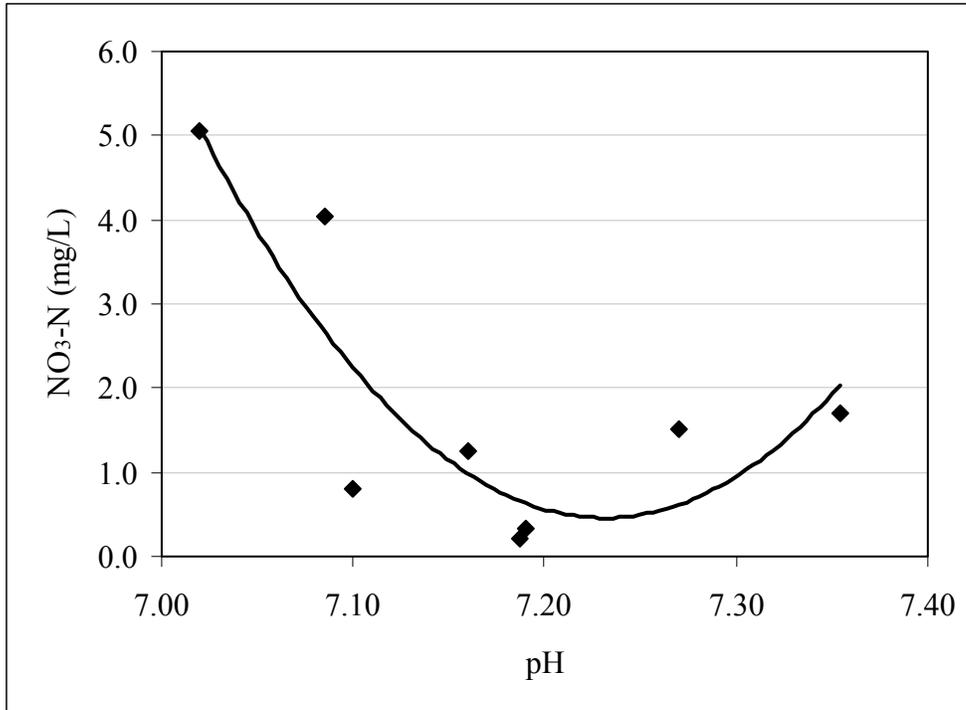


Figure 6-1. Relationship (second order polynomial), between water nitrate concentrations and water pH among eight Florida springs

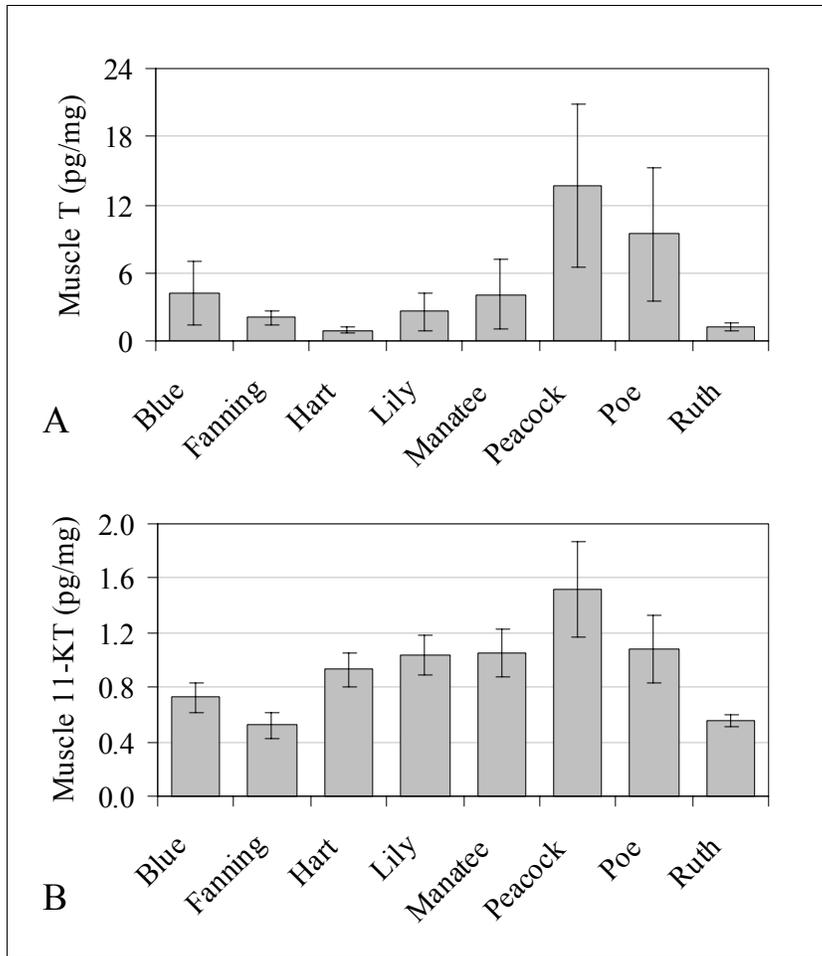


Figure 6-2. Muscle androgen concentrations for adult male mosquitofish collected from eight Florida springs. A) Testosterone. B) 11-KT. Graphs show means ± 1 SE. Note that the y-axis scales are not the same for the two androgens. Muscle 11-KT concentrations were significantly related to spring pH, oxygen concentration, and nitrate load (Table 3 and Fig. 6-6).

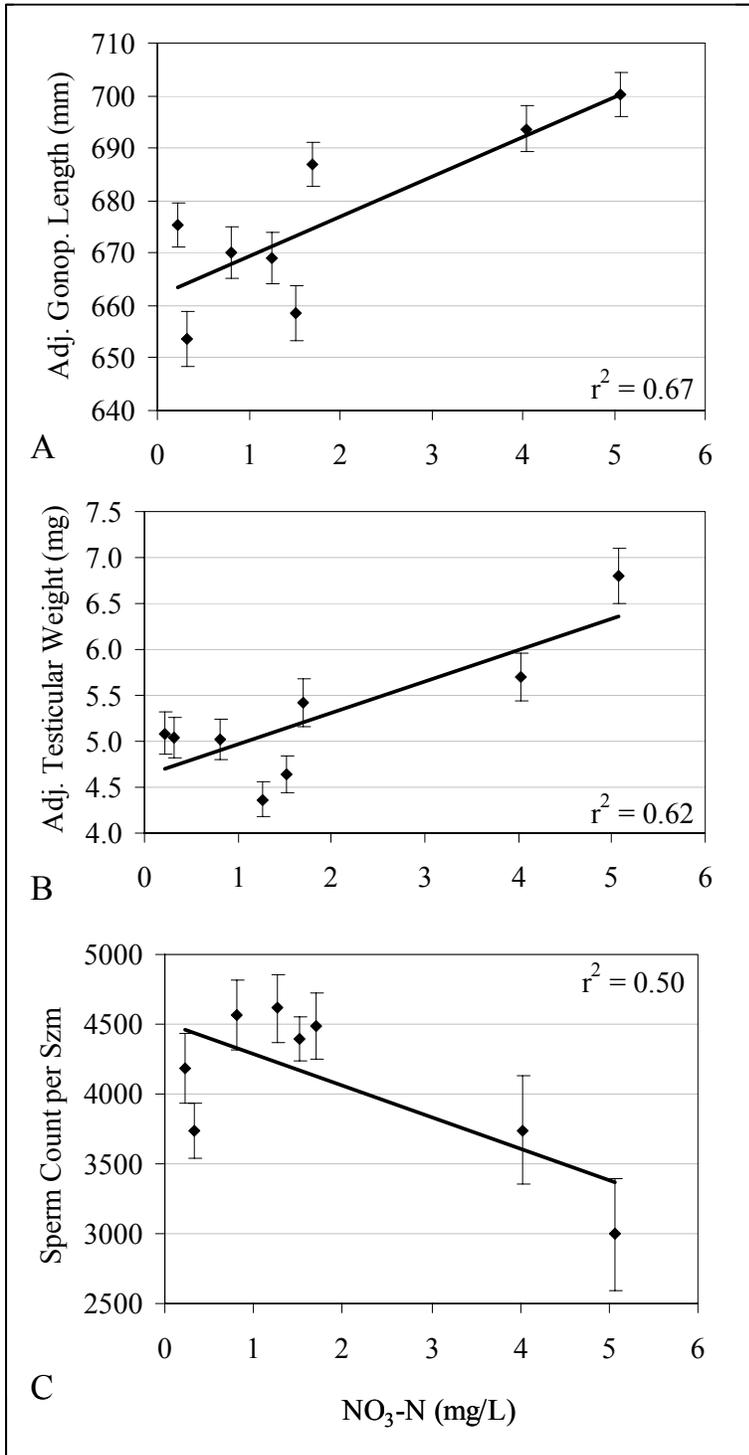


Figure 6-3. Linear relationships between water nitrate concentrations and A) adjusted mean gonopodium length; B) adjusted mean testicular weight; and C) mean sperm count per spermatozeugma of adult male mosquitofish captured from eight Florida springs. Graphs show adjusted means ± 1 SE. Regressions are significant at $p \leq 0.05$.

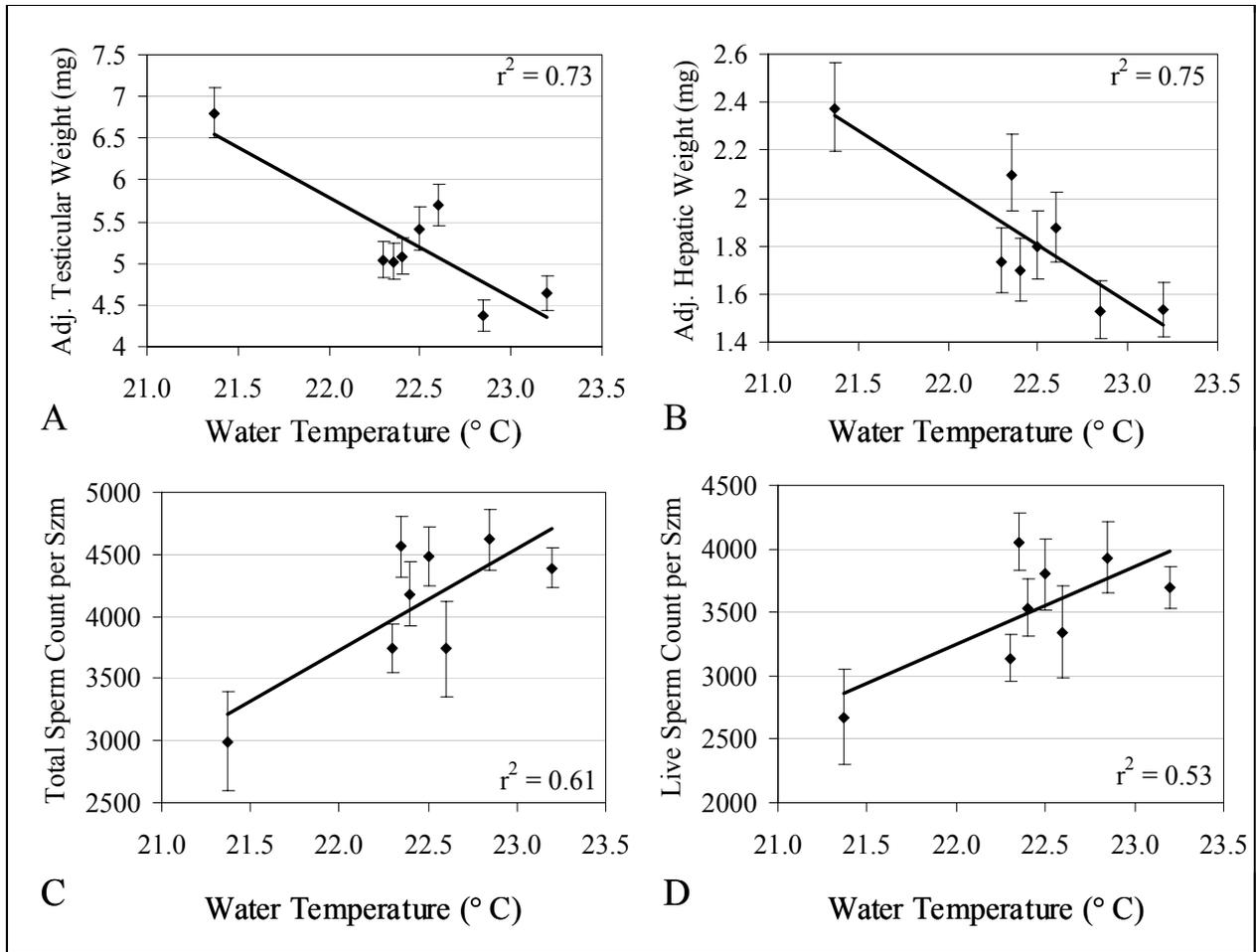


Figure 6-4. Linear relationships between water temperature (°C) and A) adjusted mean testicular weight; B) adjusted mean hepatic weight; C) mean sperm count per spermatozeugma; and D) mean live sperm count per spermatozeugma of adult male mosquitofish captured from eight Florida springs. Graphs show adjusted means \pm 1 SE. Regressions are significant at $p \leq 0.05$.

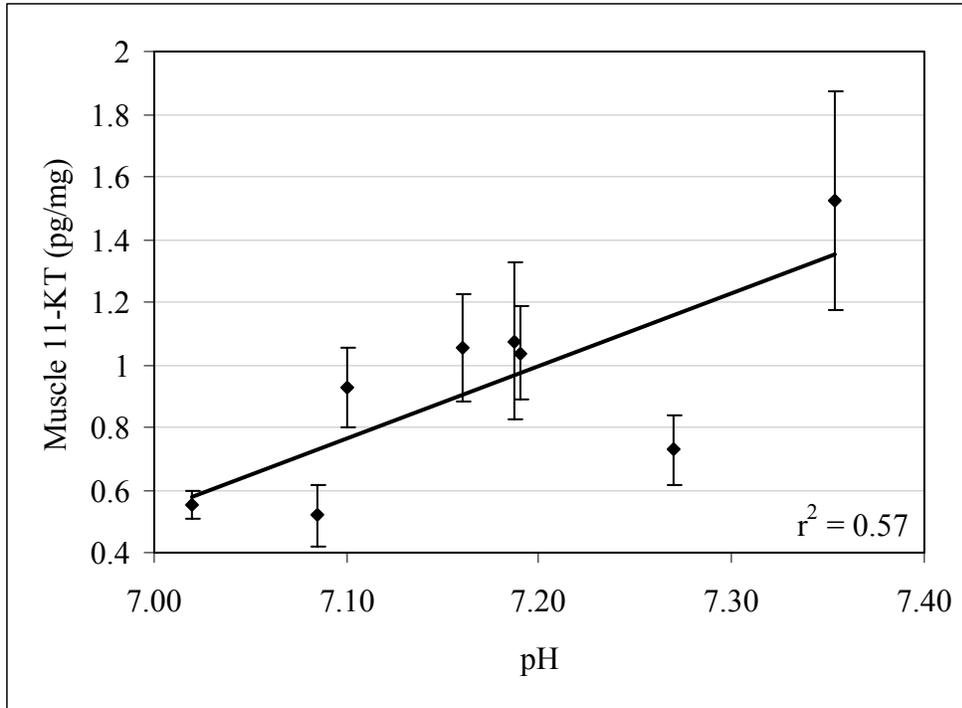


Figure 6-5. Linear relationship between muscle 11-KT concentrations and water pH for adult male mosquitofish captured from eight Florida springs. Graph shows adjusted means \pm 1 SE. Regression is significant at $p \leq 0.05$.

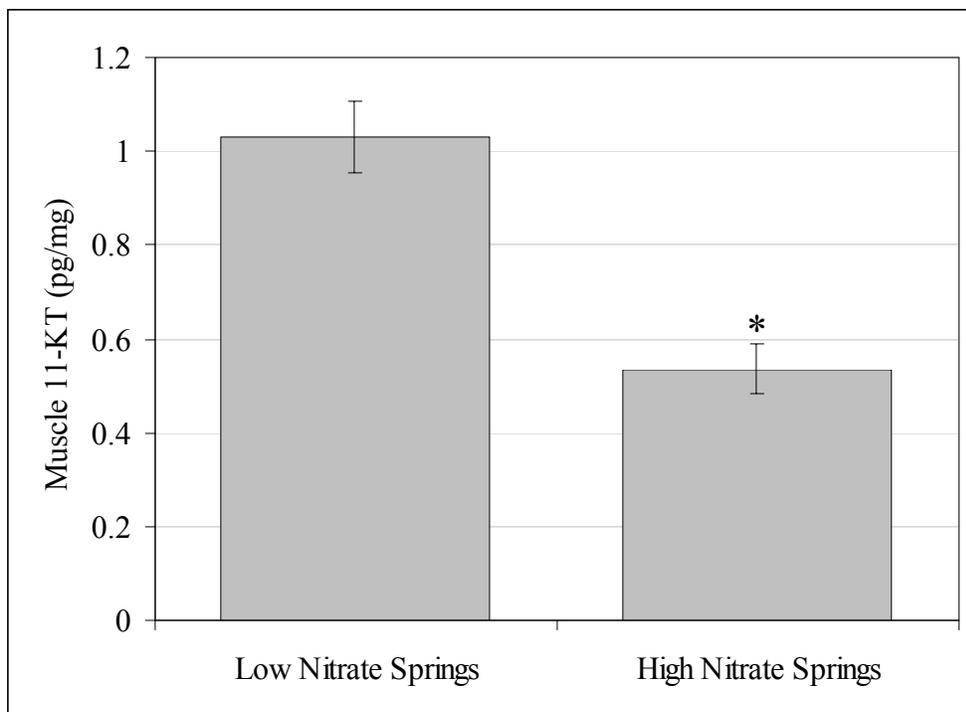


Figure 6-6. Mean muscle 11-KT concentrations (± 1 SE) of adult male mosquitofish captured from high (4.0 – 5.1 mg/L $\text{NO}_3\text{-N}$) or low (0.2 – 1.7 mg/L $\text{NO}_3\text{-N}$) nitrate springs. * $P = 0.0008$ based on ANOVA of $\log_{10}(y+1)$.

CHAPTER 7 SUMMARY AND FUTURE DIRECTIONS

Overview

This study is the first relatively long-term reproductive study of adult female and male mosquitofish to join the expanding endocrine disruption literature. It builds on a few previous endocrine disruption studies (summarized in Table 7-1) along with several available papers on *Gambusia* basic biology (i.e., Bisazza and Marin, 1995; Fraile et al., 1994; Koya et al., 2000). The result greatly expands and integrates our knowledge of *Gambusia* reproduction in terms of seasonal variation, life history, and potential influences of environmental contaminants. In addition, it forms the needed foundation on which to build a more detailed understanding of endocrine disruption, using *Gambusia* as a model species. This second idea is described in greater detail below.

Summary

Our results show that both females and males exhibit altered reproductive parameters in association with exposure to several endocrine-disrupting contaminants (EDCs), including organochlorines and nitrate (summarized in Table 7-1). In Chapters 2 through 4, we investigated seasonal reproduction of adult *Gambusia* from Lake Apopka (organochlorine pesticide contamination) and Lake Woodruff (reference). As predicted by the authors of previous studies (Table 7-1), both males and females from Lake Apopka exhibited increased hepatosomatic index relative to the population from Lake Woodruff. Apopka females also exhibited temporally altered estradiol patterns and a substantial increase in fecundity. Relative to males for Lake Woodruff, males from Lake

Apopka exhibited increased testicular size, although with no apparent benefit in terms of improved sperm production. In fact, their sperm counts and viability were significantly reduced in some months, particularly at the end of the reproductive season.

In Chapters 5 and 6, we assessed reproduction at one point during the breeding season among male and female mosquitofish captured from eight Florida springs with varying concentrations of nitrate. These last two chapters represent the first detailed reproductive studies of wild fish from aquatic systems with elevated nitrate concentrations. Our results are summarized in Table 7-1. In males exposed to high nitrate, we observed increased gonadosomatic index, reduced 11-ketotestosterone concentrations, and reduced total sperm counts (per spermatozeugma). Females exposed to high nitrate exhibited reduced embryo dry weights and a decreased rate of reproductive activity, based on the presence or absence of vitellogenic oocytes.

New Hypotheses and Development of *Gambusia* as a Model Species

As with most scientific studies, our study has generated new data, has both corroborated and contradicted previous findings (Table 7-1), and has suggested new hypotheses that logically arise from the results presented in each chapter (summarized in Table 7-2). These outcomes, coupled with the ubiquitous global distribution of *Gambusia* and their ability to tolerate polluted water (Courtenay and Meffe, 1989), suggest that *Gambusia* are well suited to be designated a sentinel species. In addition, several aspects of their biology are conducive to novel avenues of inquiry that are relevant to the fields of endocrine disruption and aquatic toxicology.

For example, unlike other established fish models used to study endocrine disruption, such as English roach (Jobling et al., 2002a), Japanese medaka (Nakayama et al., 2005), fathead minnow (Leino et al., 2005), or common carp (Gimeno et al., 1998b),

Gambusia are viviparous, and, based on our data, matrotrophic, at least through the first two thirds of gestation (Chapters 2 and 5). In Chapter 5, we observed a decreased rate of pregnancy and reduced embryo dry weights among female mosquitofish from high nitrate springs. In a viviparous species such as *Gambusia*, these observations suggest that nitrate exposure impairs maternal provisioning. A viviparous model makes it possible to differentiate between potential effects of nitrate on vitellogenesis, which occurs largely before fertilization, and effects on direct maternal provisioning during gestation. Also, the mosquitofish model promotes investigation of offspring health after maternal exposure that occurred before versus during gestation. In addition to their importance to wildlife conservation, these types of studies are compelling from the perspective of human pregnancy. They inform fetal risk assessment by comparing the effects of female body burdens obtained before pregnancy versus exposure during pregnancy.

In Chapters 3 and 6, we documented reduced sperm counts among males collected from Lake Apopka and high nitrate springs. As detailed in Chapter 3, *Gambusia* males produce and package their sperm in spherical packets (spermatozeugmata). Spermatogenesis begins with mitotic proliferation of spermatogonia within cysts that are bounded by Sertoli cells (Fraile et al., 1992). The resulting primary spermatocytes undergo meiosis and differentiation to produce spermatids and ultimately tailed spermatozoa (Fraile et al., 1992; Koya and Iwase, 2004). Because we measured sperm count per spermatozeugma, our observation of lowered sperm counts suggests specific follow-up hypotheses (Table 7-2) that are ideally addressed using the *Gambusia* model. Sperm count per spermatozeugma is most likely to be affected by altered spermatogonial mitosis or meiosis, germ cell or sperm cell apoptosis, or reduced functionality of Sertoli

cells. The clustering of all the products of cell division from a single spermatogonium into one spermatozeugma makes it possible to quantify the spermatogenic capacity of a male. In addition, cells resulting from meiosis versus mitosis are distinguishable based on size and morphology (Fraile et al., 1992), making the two processes separable in the search for mechanisms of endocrine disruption. Fraile et al. (1992) suggest that a single Sertoli cell surrounds each sperm packet. If this is the case, then the number of spermatozeugmata in the testis reflects the number of available Sertoli cells. In addition, reduced sperm number may reflect decreased capacity on the part of the Sertoli cell to support sperm cells. Investigation of these hypotheses could identify new potential mechanisms by which endocrine disruption can occur.

In Chapter 4, we observed temporal variation in the patterns of mean male body size that we interpreted as reflective of changes in adult male recruitment patterns. In Chapter 4, we speculated that males from Lake Apopka exhibited delayed puberty, an outcome that could be either beneficial or deleterious to fitness. Combined with data from *Gambusia* studies by Bisazza and Marin (1995), in which they report female preference for large males contrasted with increased copulatory success by small males (sneakers), it is clear that *Gambusia* are an interesting model with regard to the interface of endocrine disruption, sexual selection, and male reproductive strategy. For example, in Chapter 4, males from Lake Apopka were larger than males from Lake Woodruff in most months. This larger size could be due to nutrition, or it could be a response to the observed increase in female size (Chapter 2) coupled with sexual selection for larger size in males. The timing of puberty is marked by development of the gonopodium and is thus easily assessed in male *Gambusia*. If puberty were delayed in males from Lake

Apopka, it would be necessary to differentiate between the effects of organochlorines on puberty versus the effects of an energetic trade-off between growth and sexual maturation that was under selective pressure.

Another consideration that could influence *Gambusia* populations living in close contact with environmental contaminants is that they potentially adapt to these environments. Again, as a model species, *Gambusia* offers an interesting opportunity to investigate adaptation to chemical perturbation. In Chapter 2, we observed that female mosquitofish exhibited substantially greater fecundity (adjusted for body size) relative to females from Lake Woodruff. One easily tested possibility is that increased fecundity is a response to higher larval mortality. Alternatively, females from Lake Apopka may in fact be better adapted to their environment than females from Lake Woodruff. Anecdotally, adult fish from Lake Apopka generally had higher short-term survivorship in the laboratory relative to those from Lake Woodruff. In other studies, the ability of mosquitofish to excel in polluted environments has been marked by increased heterozygosity and overall genetic diversity among exposed individuals (Downhower et al., 2000; Stockwell and Vinyard, 2000; Theodorakis and Shugart, 1997). In addition, genetic diversity is supported by *Gambusia*'s mating system, in which females often mate with more than one male, resulting in broods characterized by multiple paternity (Zane et al., 1999). Thus, mosquitofish potentially provide an opportunity to study interactions among genetic diversity, adaptation, and environmental contamination. Not only are mosquitofish a model for the potentially negative effects of endocrine disruption, but also, they may provide adaptive answers that will allow long-term survival of this species in environments contaminated with anthropogenic, biologically active agents.

Class of Endocrine Disruptors [†]	Sample Compounds	Observed Alterations in Reproduction Caused By or Associated With Endocrine Disruptor Exposure	Edwards, 2005
Estrogen	Estradiol ^{cghrstx} 4-Tert-pentylphenol ^{fgh} 4-Tert-octylphenol ^{sx} Octylphenol ^l P-nonylphenol ^a 4-Nonylphenol ^{de} Nonylphenol ^w Endosulfan ^w Kepone ^b DDD ^{byz} Bisphenol A ⁱ PCBs ^{jyz} Treated sewage effluent ^{klnnop} Chlordane ^{qyz} Chronic hypoxia (1 ± 0.2 mg/L) ^v Toxaphene ^{yz} DDE ^{yz} Organochlorine mixture ^{yz*}	<p>↑Plasma vitellogenin^{ghlmnp} ♀♂</p> <p>↑Hepatosomatic index^{lt} ♂</p> <p>↑Ovotestes/intersex^{aghkmnqs} ♂</p> <p>↑Oviduct formation^{gfk} ♂</p> <p>↓Delayed puberty/persistent immature testes^d ♂</p> <p>↓Gonadosomatic index^{tux} ♂</p> <p>↔Sertoli cell structure^t ♂</p> <p>↓Gonadal development^v ♀♂</p> <p>↓Diameter of seminiferous tubules^h ♂</p> <p>↑Atrophy of germinal epithelium^h ♂</p> <p>↓Primordial germ cell number^{fg} ♂</p> <p>↔PCG distribution in developing gonad^w</p> <p>↑Malformed germ cellsⁿ (intersex fish)</p> <p>↓Delayed spermatogenesis^{ghn} ♂</p> <p>↑Loss of spermatogenic cysts^h ♂</p> <p>↓Sperm Counts^{iknz} ♂</p> <p>↓Milt volume^{knt} ♂ (intersex fishes)</p> <p>↓Sperm motility^{kv} ♂ (intersex fishes)</p> <p>↑Occluded reproductive ducts^{nt} ♂ (intersex fishes)</p> <p>↓↔Delayed gonopodial development^{cd} ♂</p> <p>↓Genital papilla length^r ♂</p> <p>↓Adult coloration^x ♂</p> <p>↓Courtship behavior^c ♂</p> <p>↓Fertilization success^{kv} ♀♂ (intersex fishes)</p> <p>↓Embryonic/larval survivorship^{ejuv} ♀♂</p> <p>↓Oocyte maturation^b ♀</p> <p>↑Oocyte atresiaⁿ ♀</p> <p>↓Embryo growth^s ♀♂</p> <p>↓Ca⁺⁺, amino acid availability to fetuses during gestation^s</p> <p>↓Delayed hatching^{iv} ♀♂</p> <p>↑E₂ binding in liver^s ♀</p> <p>↓Serum E₂, T^v ♀</p> <p>↑Plasma T, 11-KT^y ♀</p> <p>↔ Plasma E₂, Tⁿ (intersex fish)</p> <p>↑Serum E₂^{vy} ♂</p> <p>↓Serum T, 11-KT^{puv} ♂</p> <p>↑17, 20-DHP^u ♂</p>	<p>↑Body size ♀♂</p> <p>↑HSI ♀♂</p> <p>↑GSI (without increased androgen or sperm production) ♂</p> <p>Premature loss of sperm viability at end of reproductive season ♂</p> <p>↓Live/total sperm counts per spermatozeugma ♂</p> <p>↓Sperm viability (based on membrane integrity) ♂</p> <p>No strong effect on gonopodium length ♂</p> <p>↑Fecundity with stronger relationship between fecundity and body size ♀</p> <p>No apparent effect on embryo growth ♀</p> <p>↔Temporal E₂ pattern, with much higher E₂ in spring and lower E₂ in fall ♀</p>

Table 7-1 Continued

Class of Endocrine Disruptors [†]	Sample Compounds	Observed Alterations in Reproduction Caused By or Associated With Endocrine Disruptor Exposure	Edwards, 2005
Anti-Androgen	Vinclozolin ^{abc} DDE ^{abde} Flutamide ^{ab}	↓Sperm count ^{abce} ♂ ↓Fertilization success ^c ♂ ↓Adult coloration ^{abc} ♂ ↓GSI ^a ♂ ↓Courtship behavior ^{abc} ♂ ↓Delayed maturation ^b ♂ ↓Gonopodial development ^b ♂ ↑Serum E ₂ ^d ♂ ↑Plasma T, 11-KT ^d ♀	Premature loss of sperm viability at end of reproductive season ♂ ↓Live/total sperm counts per spermatzeugma ♂ ↓Sperm viability (based on membrane integrity) ♂ No strong effect on gonopodium length ↑GSI (without increased androgen or sperm production) ♂
Androgen	11-ketotestosterone ^b Paper mill effluent ^{acdef} Methyl-testosterone ^{fg}	↑Gonopodial development ^{abcdf} ♀ Male biased sex ratio ^e ↑Male coloration ^f ♀ ↓Number of reproductive females ^f ♀ ↑Intersex ^g ♀♂	
Aromatase ^{**} Inhibition	Tributyltin ^{abcd}	Male-biased sex ratio ^d ♀♂ ↓Sperm counts ^a ♂ ↑Sperm lacking flagella ^d ♂ ↓ATP content of sperm ^b ♂ ↓Lactate dehydrogenase activity in sperm ^b ♂ ↓Sperm motility ^{bd} ♂ ↓Fertilization success ^c ♀♂ ↓Hatchability ^c ♀♂ ↓Embryo survivorship ^c ♀♂	
Other	Landfill leachate ^{ab}	Male-biased sex ratio ^{ab} ↓GSI ^{ab} ♀ ↓Brain aromatase activity ^a ♀ ↓Plasma T, E ₂ ^a ♀ ↓Delayed vitellogenesis ^b ♀	

Table 7-1 Continued

Class of Endocrine Disruptors [†]	Sample Compounds	Observed Alterations in Reproduction Caused By or Associated With Endocrine Disruptor Exposure	Edwards, 2005
Other	Nitrate ^c	↓Spawning ^c ♂ ↓Egg number ^c ♀ ↓Fertilization rate ^c ♀♂ ↓Delayed hatching time ^c ♀♂ ↓Hatching rate of the eggs ^c ♀♂	↑GSI ♂ ↓11-KT ♂ ↓Total sperm count per spermatozeugma ♂ ↓Embryo dry weight ♀♂ ↓Rate of pregnancy ♀
<p>[†]Endocrine disruptor classification is general and non-exclusive. Several chemicals operate through a number of mechanisms that vary with context. For example, DDE has been called an estrogen, an anti-estrogen, and an anti-androgen. Classifications presented here depend on the papers cited.</p> <p>*Organochlorine mixture – refers to the chemical mixture detected in the plasma of juvenile alligators from Lake Apopka. The mix includes PCBs, DDE, DDD, mirex, endrin, dieldrin, trans-nonachlor, and oxychlorodane. These chemicals cause male to female sex reversal of reptile embryos (reviewed by Guillette et al., 2000)</p> <p>**Aromatase catalyzes the conversion of testosterone to estradiol, and androstenedione to estrone (Johnson and Everitt, 1995).</p>			
<p><i>Italicized descriptors</i> refer to papers on <i>Gambusia</i> species. Superscripts match sample compounds and observed effects. Citations are given in Table 1-1. Highlighted descriptors are related to hypotheses tested in our study. ♀ = female. ♂ = male. (Intersex fish(es)) = the sex of the fish(es) in the cited study was an abnormal mix of female and male. ↑ = increase. ↓ = decrease or inhibition. ↔ = altered. 11-KT = 11-ketotestosterone. ATP = adenosine triphosphate. DDD, DDE = metabolites of the insecticide DDT. GSI = gonadosomatic index. E₂ = estradiol. HSI = hepatosomatic index. PCBs = polychlorinated biphenyls. PGC = primordial germ cell. T = testosterone. T₃ = tri-iodothyronine. 17, 20-DHP = 17α-20β-dihydroxyprogesterone.</p>			

Table 7-2. Summary of New Hypotheses Suggested by Dissertation Results	
Chapter 2	Predicted reproductive variation among female <i>Gambusia holbrooki</i> living in Lake Apopka relative to Lake Woodruff
	<ul style="list-style-type: none"> ↑Vitellogenesis ↑Genetic diversity of females and offspring ↓Larval survivorship
Chapter 3	Predicted changes in sperm quality and seasonal sperm availability among male <i>Gambusia holbrooki</i> living in Lake Apopka relative to Lake Woodruff
	<ul style="list-style-type: none"> ↑Apoptosis or degeneration of germ and sperm cells. ↓Mitosis/meiosis of germ cells. ↓Sertoli cell number/function ↓Efficacy or proper timing of 11-KT or other cellular signals associated with spermatogenesis, especially during transition between breeding and winter quiescence
Chapter 4	Predicted reproductive variation (except sperm quality) among male <i>Gambusia holbrooki</i> living in Lake Apopka relative to Lake Woodruff
	<ul style="list-style-type: none"> ↓Delayed puberty among males based on temporal variation in mean body size ↑Vitellogenesis Compensatory hypertrophy or hyperplasia (most likely) of Leydig cells to ensure adequate androgen production
Chapter 5	Predicted reproductive variation among female <i>Gambusia holbrooki</i> living in high nitrate springs
	<ul style="list-style-type: none"> ↓Thyroid function Delayed puberty or onset of spring ovarian recrudescence ↓Vitellogenesis or vitellogenin sequestering by ovary ↓Maternal nutrient availability to embryos during gestation ↓Larval survivorship
Chapter 6	Predicted reproductive variation among male <i>Gambusia holbrooki</i> living in high nitrate springs
	<ul style="list-style-type: none"> ↑Sperm cell apoptosis ↓Spermatogonial mitosis/meiosis ↓Sertoli cell number/function ↓Activity of steroidogenic enzymes Compensatory hypertrophy or hyperplasia of Leydig cells to ensure adequate androgen production Possible deleterious effects from interaction between rising nitrate and low pH

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BIOGRAPHICAL SKETCH

Thea M. Edwards was born on June 30, 1970 in Harare, Zimbabwe. She spent her early childhood in the African great outdoors. Starting at the age of 8, she accompanied her adventurous parents on three trans-Atlantic voyages and an extended tour of North America. While in the United States, her family lived in Pennsylvania, New Jersey, and Virginia. In 7th grade, she dissected a clam, and decided that biology was for her. In 1988, Thea graduated from James Madison High School in Vienna, Virginia.

After high school, Thea attended Virginia Tech in Blacksburg, Virginia. She majored first in wildlife conservation, and then in horticulture, graduating with her bachelor's degree in 1991. In 1993, she was accepted to the graduate program in horticulture at the University of Florida, where she completed her master's degree on the hormonal effects of ethylene on miniature roses. She then completed a second, nonthesis master's degree in botany, with a minor in education.

In 1998, after extended hard thinking and soul searching for the next step, Thea became acquainted with the research of Dr. Louis Guillette. Dr. Guillette's research encompassed several areas that she wished to include in the development of her scientific career: comparative reproduction, mechanistic endocrinology, nontraditional species, conservation, and human impacts on the environment. During her 6 years as a graduate student in zoology, Thea developed four major research projects, wrote and received a grant to fund her research from the Florida Department of Environmental Protection, taught two courses independently, served as a teaching assistant for 9 semesters, and

mentored 22 undergraduate researchers, two of whom completed honors theses. She completely enjoyed her experience in graduate school and looks forward to a professional scientific and academic career.