EASTERN MOSQUITOFISH AS A BIOINDICATOR OF PULP AND PAPER MILL EFFLUENTS

By

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A variety of sublethal physiological effects have been reported for fish exposed to pulp and paper mill effluents. As mill processing technologies improve, mounting evidence demonstrates fewer effects potentially linked to reduction in wood extractives. Repercussions of sublethal effects at higher levels of biological organization are important questions beginning to be explored. The goal of my study was to evaluate whether sublethal effects in mosquitofish can be used reliably to indicate adverse impact of pulp and paper mill effluents. Biomarkers of anal fin morphology and whole body sex steroids were validated, then studied extensively in wild-caught mosquitofish from Florida effluent-receiving streams that varied markedly in effluent composition. These biomarkers were also examined under short-term controlled whole effluent exposures (caged in field and in tanks at 0, 10, 20, 40, and 80% dilutions); and in relation to fry production and preliminary population surveys. Extent of female anal fin masculinization, or development of male-like secondary sex characteristics, was

associated with increasing concentrations of wood extractives in water samples from field sites. Implementation of EPA's Cluster Rule at one mill was followed by a significant reduction, but not elimination, of this response. However, masculinization could not be reproduced under controlled exposure, likely due to insufficient exposure duration. Alterations in sex steroids were manifested in both sexes for wild-caught and experimentally-exposed fish: males exhibited feminized hormonal profiles will females displayed masculinized profiles. Differential responses among cage-exposed and tank-exposed fish indicated additional environmental factors (such as bacterial communities hypothesized to degrade effluent components into androgenic compounds) were influential in producing responses. However, large natural variation at unexposed sites and indication of seasonality precluded definitive interpretation. The lack of association between these biomarkers demonstrated whole body sex steroids cannot be used to predict morphological masculinization; but they can be compared as biomarkers of recent versus past exposure. Finally, reproductive success studies implied mosquitofish may adapt different reproductive strategies in effluent-receiving streams. Since neither biomarker could be linked to differences in fry production or population structure, at this point mosquitofish may not be a suitable bioindicator of adverse effects due to pulp and paper mill effluents.

CHAPTER 1
MOSQUITOFISH EXPOSED TO PULP AND PAPER MILL EFFLUENTS: USE OF A POTENTIAL INDICATOR OF EXPOSURE AND EFFECTS

Pollution from both point and nonpoint sources of human activity releases a variety of chemicals into the aquatic environment. While lethal effects have been addressed, sublethal effects of these chemicals on aquatic wildlife remain controversial. Whether observed sublethal effects lead to adverse impacts including reproductive, population, or community level effects is arguable. This question has been strongly debated about pulp and paper mill effluents and sublethal effects in fish. The controversy becomes more complex as major processing improvements are implemented by the industry. The goal of my study was to evaluate whether sublethal effects in mosquitofish can be reliably used to indicate adverse impacts of pulp and paper mill effluents.

Background of Pulp and Paper Mills

Economic Importance of Pulp and Paper Industry in the United States

Pulp and paper products (such as writing and copy paper; sanitary tissues; cardboard; linerboard; and indirect products like pill capsules, diapers; and rayon) are significant economic commodities in the United States (US). The US produces around 30% of the world's paper and paperboard (US Environmental Protection Agency (EPA) 2002). In 2000, US pulp and paper mills produced 79 billion US dollars in shipments and employed 182,000 people, while Americans consume around 300 kg of paper-based products each year (EPA 2002). Industrialization of nations increases demand for pulp

and paper products (Smook 1999), resulting in high capital investments and intensive use of forests, water, and energy.

Furnish (tree species or specifically cellulose fiber source) usually comes from harvested hardwood and softwood tree species (Table 1-1). The fiber length varies tremendously between these two tree types (longer in softwoods), so the final product determines which tree type and species is used. Alternative furnishes include recycled paper (increasingly used, especially for products like corrugated board) and nonwood sources such as bagasse, bamboo, cereal straws, cotton rags and linters, flax, hemp, and synthetic fibers (Smook 1999). Pulp and paper production consumes large amounts of forestry resources. Approximately 6 million acres of forest in the southeastern US are logged annually, mostly for paper production (Wear and Greis 2002). The process is also water and energy intensive. The pulp and paper industry is classified as the largest industrial water consumer and the third largest industrial energy consumer in the US (US Department of Commerce 2000, US Department of Energy 2000). Efforts toward more sustainable forestry practices (e.g., the Forest Stewardship Council certification program, http://www.fscus.org/), water use reduction, and increased use of wood waste material for fuel are ongoing to reduce natural resource consumption.

Production Processes and Technologies

The following summary is based on information from Smook (1999) and US EPA (2002), unless cited otherwise. Final paper products are generated in two overall steps: pulp production and paper or paperboard manufacture. Pulp and paper mills can be classified by whether they produce pulp, paper/paperboard, or both (Figure 1-1). Most US mills are nonintegrative facilities and produce paper products using pulp obtained off-

site (54%). About one-third are integrative and produce pulp and final paper products (36%), Ten percent exclusively produce market pulp.

Depending on final product, 80% of market pulp is for paper and 20% for nonpaper (Figure 1-1). Nonpaper pulps are either dissolving pulp, fluff pulp, or specialty pulp. Dissolving pulp is a "chemical cellulose" that can be converted into rayon, cellophane, cellulose acetates, cellulose nitrate, and carboxymethyl cellulose via a modified kraft or sulfite pulping process. These chemicals are used in a variety of nonpaper products ranging from synthetic clothing to pill capsules to air filters. Fluff pulp is a very soft and absorbent form of pulp used in diapers, feminine products, and hospital pads. Specialty pulp comprises the remaining nonpaper pulps that do not fit in the other two groups (final products include components of shoe soles and laminates).

Facilities involved in pulp production (integrative and market pulp facilities) face the biggest challenges of water pollution in the industry. Pulp production consists of five major steps: furnish preparation (debarking and chipping); pulping (breakdown of furnish into fibers); pulp refinement (removal of impurities, cleaning and thickening of pulp); bleaching (to whiten and brighten the pulp); and stock preparation (wet additives are integrated into pulp based upon desired end product). Two of these steps (pulping and bleaching) are considered the dominant sources of water pollution within pulp production (details next).

Pulping

Two major components of wood are cellulose (the fibers) and lignin (the glue holding fibers together). Broadly speaking, pulping unglues wood and reduces it to a fibrous mat. More specifically, the goal of pulping is to retain intact cellulose fibers while releasing all other wood components. These components include hemicellulose;

lignin; and extractives such as resin acids, fatty acids, phytosterols, turpenoids and alcohols. Pulping methodologies are generally classified as chemical, semichemical, or mechanical. Most North American pulping technologies (70%) involve chemical processes (Table 1-1). Chemical pulping digests wood chips at high temperatures and pressures, usually in an alkaline solution (called the kraft process), or historically in an acidic solution (the sulfite process).

The kraft process (also known as the sulfate process) dominates North American chemical pulping technologies (95%). Major advantages over the sulfite process are high strength pulp ("kraft" is German for strong) and recovery and reuse of digestion chemicals. Lignin removal is high, allowing for extensive bleaching without pulp degradation (via delignification). Additional advantages are the wide range of furnishes that can undergo the kraft process, and the tolerance for bark. However, pulp yield is relatively low (40-50% of furnish) compared with mechanical pulping. An additional disadvantage is pulp color: the kraft process produces a dark brown pulp that requires extensive bleaching, neutralizing the bleaching-related benefits of high lignin removal. Overall, the kraft process has proven to be the most cost-effective chemical pulping technique.

Kraft pulping is cyclical, beginning and ending with white liquor. White liquor, composed of sodium hydroxide (NaOH) and sodium sulfide (Na_2S) as the active ingredients, is the alkaline solution used to digest wood chips. Temperature and pressure is elevated using more conventional batch digesters or less common continuous digesters. Raw pulp and residual black liquor are produced. The pulp is destined for refinement, bleaching, and stock preparation, while the black liquor is concentrated and burned into

an inorganic smelt that is dissolved to produce green liquor. Some black liquor is washed away into effluent, and some carries over with the pulp. These black liquor losses impact effluent quality. Green liquor is then causticized to regenerate white liquor. This efficient chemical recovery is integral to the success of the kraft process.

Some kraft pulping by-products (turpentine and tall oil) are recovered and either reused as fuel sources or sold, depending on market prices. The rest of the noncellulose wood components are discharged (as part of the final effluent) into an aquatic receiving environment.

Bleaching

Bleaching of pulp is often desirable because it produces a whiter, brighter, softer, and more absorbent end product. Roughly half of all paper products in the US are bleached. Bleaching potential for a pulp depends on two factors.

- Inherent lignin content of furnish: higher lignin content gives a darker color, and softwoods tend to have more lignin than hardwoods.
- Pulping process: sulfite chemical pulping produces a relatively bright pulp with low residual lignin content, while kraft chemical and semichemical pulping produces a darker pulp. The brightness of mechanically produced pulp is dependent on lignin content of furnish.

In general, early stages of the bleaching sequence continue delignification begun during pulping, while later stages focus on oxidation to remove any residual color.

Modern bleaching uses a continuous sequence of alternating acidic and alkaline stages with washing between stages. Washing usually involves large amounts of water that is collected and discharged as part of the final effluent. Several bleaching agents are available (shorthand used by the industry for bleaching sequences is given in parentheses).

- Hypochlorite (H)

- Elemental chlorine (C)
- Chlorine dioxide (D)
- Oxygen (O)
- Hydrogen peroxide (P)
- Ozone (Z)
- Sodium hydroxide (E)

Historically, bleaching was accomplished using hypochlorite (Turoski 1998). Hypochlorite (as either calcium or sodium hypochlorite) was initially the only bleaching agent at the turn of the twentieth century (H or HH bleaching sequence). The addition of elemental chlorine gas commercially in 1930 was a major advance that reduced the amount of hypochlorite needed, and became the standard first stage of bleaching followed by an extraction (E) stage (CEH). A decade later, chlorine dioxide (e.g. CEHDED) and hydrogen peroxide (e.g. CEHD(Ep)D) began commercial use. Chlorine dioxide eventually replaced hypochlorite in the later stages of bleaching by the 1960s (e.g. CEDED) because of its powerful brightening combined with high selectivity for lignin. As chlorine dioxide gained popularity, oxygen and ozone bleaching were initiated (e.g. OCEDED or OZED). These latter agents have been slow to gain acceptance by the industry because of complications with low selectivity for lignin removal. However, as environmental and health concerns about chlorine began forming in the 1970s, reduced-chlorine and chlorine-free methods of bleaching (using 100% chlorine dioxide substitution, hydrogen peroxide, oxygen and ozone) have been expanded and refined. Table 1-1 compares bleaching strategies of the three mills in my study.

Water Pollution and Regulation in the US

As previously emphasized, effluent (discharge of liquid waste from a factory/plant) from integrative and market pulp facilities is of most concern to environmental and human health. Within pulp production, the pulping and bleaching stages primarily

contribute to water pollution. Four categories of water pollution are currently monitored: effluent solids, oxygen demand, color, and toxicity. In addition, major water quality characteristics of effluent-receiving waters (such as pH, temperature, dissolved oxygen, alkalinity, and conductivity) are expected to remain unchanged or minimally changed (and may be subject to regulation as well).

Abatement efforts to control these variables occur within plant processing systems and post-processing. More efficient use of raw materials, reuse of mill waters to create a more closed system, and reduced effluent volume are strategies within mill operations that are very effective at increasing profits and at restricting contaminants produced. Additionally, external or end-of-pipe treatment of effluent helps reduce or remove contaminants. Primary external treatment entails sedimentation in settling basins to remove suspended solids. Secondary treatment reduces biochemical oxygen demand using biological degradation/oxidation. Occasionally, mills also apply a tertiary treatment to reduce color (turbidity), but this step is costly.

Since pulp production releases disproportionate amounts of chemicals into air and water (compared to other industries releasing primarily to land), health-related concerns center on air emissions and aquatic toxicity. With the discovery of dioxins and furans in fish collected downstream of a pulp and paper mill in 1985 (Smook 1999) and related evidence for biological effect in fish by the Environment-Cellulose project of Sweden in the late 1980s (Lehtinen 2004), public attention focused on environmental impacts of pulp and paper mills. Dioxins and furans are a class of chlorinated organic compounds produced mainly by incomplete combustion of organic compounds. Natural sources are forest fires and volcanic eruptions, while human sources include waste incinerators, coal

and oil-fired power plants, vehicle exhaust, chlorinated pesticide and herbicide production, and chlorine bleaching during pulp production. Although pulp and paper mills represent a minor source of chlorinated organics, these compounds are lipophilic and persistent in the environment. Hence, they have the potential to biomagnify up the food chain through fish and potentially to humans, causing sublethal, chronic toxicity (which is not traditionally monitored). The most toxic congeners 2,3,7,8-tetrachlorodibenzodioxin and 2,3,7,8-tetrachlorodibenzofuran (TCDD and TCDF respectively) are classified as probable human carcinogens by the EPA.[1] Release and exposure predominately occurs as mixtures of chlorinated compounds (measured as adsorbable organic halides or AOX) including dioxins and furans, which can enhance toxicity. So pollution prevention efforts have focused on reducing release of chlorinated compounds as a group.

To reduce the toxic release of chlorinated compounds to both air and water, EPA enacted a landmark regulation deemed the Cluster Rule in April 1998. The rule set new baseline limits for toxic and nonconventional pollutant releases; and aims for approximately 60% reduction in air emissions, and virtual elimination of chlorinated organic compounds in water (US EPA 1997). Individual mills are allowed flexibility in tailoring pollution prevention technologies to their specific situations. A voluntary incentive program for technologies above and beyond the rule grants mills a variable compliance period (3 to 8 years). Paper-grade bleached kraft and sulfite mills are most affected by the Cluster Rule, requiring 100% chlorine dioxide substitution for elemental

[1] The US Department of Health and Human Services issues a Toxicological Profile for Chlorinated Dibenzo-*p*-Dioxins containing a detailed survey of human exposure and effects.

chlorine in the bleaching sequence, rendering these mills Elemental Chlorine Free (ECF). In addition to this source control, spill control of black liquor is required. Beyond these two requirements, mills develop their own approved plan to meet the new limits, potentially including voluntary measures such as extended delignification, closed loop technologies, or Total Chlorine Free (TCF) bleaching. Table 1-1 shows different pollution prevention strategies adopted by the mills in my study. Ultimately regulating both media (air and water) at the same time creates a synergistic reduction in pollution.

Once fish living downstream of pulp and paper mills were discovered with measurable dioxins and furans in their tissues, intensive research into exposure and effects on fish was initiated. Concerned with biomagnification to humans and carcinogenicity, regulatory agencies were also interested in potential adverse effects on aquatic life. As the next section shows, that research led to questions about reproductive impairment as an effect of pulp and paper mill effluent exposure.

Effects of Pulp and Paper Mill Effluents on Fish

The interaction between industry and government regulatory agencies (primarily in North America and Scandinavia) produced a large body of knowledge (centered upon fish) concerning aquatic toxicity of pulp mill effluents. In general, effects have shifted from gross alterations in growth and acute, lethal toxicity; to more subtle sublethal effects influencing development, maturation and reproduction. In Canada, regulation passed in the early 1990s (Environmental Effects Monitoring Program) produced a decade of consistent fish research at all Canadian mills (McMaster et al. 2003). Also, since 1991, five international conferences have provided a forum to discuss research on the environmental impacts of pulp and paper mill effluents, all of which have published

proceedings (Sodergren 1991, Servos et al. 1996, Ruoppa et al. 2000, Stuthridge et al. 2003, Borton et al. 2004).

Many of these studies are field-based, precluding an identification of causative agents in effluent. Conversely, studies using controlled exposure to whole effluent dilutions preclude isolation of bioactive effluent components, yet retain environmental relevance. Several research efforts have addressed controlled exposure to specific effluent components that are not easily extrapolated to observed effects in the field. Most recently, efforts to elucidate bioactive compounds have used bioassay-based fractionation studies. While appealing in theory, van den Huevel (2004a) points out two major drawbacks of these studies.

- Isolation of bioactive compounds within mill processes as opposed to in final effluent.
- Dependence on receptor-binding studies (some of which use human receptors as opposed to fish receptors) when a receptor-mediated mechanism has not been firmly established.

Chlorinated organics were thought to be key components causing toxicity. However, their virtual removal from effluent has not been associated with removal of chronic, sublethal effects (Lehtinen 2004). Importantly, implementation of the Cluster Rule has also led to large reductions in nonchlorinated, nonconventional pollutants such as wood extractives, which could be tied to reduction in effects. Despite the above caveats, bioassay-based fractionation studies have provided the most specific attempts at identifying which portions of the nonconventional pollutants may be causing effects. For example, in association with studies on reduced steroidogenesis, lignin derivatives such as polyphenolics were identified as bioactive agents in condensates of black liquor

(Hewitt et al. 2002). On the other hand, phytosterols were not determined to be causing observed effects (Dube and MacLatchy 2001).

Other mechanistic-based studies found ligands for the estrogen receptor and sex steroid binding protein present in pulp mill effluents, implying an estrogenic cause for well-documented reproductive effects (Hewitt et al. 2000, Pryce-Hobby et al. 2003). In support of these findings, known (weakly) estrogenic compounds were recently identified in effluents such as genistein (Kiparississ et al. 2001) and industrial nonionic surfactants (nonylphenol ethoxylates) (Lee and Peart 1999). Additional studies showed significant estrogenic properties of the phytosterol β-sitosterol in fish (MacLatchy and Van der Kraak 1995, Tremblay and Van der Kraak 1998).

Androgenic properties of pulp and paper mill effluents have also been reported (Svenson and Allard 2004). *In vitro* assays using the human androgen receptor showed effluents from softwood furnish, but not hardwood, produced low levels of androgenic activity. Biological treatment of effluent had no effect on androgenicity. Uptake of these androgenic compounds by fish and conjugation in bile was also demonstrated.

However, researchers have had little success in associating androgenic compounds with masculinization effects. For instance, androstenedione and human androgen receptor binding was detected in water and sediment samples downstream from one of the mills in my study and associated with masculinized fish (Parks et al. 2001, Jenkins et al. 2003). Evidence for androstenedione as a bioactive effluent component was then refuted by more quantitative analysis showing androstenedione was present only in fractions that did not induce human androgen receptor activity and expression (Durhan et al. 2002). Phytosterol degradation analysis of effluents from these same waters did not

reveal androstenedione metabolites but detected androsteneone (Quinn 2004). Separate studies also refuted androstenedione and testosterone as the active androgens causing masculinizing effects (Ellis et al. 2003), and showed *in vitro* fish receptor binding responses that do not correlate with *in vivo* effects. Follow up studies with this mill effluent (van den Huevel et al. 2004b), using more accurate measures of both masculinization and fish receptor binding, failed to produce any response. Although no major process changes occurred between studies, treatment system maintenance was improved as indicated by gradual reduction in total suspended solids.

In reality, the effluent components responsible for causing sublethal toxicity in fish may never be determined, even though specific mechanisms of action may be narrowed down. As in other technology sectors, pollution prevention technology in the pulp and paper industry rapidly progresses. For this industry, the movement is toward a closed system with recycling and reuse of all materials (Lehtinen 2004). Implementation of these technologies is the limiting factor, since the industry is capital-intensive; but voluntary incentive programs (such as the one associated with the Cluster Rule) help offset investment risks. As the monitored effects diminish and potentially disappear with improving pollution prevention technologies, identification of the specific bioactive compounds may not be necessary.

The following literature review of effects in fish exposed to pulp and paper mill effluent begins with nonreproductive effects, followed by general reproductive effects. It ends with specific reproductive effects indicating masculinization or femininization of fish.

Nonreproductive Effects

A variety of sublethal, nonreproductive physiological effects have been reported in fish exposed to pulp and paper mill effluents. Alteration of liver function (mainly induction of the detoxifying cytochrome P450 system as measured by ethoxyresorufin-o-deethylase (EROD) activity) was the most consistently reported nonreproductive effect in fish. Additional work with conjugating detoxification systems, stress, hematology, and immunological responses received comparatively scant attention and effects are conflicting. As a more general measure of health, growth rates were also examined, although results are equally difficult to interpret.

Significant EROD induction (typically a 2- to 4-fold increase), usually accompanied by an increase in liver somatic index, is often considered a nonspecific marker of effluent exposure (Rogers et al. 1989, McMaster et al. 1991, Servos et al. 1992, Gagné and Blaise 1993, Ahokas et al. 1994, Munkittrick et al. 1994, Gagnon et al. 1995, Soimasuo et al. 1995, Martel et al. 1996, Martel and Kovacs 1997, Soimasuo et al. 1998, Sepúlveda et al. 2002, van den Huevel et al. 2002). Yet the plethora of compounds known to induce this detoxification response (combined with the variable nature of effluent composition within and among mills) makes it difficult to link this biomarker to specific chemical compounds. Dioxins and furans are strong inducers (Servizi et al. 1993), although EROD induction was also detected when these compounds were not present (Munkittrick et al. 1992). Unfortunately EROD activity could not be consistently tied to reproductive effects (Munkittrick et al. 1999).

Induction of conjugating detoxification systems (as well as stress, hematological changes, and immunological responses) has also been addressed in the literature, albeit with much less intensity compared to EROD activity. Results are often mixed; and most

of this work has not been linked to adverse effects at higher levels of biological organization, so the consequences of these physiological changes remain unclear. For example, oxidative stress has been reported in fish due to induction of hepatic enzymatic (glutathione peroxidase, glutathione *S*-transferase, catalase) and nonenzymatic (glutathione and metallothein) antioxidants, as well as lipid peroxidation in gill and kidney (Oikari et al. 1988, Stephensen et al. 1998, Ahmad et al. 2000, Fatima et al. 2000). However, hepatic antioxidants (mainly glutathione *S*-transferase and glutathione) were not induced by other studies (Mather-Mihaich and DiGuilio 1991, Bucher et al. 1992, Larsson et al. 2002). Similarly, effects on activity of another conjugating detoxification enzyme (uridine diphopsphate glucuronosyltransferase) range from induction to inhibition (Oikari et al. 1983, Förlin et al. 1985, Lindström-Seppa and Oikari 1988, Andersson et al. 1988b, Lindström-Seppa et al. 1989). These mixed results on conjugating detoxification systems are likely due to differences in effluent quality, experimental design, exposure conditions and life stage at time of exposure. They are representative of results for the inter-related responses in stress, immune, and hematological functions (see Sepúlveda 2000 and van den Huevel 2004a for discussion of these latter parameters).

Likewise, conflicting results exist for growth patterns of fish exposed to pulp and paper mill effluents. For instance, Warren et al. (1974) and Munkittrick et al. (1991) detected reductions in growth of fish in laboratory and field collections respectively. Other field and laboratory studies found no effects of pulp and paper mill effluents on growth rates in fish (Servizi et al. 1993, Swanson et al. 1992). Additionally, some field collections documented increased growth at effluent-exposed sites (McLeay and Brown

1974, Sandstrom et al. 1988). Explaining some of these discrepancies, Gagnon et al. (1995) found accelerated growth characteristic of downstream nutrient loading from both natural and anthropogenic sources, independent of effluent exposure. Despite rapid growth, fish collected downstream of the bleached kraft mill did not have concomitant increases in reproductive effort; rather, they exhibited greater length at maturity, reduction in gonad size and highly variable fecundity compared to reference fish. Similar reproductive impairment was detected by Munkittrick et al. (1991) in fish with reduced growth. Such findings focused researchers toward evaluating reproductive impacts of pulp mill effluents on fish.

Reproductive Effects

Dominant reproductive effects of pulp and paper mill effluent exposure include depressed circulating sex steroids associated with alterations in steroidogenic capacity; reduced gonadal development; delayed sexual maturation; and negative impacts on egg and fry quality. Effects on egg production and size have been debatable, probably due to differences in the quality of effluent tested. Although many of these reproductive parameters have improved with changing effluent technologies, the virtual removal of chlorinated organics from effluent has not eliminated responses. This finding leads researchers away from dioxins and furans as causative agents; and toward wood extractives such as phytosterols, which have been reduced but not eliminated by pollution prevention technologies.

The most compelling evidence for physiological reproductive alteration comes from a series of Canadian studies over the past 10 years (summarized by McMaster et al. 2003). Extensive work on white sucker (*Catostomus commersoni*) and many other fish species such as lake whitefish (*Coregonus clupeaformis*) and longnose sucker

(*Catostomus catostomus*) showed inhibited gonadal development (primarily decreased gonadosomatic index) and depressed circulating sex steroids (primarily 17β-estradiol and 11-ketotestosterone) in both sexes (Munkittrick et al. 1998). However, the steroid response was not 100% consistent, especially at more recent, modernized mills. Importantly, steroid effects not observed in the field at a modern mill (Servos et al. 1992) occurred in laboratory exposures of another species at concentrations higher than observed in the receiving environment (Robinson 1994). Thus, bioactive compounds were still being produced, but differential species sensitivity, effluent dilution, and/or conditions of the receiving environment protected wild fish. This emphasized the importance of field studies, despite inherent problems with identifying causative effluent components.

Along with the more persistent reduction in gonadal development of these fishes, a concerted effort was launched to identify mechanisms behind depression of sex steroids. Several sites along the pituitary-gonad axis appeared to be affected by exposure to bleached kraft mill effluents: pituitary function was decreased, ovarian biosynthetic capacity was reduced, and peripheral steroids metabolism was inhibited (Van der Kraak et al. 1992, McMaster et al. 1995, 1996). Conflicting evidence was presented by Gagnon et al. (1994a), who concluded that increased steroid metabolism may reduce steroid levels. These discrepancies may have occurred from capture and handling stress (Jardine et al. 1996). Regardless of the controversy over mechanism, improved processing technologies were followed by partial recovery of reproductive function: steroids and potential mechanistic responses along the biosynthetic pathway were either less impacted

or no longer significant in both wild and cage-exposed fish, although gonad size was often still reduced (Munkittrick et al. 1997, van den Huevel et al. 2004b).

Whether these physiological effects translate into effects on the production, survival, and development of young has been more controversial. Early Scandinavian studies showed dramatic effects of pulp mill effluent on eggs and fry including reduced fecundity, smaller egg size, poorer fertilization of eggs, and decreased viability of fry (Vuorinen and Vuorinen 1985). More recent Scandinavian experiments exposing fish to wood-derived phytosterols (primarily β-sitosterol) showed increased egg mortality, larval deformities, and maternal transfer of phytosterols to offspring (Lehtinen et al. 1999, Mattson et al. 2001). In contrast, exposure to another phytosterol (stigmastanol) unequivocally had no effect on egg, larval, or juvenile survival and quality (NCASI 1999). Similar to Canadian reports of reduced steroids at effluent concentrations higher than receiving stream concentrations, NCASI (1996) documented reduced egg production in 18-100% v/v effluent from a bleached kraft mill. Unlike the Canadian steroid work, though, impacts in the field were not addressed to verify a lack of observed laboratory effects. Initial Canadian reports on sex steroids and gonadal development also indicated reduced egg size and fecundity (Munkittrick et al. 1991). However, further research found fecundity to be quite variable (Gagnon et al. 1994b) and fertility of eggs and sperm not affected at exposed sites (McMaster et al. 1992), despite the well-documented physiological responses.

Research has been conducted on egg and fry characteristics of fish exposed to effluent from the primary mill investigated in our study, before implementation of Cluster Rule process changes. The NCASI (2000a) found egg production (but not hatchability)

significantly reduced at 23% v/v effluent, well within instream concentrations (yearly averages aprroximately 60%).[2] Additionally, exposure to 10% or greater whole effluent dilutions did not result in effects on fecundity, egg size, or hatchability; but caused reduction in fry growth and survival (Sepúlveda et al. 2003). Parent fish also had reduced circulating sex steroids and gonad size at 20-40% effluent dilution, similar to previous findings (Sepúlveda et al. 2001) and in support of Canadian research. These results are perhaps the most convincing link between physiological reproductive impact and more subtle influences on offspring, although effects in fry could have originated from maternal transfer of bioactive effluent components instead of (or in addition to) direct impairment of parental reproductive systems.

Masculinization and Femininization Effects

Studies of masculinization and femininization of fish began with the desire to control sex ratios in the aquaculture industry (Yamazaki 1983). A number of fish species have an innate capacity to regulate sex ratios in the population triggered by subtle social and environmental contexts (Baroiller et al. 1999). On this level, masculinization refers to complete sex reversal (females to males) at the gonad level; and feminization, vice versa. Control of sex ratios is accomplished by careful exposure to sex steroids, altering ratios developmentally or in adult fish (the latter sometimes resulting in sterility) (Pandian and Sheela 1995). Androgens (mainly the synthetic 17α-methyltestosterone) have been used to shift sex ratios to males. Natural estrogens (mainly 17β-estradiol) have been used to shift sex ratios to females. However, male-biased sex ratios have also

[2] Since implementation of process changes, NCASI has repeated its fish full life-cycle exposure and showed improvement in egg production (DL Borton, pers. comm.).

been induced successfully, using aromatase inhibitors (Jalabert et al. 2000, Kwon et al. 2000). (Aromatase converts androgens to estrogens in many tissues of both mammals and fish.) Effective doses vary drastically among species, with Poeciliids often requiring the largest doses compared to salmonids, cyprinids, cichlids and anabantids (Pandian and Sheela 1995). Environmental pollution has also been linked to unintentional shifts in sex ratio of fish (Jalabert et al. 2000).

The terms masculinization and femininization have also been used to describe changes in secondary sex characteristics. In this sense, masculinization refers to external appearance of male secondary sex characteristics in a female fish (i.e., she retains ovaries), and feminization vice versa. This phenomenon can occur naturally in the wild: arrhenoidy, or masculinization of older, reproductively senescent females, has been documented at low levels in wild Poeciliid populations (Constanz 1989). Changes in secondary sex characteristics can be induced by administration of sex steroids (Turner 1941a, Turner 1942a, Turner 1942b, Hildemann 1954, Borg 1994). Androgens and estrogens are assumed to be key players, however progestins may also play a significant role (Jalabert et al. 2000). Changes in secondary sex characteristics have also been associated with human impacts on the environment (Jalabert et al. 2000).

As endocrine disruption became a controversial issue in toxicology, the distinction between these levels of masculinization and femininization was important. Changes in appearance could behaviorally affect reproduction and population size, or reproduction could be unaffected. On the other hand, a significant shift in sex ratios could impact reproduction and population size more overtly. The difficulty with sex ratios is determining how much of a shift is significant. Hence, for the purpose of my study,

masculinization and femininization will refer to alterations in secondary sex characteristics only. Shifts in sex ratios (implying alteration at the gonad level) will be referred to as such. Both of these endpoints have been associated with pulp and paper mill effluent exposure in fish.

Effects on sex ratio varied by effluent exposure and species. Research on an indigenous species living near a TCF Swedish kraft mill demonstrated slight yet statistically significant male-biased sex ratios in embryos (55 to 58% male) compared to pooled reference sites (Larsson et al. 2000). Further, temporary mill shutdown allowed recovery of normal sex ratios, and the male bias reappeared after mill processes were restored (Larsson and Förlin 2002). Short-term (42 day) laboratory exposure of this effluent to a livebearing species did not reflect field results, failing to induce any change in sex ratios (Larsson et al. 2002). Full life-cycle exposure to bleached sulfite mill effluent demonstrated the opposite response in yet another species: sex ratios were female-biased at 30% effluent and greater (Parrott et al. 2004). In addition, egg production was reduced at 10% effluent and failed at 30% effluent or greater. Based upon these findings, egg production but not sex ratios may be impacted in the wild, since effluent concentrations vary from 1 to 15% by season and river flow. Finally, multigenerational laboratory exposure to environmentally relevant levels of phytosterols, primarily β-sitosterol, revealed male-biased sex ratios in the first offspring generation and female-biased sex ratios in the second (Nakari and Erkomma 2003). Clearly, bias toward one sex or another cannot be generalized in response to pulp and paper mill effluent exposure, although changes in sex ratio may be a useful indicator of impacts on fish reproduction (Parrott et al. 2004).

Changes in secondary sex characteristics of fish exposed to different types of pulp and paper mill effluent include precocious and delayed maturation, femininization, and masculinization. Among these alterations, masculinization is the most consistently reported effect across field and laboratory studies. Precocious maturation (or early development of secondary sex characteristics) has been reported in fish collected from a bleached kraft effluent receiving stream investigated in my study (Caruso and Suttkus 1988). Precocious maturation at 32% or greater effluent, masculinization (at 10% or greater) and femininization (at 32% or greater), were reported in fish exposed to dilutions of bleached sulfite effluent (Parrott and Wood 2002, Parrott et al. 2003, 2004). As with sex ratios and egg production reported by this group, the only environmentally significant response may be masculinization. Using the same model species, NCASI (2000b) found delayed maturation resulting from exposure to bleached kraft mill effluent. Masculinization occurred in this species from exposure to a different bleached kraft mill effluent (Kovacs et al. 1995b) but not to effluent from a thermomechanical pulp mill (Kovacs et al. 1995a). In the study by Larrson et al. (2002a) on a livebearing species, although sex ratios were not altered, masculinization was weakly indicated by male-like coloration. Perhaps the strongest case for masculinization lies with effects on mosquitofish, initially documented by Howell et al. (1980). This species is elaborated upon in the next section.

Examining these responses as a whole, in order to be useful biomarkers any of the suborganism level effects must be linked to exposure, as many studies included; and to effects on reproductive success, and if possible on populations. Full life cycle tests are very useful to this end, but these tests must be comparable to responses in wild fish

actually living under exposure conditions. Hence a two-pronged approach pairing field and laboratory exposures is ideal, especially if the same species can be used for both types of studies. The mosquitofish has potential for both types of exposures, as shown in the following section. The remainder of this chapter examines mosquitofish in relation to pulp and paper mill effluent, as a model species, and as a potential bioindicator of pulp and paper mill effluents.

Effects of Pulp and Paper Mill Effluents on Mosquitofish

The Eastern mosquitofish, *Gambusia holbrooki*, was the first species recorded as masculinized by pulp and paper mill effluent exposure (Howell et al. 1980). Since then, improved analysis of field collections and laboratory exposure to degraded effluent components have supported the original observational response. The degree of masculinization was highly variable within a site and by season, yet considered comparable among three Florida mills (all of which were examined in my study). In contrast, controlled exposure to whole effluent dilutions provided mixed evidence for masculinization in Western mosquitofish, *Gambusia affinis* (McCarthy et al. 2004). Beyond the masculinization response, precocious maturation, behavior, and aspects of reproduction (mainly brood size) were also addressed without significant observable impacts. So far, attempts to isolate potential mechanism(s) of masculinization were inconclusive.

Masculinization

Sampling of Eastern mosquitofish in Elevenmile Creek, FL, USA revealed the first known occurrence of masculinization associated with pulp and paper mill discharge (Howell et al. 1980, p. 676). Lacking quantification of data, the authors reported the following.

> All females within this stream are strongly masculinized, possessing a male-like gonopodium and displaying male reproductive behavior. All males exhibit precocious secondary sex characters and reproductive behavior.

Photomicrographs indicated elongation, segmentation, and intermittent terminal differentiation of female anal fins similar to the male gonopodium (the copulatory organ used to inseminate females in this livebearing species). Apparently equivalent responses were detected in another effluent-receiving stream in Florida, the Fenholloway River (Bortone and Drysdale 1981). After significant process changes at the Elevenmile Creek mill (including conversion to ECF bleaching and oxygen delignification), quantification of the response (anal fin length) and statistical comparison to females from a reference stream showed masculinization remained (Cody and Bortone 1997). However photographs qualitatively indicated reduced elongation and lack of terminal differentiation. Season (winter versus summer months, based upon seasonal drought conditions) also influenced anal fin length significantly: greater elongation occurred in summer months. As further evidence, Bortone and Cody (1999) detected a statistically significant increase in the ratio of anal fin length to fish standard length (finally accounting for the influence of body size on this morphological feature), and inferred a distance/dose-dependent response downstream of pulp mill effluent discharge in Rice Creek, FL. In light of seasonal effects reported previously (Cody and Bortone 1997), the inference was tenuous: they compared one upstream and three downstream sites collected in summer with a fourth, furthest downstream site collected twice; once in winter, and once several years previously by separate researchers. Significant increase of anal fin elongation in females from the first two downstream sites was statistically comparable to Fenholloway River females collected several years before. Variation was high (highest in the Fenholloway collection), even at the upstream (200 m above outfall) site. The

authors speculated tidal influence may draw effluent above the discharge point, accounting for this unexpected result. Exposure to pulp mill effluent, either potential or actual, was never documented in these collections.

Based upon detailed observations of exposure to androgens by Turner (1941a, 1942a, 1942b) and steroid production studies using bacterially degraded phytosterols by Marsheck et al. (1972), it was hypothesized that female mosquitofish may be masculinized when exposed to androgens formed by degradation of phytosterols present in pulp and paper mill effluents. Subsequently, female mosquitofish were exposed to high concentrations of phytosterols (approximately 0.1-0.5 g/L of stigmastanol and β-sitosterol) combined with a bacterium (*Mycobacterium smegmatis*) not common to effluent-receiving streams (Denton et al. 1985, Howell and Denton 1989). Although presence of androgens was not monitored to verify androgen exposure, females developed male-like gonopodial structures within two weeks. Stigmastanol produced a more potent effect. The male-like gonopodial structures did not elongate to the length of normal male gonopodia, but developed terminal differentiations. Lacking quantification and statistics, nonetheless this mechanism was proposed to explain observed effects of masculinization. In support of these findings, Angus et al. (2001) detected rapid onset of anal fin elongation: 14 days at 60 μg 11-ketotestosterone/g food, development of terminal differentiations by 20 days in the high exposure groups (80 and 100 μg/g), and average of 40 days in the low exposure groups.

Analysis of various morphological endpoints revealed the most sensitive measures of masculinization. Bortone et al. (1989) determined an unranked suite of about ten morphological measures of body and fin sizes—including only one measure of the anal

fin—statistically differentiated effluent-exposed fish from reference fish. These variables were proposed as a rapid bioassay to detect effects of pulp mill exposure, but were never fully developed. Howell and Denton (1989) developed five stages of increasing gonopodial development in females exposed to bacterially-degraded phytosterols. More recently, quantitative measures of anal fin morphology were compared from wild-caught and androgen-exposed females (Angus et al. 2001, Bradley et al. 2004). The length ratio of ray 4 to 6 was the most sensitive measure of masculinization of the anal fin. While the number of segments along rays 3 and 4 and the width ratio of ray 3 to 4 were also sensitive measures, they were more subject to variability.

Controlled exposure to whole effluent dilutions produced inconsistent masculinization results. Initially in support of field collections, static renewal exposure of newborn mosquitofish to water collected 3.6 km downstream from Elevenmile Creek induced elongated anal fins (measured as anal fin length) in females upon maturity (Drysdale and Bortone 1989). While my study research was being conducted, researchers in Canada and New Zealand were also studying masculinization of adult female mosquitofish using controlled (mainly static renewal, one flow-through) exposures to 15%, 70% or 100% effluent (McCarthy et al. 2004 summarizes results across separate studies). Out of seven pulp mills and one sewage treatment facility tested, four of the pulp mill effluents and the sewage effluent[3] induced masculinization (all static renewal exposures). Among these four pulp mill effluents, two induced masculinization relatively quickly (within 3 weeks) while the other two required 24 weeks of exposure. No association between induction and type of mill or concentration

[3] In contrast, collection near other sewage discharges found effect on males, not females (Batty and Lim 1999, Angus et al. 2002). Bradley et al. (2004) also found no effect on females

of β-sitosterol could be established. Apparently, duration of exposure required to produce effect varies widely. However, three important caveats exist for these studies.

- Other than Elevenmile Creek, comparative field studies were not conducted to determine if effects existed in wild mosquitofish exposed to these effluents.
- All but one exposure required holding and transport of effluent back to the exposure system, with unknown consequences to effluent composition.
- Masculinization was measured qualitatively, as presence/absence or staged using categories established by Howell and Denton (1989).

One of these controlled exposures detected differences in masculinization due to effluent treatment and filtration (Ellis et al. 2003). Secondary treatment of effluent at environmentally relevant concentration reduced gonopodial development by 25%, yet masculinization remained significantly greater than controls. Filtration of treated effluent, removing organic extractives adsorbed to particulates, also removed the response. Exposure was repeated two years later, after treatment system maintenance was improved as indicated by gradual reduction in total suspended solids. Using the more specific ray 4 to 6 length ratio, masculinization was not induced (van den Huevel et al. 2004b). Since exposure duration remained the same (3 weeks), it is unknown if the effect was entirely removed or if time to manifestation was extended. Regardless, these experiments strongly correlate masculinization with adsorbable organic effluent components, such as low molecular weight wood extractives.

Precocious maturation

Precocious (early) maturation of male mosquitofish exposed to pulp and paper mill effluent has been examined briefly. Effects were associated with bleached kraft effluent from Elevenmile Creek before major process changes (Howell et al. 1980, Drysdale and Bortone 1989), but not with effluent from a thermomechanical/kraft/newsprint mill in

Ontario (McCarthy et al. 2004). Males of this species grow steadily until maturation at which point growth plateaus, as opposed to females who grow steadily throughout life allowing body length to roughly approximate age (Snelson 1989). Howell et al. (1980) reported small males (12-13 mm standard length) began to develop gonopodia, and fully-differentiated gonopodia occurred in males measuring 13-18 mm standard length. Compared to males in unexposed sites with mature gonopodia at 18 mm or longer, they concluded males developed earlier due to effluent exposure. However, neither data nor statistics was provided to support this conclusion. Static renewal exposure of newborn mosquitofish to water collected 3.6 km downstream from Elevenmile Creek provided more compelling, statistical evidence of precocious maturation in males (Drysdale and Bortone 1989). Exposed males began anal fin elongation approximately one month before unexposed males, with groups evening out during late-stage gonopodial growth. Surprisingly, this endpoint was virtually ignored by researchers until recently. In contrast, in the Ontario study, continuous flow-through exposure of mosquitofish to 15% and 100% effluent for 21 weeks did not affect male gonopodial length relative to body size (McCarthy et al. 2004).

Behavior

Investigation of male-like reproductive behavior was a logical step once male-like secondary sex characteristics were discovered in female mosquitofish. As implied previously, changes in secondary sex characteristics could potentially lead to behavioral changes that keep females from copulating and reproducing. Initial preliminary investigation, lacking statistical comparison, indicated both masculinized females and precocious males displayed more aggressive reproductive behavior (Howell et al. 1980). Behavioral evaluation of masculinized females in the presence of normal females

supported increased aggressive, but not reproductive, behavior (Ellis et al. 2003). Analyzing the ability of a suite of reproductive behavioral changes to detect pulp mill effluent exposure, Bortone et al. (1989) determined behavior did not adequately discriminate effluent exposure from unexposed groups. In support of this conclusion, Krotzer (1990) performed a thorough reproductive behavioral analysis of mosquitofish females exposed to bacterially-degraded phytosterols. Masculinized females displayed aggressive male behaviors toward nonmasculinized females only in a noncopulatory fashion. Paired with males or other masculinized females, they behaved normally. Thus, masculinization likely does not impact mosquitofish populations in a behavioral sense.

Reproduction

Potential for reproduction does not appear impacted by pulp and paper mill effluent exposure in female mosquitofish. Except for histological evaluation of gonads, reproductive parameters were never directly compared with measures of masculinization. True to the distinction between masculinization and actual sex reversal, normal ovaries lacking any testicular tissue were consistently reported in masculinized females (Howell et al. 1980, Hunsinger et al. 1988, Ellis et al. 2003, McCarthy et al. 2004). Fecundity (inferred by brood size or number of eyed embryos plus mature eggs) relative to body length was depressed in females collected below effluent discharge in Elevenmile Creek (Rosa-Molinar and Williams 1984, p. 122). However, the authors state:

> "Estimated fecundities in reference to length in the arrhenoid [masculinized] fishes were not found to be similar to those found in other studies…although the fecundity of the normal *G. a. holbrooki* [in the current study] was similar."

In contrast, static renewal exposure to sediments and waters of bleached and unbleached kraft effluent receiving streams for 56 days produced no statistical differences in several measures of fecundity compared to reference sites (Felder et al. 1998, D'Surney et al.

2000). However, variation was high between reference sites. One reference site was a research station, a well-documented habitat without pollution impacts but obviously very different from the receiving stream (e.g. very low water hardness contributing to increased skeletal abnormalities). Thus the selection of unexposed sites is important, and should include an upstream site at the minimum and sites belonging to the same watershed. In support of this overall lack of histological and embryological impairment in effluent-exposed females, McCarthy et al. (2004) did not detect alteration in sex ratios of mosquitofish reared in 100% effluent under laboratory conditions. The assumption must be made for all above data on fecundity and sex ratios that at least a portion of exposed females analyzed were masculinized as well.

Mechanism of Action

Since mosquitofish research has produced the only specific hypothesis of causation linking one class of effluent components to potentially adverse effects, several researchers have attempted to isolate potential mechanism(s) of action. In support of the bacterial degradation hypothesis forming androgens, Jenkins et al (2001) detected low levels of androstenedione (0.14 nM) in the Fenholloway River downstream of effluent discharge. However bioassay-based fractionation studies, discussed previously under effects of pulp mill effluent on fish, have not supported androstenedione and testosterone as active androgens that bind the androgen receptor and masculinize females (Jenkins et al. 2001and2003, Parks et al. 2001, Durhan et al. 2002, Ellis et al. 2003, van den Huevel et al. 2004b). Orlando et al. (2002) investigated an alternative mechanism used in the aquaculture industry, aromatase inhibition. Contrary to their hypothesis, aromatase activity was elevated in both brain and ovarian tissue of females collected downstream of effluent discharge in the Fenholloway. Masculinization was indicated by increased

segmentation of anal fin rays, but not increased anal fin length. The authors concluded aromatase inhibition was not a likely mechanism to account for masculinization. However, impaired activity could potentially upregulate enzyme production. Measurement of endogenous steroid levels may provide more insight into this potential mechanism.

Mosquitofish as a Model Species

Several life history and ecological characteristics of mosquitofish (the closely related Eastern and Western species (*Gambusia holbrooki* and *G. affinis*) of the family Poeciliidae), make these species an ideal model for both field and laboratory toxicological studies. Meffe and Snelson (1989) compiled the most comprehensive overview of Poeciliids, from which the following summary was derived unless noted otherwise.

Occurrence and Availability in Effluent-Receiving Systems

Mosquitofish are opportunistic, omnivorous feeders that can exploit diverse foods ranging from planktonic invertebrates and fish fry to detritus and algae; so food source should not limit their occurrence in effluent-receiving streams. Similarly, mosquitofish inhabit a diverse range of shallow habitats with the ability to occupy "fringe" habitats characterized by environmental extremes. Combined with their tolerance to high salinity (up to 50% seawater in the Western mosquitofish, *G. affinis*), broad thermal range, and tolerance to low dissolved oxygen (mosquitofish gulp air at the surface in response to hypoxia), mosquitofish should tolerate water quality of effluent-receiving streams. Their home range is small (several meters) making chronic exposure likely. In addition, they readily colonize new populations via migration of a single gravid female, and have

become ubiquitous around the world caused by deliberate introductions in attempt to control mosquitoes and associated mosquito-borne illnesses.

In addition to their suitability as a field model, mosquitofish can be maintained under laboratory conditions with ease relative to other livebearing fish species. Also compared to other livebearers, much more is known about mosquitofish reproduction.

Reproductive Characteristics

As members of the livebearing fish family, Poeciliidae, mosquitofish develop eggs internally and appear to give birth to fry (ovoviviparity). This is in stark contrast to most fish that lay eggs (oviparity). Males inseminate females with packets of sperm called spermatozeugmata, and females can store sperm in ovarian folds and gonoduct for up to eight months/broods. Fertilization and embryological development occur directly in the ovarian follicle, and ovulation is immediately proceeded by parturition. Estrogen (as opposed to prostaglandins in other fish species) stimulates postovulatory sexual receptivity of females.

Livebearers exhibit a spectrum of maternal-embryo nutrient exchange, from more fish or reptilian like yolk loading before fertilization (lecithotrophy) to more mammalian like continuous provisioning throughout embryological development by the mother (matrotrophy). Mosquitofish represent the former group. In addition, mosquitofish carry one fertilized brood at a time, as opposed to many other livebearers that harbor several broods at different stages of development (superfetation). Brood size is dependent on female body size, with larger fish producing larger broods. Reproduction is asynchronous and seasonal in temperate to subtropical climates such as Florida. The reproductively active period is during spring and summer months followed by reproductive senescence in the fall and winter. Temperature and photoperiod are

considered dominant environmental cues controlling reproductive season in both sexes (Koya and Kamiya 2000, Koya and Iwase 2004).

Mosquitofish begin life as hermaphrodites, containing both ovarian and testicular tissue (Koya et al. 2003). Hermaphroditisim (Teh et al. 2000) has also been detected in adult farm-reared albino mosquitofish. Within 10 days, gonads differentiate into paired, fused testes or ovaries. Gonadal maturity is reached in approximately 3 months, with males maturing 2-3 weeks earlier than females.

Mosquitofish, similar to many other livebearers, are sexually dimorphic. Male and female mosquitofish have several gender-specific traits: females are larger and possess an anal/gravid spot and urogenital papilla, while males are smaller and possess a gonopodium. The gonopodium facilitates internal fertilization and is formed by the elongation of rays 3, 4, and 5 of the anal fin. Formation of the gonopodium is controlled by androgens, and a fully-developed gonopodium (marked by terminal differentiations on the tips of rays 3, 4, and 5) signifies complete maturity (Turner 1941b). Turner (1941a, 1942a, 1942b) also documented formation of the fully mature gonopodium in females exposed to androgens such as ethynyl and methyl testosterone. Thus, female mosquitofish could be a useful model of exposure to environmental androgens.

Mosquitofish as a Bioindicator of Pulp and Paper Mill Effluent

Mosquitofish have been repeatedly proposed as an indicator of environmental disturbance by pulp and paper mill effluents (Davis and Bortone 1992, Bortone and Davis 1994, Cody and Bortone 1997). At the state level, the Florida Department of Environmental Protection has explored this possibility (T.S. Gross pers. comm.) without implementation. Federally, the US EPA is developing the mosquitofish as an androgenic model of endocrine disruption (Angus et al. 1997). While mosquitofish have potential

for use in regulatory testing and screening of pulp mill effluents, they have not been adequately assessed as an indicator species.

Definitions: Bioindicator and Biomarker

For every critical review of bioindicators and biomarkers there exists a slightly different definition. The term biomarker is more consistently defined as a measurable biological response to environmental pollution observed below the organism level of biological organization (Foster et al. 1992, Peakall 1992, Jamil 2001). Biomarkers encompass changes at the molecular, biochemical, physiological, histological, morphological, or behavioral levels. The major premise for use of biomarkers in environmental regulation is the bridge they form between chemical exposure and adverse effect. However, biomarkers are usually classified as more indicative of either exposure or effect. Current challenges for biomarker use include questions of natural variability, use in the field, and extrapolation to higher levels of biological organization and to humans. Though the challenges appear formidable, researchers strive to meet these important demands of regulatory application (e.g. analysis of suites of biomarkers and increasing inclusion of biomarker analyses in population and community studies). Unfortunately, as a society we have little patience for the pace of science and often biomarkers are misjudged or overinterpreted.

In contrast to biomarkers, the term bioindicator usually implies changes at the organism level or above, including individual reproduction, populations and communities. For example, US EPA (2004, website) states the following:

> Environmental scientists have determined that the presence, condition, and numbers of the types of fish, insects, algae, and plants can provide accurate information about the health of a specific river, stream, lake, wetland, or estuary. These types of plants and animals are called biological indicators.

Ecological bioindicators have been developed and used extensively by the US EPA to assess ecosystem health. Jamil (2001, p. 4) defines bioindicators for evaluating ecological health "as a species or groups of species (plants or animals) that, by their presence and/or abundance, play an important role in the ecosystem to which they belong." Further, he distinguishes two classes of bioindicators used to evaluate environmental quality: bioaccumulator and sentinel species. Similar to separation of biomarkers into exposure and effect groups, bioaccumulators represent organisms that bioconcentrate toxicants from the surrounding media and biomagnify up the food chain, while sentinels indicate toxic effect that allows judgment of effects on human and/or environmental health. Sentinel species are further characterized as species with field application, either preexisting at sites of interest or capable of *in situ* exposure (such as caging onsite). Ideally, extensive knowledge exists about normal states measured in sentinel species. A final condition is the surrogate nature for species at risk, i.e., sentinels substitute for sampling of already imperiled species that are difficult to study directly and could potentially be harmed by intensive research.

For the purposes of my study, bioindicator is defined as a species possessing measurable changes in biomarkers that correspond to impacts at higher levels of biological organization. Similar to applicability requirements for sentinel species, a bioindicator should be a versatile subject in the field, not only in the laboratory. Thus the bioindicator retains environmental relevance while affording analysis of time and dose-dependent responses.

Bioindicator Criteria for Success

Criteria, like definitions, abound for rating success or failure of potential bioindicator organisms (Peakall 1992, Jamil 2001). Regardless of variable definitions, several common criteria for success exist and overlap with criteria for biomarkers.

Practicality

- Sufficient scientific knowledge of model species under normal, unexposed conditions.
- Ease of training and use by personnel.
- Cost- and labor-effective.
- Availability of model species for both field and laboratory study.

Variability

- Intrinsic or natural variability of biomarkers such as seasonality and gender differences.
- Exposure variability especially sensitivity and tolerance/acclimatization.
- Method variability such as observer bias and instrumentation bias.

Predictability

- Extrapolation to organism level or higher adverse effects, i.e., reproductive or population impacts.
- Extrapolation to other species living in exposure conditions.
- Extrapolation to humans.

Applying these criteria to the existing research on mosquitofish exposed to pulp and paper mill effluents reveals practicality, but not variability and predictability, has been adequately addressed. Practicality was supported in the previous section about mosquitofish as a model species. The mosquitofish is one of the most intensely studied livebearing species because of its use in mosquito control. Masculinization studies are not expensive, especially compared to molecular and biochemical research.

Measurements require basic lab skills using dissecting scopes and ideally computer measurement software. The most expensive aspect for these studies is exposure facility construction, a common expense for any bioindicator. Similarly, exposures are the most labor-intensive aspect of masculinization studies. Perhaps the greatest strength for mosquitofish as a bioindicator is its global distribution (again because of mosquito control), making collection of large numbers in effluent-exposed and reference sites very easy.

In terms of variability, natural fluctuation has been indicated by season (in Florida) and the response is gender specific, although precocious maturation remains questionable in Florida streams. Exposure variability has been implied by the work in Canada and New Zealand, among mills and within mills with improving technologies and maintenance of systems. Research in Florida has not directly addressed this type of variability, but a consistent response to variable exposure is implicated. Since specific bioactive agents and mechanism of action have not been isolated, sensitivity is difficult to address and can only be viewed from whole effluent exposures. Compared to binding of fish androgen receptor (Ellis et al. 2003), masculinization is a less sensitive, but potentially more relevant response. Specificity of masculinization for pulp and paper mill effluent versus sewage effluent appears high, with the abnormal exception to treated sewage effluent by McCarthy et al. (2004). Yet laboratory exposures to other chemicals (such as the hypertensive drug spironolactone and the agricultural insecticide endosulfan) have also induced masculinization (Howell et al. 1994, Park et. al 2004). In addition, Bradley et al. (2004) found masculinized females living in the retention pond of an urban parking lot; therefore, nonpoint sources of pollution may confound results. So

specificity, once considered high for pulp mill effluents, has become questionable. At the same time, exploration of how these alternative compounds masculinize may catalyze isolation of bioactive compounds. Tolerance and acclimatization have not been studied. A handful of anecdotal reports (Davis and Bortone 1992, Bortone and Davis 1994, Cody and Bortone 1997) indicate masculinization is reversible when females are transferred to clean water; McCarthy et al. (2004) did not observe resorption of anal fin elongation under controlled exposure. Finally, method variability, other than determination of the more sensitive measures of anal fin morphology, has not been addressed.

The final major criterion for a successful bioindicator, predictability, has begun to be examined with studies of reproductive potential. As previously discussed, gonad condition, fecundity, and sex ratios do not appear affected by effluent exposure. A major drawback to most of this work is the lack of masculinization measures. Actual fry production, quality and survival have not been examined either as a more accurate measure of reproductive success. Regarding extrapolation to the fish community, Bortone and Cody (1999) attempted to examine masculinization of other livebearing fish species in their Rice Creek collection, but failed to obtain adequate fish numbers for analysis. The link to humans has yet to be determined as well.

Obviously, further testing of mosquitofish is required to determine if this species would be a successful bioindicator of pulp and paper mill effluents. Practically speaking, mosquitofish are very promising, especially on a worldwide scale but perhaps less useful in nations with advanced processing technology.

Contribution of My Study

My study addresses two of the three major bioindicator criteria for mosquitofish: variability and predictability. Industry and regulatory agencies alike will thus have a

better understanding of the potential for mosquitofish as a bioindicator of pulp and paper mill effluent exposure. In addition to anal fin morphology as a biomarker, sex steroids were investigated for two reasons: 1) masculinization as an androgenic model innately assumes alteration of steroid levels, either peripherally or systemically; 2) sex steroid levels are generally depressed in other fish species exposed to pulp and paper mill effluents. Variability by season, method, and exposure were addressed directly. Method and seasonal variability, while not a stated objective of the original research proposal, were necessary precursors in the development of techniques and thus were included in my study results. Exposure variability studies focused upon the three Florida freshwater systems (Elevenmile Creek, Fenholloway River, and Rice Creek) for which masculinization has been reported and considered equivalent. Predictability was addressed by evaluating the relationship among biomarkers and reproductive success. Preliminary examination of population structure was also conducted during the reproductive experiments.

Explicitly stated objectives for my study were divided into two specific aims with associated hypotheses. Within each specific aim, three sub-aims were identified and studies developed for each.

Specific Aim 1

Our first specific aim was to determine the effects of improved mill technology on masculinization of female mosquitofish. We hypothesized that reduction in brown side effluent components (i.e., wood extractives such as phytosterols and resin acids) would reduce anal fin elongation and hormonal alteration in female mosquitofish.

- Aim 1A: Assess induction of masculinization in female mosquitofish under short-term controlled exposure to effluent at one mill throughout process changes and at two mills using different processing techniques. Expected outcomes were:

induction would be rapid; degree of response would reflect differences in concentration of wood extractives; and effect would be reduced following major process changes.

- Aim 1B: Compare and contrast anal fin morphology and sex hormone concentrations in female mosquitofish at one mill throughout process changes. The expected outcome was that process changes would reduce masculinization in wild fish to a similar degree as induction studies.

- Aim 1C: Compare and contrast anal fin morphology and sex hormones in female mosquitofish collected from systems exposed to different types of mill effluent. The expected outcome was degree of response would reflect differences in concentration of wood extractives, similar to differences expected with induction studies.

Specific Aim 2

Our second specific aim was to evaluate reproductive success of mosquitofish exposed to pulp and paper mill effluents. We hypothesized that exposure to pulp and paper mill effluents would not impair reproductive success of mosquitofish.

- Aim 2A: Determine population structure of mosquitofish living in Fenholloway River. The expected outcome was that population structures would reflect sex ratios and recruitment (juveniles) found in unexposed references.

- Aim 2B: Characterize organism level responses to effluent exposure associated with reproduction. The expected outcome was that adult females would display normal reproductive status regardless of exposure and masculinization.

- Aim 2C: Quantify offspring production of female mosquitofish exposed to pulp and paper mill effluents. The expected outcome was that offspring production would not vary regardless of exposure or masculinization.

Studies to address these aims were designed with certain limitations and assumptions in mind.

- Home ranges do not overlap between sites sampled.

- However, the above implies field studies may be comparing genetically different populations of mosquitofish.

- Mosquitofish caught at field sites have lived there throughout life (birth, pre- and post-maturation).

- Mosquitofish (*G. affinis* and *holbrooki*) respond in the same manner.
- Masculinization of the anal fin is finite and permanent (as indicated by Turner 1942b), i.e., gonopodial development does not vary between acute and lifetime exposures.
- Fry production studies use wild-caught fish therefore reproductive history (i.e., fertilization, past broods) is not controlled and is unknown.
- Cannot expose to whole effluent by transfer back to the laboratory without potentially altering its composition.
- Composition of effluent changes drastically when mills cycle through different types of trees.
- Potential (as opposed to actual) exposure is sufficient to document toxicant exposure, since the masculinization hypothesis predicts actual exposure is to as yet unknown androgens and not parent effluent components.
- Specific changes in mill technology are not always known or available to investigators.

Table 1-1. Select characteristics of the mills in my study according to receiving stream.

Mill Characteristic	Elevenmile Creek	Fenholloway River	Rice Creek
Furnish	75% hardwood, 25% softwood	100% softwood	50% hardwood, 50% softwood
Pulping	Chemical/kraft	Chemical/ dissolving kraft	Chemical/kraft
Bleaching	ECF[a] (1995)	Sodium hypochlorite[b]	ECF[a] (May 2001)
Product	White copy paper, return postcards, market (paper) pulp	High grain cellulose (dissolving nonpaper pulp)	Paper towels, tissue paper, kraft bag, linerboard
Effluent treatment	Aeration with microbial degradation, chemical flocculation, oxygen delignification	Aeration with microbial degradation	Aeration with microbial degradation, activated sludge
Effluent volume	21-26 mgd[c]	43 mgd	28 mgd

[a] Elemental chlorine free; conversion date in parentheses
[b] Note this mill was not subject to many Cluster Rule requirements, since it is not a papergrade bleached kraft or sulfite mill
[c] million gallons per day

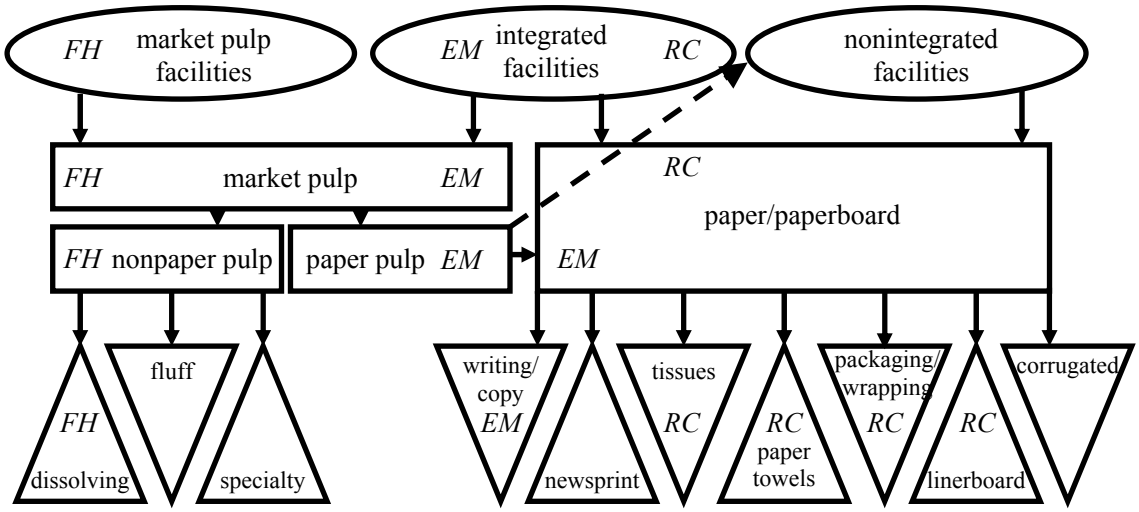


Figure 1-1. Categories of pulp and paper mill facilities. Mills associated with my study are abbreviated by receiving stream: *EM* = Elevenmile Creek, *FH* = Fenholloway River, *RC* = Rice Creek.

CHAPTER 2
VALIDATION OF MOSQUITOFISH ENDPOINTS USED TO ASSESS EFFECTS OF PULP AND PAPER MILL EFFLUENT EXPOSURE

Mosquitofish have been proposed as a bioindicator of pulp and paper mill effluents, although several aspects concerning variability in masculinization responses have not been addressed. Eastern mosquitofish were collected in summer and winter months from Rice Creek, the receiving stream for effluent from the Georgia-Pacific bleached/unbleached kraft mill in Palatka, FL. In this study, a series of validations were performed for morphological measurements and for a new biomarker in this species, whole body sex steroid concentrations. Seasonality in responses was also addressed. Gender identification using the urogenital papilla was successfully validated against internal examination of gonads. Morphological measurements validated adequately, and computer-aided measurement was a preferred alternative to manual measurement. Sex steroids also validated adequately considering the unavoidable limitations using whole body analysis. Greater masculinization was associated with effluent-exposed sites for females, while males were not overtly affected. Females displayed seasonal effects on sex steroids, especially steroid ratios, but not anal fin morphology. Surprisingly, sex steroids were not altered in females collected from 100% final effluent before discharge, indicating a complex interplay of environmental factor(s) may produce responses as opposed to effluent alone, such as differential bacterial degradation of phytosterols.

Introduction

Concerns over release of chemicals into the environment have shifted from lethal to sublethal effects on nontarget wildlife species (Lehtinen 2004). Reported sublethal effects of pulp and paper mill effluents on fish include induction of liver detoxification systems, alterations in sex steroids concentrations and production/metabolism, reduced gonadal development, decreased egg production and decreased fry survival (Van Der Kraak et al. 1992, Gagnon et al. 1994a, Munkittrick et al. 1999, NCASI 2000a, Sepulveda et al. 2003, Parrott et al. 2004, McMaster et al. 2003). Degree of these effects is often mill-specific with some effluents producing no effect at all (Kovacs et al. 1995a, McCarthy et al. 2004). No clear pattern existed between effect and type or quality of effluent, other than reduced responses with improved mill technologies (Munkittrick et al. 1997, van den Huevel et al. 2004b). Further complicating the matter, bioactive effluent components have not been strictly identified yet and most likely different compounds and conditions influence response pathways.

Several responses imply androgen-induced mechanisms in fish. For example, pulp mill effluents have been associated with male-biased sex ratios and development of male-like secondary sex characteristics in females or masculinization (Kovacs et al. 1995b, Larsson et al. 2000, Larsson and Förlin 2002, Larsson et al. 2002, Parrott and Wood 2002, Parrott et al . 2003). The masculinization response has been frequently reported in female mosquitofish as an elongation of the anal fin into a male-like gonopodium, the copulatory organ in this livebearing species (Howell et al. 1980, Cody and Bortone 1997, Bortone and Cody 1999, Parks et al. 2001, Ellis et al. 2003, Bradley et al. 2004, McCarthy et al. 2004). Further, this species has been repeatedly proposed as a bioindicator of pulp and paper mill effluents (Davis and Bortone 1992, Bortone and

Davis 1994, Cody and Bortone 1997). In a practical sense mosquitofish are a suitable model, yet several aspects require more thorough evaluation to warrant this broad application as a bioindicator of pulp and paper mill effluents.

In collaboration with the National Council for Air and Stream Improvement (NCASI), Southeastern Aquatic Biology Program, New Bern, NC, techniques to assess masculinization were validated and refined. Method validation involved a new gender identification technique, anal fin morphological measurements, and development of a novel assay for measurement of whole body sex steroids in mosquitofish. NCASI's validation results have been reported in the proceedings of the 5th International Conference on Environmental Fate and Effects of Pulp and Paper Mill Effluents (Bradley et al. 2004).

Materials and Methods

Mill Characteristics and Field Collection

Adult mosquitofish were collected along shallow vegetated banks in Rice Creek, the receiving stream for effluent discharge from Georgia-Pacific's Palatka, FL, USA operation (Figure 2-1). Collections occurred in both summer (March and June 2000, July 2001: n = 174, n = 141, n = 368 respectively) and winter (November 2000, n = 899), corresponding to reproductive and nonreproductive periods respectively. All fish were measured for standard length ($\pm$ 0.01 mm) using digital calipers and weighed ($\pm$ 0.001 g) using a digital scale before preservation. Chapter 3 gives details about the mill, and field collection techniques (site descriptions and latitude/longitude can be found in Appendix A).

Gender Identification Using the Urogenital Papilla

For all collections, gender was determined by external examination of the urogenital sinus. Most masculinization studies have used either the gonopodium (typically male-specific) and/or anal spot (female-specific) as external indicators of sex. Gender identification should not be based upon the gonopodium, since it is the primary measure of masculinization. This laboratory found the anal spot a difficult indicator as well, since many female fish have a partial anal spot and some females, mostly collected from effluent-exposed sites, have very light, brownish anal spots.[4] Hence, a new gender identification technique other than examination of gonads, which is very labor-intensive, was developed. Females have a gender-specific urogenital papilla that protrudes from the urogenital sinus, and the urogenital opening is located on the tip of the papilla (Meffe and Snelson 1989). During copulation, males hold on to the papilla using terminal differentiations of the gonopodium.

Gender identification via the urogenital papilla was validated using the winter 2000 and summer 2001 collections on several levels. First, variation among personnel was evaluated by having three technicians determine sex using this new technique (n = 200 fish). Second, the new method was verified against gross internal examination of gonads by NCASI (n = 200 fish). Third, the new method was verified against histological identification of gonads within USGS using a separate group of fish (n = 354).

Anal Fin Morphology

Several measures of anal fin elongation have been employed by researchers, from qualitative scores of presence/absence and categorizing degree of gonopodial

[4] Investigation did not reveal any dead embryos or abnormal ovaries within the body cavity, as initially suspected (pers. obs.). Cause(s) of brown anal spot remains unclear.

development to quantitative measures such as total anal fin length, gonopodium/extension length, number of terminal differentiations, length ratio of Rays 4 and 6, thickness ratio of Rays 3 and 4, and number of segments on Ray 3. Measurement of anal fin and gonopodium/extension length has been shown to be dependent on body size (standard length) and thus must be accounted for during statistical analysis (Bortone and Cody 1999, Bradley et al. 2004). For this reason, several studies restricted analysis of anal fin morphology to specific size classes. However, length ratio of Rays 4 and 6 and width ratio of Rays 3 and 4 are independent of body size and such a restriction is not necessary (Angus et al. 2001). Ray 3 segment counts, surprisingly, did not correlate with either of these ratios and was not suggested as an accurate measure of masculinization. Bradley et al. (2004) distinguished length ratio of Rays 4 and 6 as more sensitive than either width ratio of Rays 3 and 4 or segment number of Ray 3.

Length ratio of Rays 4 to 6 was used to assess masculinization in our study. Body weight ($\pm$ 0.001 g) and standard length ($\pm$ 0.01 mm) were also measured before preservation using a digital scale and a pair of digital calipers for all fish. For the winter 2000 collection, potential variation in both sexes was addressed due to preservation state (fresh versus fixed) and observer bias within and among laboratories (n = 200 fish for each comparison). Manual measurements of the linear distance from base to tip of Rays 4 and 6 of the anal fin ($\pm$ 0.1 mm) were made using a dissecting scope with ocular micrometer, before and after preservation in 10% neutral-buffered formalin. Preserved fish were additionally measured independently by two other technicians using the same equipment, and then shipped to NCASI for measurement. Influence of size class for females was also investigated in this collection, dividing females into four 5 mm groups

(20–24.99 mm, 25–29.99 mm, 30–34.99 mm, 35–39.99 mm). The summer and winter 2000 collections were compared to address seasonal variation for both sexes (summer 2000 fish were measured after preservation). Finally, the summer 2001 collection was used to test observer bias within the laboratory (n = 200 fish) using newly developed computer-aided measurements (± 0.01 mm, SigmaScan Pro 5.0, SPSS, Inc.) of digital images taken of fish before preservation. Bradley et al. (2004) evaluated the use of computer-aided versus manual measurements and determined although they were comparable, computer-aided measurements were more accurate and useful for archiving data.

Sex Steroids

In addition to anal fin morphology as a biomarker of masculinization, sex steroids were investigated as a second, physiological biomarker. Circulating sex steroids are generally depressed in other fish species exposed to pulp and paper mill effluents (McMaster et al. 2003, Sepulveda et al. 2001). Masculinization as an androgenic model innately assumes more specific alteration of steroid levels, either peripherally or systemically in favor of androgens. Orlando et al. (2002) hypothesized inhibition of aromatase resulted in a masculinized hormone profile. Although they tested the first half of this proposed mechanism and found aromatase activity actually elevated, the second half, sex steroid profile, was not measured. Examination of sex steroids in mosquitofish would potentially shed light on hypothesized mechanisms.

Primary sex steroids were analyzed using a modified radioimmunoassay (RIA) method originally developed for serum and plasma samples of common carp, *Cyprinus carpio* (Goodbred *et al* 1997), and since adapted for use in a variety of other aquatic species and tissue media such as plasma of largemouth bass, *Micropterus salmoides*

(Gross et al. 2001) and mantle of freshwater invertebrates (Gross et al. 2000). Mosquitofish were initially analyzed for 17β-estradiol, 11-ketotestosterone and testosterone, considered the most active reproductive hormones in fish. However, Borg (1994) demonstrated testosterone, and not 11-ketostosterone, as the only dominant androgenic hormone in poeciliid fishes. Analysis of 459 fish (both sexes) revealed nondetectable levels of 11-ketotestosterone, confirming Borg's finding. Therefore, testosterone is assumed to be the dominant androgen in mosquitofish and analysis of 11-ketotestosterone was discontinued. Ten fish of each sex from each site were analyzed for sex steroids in the summer 2000 collection; 42–50 females and 16–28 males per site were analyzed in the winter 2000 collection.

RIA steroid analysis involves chemical digestion of an entire fish, followed by extraction, radiolabeling, and a competitive binding assay to quantify steroid levels (see Appendix B for laboratory protocols). Whole body chemical digestion is accomplished by boiling individual fish in potassium hydroxide (30% w/v) at a volume three times the individual fish weight. Fifty microliters of resultant homogenate are removed in duplicate for extraction. Diethyl ether added in excess (4 mL) is used to extract lipophilic compounds, including sex steroids, from the digestion homogenate. Extraction and evaporation is performed twice to increase extraction efficiency. A reaction solution is prepared composed of evaporated extract, tritiated hormone and a corresponding hormone-specific antibody. This solution incubates overnight to allow unlabeled hormone from the extract sample (at unknown concentration) and radiolabeled hormone (at a known concentration) to compete for antibody binding sites. After incubation, the reaction solution is centrifuged with charcoal dextran to remove any hormone not bound

to antibody. Radioactivity is measured using scintillation spectrophotometry. Standard curves (at 1, 5, 10, 25, 50, 100, 250, 500, and 1,000 pg hormone) are generated for each hormone using known concentrations of radioinert hormone in buffer. Steroid concentration in samples is then calculated by aligning values of an inhibition curve, generated from the competitive displacement of radiolabeled hormone in the sample, to the standard curve. Validation and characterization of this procedure entailed determination of digestion and extraction efficiencies by reproductive status and exposure (n = 5 each group for each hormone: males, gravid and nongravid females, juveniles, and effluent-exposed males and females); minimum detection limits on the standard curve; cross-reactivities of antiserum with other steroids; and inter- and intra-assay variation.

Statistics

Body weight and standard length were used to calculate condition factor, $K = \text{weight} / \text{length}^3 \times 100$, as an indication of overall health used by the aquaculture industry (values at least 1 are considered healthy). The length ratio of anal fin Rays 4 and 6 was calculated as an index of anal fin elongation. Estrogen and testosterone concentrations were used to calculate a ratio indicating masculine hormone profile (E:T<1) or feminine hormone profile (E:T>1).

Gender identification and anal fin morphological measurements used for validations were analyzed using Pearson's product moment correlations or calculation of coefficients of variance. Anal fin morphology and sex steroid data were analyzed within sex using two-way analysis of covariance (ANCOVA) to test for significant variation by site and season for the summer and winter 2000 data, or by site and size class within the winter 2000 female anal fin data only. Size class was also analyzed by one-way ANOVA within site. Any data failing tests for normality and homogeneity of variance were

transformed using log transformations. Angus et al. (2001) determined length ratio of Rays 4 to 6 is appropriately analyzed using parametric statistics after log transformation of the ratio data. Significant differences in the ANCOVA and ANOVA tests were analyzed for multiple comparisons using Tukey's HSD. Within site, differences between seasons were analyzed by t-test. Statistical significance was attained at $\alpha<0.05$ for all tests. All statistical analyses were conducted using SAS © version 9.0.

Results and Discussion

Water Quality

As expected, conductivity, salinity, turbidity and pH were elevated at effluent-exposed sites compared to unexposed sites (Table 2-1). Water temperature was highest at the reference site and predischarge pond, and increased along the length of Rice Creek. Reference and upstream sites were comparable, other than water temperature. Dissolved oxygen remained high enough to support fish at all sites (>4 mg/L).

Validation of Gender Identification Using the Urogenital Papilla

Agreement about gender was significant at all three levels for which it was tested: among personnel within the USGS laboratory ($r^2 = 0.899$); between USGS and NCASI laboratories ($r^2 = 0.93$); and against histological evaluation ($r^2 = 0.99$). Between laboratories, females were agreed upon slightly more than males and the error rate for the new technique was 3.5%. Disagreement occurred for 15 fish (7.5% of all fish examined, Figure 2-2): of these fish, half (7) were incorrectly identified by the urogenital papilla, whereas the other half (8) could not be accurately identified by inspection of gonads. Incorrectly identified fish were equally from both exposed [DIS] and unexposed [REF2, U(8)] sites, precluding bias against masculinized fish. Comparison against histological identification of gonads was even more accurate with an error rate less than 1%.

Disagreement occurred for 7 fish (2% of all fish examined Figure 2-3): of these fish, over half (5) could not be accurately identified by histological inspection, while the remaining two fish were incorrectly identified by the urogenital papilla. Therefore the urogenital papilla is a reliable indicator of internal sex. This new noninvasive external gender identification technique could be very useful for a multitude of studies utilizing mosquitofish repeatedly sampled over time.

Morphology

Morphological measurements validated well, with computer-aided measurements preferable to manual measurements. Body size in winter 2000 was not impacted by effluent exposure relative to site; both sexes were in good general health judged by condition factor higher than 1. Increased gonopodial length was weakly indicated for males at effluent-exposed sites. Precocious maturation in males was not apparent, since smallest males did not have fully developed gonopodia to afford statistical analysis. In females, analysis by size class did not reveal specific size class(es) associated with anal fin elongation, although caution was implied for using appropriate sample sizes by females from the outfall site. Female anal fin elongation was significantly elevated at the discharge and first downstream site in both fall and winter. Seasonality was not evident for anal fin elongation in either sex.

Validations

Measurements on fish before and after preservation in formalin were significantly correlated (Table 2-2), although measurements on formalin-preserved fish were consistently smaller than on fresh fish (data not shown). This bias was expected since preservation tends to dehydrate and shrink specimens. Observer bias between NCASI and USGS laboratories measured differences not only between two observers, but also

differences between equipment. All measurements were significantly correlated (Table 2-2), although correlation coefficient for Ray 4 indicates somewhat inconsistently larger measurements by USGS. NCASI reports data to the nearest 0.01 mm for manual anal fin measurements (Bradley et al. 2004), while USGS reports data to the nearest 0.1 mm. Therefore, instrument bias is indicated. Significant differences occurred between manual and computer-aided measurements (Table 2-2). This discrepancy is likely due to the more accurate measurement by computer ($\pm$ 0.01 mm), similar to the nonsignificant differences between laboratories. Manual and computer-aided measurements of fish are thus not comparable when examining data across studies.

Variation within one observer and among several observers was also addressed for both types of measurements. Coefficients of variation at 10% or less were considered acceptable. Repeated manual measurements by one observer were consistent, as were measurements among observers (Table 2-3). Repeated computer-aided measurements by one observer were even more consistent than for manual measurements, but this was not quite the case among observers. This result means all computer-aided measurements should be (and were) made by the same observer for masculinization studies. With the greater accuracy afforded by computer-aided measurements (the Ray can be traced along exact curvatures, as opposed to linear distances with manual measurements), this technique is preferable. As Bradley et al. (2004) also note, computer-aided measurements are ideal for archiving data, but are time-intensive with the extra step of photography involved.

Body Size for Fall 2000 Collection

Body size data is presented for winter 2000 in this chapter (Table 2-4), while summer 2000 and 2001 data is presented in Chapters 3 and 4, respectively. Overall

males were not impacted by exposure site. Weight decreased with increasing distance from outfall (i.e. decreasing exposure), and the longest males were found at the upstream site even compared to the reference site [REF1]. Condition factor, above one and indicating healthy males, significantly varied inversely with exposure similar to weight: highest for all sites at the outfall [DIS]; lower than outfall and unexposed sites but higher than furthest downstream site at the first downstream site [D(1)]; and lowest for all sites at the furthest downstream sites [D(3+6)]. However this index is not appropriately interpreted beyond the benchmark of above or below a value of one. Female body size was not affected by exposure site. The only significant difference was the presence of longest females in the lower half of Rice Creek. Condition factor was also above one for females at all sites, indicating adequate overall health.

Influence of Body Size on Anal Fin Morphology

Since body size (length) has been associated with anal fin length in masculinized females and precociously matured males, fish were divided into 5 mm size classes and analyzed as a covariate with site. Size classes were also analyzed within each site. When males were divided into size classes, the majority fell into the 20–24.99 mm class, a minority fell into the 25–29.99 mm, and less than 10 fell into the <20 mm class. This paucity of mature males in the smallest size class precluded evaluation for precocious maturation. Precocious maturation may not be occurring in these males during the winter, since small males with fully differentiated gonopodia were not found in significant abundance. Precocious maturation is addressed in the summer 2001 collections presented in Chapter 4.

Standard length significantly correlated with total anal fin length (linear distance of Ray 4) for female mosquitofish in winter 2000 ($r^2 = 0.70$, data not shown). However,

index of anal fin elongation (Ray 4 to Ray 6 length) was statistically independent of standard length ($r^2 = 0.222$, data not shown). Overall across size classes (Figure 2-4), the first two exposure sites [DIS and D(1)] were significantly longer by the index of anal fin elongation (Ray 4 to Ray 6 length) compared to nonexposed sites and the lower half of Rice Creek [REF1, U(8), D(3+6)] . Size class did not significantly covary with site. For size classes within each site, the only statistically significant difference was at the outfall [DIS]: females in the middle two size classes had significantly longer anal fin elongation than the largest size class (Figure 2-3). This result negates predicted responses of size classes due to drought: in light of the drought faced by females in 1999 and early 2000 (Appendix A), the older/larger females should have greater elongation. Instead, this result may imply anal fin elongation is induced at a sensitive life stage and/or represents a dynamic exposure to bioactive compounds. (Dynamic exposure refers to variable concentrations of effluent components over time, dependent on factors such as tree species for furnish, within plant processing spills, rainfall/dilution, and bacterial degradation.) Without specific exposure data for these fish, these conclusions remain speculative.

Seasonality

Female anal fin elongation at the first two exposed sites was significantly elevated compared to unexposed sites for both seasons (Figure 2-5A). Elongations were more similar to a developing male gonopodium in length and lacked terminal differentiations (data not shown, see Chapter 3 for photographs of anal fins of females collected in summer 2000). Site and season did not covary for any of these data. Season alone influenced the latter two downstream sites [D(1) and D(3+6)], with opposite trends: at the first downstream site [D(1)] elongation was larger in winter than summer and vice versa

for the lower half of Rice Creek [D(3+6)]. Therefore, season was not consistently influencing presence of anal fin elongation in females from Rice Creek. This result is in contrast to the seasonality study from Elevenmile Creek (Cody and Bortone 1997), where winter months demonstrated a reduction in elongation compared to summer months. Granted, the Elevenmile Creek study was conducted over the entire year, while the current study compares one month from each season. Variation within a season was not reported for Elevenmile Creek by Cody and Bortone (1997). Both reports agree the response is present regardless of season.

Increased anal fin elongation, or greater gonopodial length, was not as apparent for males as for females (Figure 2-5B). The summer collection revealed males with shorter gonopodia at the upstream site [U(8)] compared to the rest. The winter collection demonstrated significantly longer gonopodia at the first downstream site compared to the reference [REF2] but not the upstream site [U(8)], and longer gonopodia further downstream [D(1) and D(3+6)] compared to both the upstream and reference sites. Hence selection of unexposed site(s) is of vital importance for interpreting results. Season had no effect on gonopodial length as either a covariate with site or alone. Overall the evidence is weak for increased gonopodial length in males due to pulp mill effluent exposure.

Sex Steroids

Radioimmunoassay of primary sex steroids was validated for whole mosquitofish. To date, only one other study has reported this biomarker in mosquitofish for males only (Toft et al. 2003). Elevated testosterone was associated with instream effluent-exposed sites in females regardless of season. However seasonality, both alone and relation to site, was detected. Estrogen to testosterone ratios were masculinized (i.e., greater than 1)

at impacted sites for the summer collection only. Importantly, neither hormone concentrations nor their ratio were altered in 100% final effluent before discharge, indicating additional environmental factors interact to produce the response. For males, seasonality of sex steroids was indicated, and effluent exposure did not appear to alter concentrations and ratio.

Validations

Validations were completed for 17β-estradiol and testosterone. Since 11-ketotestosterone was not detected in these fish samples, only partial validation was possible and reported elsewhere (Gross et al. 2001).

Digestion and extraction efficiencies were not influenced by reproductive status or exposure to pulp mill effluent (see Table 2-5), but greater efficiency was consistently achieved for 17β-estradiol than testosterone. For 17β-estradiol overall, digestion efficiency averaged 70 $\pm$ 4.9% while extraction efficiency averaged 65 $\pm$ 6.4%. For testosterone overall, digestion efficiency averaged 63$\pm$ 3.9% while extraction efficiency averaged 51 $\pm$ 12.5%. While these values would be considered low for plasma or serum samples, efficiencies at 60% or greater are high for whole body samples. Data was corrected for both digestion and extraction efficiencies.

Minimum detection limits on standard curves were 6.4 pg/mL and 9.3 pg/mL for 17β-estradiol and testosterone, respectively. Cross-reactivities of 17β-estradiol antiserum (produced and characterized by T. S. Gross, University of Florida) with other steroids were: 11.2% for estrone, 1.7% for estriol, less than 1% for 17α-estradiol and androstenedione, and less than 0.1% for all other steroids examined (ICN Biomedicals). Cross-reactivities of testosterone antiserum (produced and characterized by T. S. Gross,

University of Florida) with other steroids were: 17.6% for dihydrotestosterone, 2.3% for androstenedione, 1.4% for 11-ketotestosterone, <1.0% for androstenediol, and <0.1% for all other steroids examined (ICN Biomedicals). A pooled sample (1.25 g of unexposed males and females randomly selected and approximately 135 pg 17β-estradiol/mL and 176 pg testosterone/mL added) was assayed serially in 10, 20, 30, 40, and 50 µL volumes (final volume of 50 µL with boiled KOH). Resulting inhibition curves were parallel to respective standard curve based upon tests for homogeneity of regression indicating curves did not differ. Finally, average inter-assay and intra-assay coefficients of variation were 7.8% and 9.4% for 17β-estradiol and 8.7% and 10.1% for testosterone. All values are reported as pg hormone per g body weight.

Seasonality

Both site and season significantly covaried for 17β-estradiol in female mosquitofish from the discharge [DIS] and first downstream [D(1)] sites in Rice Creek (Figure 2-6A). However, examination of site and season separately does not reveal a consistent pattern. 17β-estradiol appeared seasonally elevated in winter, although the opposite occurred for the lower half of Rice Creek. During the summer, 17β-estradiol was depressed in females from upstream and downstream sites [U(8), DIS, D(1)] compared to remaining sites. Notably, 17β-estradiol in females was not depressed in 100% effluent before discharge [PRE-DIS]. Females collected in the winter revealed a different pattern: 17β-estradiol was significantly elevated at the upstream site [U(8)] compared to the discharge and first downstream sites [DIS and D(1)]. However, this hormone was not significantly different at effluent-exposed sites compared to reference fish [REF2], reiterating the influence of unexposed site selection. While it is ideal to collect upstream of effluent

outfall, additional references provide insight into natural variability. Based upon inherent variation implied by these data within sites and between unexposed sites, 17β-estradiol alone does not appear influenced by effluent exposure but may be seasonally influenced.

Site and season also significantly covaried for testosterone in female mosquitofish from the discharge [DIS] and first downstream [D(1)] sites in Rice Creek (Figure 2-6B). Patterns for this hormone are clearer. Testosterone was seasonally depressed in winter across all sites. For both seasons, testosterone was elevated at either the first downstream site (summer) or both the discharge and first downstream sites (winter). Like 17β-estradiol, testosterone was not impacted at the 100% final effluent before discharge [PRE-DIS]. Thus, elevated testosterone concentrations were associated with instream effluent exposure but not at highest effluent concentrations before discharge.

Figure 2-7 illustrates percentage of females with normal, feminine sex steroid ratios (> 1) versus masculine steroid ratios biased toward testosterone (< 1). Estrogen to testosterone ratios were significantly masculinized for females in the summer (DIS and D(1) which averaged 0.8 and 0.3, respectively). In the winter masculine versus feminine sex steroid ratios were not significantly different among sites. Thus, masculinized steroid profiles appear seasonally affected by effluent exposed sites. A low background level of masculine ratios existed in females from unexposed sites [U(8) and REF1 for summer, REF2 for winter], inferring this type of hormone profile can occur naturally in the population. In 100% effluent before discharge (summer collection), no females had masculinized estrogen to testosterone ratios, emphasizing the difference in response between predischarge and instream effluent-exposed sites.

Sex steroids in male mosquitofish covaried by site and season for 17β-estradiol but not for testosterone (Figure 2-8). Within sites, seasonality was inferred at the upstream and outfall sites [U(8) and DIS] for 17β-estradiol and at the outfall and lower half of Rice Creek [DIS and D(3+6)] for testosterone. 17β-estradiol was elevated and testosterone was depressed in the winter for these sites, respectively. By site alone, in the winter 17β-estradiol significantly peaked at the outfall and testosterone peaked at the upstream site. No differences were detected by site in the summer. Testosterone data reveal large variation within site and between unexposed sites, similar to 17β-estradiol in females and again stressing importance of reference site selection and an inherent natural variability (although mosquitofish have a reproductively active season they are asynchronous breeders). Estrogen to testosterone ratios were dominated by testosterone for all males across all sites for both seasons (significantly less than one, approximately 0.01 to 0.001). Overall, sex steroids in males did not appear impacted by effluent exposed sites and seasonality was inconsistently indicated.

Recently Toft et al. (2003) examined whole body sex steroids in male mosquitofish collected in lakes contaminated with agricultural pesticides. Technique was similar to the radioimmunoassay used for our study, although mechanical as opposed to chemical digestion of fish preceded extraction. Steroids were monitored December to May and seasonality was implied, although statistical relevance was unstated. Overall concentrations for both steroids were much higher than concentrations observed in this study: 17β-estradiol was five times higher and testosterone was approximately 2 times higher. Extraction efficiencies were higher for the pesticide lake study (83% and 111% for 17β-estradiol and testosterone, respectively) and were not corrected, meaning actual

concentrations were even higher for 17β-estradiol but lower for testosterone. Digestion efficiencies were not reported, nor were cross reactivities with other sex steroids. Thus direct comparison of results is difficult and laboratory differences, in addition to habitat and seasonal variations, may explain discrepancies.

Conclusions

Validations of gender identification, morphological and steroidal measurements support the use of mosquitofish as a bioindicator of pulp and paper mill effluent. Site and seasonal differences were not readily apparent in morphological measurements of male mosquitofish, especially in association to effluent-exposed sites. Precocious maturation could not be evaluated in the winter collection because small mature males were not captured in adequate numbers for statistical comparison. Therefore, this aspect must be re-examined.

For females, size class was not a complicating factor for the index of anal fin elongation overall, although differential life stage exposure or dynamic exposure could be speculated at the outfall site. Masculinization at sites closest to effluent discharge was evident for both anal fin morphology and steroids in females: anal fin elongation was independent of season, while effects on steroids were seasonal. This difference in seasonality may be the product of separate mechanisms, exposure to bioactive compounds, and/or perhaps differential exposure due to drought (see discussion below). However, both anal fin elongation and sex steroids were not measured in the same fish. The development and validation of computer-aided measurements allowed both endpoints to be measured in the same fresh fish for research presented in subsequent chapters.

Importantly, neither hormone concentrations nor ratio were altered in 100% final effluent prior to discharge. Anal fin elongation at this site was not examined until 2001 and 2002, and data is presented in Chapters 4 and 3, respectively. This result points toward the presence of bioactive compounds in the receiving stream below discharge and not in the effluent by itself, indicating additional environmental factors interact to produce the response. Which environmental factors are influential is pure speculation without chemistry, controlled exposure or long-term biomonitoring data associated with this study.

With that caveat, there is one factor that is often overlooked yet crucial to the hypothesized mechanism of anal fin masculinization via degraded phytosterols: bacteria. If the bacterial degradation hypothesis is correct, instream bacterial communities may be more efficiently converting phytosterols to androgens than communities living in retention ponds. For example, aerobic microorganisms degraded 17β-estradiol 60 to 130 days faster and by first-order kinetics compared to anaerobic microorganisms (Quinn 2004). Natural variation in bacterial communities may also alter degree of response for that matter. Only one type of bacteria (*Mycobacterium smegmatis*) has been examined in laboratory exposures to degraded phytosterols (Denton et al. 1985, Howell and Denton 1989, McCarthy et al. 2004), and the *Mycobacterium* genus is more efficient at transforming phytosterols to androgens than other bacterial species (Marcheck et al. 1972).

A potentially confounding factor in these studies was the variation in some responses at unexposed sites. Significant differences between reference and upstream sites existed for male gonopodial length and testosterone, and for 17β-estradiol in

females. If collection had been restricted to one unexposed site, responses would have been either masked or abnormally inflated. Unfortunately the ideal reference site does not exist: often an upstream site is the best at representing environmental habitats at exposed sites, although habitat can vary substantially along a stream. Therefore multiple reference sites within the receiving system region are imperative to document natural variation in response and allow more relevant interpretation. Overall, there was no difference in female anal fin elongation between unexposed sites, in agreement with the geographic study by Bradley et al. (2004). Their study collected at dozens of reference and polluted sites (not just pulp and paper mill effluent), and they pooled reference data for analysis since reference values were not significantly different. Since our study investigated several other endpoints in addition to female anal fin elongation, multiple reference sites were ideal when funding and efforts allowed.

Another confounding situation involved weather during this study. Summer 2000 fish were living under drought conditions, while winter 2000 fish were collected after relief from drought with a slightly higher than normal rainy season (Appendix A). According to Davis and Bortone (1992) and Cody and Bortone (1997), anecdotal observation of mosquitofish during times of drought corresponded to increased masculinization of the anal fin. The logical cause was a concentration of effluent, a plausible assumption for the low-flow streams characteristic of the Florida mills under investigation. This argument was used to support seasonal differences detected in anal fin length of females (Cody and Bortone 1997) since precipitation follows a seasonal pattern. However, since wild females survive 1 to 2 years, anal fin elongation among summer populations would still be present through the winter. According to McCarthy et

al. (2004), gonopodial development in female mosquitofish does not regress once effluent exposure ceases. This controversial point, however, has not been well documented. Sex steroid levels, in contrast, are more labile and subject to recovery in effluent-exposed fish (McMaster et al. 2003), and this study documented normal steroid ratios in the winter collection. Thus sex steroids may be a more sensitive marker of differential effluent exposure, while anal fin elongation is a more static and durable marker. Condition factor data cannot support or refute the potential influence of drought due to the experimental bias. A critical limitation of this drought hypothesis is a lack of exposure data to support concentration of effluent during times of drought. As an estimate of relative concentration of effluent, conductivity was greater in the summer than winter, providing some support to the drought hypothesis. Regular monthly field collections could shed more light on apparent trends. However, effects of drought should be considered when using mosquitofish as a bioindicator.

Two aspects of previously defined bioindicator criteria were addressed in this chapter: method and seasonal variation. Method variation was low, supporting the use of mosquitofish as bioindicators. Wild-caught females demonstrated greater effects than wild males. In females, seasonal variation related to exposure occurred for sex steroids but not anal fin elongation, indicating greater sensitivity of steroids than anal fin morphology. Ideally, both of these markers are measured for individual fish to better evaluate the hypothesized link between anal fin elongation and altered sex steroid profiles. An expanded year long seasonality study of mosquitofish living in pulp mill effluents would allow better interpretation of absolute hormone concentrations, and changes in anal fin morphology, especially since the present results are in conflict with

reported seasonal effects at Elevenmile Creek (anal fins) and reported concentrations of sex steroids in males.

Table 2-1. Water quality parameters of Rice Creek field collection sites in winter 2000

Site	REF2	U(8)	PRE-DIS	DIS	D(1)	D(3+6)
Winter 2000						
Water temperature (°C)	17.7	14.9	16.4	15.2	15.0	18.5
Conductivity (µS)	167	173	2132	1112	1315	1336
Salinity (ppt)	0.1	0.1	1.1	0.6	1.3	0.7
Dissolved Oxygen (mg/L)	7.60	7.00	4.14	7.23	11.90	8.44
Turbidity (ntu)	1.90	3.34	32.5	18.9	18.4	13.5
pH	6.73	6.45	7.35	7.23	7.00	7.15

Table 2-2. Correlation coefficients (r^2) for morphological measurements made before and after preservation in formalin; between USGS and NCASI laboratories; and between manual and computer-aided measurement by the same observer. Standard Length (SL) was only measured manually using digital calipers. All correlations were statistically significant at $p < 0.05$ unless noted.

Measurement	Ray 4	Ray 6	SL
Preservation	0.93	0.94	0.94
Between laboratories	0.54	0.80	0.97
Manual vs. computer-aided	0.27*	0.33*	NA[a]

[a] NA = not applicable

*not significantly correlated ($p > 0.05$)

Table 2-3. Average coefficients of variation for manual and computer-aided measurements by observer and among observers (three measurements per trait). Standard Length (SL) was only measured manually using digital calipers.

Measurement	Ray 4	Ray 6	SL
Manual			
By observer	3.1%	3.6%	1.4%
Among observers	8.8%	9.4%	5.6%
Computer-aided			
By observer	1.1%	0.9%	NA[a]
Among observers	12.3%	10.1%	NA[a]

[a] NA = not applicable

Table 2-4. Body size parameters (ave ± se) for mosquitofish collected in winter 2000

Site	REF2	U(8)	DIS	D(1)	D(3+6)
Winter 2000					
♂ Sample Size	45 (29,16)[e]	89 (61,28)	73 (73,16)	46 (29,17)	52 (32,20)
♂ Body Weight	0.255±0.010	0.289±0.008	0.255±0.013	0.221±0.006[a]	0.169±0.009[a]
♂ Standard Length (mm)	22.74±0.31	24.08±0.20[b]	22.24±0.31	22.95±0.25	23.78±0.33
♂ Condition Factor (g/cm^3)	2.13±0.03	2.03±0.02	2.24±0.02[c]	1.84±0.04[c]	1.22±0.03[c]
♀ Sample Size	141 (91,50)[e]	106 (64,42)	106 (53,53)	128 (99,29)	112 (65,47)
♀ Body Weight (g)	0.567±0.029	0.527±0.029	0.513±0.025	0.471±0.022	0.479±0.033
♀ Standard Length (mm)	28.97±0.48	28.67±0.46	28.18±0.44	27.37±0.40	29.13±0.59[d]
♀ Condition Factor (g/cm^3)	2.08±0.01	2.05±0.02	2.15±0.03	2.11±0.02	1.62±0.04

[a] D(1) statistically different than D(3+6); D(3+6) statistically different from all other sites

[b] U(8) statistically different than REF2, DIS, and D(1) ($p < 0.05$)

[c] DIS; D(1); and D(3+6) statistically different than rest ($p < 0.05$)

[d] D(3+6) statistically different than rest ($p < 0.05$)

[e] sample sizes displayed as: total sample size (preserved anal fin measurements, hormone and computer-aided measurements)

Table 2-5. Digestion and extraction efficiencies, and coefficients of variation (CV), by exposure and reproductive status for mosquitofish whole body hormone analysis.

	Digestion		Extraction	
	Efficiency	CV	Efficiency	CV
17β-estradiol				
Exposed ♂	70±4.7%	6.7%	66±6.6%	10%
Unexposed ♂	63±3.3%	5.2%	61±7.2%	11.7%
Exposed ♀	75±5.8%	7.7%	71.7±9.5%	13.2%
Unexposed ♀	76±7.6%	10%	60±5.8%	9.7%
Adult ♂	67±6.4%	9.6%	62±6.2%	10%
Adult gravid ♀	74±4.5%	8.9%	75±5.8%	12.7%
Adult nongravid ♀	72±4.1%	9.2%	60±6.3%	10.9%
Juvenile	69±2.8%	5.7%	67±4.9%	10%
Testosterone				
Exposed ♂	64±2.5%	3.9%	41±4.4%	14%
Unexposed ♂	57±3.9%	7.0%	44±2.5%	9.9%
Exposed ♀	58±3.9%	6.8%	45±4.1%	9.1%
Unexposed ♀	50±2.9%	5.7%	46±6.1%	13%
Adult ♂	89±3.1%	3.1%	66±3.7%	14%
Adult gravid ♀	61±4.5%	7.3%	61±4.6%	15%
Adult nongravid ♀	67±3.8%	5.4%	66±5.3%	10%
Juvenile	62±7.1%	11%	40±5.9%	15%

Figure 2-1. Maps of Rice Creek, a tributary of the Saint Johns River, FL, USA. A) Relative location in Florida. B) Summer 2000 field collection sites. C) Winter 2000 collection sites. D) Summer 2001 collection sites. Site symbols distinguish sites exposed to effluent: circles = unexposed and triangles = exposed. Site abbreviations denote upstream (U) or downstream (D) of discharge, followed by approximate distance (km) from discharge in parentheses. PRE-DIS indicates site before discharge into the creek; DIS denotes site at discharge into creek; and REF indicates reference site, followed by identifying number.

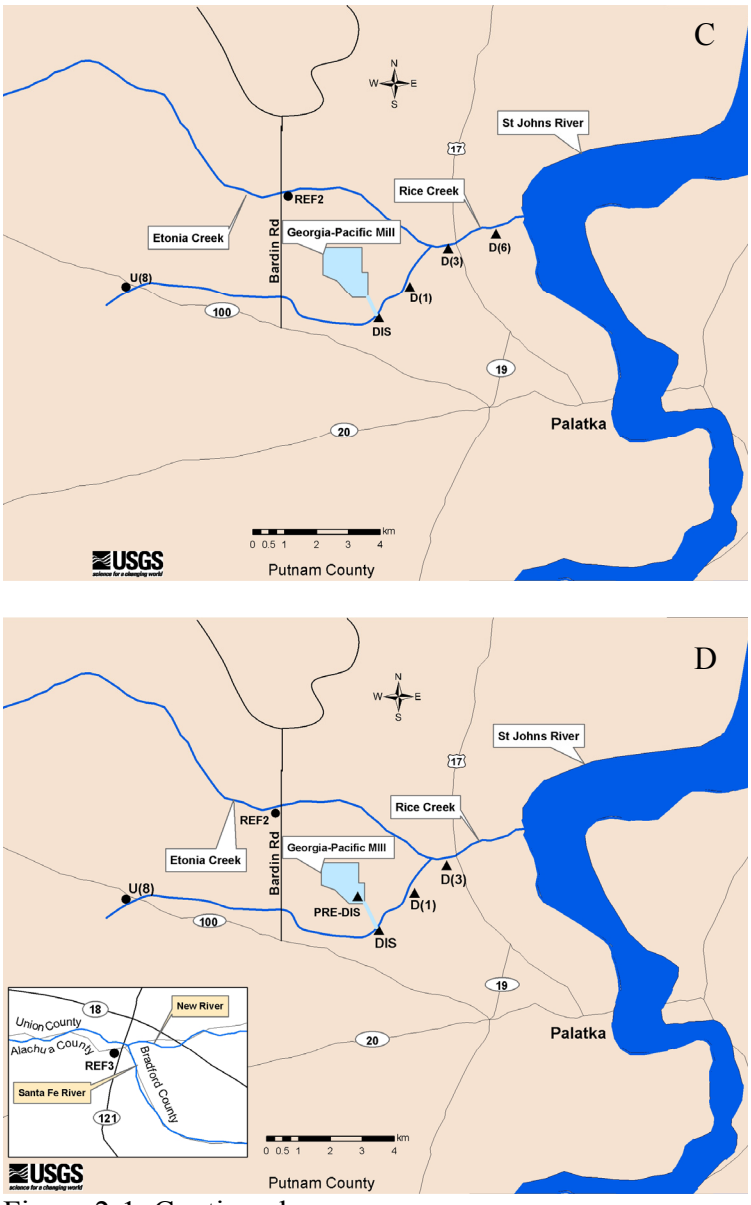


Figure 2-1. Continued

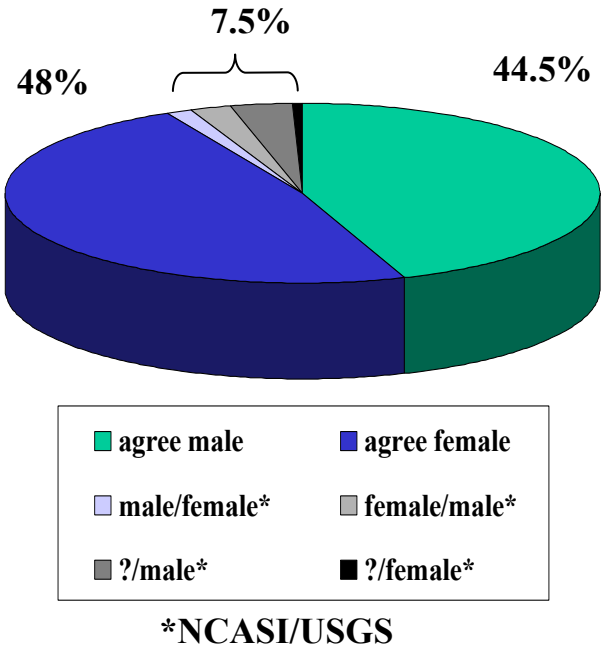


Figure 2-2. Gender agreement between NCASI and USGS laboratories. Fish were sexed externally by USGS using the urogenital papilla then gonads were grossly identified by NACSI. Question marks corresponding to NCASI were unable to be reliably sexed by gross gonad examination.

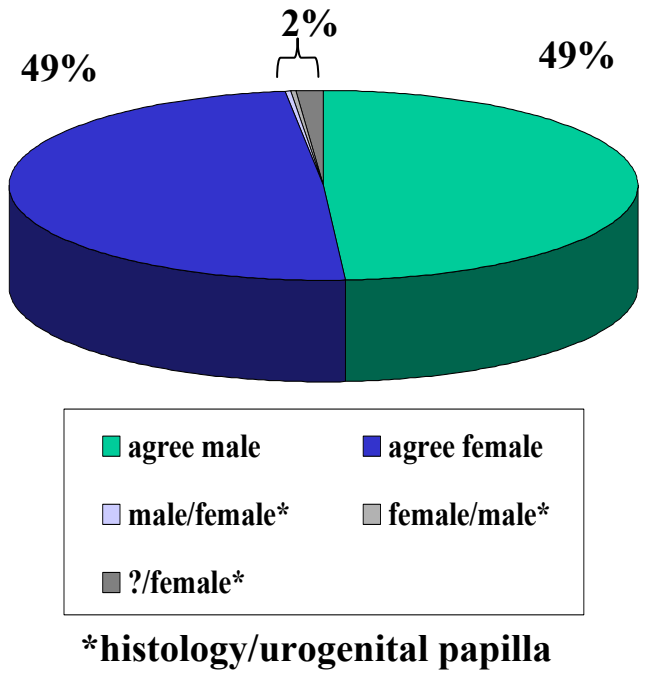


Figure 2-3. Gender agreement within USGS laboratory. Fish were sexed externally using the urogenital papilla then gonads were examined histologically. Question marks indicate gender could not be reliably identified by histological techniques.

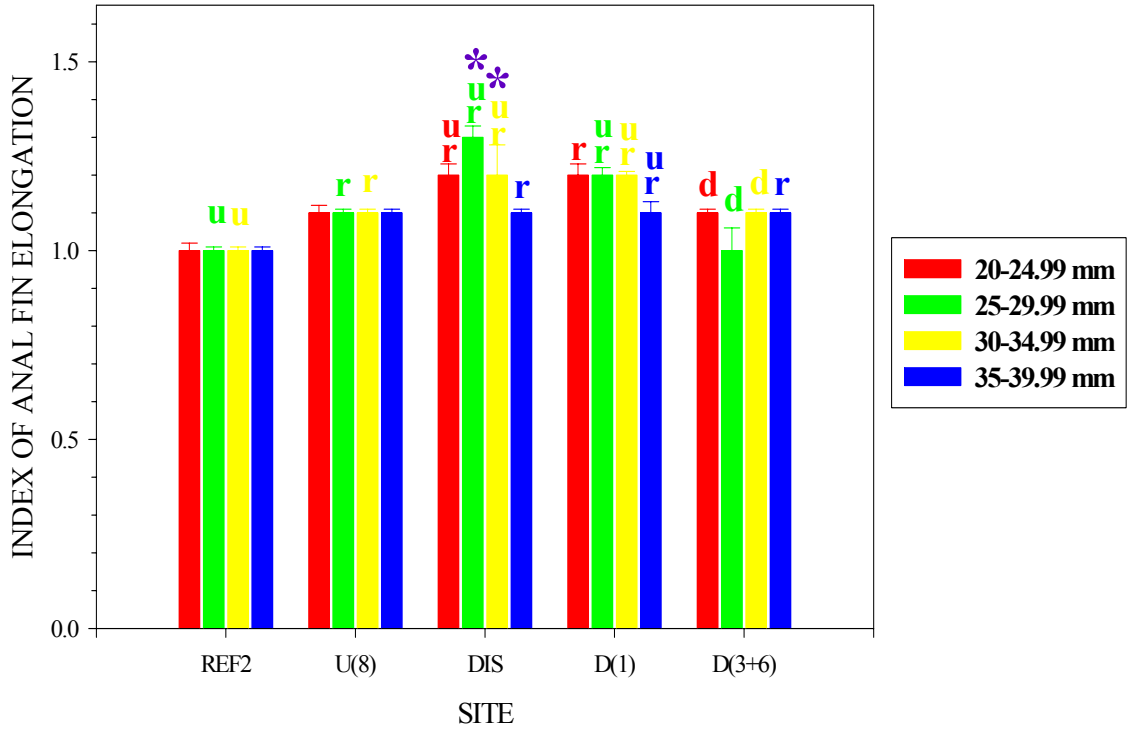


Figure 2-4. Female index of anal fin elongation (linear Ray 4 / Ray 6, manually measured on preserved fish) for each site by 5 mm increments (winter 2000). Significant differences ($p < 0.05$) are color coded within size class: u = different from upstream site [U(8)]; r = different from reference site [REF2]; and d = different from other exposed sites [DIS, D(1)]. Purple asterisks indicate significant differences to the largest size class within site ($p < 0.05$).

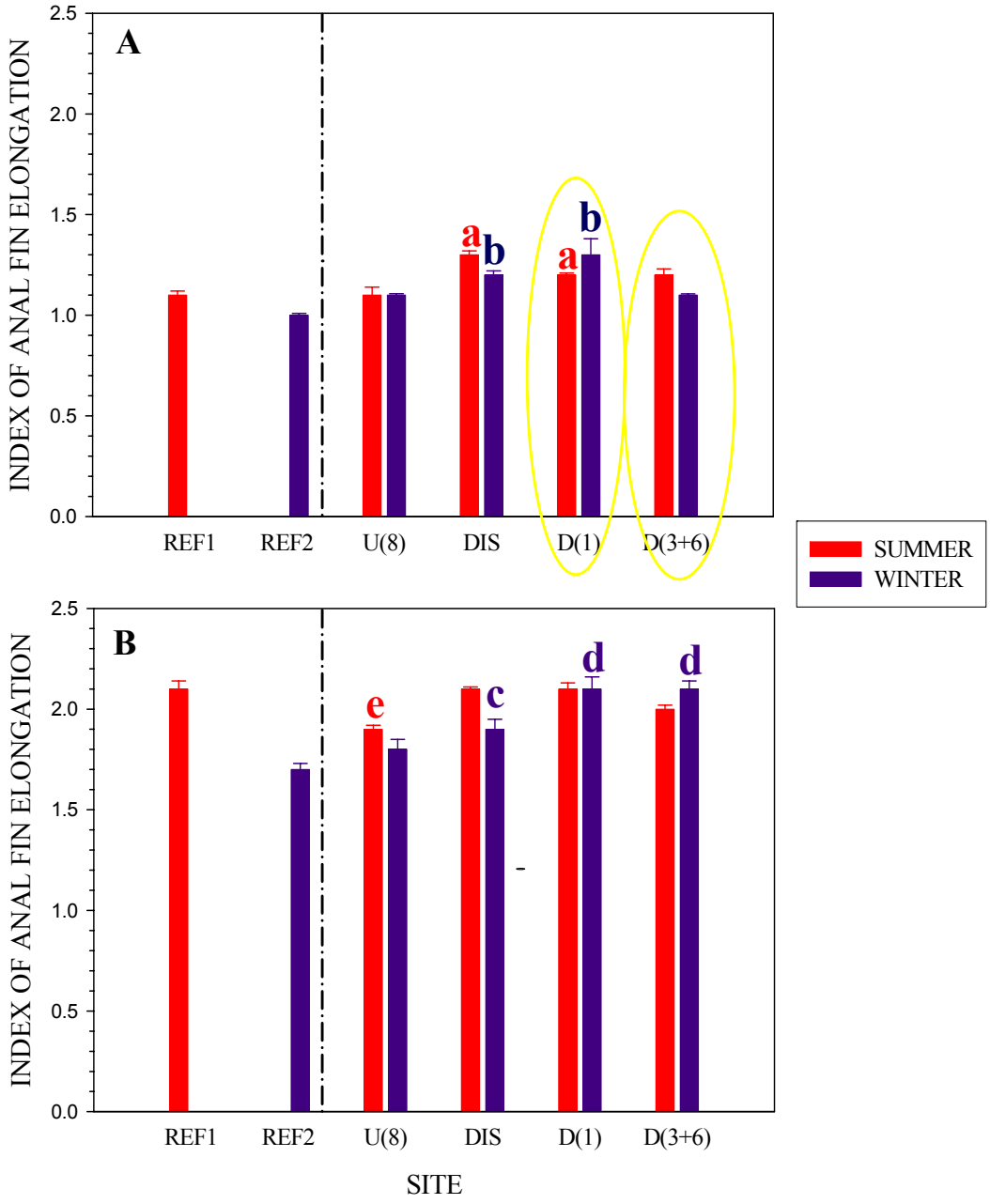


Figure 2-5. Index of anal fin elongation (linear Ray 4 / Ray 6, manually measured on preserved fish) for winter and summer months in 2000. A) Females. B) Males. Dashed lines separate sites not involved in ANCOVA analysis by site and season. Letters indicate significant differences among sites within season ($p < 0.05$): "a" denotes differences to nonlettered sites except D(1) was not different than REF1; "b" denotes differences to nonlettered sites; "c" denotes differences to all but REF1; "d" denotes differences to REF1 and U(8); "d" denotes differences to DIS, D(1), and REF1. Season was significant within site ($p < 0.05$) for females at the two downstream sites only [D(1) and D(3+6), circled in yellow].

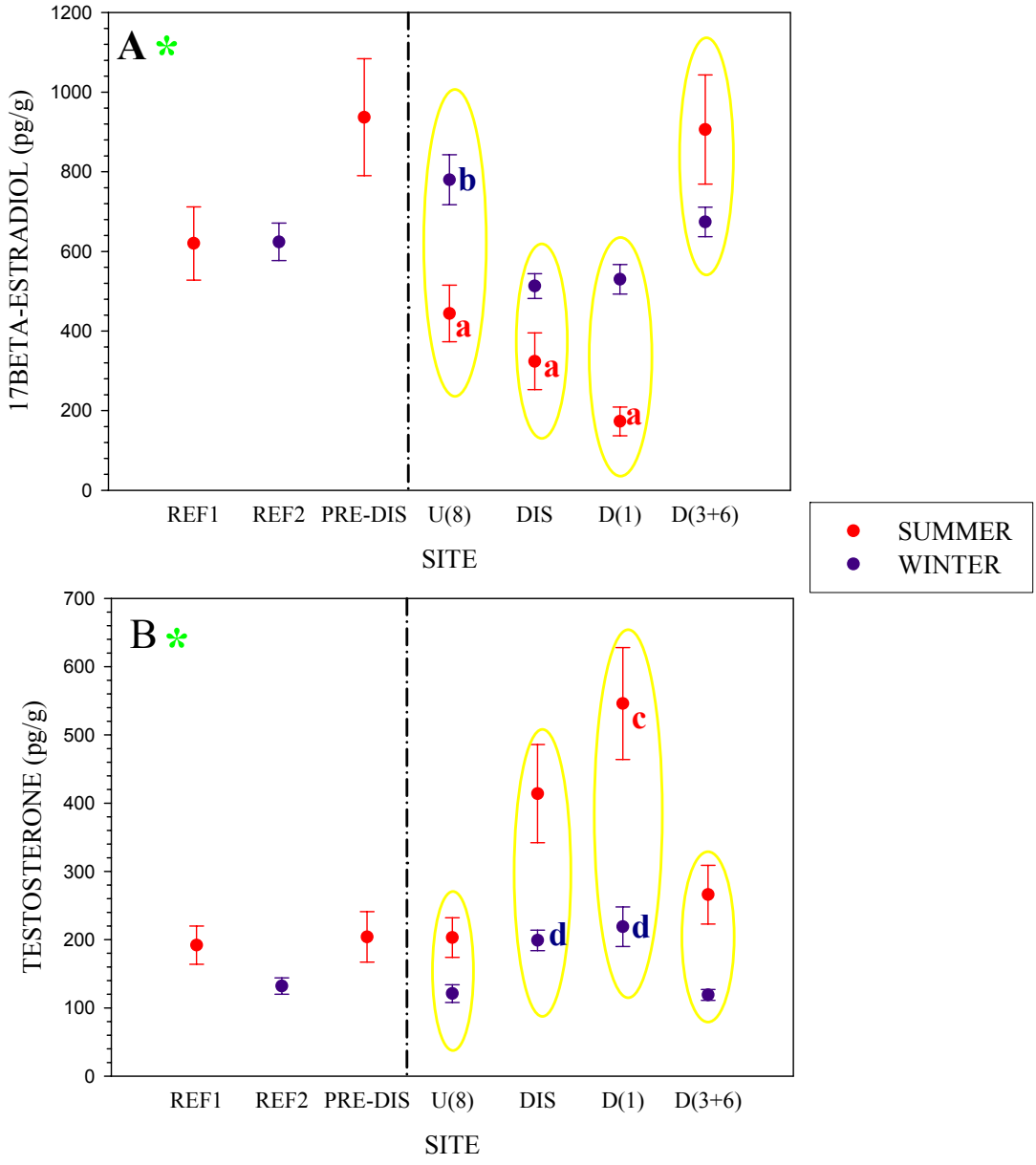


Figure 2-6. Female whole body sex steroids from collections made in the summer and winter of 2000 (ave + se). A) 17β-estradiol. B) Testosterone. Dashed lines separate sites not involved in ANCOVA analysis by site and season. Letters indicate significant differences by site within season ($p < 0.05$): "a" denotes differences to nonlettered sites; "b" denotes differences to DIS and D(1); "c" denotes differences to all but DIS; and "d" denotes differences to all other sites. Yellow circles demonstrate differences between seasons ($p < 0.05$). Green asterisk show that both hormones covary by site and season ($p < 0.05$).

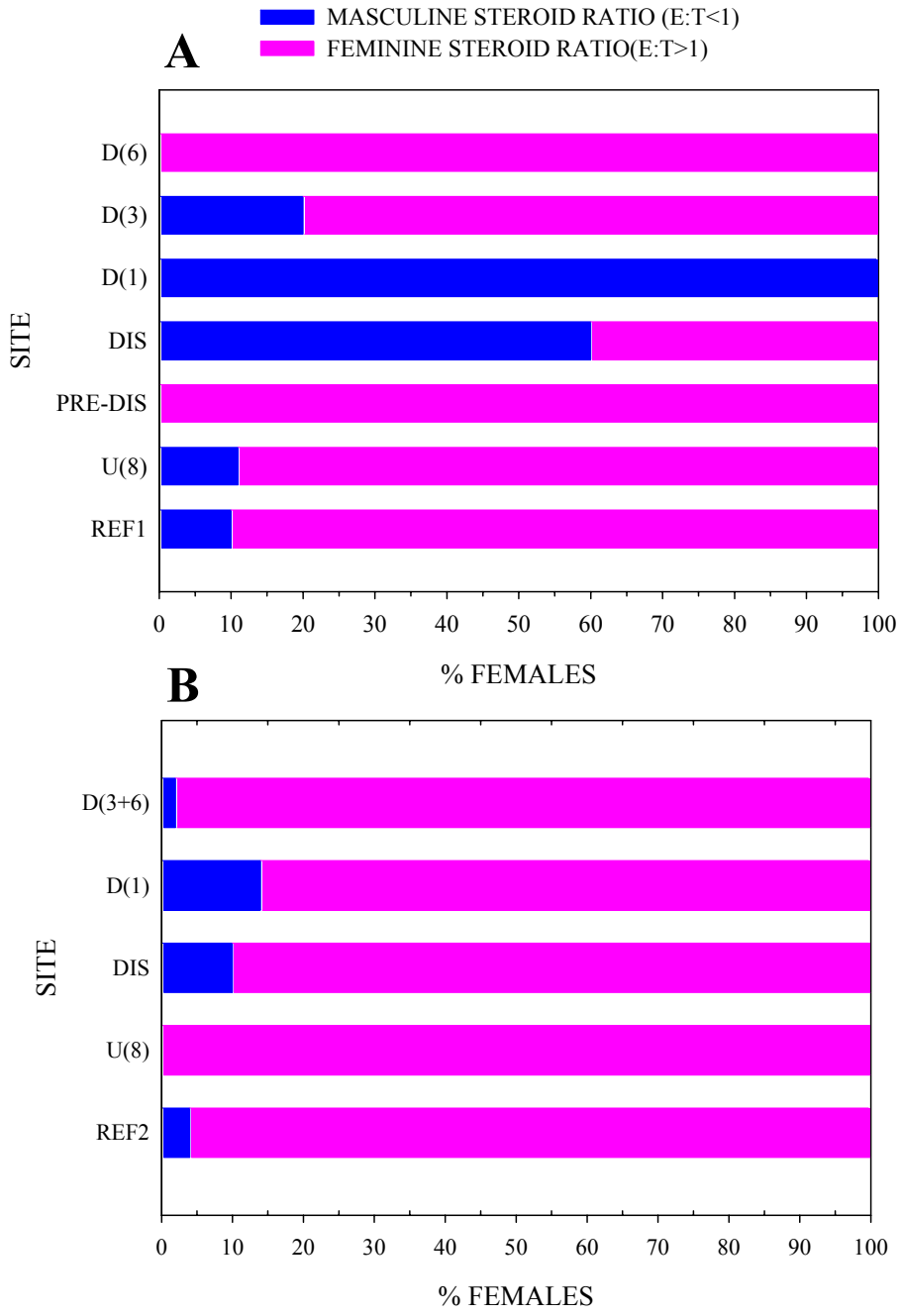


Figure 2-7. Percentage of female mosquitofish with masculine and feminine sex steroid ratios collected in 2000. A) Summer ($\chi^2 = 40.12$, df = 6, $p < 0.05$). B) Winter ($\chi^2 = 8.270$, df = 4, $p < 0.05$).

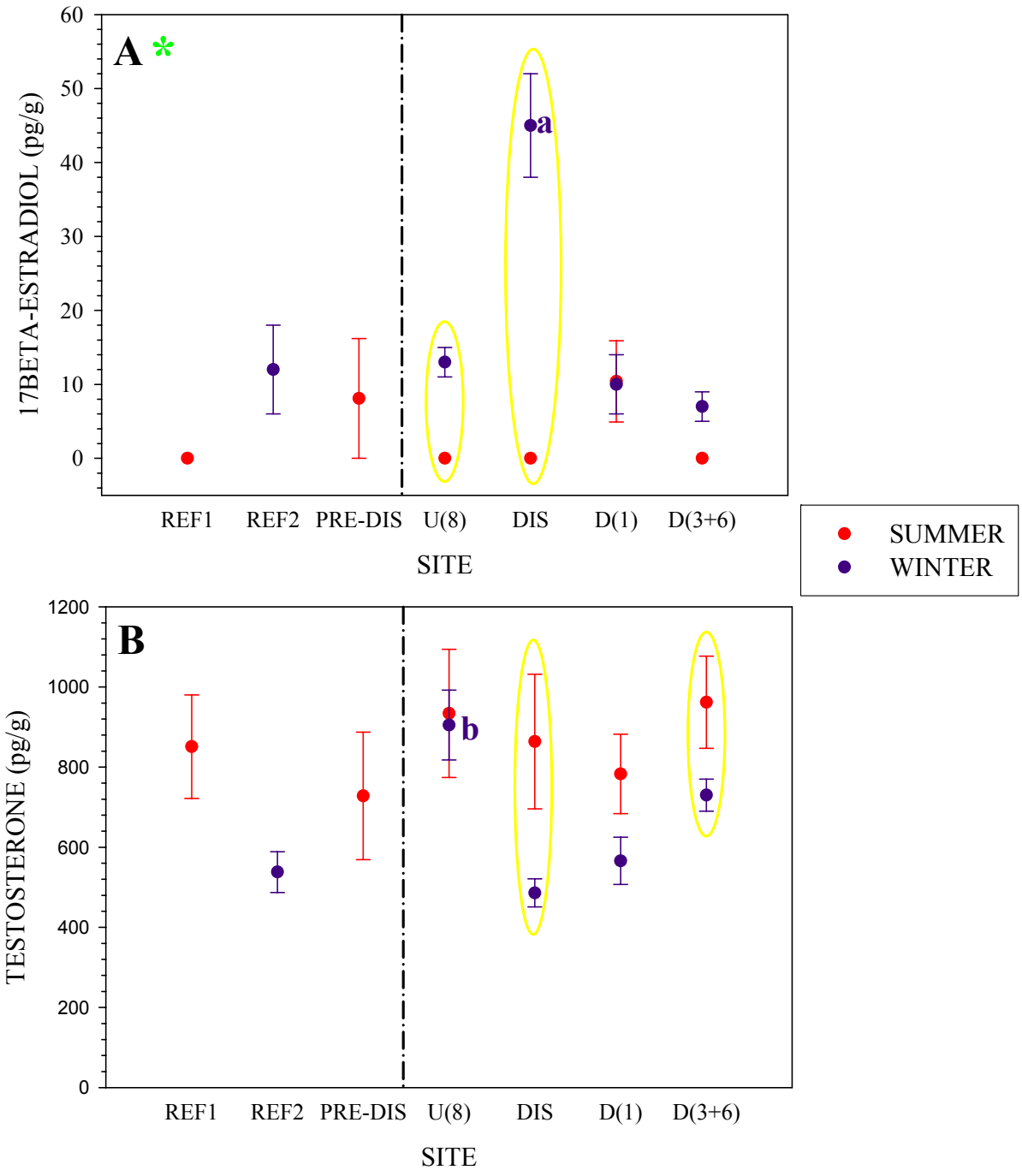


Figure 2-8. Male whole body sex steroids from collections made in the summer and winter of 2000 (ave + se). A) 17β-estradiol. B) Testosterone. Dashed lines separate sites not involved in ANCOVA analysis by site and season. Letters indicate significant differences by site within season ($p < 0.05$): "a" denotes differences to all other sites; "b" denotes differences to REF2, DIS and D(1). Yellow circles demonstrate differences between seasons ($p < 0.05$). Green asterisk signifies 17β-estradiol covaried by site and season ($p < 0.05$).

CHAPTER 3
DIMINISHED EFFECTS OF PULP AND PAPER MILL EFFLUENT ON EASTERN MOSQUITOFISH BEFORE AND AFTER MAJOR PROCESS IMPROVEMENTS

The US Environmental Protection Agency's (EPA) Cluster Rule was enacted in 1998 to regulate both air and water pollution by the pulp and paper industry. Final implementation of this rule was carried out in 2001 by the Georgia-Pacific bleached kraft mill in Palatka, FL. Comparison of mosquitofish collections before and after these major process changes was conducted to determine if responses were reduced or eliminated. Males and females were evaluated for anal fin morphology and whole body sex steroids, although male responses seemed more affected by potential seasonality as opposed to effluent exposure. Female anal fin elongation was consistently reduced, but not eliminated, in effluent-exposed sites. Female sex steroid concentrations were difficult to interpret and may be seasonally affected; however the ratio of 17β-estradiol to testosterone indicated a masculnized hormone profile remained despite processing improvements. No association between anal fin elongation and sex steroid levels or ratios was apparent. Anal fin morphology likely portrays longer term, chronic exposure whereas sex steroids provide a snapshot of more recent exposure. Overall, reduced anal fin elongation associated with major process changes supports the use of mosquitofish as a bioindicator of effluent exposure. Seasonal effects on sex steroids need to be clarified before use of this biomarker.

Introduction

Pulp and paper mills release a complex mixture of chemical compounds into the aquatic environment that has been a source of environmental concern for the past two decades (Borton et al. 2004). Compounds such as chlorinated organics, metals, and wood extractives (e.g. resin acids, fatty acids, phytosterols, and lignin) have been shown to cause an array of effects in fish (EPA 2002 and Borton et al. 2004). Acute lethal toxicity concerns are no longer an issue, while chronic sublethal toxicity remains controversial. Linking actual adverse effects to effluent component(s) has been a challenge since effluents are complex by nature, and their variation in effluent composition among mills and even within a single mill. Within this effort, a wealth of information has been generated about effects in fish.

Pulp and paper mill effluents have been shown to alter reproductive function in indigenous fish species. For example, white sucker (*Catostomus commersonii*) collected near Canadian mills had reduced ovarian steroid biosynthesis, delayed sexual maturity, decreased gonad size, and reduced expression of secondary sex characteristics (McMaster et al. 1991, Munkittrick et al. 1991, McMaster et al. 1995, Van der Kraak et al. 1992). Largemouth bass (*Micropterus salmoides*) exposed to whole effluent dilutions at the same mill examined in our study exhibited depressed sex steroids, vitellogenin, GSI, fry production and fry survival in largemouth bass (Sepulveda et al. 2001, Sepulveda et al. 2003).

Several reported effects on fish reproduction imply an androgenic effect. A Finnish totally chlorine free (TCF) kraft mill was associated with male-biased sex ratios in wild eelpout (*Lycodes* sp.) (Larsson et al. 2000). In a study of the same mill, increased male coloration was observed under laboratory exposure of livebearing guppies (*Poecilia*

reticulate) (Larsson et al. 2002). Full life cycle testing using the fathead minnow (*Pimaphales promelas*) in Canada demonstrated both masculinization of females and feminization of males (Parrott and Wood 2002, Parrott et al. 2003). Pulp mills in Florida have been associated with development of male secondary sex characteristics in female mosquitofish (Howell et al. 1980, Bortone and Drysdale 1981, Cody and Bortone 1997, Bortone and Cody 1999, Jenkins et al. 2001, Parks et al. 2001) and possibly precocious maturation in male Eastern mosquitofish (*Gambusia holbrooki*) (Howell et al. 1980, Drysdale and Bortone 1989). Unique among the vast majority of these effects-based studies, the mosquitofish work has led to a proposition of bioactive compounds: androgens formed by bacterial degradation of phytosterols. This hypothesis has stimulated controversial discussions about mosquitofish as a bioindicator or sentinel species for pulp and paper mill effluents.

Eastern mosquitofish have been considered for use in regulatory testing and screening of pulp mill effluent toxicity at both the state (Florida Department of Protection, T. S. Gross, pers. comm.) and federal levels (Angus et al. 1997). Mosquitofish have been proposed since most other wild small fish species found in effluent-receiving streams cannot be collected in adequate numbers or maintained in the laboratory for use in research.

As data on reproductive effects in fish has been generated, pulp and paper mills have been improving and refining process technologies. Upgrades in pulp production processes have the potential to reduce if not abolish reported effects. For example, short-term laboratory exposures of goldfish (*Carassius auratus*) revealed a recovery of steroid function following unknown process changes (McMaster et al. 1996). More recently,

temporary shutdown of the TCF mill associated with male-biased sex ratios in eelpout allowed recovery of normal sex ratios in the exposed population (Larsson and Förlin 2002).

With the implementation of US EPA's Cluster Rule in 2001, the Georgia-Pacific mill located in Palatka, FL, USA has modified its pulping and bleaching processes. The objective of this study was to evaluate the influence of these process changes on a native small fish species, the Eastern mosquitofish, specifically examining effects on two endpoints: anal fin morphology and sex steroids.

Materials and Methods

Mill Characteristics

Georgia-Pacific's mill in Palatka, Florida, USA is a paper grade bleached kraft mill established in 1947. It has two bleached (40% product) and one unbleached line (60% product). The bleaching lines manufacture paper towels and tissue paper, whereas the unbleached line produces kraft bags and linerboard. Wood furnish for this mill typically consists of 50% softwood (slash, sand and loblolly pines) and 50% hardwood (gums, tupelo, magnolia and water oaks) cycled back and forth between the two types of furnish. Effluent receives secondary treatment consisting of anaerobic followed by aerobic degradation with a retention time around 40 days.

Effluent discharges into Rice Creek, nearly 6 km upstream of the confluence with the Saint Johns River (Figure 2-1). Rice Creek is a low-flow, tannic stream, so dilution factor for effluent is low until it reaches the Saint Johns River. Before process changes the average yearly instream effluent concentration was approximately 60% until reaching the St Johns River, where concentration dropped below 10%. In contrast, most North

American mills average less than 5% yearly instream effluent concentration by discharging into larger and/or faster-flowing bodies of water.

Before major process improvements, the Palatka mill released approximately 36 million gallons of effluent per day (mgd). Bleaching pre-process modifications used elemental chlorine and up to 10% chlorine dioxide substitution. The bleaching sequences were $C_{90}d_{10}$EopHDp and CEHD for the softwoods and hardwoods, respectively. Process modifications in May 2001, to meet EPA the Cluster Rule, involved: 1) conversion to ECF bleaching via 100% chlorine dioxide substitution; 2) reduction in black liquor losses; 3) added condensate stripping; 4) conversion of all retention ponds to aerobic degradation; and 5) reduction in water use resulting in release of approximately 28 mgd effluent. The current bleaching sequence is DEopD for both types of furnish.

Field Collections

Field collections of adult mosquitofish occurred during the reproductively active summer months for this species one year before (March and June 2000, n = 174 and n = 141 respectively) and one year after (April 2002, n = 363) process modifications. Water quality parameters typically affected by pulp and paper mill effluents were measured before fish collection at each site: dissolved oxygen, temperature, pH, conductivity, salinity, and turbidity. Adult Eastern mosquitofish were collected along shallow vegetated banks using dip nets and a backpack electroshocker at several locations upstream and downstream of effluent discharge in Rice Creek, and at reference sites lacking effluent exposure or any other known point sources of pollution (Figure 3-1 and Appendix A). Fish were transported back to the laboratory in oxygenated bait buckets then euthanized with a terminal dose of buffered tricaine methanesulfonate (Tricaine-S, Western Chemical Inc., Ferndale, WA, USA).

Morphology

Once euthanized, fish were examined under a dissecting scope to determine gender using the urogenital papilla (Chapter 2). General measurements of body size and selected measurements of anal fin morphology were taken for all adult fish collected. Body weight (± 0.001 g) and standard length (± 0.01 mm) were measured using a digital scale and a pair of digital calipers. In 2000, the March collection was preserved in 10% neutral-buffered formalin for anal fin measurements while the June collection was frozen and stored at -80°C for subsequent radioimmunoassay (RIA) of sex steroids. Linear distance from base to tip of Rays 4 and 6 of the anal fin (± 0.1 mm) were measured for formalin-preserved fish under a dissecting scope using an ocular micrometer. In 2002, a subset (n = 87) was preserved in 10% neutral-buffered formalin for comparison to 2002 anal fin data. Remaining fish were photographed digitally before freezing for sex steroid analysis. Digital photographs of anal fins for these fish were measured using a computer software program (SigmaScan Pro 5.0, SPSS, Inc.), tracing along the lengths of Rays 4 and 6 (± 0.01 mm). Chapter 2 gives validation of these morphological measurements.

Sex Steroids

Whole body primary sex steroids (17β-estradiol and testosterone for this species) were analyzed using a modified RIA method originally developed for serum and plasma samples of common carp, *Cyprinus carpio*, (Goodbred et al. 1997), and since adapted for use in a variety of other aquatic species and tissue media such as plasma of largemouth bass (Gross et al. 2001) and mantle of freshwater invertebrates (Gross et al. 2000). Chapter 2 gives methods and validation of this assay.

Statistics

Body weight and standard length were used to calculate condition factor, K = weight / length^3 x 100 (g/cm^3), as an indication of overall health used by the aquaculture industry (values greater than 1 are considered healthy; less than one are considered poor). The length ratio of anal fin Rays 4 and 6 was calculated as an index of anal fin elongation. Estrogen and testosterone concentrations were used to calculate a ratio indicating either a masculine hormone profile (E:T < 1) or a feminine hormone profile (E:T > 1).

Any data failing tests for normality and homogeneity of variance were log transformed. Anal fin morphology and sex steroid concentrations were analyzed separately within sex using two-way analysis of covariance (ANCOVA) to test for significant variation by site and year. Site differences within year were also analyzed by one-way ANOVA. Significant differences in the ANCOVA and ANOVA were followed by multiple comparison tests using Tukey's HSD. Within site, differences between years were analyzed by Student's t-test. Fish measured for both anal fin morphology and sex steroids (2002 data only) were analyzed in two ways: first, by examining Pearson's correlations of the index of anal fin elongation to sex steroid concentrations and ratio, then by t-test for differences in index of anal fin elongation between females with masculine versus feminine E:T ratios. Statistical significance was set at $\alpha < 0.05$ for all tests. All statistical analyses were conducted using SAS © version 9.0.

Results and Discussion

Water Quality

As expected, conductivity, salinity and turbidity were higher at effluent-exposed sites compared to the upstream site (Table 3-1). The reference site REF1 was more

similar to effluent-exposed sites than the upstream site in terms of water quality parameters. Dissolved oxygen remained high enough to support fish at most sites (> 4 mg/L), except in 2002 at the predischarge site [PRE-DIS] where levels were extremely low (< 1 mg/L). The fact that mosquitofish were present in this low oxygen environment demonstrates their tolerance to extreme environmental conditions. After process changes, conductivity was reduced at downstream sites sampled both years [DIS, D(1), D(3)] as a gross indication of reduced effluent concentration and/or improved effluent quality.

Body Size and Condition

Effluent exposure, regardless of process changes, was not associated with alterations in body size or condition in both male and female mosquitofish (Table 3-2). Rather, differences in condition between years were drastic (poor condition with CF < 1 in 2000 compared to good condition with CF > 1 in 2002), likely caused by fixation of 2000 fish before body size measurement (thereby decreasing weight of fish). For the 2000 collection, a statistically significant difference in these parameters occurred between populations in Rice Creek compared to Saint Johns River, therefore river sites were excluded from sampling in 2002.

Males

Before process changes, males at the first downstream site [D(1)] were statistically longer than males from the upstream site [(U8)] (Table 3-2). Otherwise, males collected at effluent-exposed sites [DIS and D(3)] did not show any significant variation from unexposed sites. Males living in Saint Johns River [D(6) and REF1] were in the poorest relative condition of all sites collected, indicating these populations may be significantly different than creek populations. Condition factor cannot be assessed in terms of general

health status since this collection was preserved before measurement of body size. After process changes, effluent exposure had no obvious effect on size or condition of males. Although length and weight of males at the discharge site [DIS] was significantly less than upstream males [U(8)] (length) or all other sites (weight), condition factor was statistically equivalent and above one indicating good overall health.

Females

Before process changes, female body size and condition was not overtly affected by site when comparing exposed to nonexposed sites (Table 3-2). Statistically significant variation occurred between females from unexposed sites [U(8) and REF1] for length, weight and condition factor. In addition, condition factor was statistically different between the upstream site [U(8)] and the confluence of Rice Creek and Saint Johns River [D(6)]. Together with data on males, these data indicate variation between populations inhabiting the creek compared to the river. Thus, these river sites were excluded from collection in 2002.

Similar to males, female body size and condition in 2002 were greater than what was observed in 2000 most likely caused by preservation state. Overall variability in length was reduced. Females collected from retention ponds before discharge into Rice Creek [PRE-DIS] were statistically larger in weight and length while condition factor was less, compared to the upstream site [U(8)]. Although statistically significant differences in condition factor were detected (reduced in PRE-DIS and elevated in DIS), these differences are not appropriately interpreted beyond the benchmark of above or below a value of 1. All values were above one indicating good general health.

Anal Fin Morphology

Major process changes at the mill in 2001 were associated with a reduction, but not elimination, in the masculinization response for female mosquitofish. Further, 2002 data implied a dose-dependent response. Length of gonopodia in males was questionably affected before process changes, and definitely not affected after improvements. Rather, an overall shift toward greater gonopodial length was evident after processing modifications, perhaps reflecting environmental stress on these animals in 2000 separate from effluent exposure. Variation in response at reference sites occurred for males but not females (2000 data only, additional reference site was not included in 2002 collection).

Males

Figure 3-2 shows the index of anal fin elongation for male gonopodia before (2000) and after (2002) major process modifications. For both years, gonopodia (Rays 3, 4 and 5) extended at least twice as long as the rest of the fin. At the tips, the gonopodia were marked by terminal differentiations (hooks, serrae and blade; in the photographs, hooks are the most visible of these structures). These terminal structures signify complete maturation and the end of gonopodial development.

Before process changes, male index of anal fin elongation was significantly different among sites (Figure 3-3A); however, the relationship to effluent exposure was unclear. Compared to males from the upstream site [U(8)], gonopodia were longer at the discharge [DIS] and at the first downstream site [D(1)]. Yet the reference site [REF1] also had males with significantly longer fins in relation to upstream males. Therefore, significant differences were dependent on both unexposed and effluent-exposed sites.

After process changes, males no longer had any statistically significant differences in the index of anal fin elongation among sites (Figure 3-3B). Within each site, distribution of the index was normal, as opposed to the nonnormal distributions that characterized 2000 male data (compare Figure 3-3A and B). Interaction of site and season was not statistically significant for male gonopodial length. For all sites collected both years [U(8), DIS, D(1)], males had significantly longer gonopodia in 2002 regardless of effluent exposure. Since observer and experimental bias was not an issue like the body size data (Chapter 2) these data may allude to environmental stress such as the 1999/2000 drought (Appendix A). Unfortunately, the potential stress of a drought obscures any conclusions that could have been made about precocious maturation, or the development of gonopodia in younger, smaller males. Under ideal environmental conditions, precocious maturation would be demonstrated by consistently longer gonopodia relative to standard length at exposed sites versus unexposed sites and at exposed sites before process changes versus after modification.

Females

Figure 3-4 shows differences in female index of anal fin elongation among field collection sites before (2000) and after (2002) major process modifications. For both years, anal fin elongation resembled a developing male gonopodium, as opposed to a mature gonopodium, in both length of elongation (averaging 1.2 in 2000 and 1.1 in 2002 for females versus 2.0 and 2.5 for males) and complete lack of terminal differentiations (Figure 3-2).

In 2000, female mosquitofish had significant anal fin elongation at the discharge [DIS] and first downstream site [D(1)] compared to all other sites, as displayed in Figure 3-5A. Further downstream, elongation of the anal fin was not significant [D(3) and

D(6)]. In contrast to body length and weight measurements of females, there was no statistical difference in anal fin elongation between the reference and upstream sites [REF1 and U(8)].

In 2002, anal fin elongation remained at effluent-exposed sites [PRE-DIS, DIS, D(1)] compared to the upstream site (Figure 3-5B). Dose-dependence was implied by decreasing elongation with increasing distance from the discharge point. Anal fin elongation before discharge was not significantly different to elongation at the discharge site, while elongation was different between the discharge and first downstream sites. Interestingly, anal fin elongation was present at the 100% final effluent site before discharge, despite no effects observed on sex steroid ratios in 2000 (Chapter 2 and Figure 3-7). Similar to males, the combination of site and year did not covary significantly. However, anal fin elongation in females was significantly reduced (average 8% reduction) after process changes for effluent-exposed sites [DIS, D(1), D(3)] but not the upstream site. Thus the masculinization response appears reduced, but not eliminated, in regard to mill process improvements.

Sex Steroids

Absolute hormone concentrations between years for both males and females indicated seasonal changes as opposed to mill processing-related changes. The exception was elevated testosterone in females exposed to effluent in Rice Creek in 2000 but not 2002, implying a reduction in masculinized hormonal response. However, masculinized or testosterone-biased hormone profiles remained dominant further downstream at effluent-exposed sites in 2002. For both years, sex steroids in females living in 100% final effluent before discharge were not altered similar to instream females, supporting the contribution of additional environmental factors to produce an endocrine response or

indicating a differential or dynamic exposure to bioactive compounds that allows steroid levels to recover. (Dynamic exposure refers to variable concentrations of effluent components over time, dependent on factors such as tree species for furnish, within plant processing spills, rainfall/dilution, and bacterial degradation.) Large individual variation within sites and significant differences between unexposed sites, indicate a naturally high variation in this biomarker.

Males

Male sex steroids before process changes were not affected by effluent exposure (Figure 3-6). 17β-estradiol concentrations were between nondetectable levels to 50 pg/g, while testosterone values ranged between 500-1200 pg/g and displayed large variation among individuals. Estrogen to testosterone ratios were extremely dominated by testosterone across all sites (less than one, approximately 0.01 to 0.001). After process changes in 2002, male sex steroids varied among effluent exposed sites but not to the upstream site. There was no statistical interaction or covariance between site and year.

Comparing years, a marked overall increase of 17β-estradiol in males characterized post-process changes, approximately five times higher than concentrations measured before improvements and within range of concentrations reported by Toft et al. (2003). Testosterone, on the other hand, remained within the same range and variation before and after process improvements. As further evidence of the shift in 17β-estradiol, estrogen to testosterone ratios were much closer to one (0.36 to 0.69), while still retaining a masculine profile in the majority of males. Since this change was independent of effluent exposure, most likely this is further evidence for seasonality of sex steroids in male mosquitofish (compared to winter and summer 2000 analyses in Chapter 2).

In partial support of this evidence for seasonality, Toft et al. (2003) measured sex steroids in male mosquitofish from December to May in reference and pesticide-contaminated lakes and found a decrease in 17β-estradiol concentrations from December (300 pg/g) to March (100 pg/g), with levels beginning to rise in April back to December values by May (regardless of exposure). Since 2000 and 2002 collections occurred in April and June, respectively, 17β-estradiol levels may reflect this normal rise during the beginning of reproductive season. Unfortunately the other half of the cycle, from June to November, was not included in Toft et al. (2003), nor were hormones monitored continuously throughout one year for our study. Thus there is an incomplete inderstanding of the seasonality of hormone concentrations in males.

Females

Before process changes, 17β-estradiol was depressed in females from upstream and downstream sites [U(8), DIS, D(1)] compared to remaining sites (Figure 3-7A). Notably, 17β-estradiol in females was not depressed in 100% effluent before discharge [PRE-DIS]. As explained in Chapter 2, the differences between unexposed sites imply a natural variation for this hormone and do not consistently indicate impacts from effluent exposure. Testosterone was elevated at the first downstream site in 2000, but was not impacted in 100% final effluent before discharge [PRE-DIS] (Figure 3-7B). Thus, elevated testosterone concentrations were associated with initial instream effluent exposure but not at highest effluent concentrations before discharge. Estrogen to testosterone ratios were masculinized for most females (less than one, in favor of testosterone) at the discharge [DIS] and first downstream [D(1)] sites (average ratios of 0.8 and 0.3, respectively). Average ratios were normal for females (above one, in favor

of estrogen) for most females from all other sites, although a low background level of masculine ratios existed at unexposed sites. In Chapter 2, Figure 2-6A depicts the percentage of females collected during the summer 2000 with normal estrogen to testosterone ratios (> 1) and those females with masculinized ratios in favor of testosterone.

Sex steroids for females did not vary among sites for 2002, and were significantly reduced for both hormones at all but one site [17β-estradiol at DIS] compared to 2000 hormone data (Figure 3-7). Consistent differences between years regardless of effluent exposure implied seasonality of a different nature than males, although a decrease in both hormones is counter-intuitive to the onset of reproductive season. Lacking basic knowledge of year to year seasonality in unexposed females, these hormone data remain difficult to interpret.

Estrogen to testosterone ratios, conversely, clearly indicated hormonal masculinization (E:T > 1 for greater than 50% of females) at instream effluent exposed sites (Figure 3-8). Average sex steroid ratios were testosterone-biased at the two furthest downstream sites [0.7 at both D(1) and D(3)], but not before or at the discharge sites [1.3 at PRE-DIS and 1.0 at DIS]. This shift in masculinized hormone profile farther downstream of effluent outfall following processing improvements may also indicate that additional environmental factors influence hormonal response as predicted by the bacterial degradation hypothesis for anal fin elongation.

Association Between Anal Fin Morphology and Sex Steroids

Females collected for sex steroid analysis in 2002 were also photographed for computer-aided measurement of anal fins (separate from manual measurements made for

comparison to 2000 anal fin data). No correlation existed for the index of anal fin elongation to estrogen, testosterone, or the estrogen to testosterone ratio ($r^2 < 0.5$ and $p>0.05$ for correlations). As further proof against relationship between these endpoints, Figure 3-8 gives average (±se) index of anal fin elongation for females with masculine and feminine hormone ratios. Across all sites and within site, elongation did not differ between the two groups. Females with normal, feminine hormone profiles were just as likely to have masculinized anal fins as females with masculine hormone profiles. This does not rule out alterations in sex steroid ratios contributing to elongation of the anal fin, since sex steroids were measured after onset of elongation. Seasonal changes in sex steroids, but not anal fin elongation, support this point (Chapter 2). However, presence of the hormonal alteration cannot be used to predict occurrence of anal fin elongation in individual females living in effluent-receiving streams, based upon these data.

Conclusions

Major processing improvements implemented in 2001 by the Georgia-Pacific Palatka, FL mill to meet Cluster Rule requirements did not eliminate responses in wild female mosquitofish inhabiting Rice Creek, while males are likely not impacted. Female anal fin elongation was significantly reduced at downstream sites (average 8% reduction), while masculinized sex steroid ratios remained with a majority bias shifted further downstream. No clear relationship exists between anal fin elongation and whole body primary sex steroid concentrations or ratios. Importantly, anal fin elongation at the predischarge 100% effluent site was equivalent to elongation detected in the creek, even though hormone profiles were not affected.

The 2002 female anal fin data alludes to a dose-dependent response in the creek, however an increased effect is not observed at the site of highest effluent concentration

(before discharge into the creek). Combined with the 2000 anal fin data, anal fin elongation appears to be a threshold response. Angus et al. (2001) concluded an all-or-none response of female anal fins to dietary 11-ketotestosterone exposure (at 0, 20, 40, 60, 80, and 100 µg/g). The highest concentration produced elongation and terminal differentiation the fastest, while all other treatments were comparable in terms of rate of elongation (but not rate of differentiation which varied inconsistently). Extent of elongation was less in the lowest dose, but similar among all other doses. Also, length ratio of Rays 4 and 6 (measured by computer) ranged from 1.35 to 1.50. An actual threshold could not be calculated since all treatments responded, and can only be stated as less than 20 µg/g feed. Comparing current data to these results, mosquitofish in Rice Creek are probably exposed below test concentrations of androgenic compounds but above the actual threshold. Average index of elongation at exposed sites was 1.2, and no terminal differentiations were recorded. This implies a partial or incomplete response at Rice Creek and exposure may be near the threshold concentration.

Hormone data reveal within season differences in addition to effluent exposure likely influenced response. Without baseline knowledge of seasonality in hormones, interpretation of effects due to exposure is tentative. An acclimation of response in the highest exposure group (before discharge) may be possible, since both years revealed no effects of exposure on estrogen to testosterone ratios. Equally possible is the short-term recovery of normal steroid ratios during periods of exposure below threshold.

Concentration of bioactive compounds may be cycling above and below the threshold. Wood extractives present in final effluent can vary widely over short periods of time (Chapters 5 and 6). The Rice Creek mill cycles between hardwood and softwood

tree species, and softwoods generally contain more wood extractives such as phytosterols (Smook 1999, Svenson and Allard 2004). In addition, there is the "black box" of bacterial communities and how they change over time in the predischarge secondary treatment lagoons and the receiving stream. Even more exposure complexity may occur in these low-flow systems from precipitation and periods of drought and flood (Chapter 2 and Appendix A). A scenario of dynamic exposure appears plausible.

This concept would also explain the lack of relationship between anal fin elongation and sex steroids. Lister and Van der Kraak (2001) pointed out a similar lack of relationship between sex steroids and other measures of reproductive function (gonad size, age at maturity) among several species exposed to pulp mill effluents in Ontario. As mentioned in Chapter 2, sex steroids are likely more sensitive and labile biomarkers than anal fin elongation which appears more static.

Unfortunately, lacking exposure data specifically for these studies limits validity of this concept of dynamic effluent exposure and complete demonstration of improved effluent quality due to process changes. Research on other fish species has also been conducted for the Rice Creek system using controlled effluent exposures, providing indirect information on exposure before and after process changes. Life cycle exposure to fathead minnows by NCASI occurred before (1998) and after (2002) mill process changes (NCASI 2000a and NCASI, unpublished data). Based upon chemical analyses of 100% whole effluent samples, before process changes this mill had some of the highest concentrations of organic compounds compared to other kraft mills studied by NCASI. For example, 615$\pm$369 µg/L for three fatty acids, 4,008$\pm$1,675 µg/L for nine resin acids, 174$\pm$33 µg/L for three chlorinated resin acids and 380$\pm$169 µg/L for four

phytosterols. These concentrations dropped substantially after process improvements: chlorinated resin acids dropped by 97% while resin acids, fatty acids and phytosterols dropped an average of 80%. The fact that we observed relatively modest reduction in effects in the face of these significant reductions to effluent components further supports the concept of a threshold effect as opposed to a dose-response effect. Virtual removal of chlorinated compounds also implies they are not the bioactive agents causing anal fin elongation in mosquitofish.

Additional data on resin acids and phytosterols (campesterol mainly) in bile of largemouth bass exposed just before process changes in 2001 and after process changes in 2002 similarly indicates reduction in exposure (Quinn 2004). Further, excretion of these compounds in bile dropped at 40% and 80% exposure indicating inhibition of detoxification pathways. This inhibition of excretion correlated to significant reproductive effects in these bass such as depressed sex steroids and GSI (Noggle et al. 2004). Thus a threshold effect in mosquitofish, perhaps caused by overwhelming of the detoxification system, is supported by the largemouth bass work.

This study focused upon bioindicator criteria related to wild exposure and, inadvertently, seasonal variability. Specifically, wild female mosquitofish appear sensitive to improved effluent quality in regards to anal fin masculinization. Effect of upgrades on sex steroids was unclear because of: 1) large natural variation, and 2) unknown seasonal impacts hindering interpretation. While masculinized sex steroid ratios may be associated with effluent exposure in the receiving stream, they are not predictive of anal fin responses so these biomarkers cannot be associated based upon these data. However the two biomarkers together may be useful to contrast static or long-

term versus dynamic or short-term responses. This would be especially useful if the concept of dynamic exposure proves to be correct. Exposure history is crucial to support the latter proposition. Finally, the unique response pattern in females living in the predischarge site may be key to defining mechanisms in future studies.

Table 3-1. Water quality parameters of field collection sites before (2000) and after (2002) process changes at the Georgia-Pacific Palatka mill.

Site	REF1	U(8)	PRE-DIS	DIS	D(1)	D(3)	D(6)
2000–before							
Temperature (°C)	23.7	18.7	NA[a]	23.5	23.1	24.9	25.1
Conductivity (µS)	955	240.4	NA	1909	1815	1091	684
Salinity (ppt)	0.5	0.1	NA	1.0	0.8	0.5	0.5
Dissolved Oxygen (mg/L)	6.84	6.31	NA	5.56	7.23	3.72	6.83
2002–after							
Temperature (°C)	NA	23	26.2	25.7	28.5	28.7	NA
Conductivity (µS)	NA	230.6	2000.2	1814	1340	997	NA
Salinity (ppt)	NA	0.1	1.0	0.9	0.7	0.5	NA
Dissolved Oxygen (mg/L)	NA	6.58	0.74	10.1	8.27	4.60	NA
Turbidity (ntu)	NA	2.43	17.0	18.0	9.92	7.04	NA
pH	NA	7.41	7.85	7.45	7.32	7.18	NA

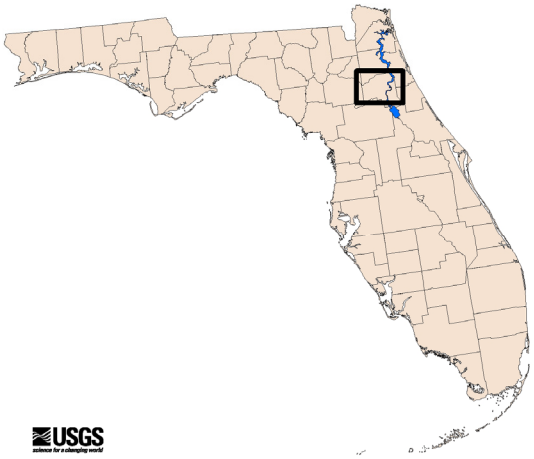
[a]NA = not available

Table 3-2. Body size parameters (ave ± se) and sample sizes for mosquitofish collected before (2000) and after (2002) process changes.

Site	REF1	U(8)	PRE-DIS	DIS	D(1)	D(3)	D(6)
2000 – before							
♂ Sample Size[g]	30 (20,10)	18 (8,10)	NA[a]	29 (19, 10)	30 (20, 10)	26 (16,10)	21 (11,10)
♂ Body Weight (g)	0.119±0.010	0.114±0.001	NA	0.125±0.008	0.146±0.009	0.134±0.009	0.129±0.018
♂ Standard Length (mm)	26.43±0.55	24.70±0.58	NA	26.23±0.43	28.24±0.51[b]	26.98±0.48	27.94±1.04[b]
♂ Condition Factor * (g/cm^3)	0.62±0.02[b]	0.74±0.02	NA	0.68±0.02	0.64±0.01	0.67±0.03	0.56±0.02[b]
♀ Sample Size[g]	27 (17,10)	17 (7,10)	NA	29 (19,10)	30 (20,10)	22 (12,10)	14 (4,10)
♀ Body Weight (g)	0.217±0.019	0.584±0.072[c]	NA	0.337±0.035	0.589±0.046[c]	0.419±0.085	0.550±0.292
♀ Standard Length (mm)	30.85±0.76	40.14±1.28[d]	NA	33.55±0.91	39.52±0.86[d]	34.81±2.40	39.09±5.54
♀ Condition Factor * (g/cm^3)	0.71±0.01	0.88±0.04[e]	NA	0.84±0.02[e]	0.91±0.02[e]	0.81±0.03[e]	0.70±0.06
2002 - after							
♂ Sample Size[g]	NA	40 (20,20)	40 (20,20)	40 (20,20)	40 (20,20)	15(0,15)	NA
♂ Body Weight (g)	NA	0.186±0.010	0.193±0.008	0.147±0.007[f]	0.204±0.012	0.179±0.022	NA
♂ Standard Length (mm)	NA	21.46±0.36	22.30±0.31	20.32±0.32[b]	22.05±0.36	21.40±0.85	NA
♂ Condition Factor (g/cm^3)	NA	1.82±0.03	1.71±0.03	1.71±0.03	1.82±0.03	1.71±0.04	NA
Sample Size[g]	NA	40 (20,20)	40 (20,20)	40 (20,20)	40 (20,20)	27 (17, 20)	NA
♀ Body Weight (g)	NA	0.557±0.036	0.851±0.053[b]	0.646±0.162	0.620±0.052	0.822±0.059[b]	NA
♀ Standard Length (mm)	NA	29.17±0.54	34.79±0.71[b]	29.69±0.85	30.27±0.66	33.09±0.81[b]	NA
♀ Condition Factor (g/cm^3)	NA	2.25±0.03	2.02±0.02[f]	2.47±0.03[f]	2.24±0.02	2.27±0.04	NA

*2000 fish were measured for length and weight after preservation.
[a]NA = not available.
[b]statistically differs from U(8) ($p < 0.05$).
[c]statistically differs from REF1 (D(1) also from DIS) ($p < 0.05$).
[d]statistically differs from REF1 and DIS ($p < 0.05$).
[e]statistically differs from REF1 and D(6) ($p < 0.05$).
[f]statistically differs from all other sites ($p < 0.05$).
[g]sample sizes displayed as: total sample size (preserved anal fin measurements, hormone and computer-aided meausurements)

A



B

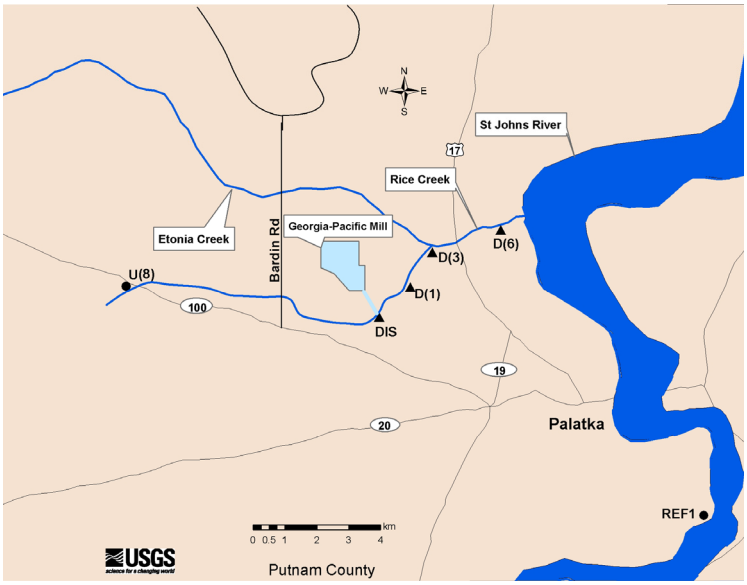


Figure 3-1. Maps of Rice Creek and Saint Johns River, USA. A) Relative location in Florida. B) Field collection sites sampled before process changes (2000). C) Field collection sites sampled after process changes (2002); asterisks indicate sites also collected in 2000. Site symbols distinguish sites exposed to effluent: circles = unexposed and triangles = exposed. Site symbols denote upstream (U) or downstream (D) of discharge, followed by approximate distance (km) from discharge in parantheses. PRE-DIS indicates site before discharge into the creek; DIS denotes site at discharge into creek.

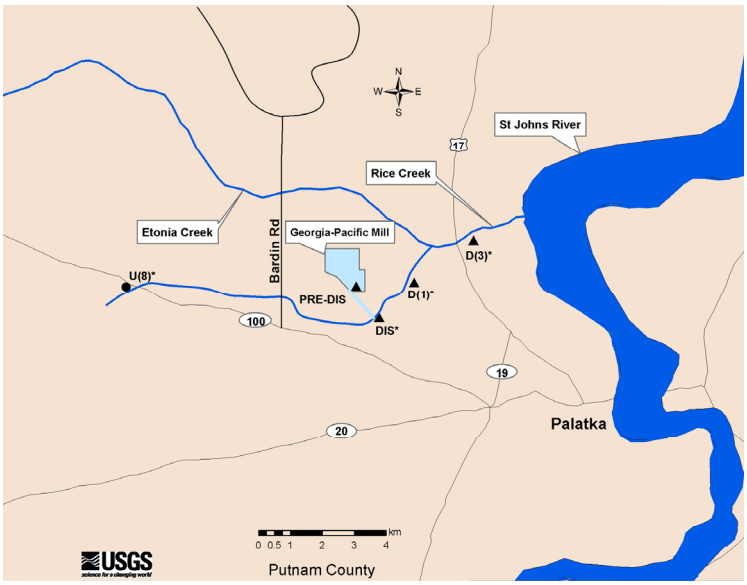


Figure 3-1. Continued

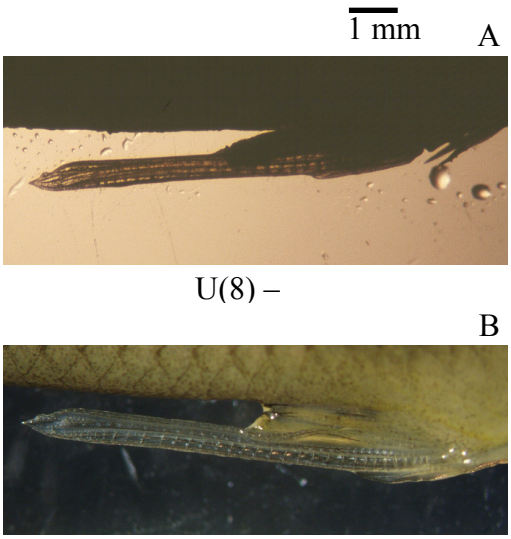


Figure 3-2. Representative male gonopodia from the upstream site collected before and after process changes. A) Before process changes (2000); preserved male photographed using 35 mm camera. B) After process changes (2002); fresh male photographed using digital camera. Upstream site abbreviation is followed by that year's average index of anal fin elongation represented by photograph. Notice terminal differentiations on tips of gonopodia.

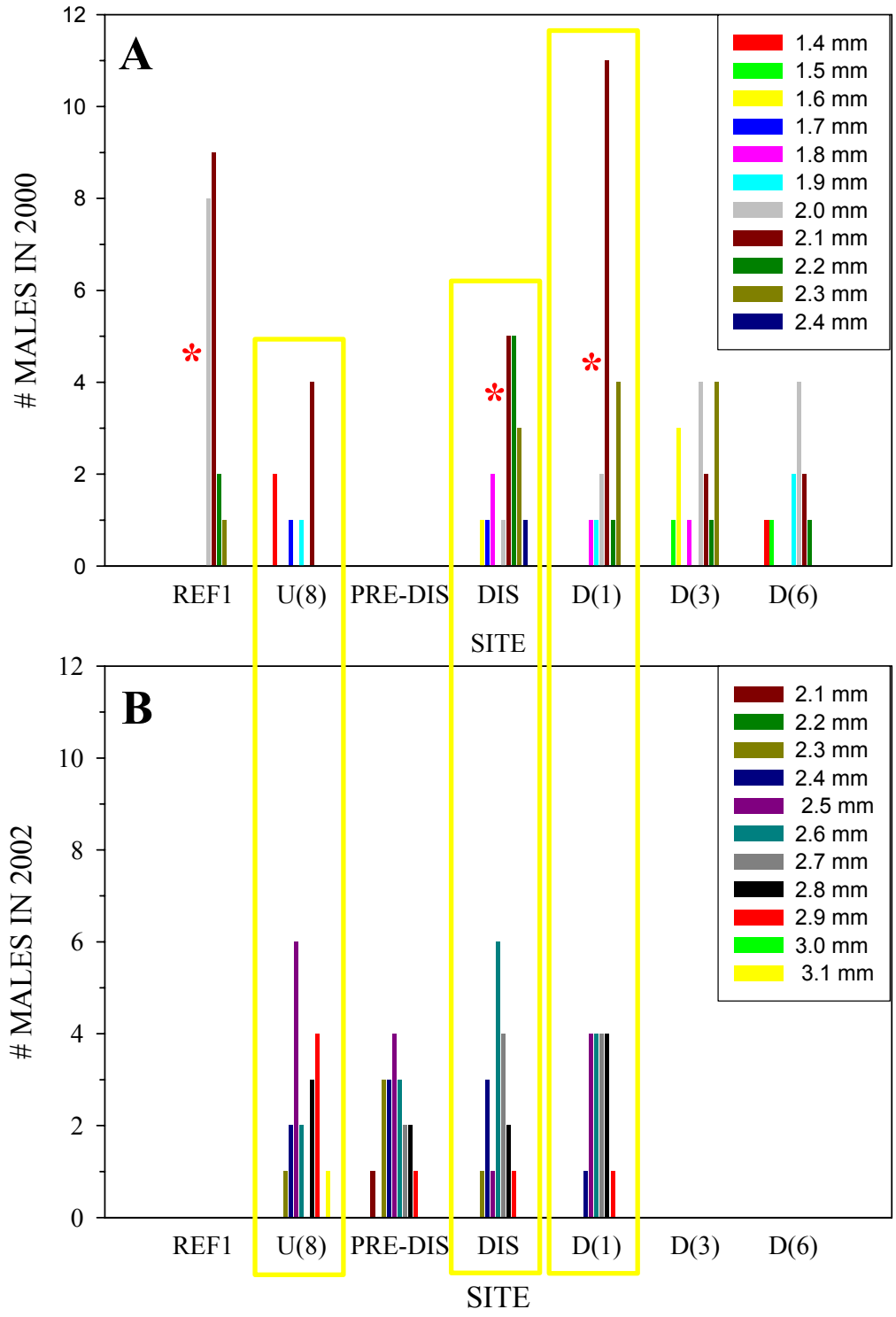


Figure 3-3. Male index of anal fin elongation (linear Ray 4 / Ray 6, manually measured on preserved fish) for each site by 0.1 mm increments. A) Before process changes (2000). B) After process changes (2002). Red asterisks indicate significant difference from upstream site [U(8)] within year ($p < 0.05$). Yellow boxes indicate significant differences between years.

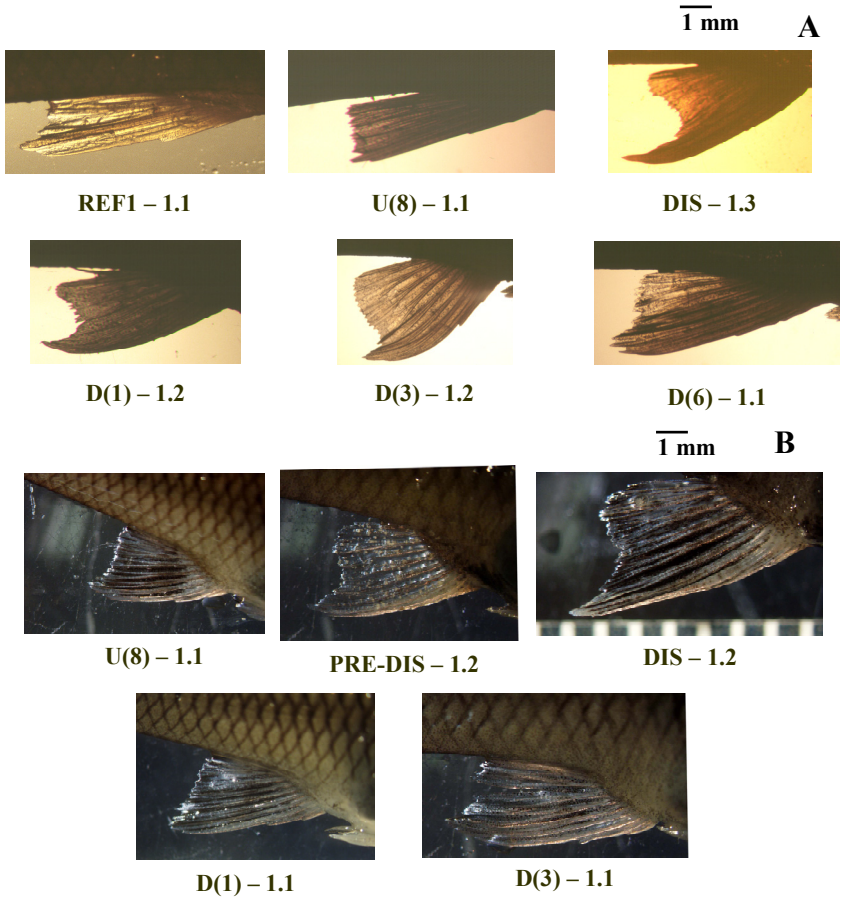


Figure 3-4. Representative female anal fins from collections made before and after process changes. A) Before process changes (2000); preserved fish photographed using 35 mm camera. B) After process changes (2002); fresh fish photographed using digital camera. Fins listed by site abbreviation followed by average index of anal fin elongation represented by photograph.

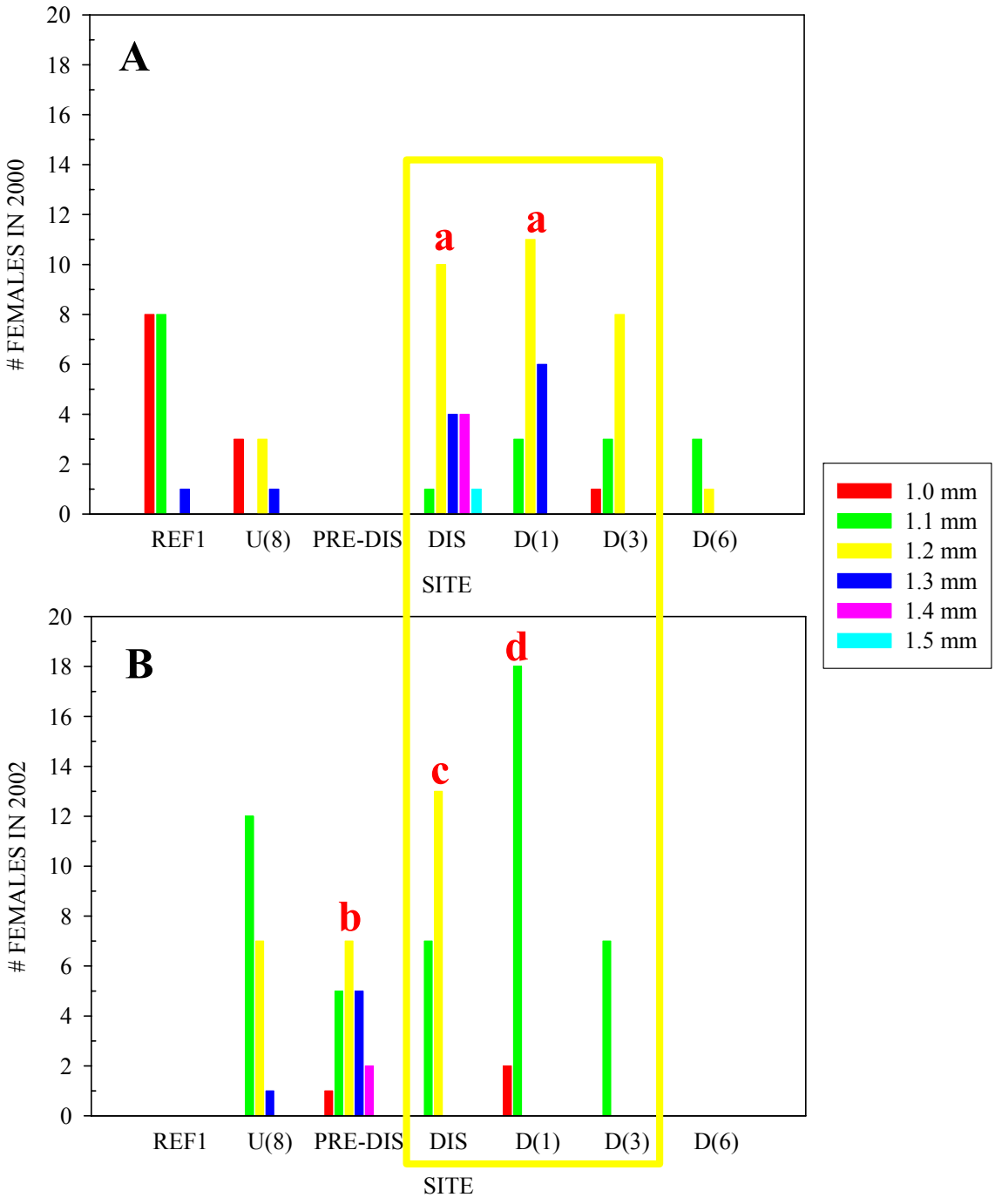


Figure 3-5. Female index of anal fin elongation (linear Ray 4 / Ray 6, manually measured on preserved fish) for each site by 0.1 mm increments. A) Before process changes (2000). B) After process changes (2002). Letters indicate statistically significant differences within year ($p < 0.05$): "a" denotes differences to nonlettered sites; "b" denotes differences to U(8) and D(3); "c" denotes differences to all sites except PRE-DIS; and "d" denotes differences to U(8). The yellow box surrounds sites with significantly reduced anal fin elongation from 2000 to 2002.

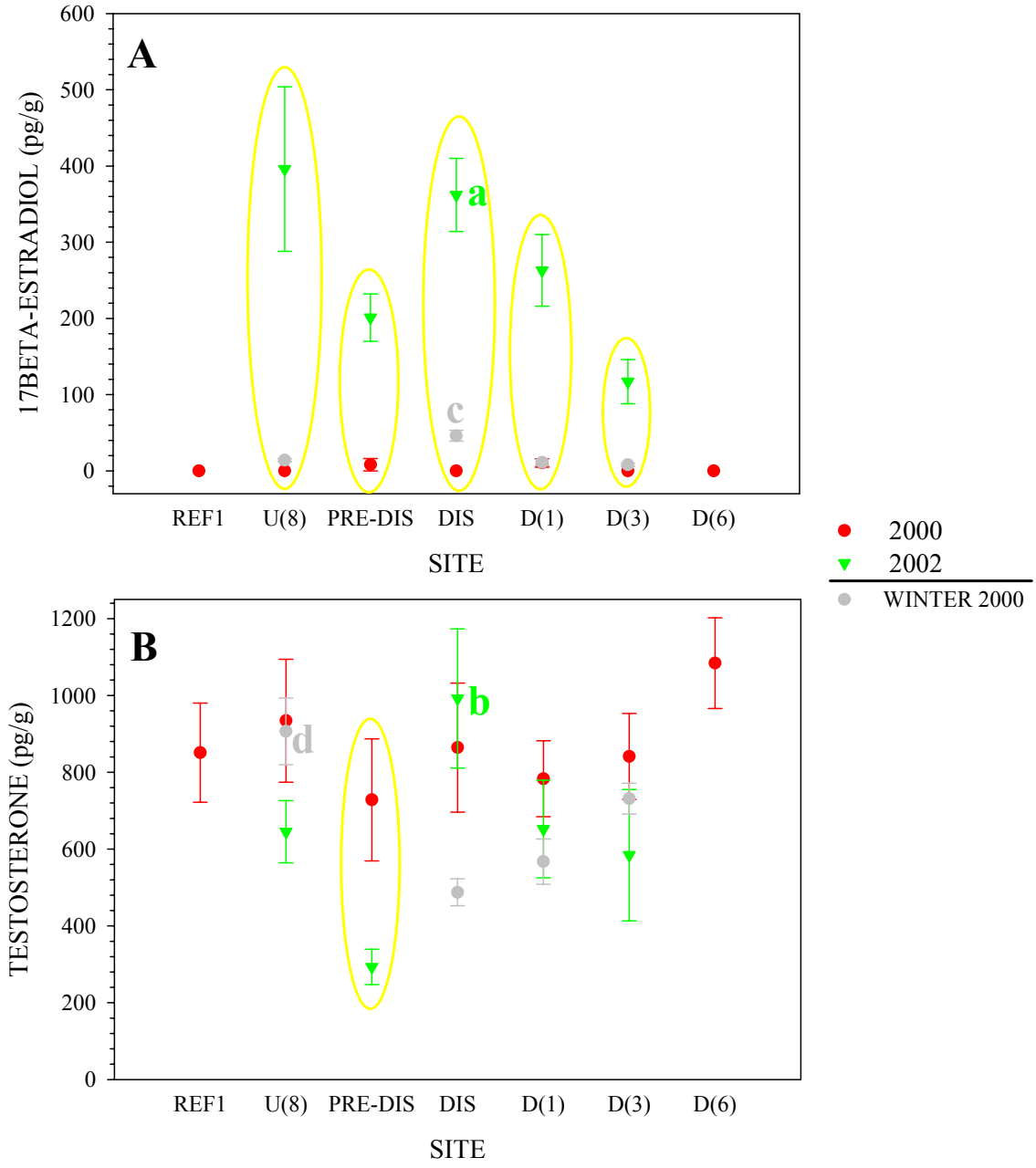


Figure 3-6. Male whole body sex steroids (ave ± se) from collections made before (2000) and after (2002) process changes. A) 17β-estradiol. B) Teststosterone. Letters indicate significant differences by site within season ($p < 0.05$): "a" denotes differences to D(3); "b" denotes differences to PRE-DIS, D(1), D(3); "c" denotes differences to all other sites; "d" denotes differences to REF2, DIS and D(1). Yellow circles demonstrate differences between years. Site and year did not covary for either hormone. Winter 2000 collection hormone values are given in grey for comparison.

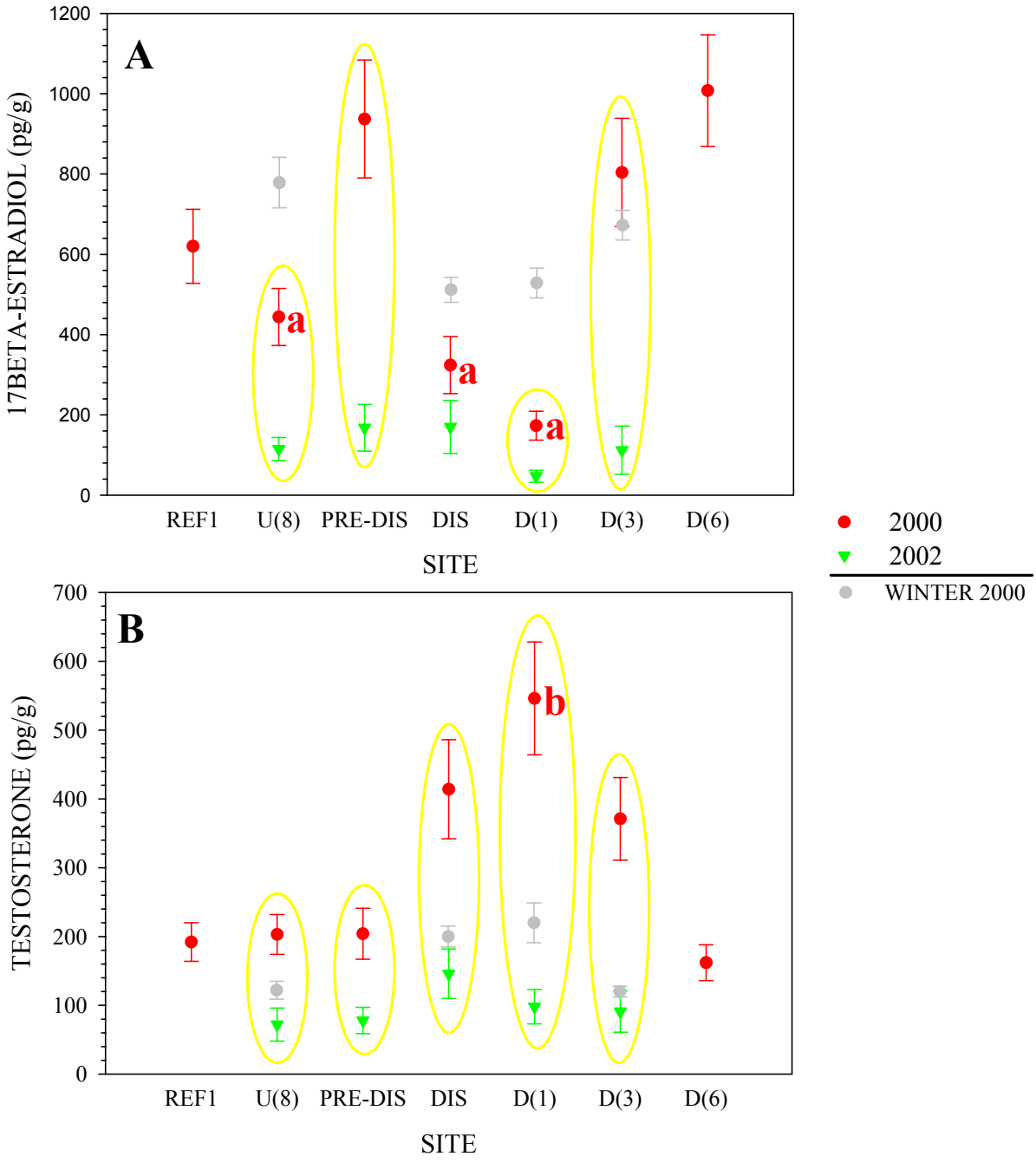


Figure 3-7. Female whole body sex steroids (ave ± se) from Rice Creek collections made before (2000) and after (2002) process changes. A) 17β-estradiol. B) Teststosterone. Letters indicate significant differences by site within season ($p < 0.05$): "a" denotes differences to nonlettered sites; "b" denotes differences to all but DIS. Yellow circles demonstrate differences between years. Site and year did not covary for either hormone. Winter 2000 collection hormone values are given in grey for comparison.

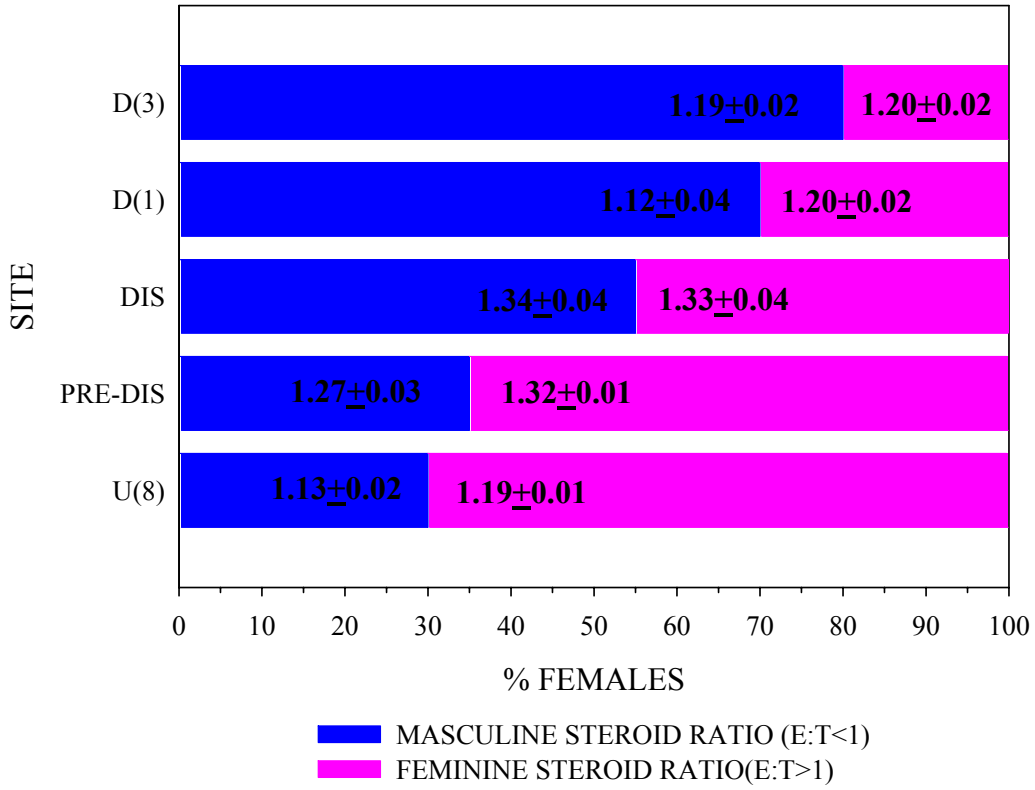


Figure 3-8. Percentage of female mosquitofish with masculine and feminine sex steroid ratios collected after process changes in 2002 ($\chi^2 = 8.270$, df = 6, $p < 0.05$). Index of anal fin elongation (ave ± se) is given for fish in each of these groups by site (no significant differences at $p < 0.05$).

CHAPTER 4
VARIABLE EFFECTS OF EFFLUENT ON EASTERN MOSQUITOFISH COLLECTED BELOW THREE FLORIDA PULP AND PAPER MILLS

Masculinization, or development of male secondary sex characteristics, in female mosquitofish was considered equivalent among pulp and paper mills in Florida (FL) where this phenomenon was studied most extensively (Howell et al. 1980, Drysdale and Bortone 1981, Cody and Bortone 1997, Bortone and Cody 1999, Jenkins et al. 2001, Parks et al. 2001). However, upgrades in processing technology have improved effluent characteristics, and reduction in response to upgrades at two of the three mills were documented (Cody and Bortone 1997, Chapter 3). In this study, field surveys within the same time period and using the same experimental design were conducted to assess differences in male and female mosquitofish responses among the three FL mills classically studied. Body size and anal fin morphology were measured, and sex steroids to provide insight into potential physiological disturbances. Female, and not male, anal fin morphology was significantly affected by effluent exposed site for all three systems. Despite high background and potential seasonal variation, hormone data indicated an androgenic or masculinized hormonal profile in females and possible estrogenic or feminized hormonal profile in males. Degree of response in females mirrored relative concentrations of effluent components in water: greatest response and concentrations at Fenholloway River, intermediary responses and concentrations at Rice Creek, and lowest responses and concentrations at Elevenmile Creek. These data support the use of

mosquitofish as a bioindicator species to detect extent of exposure and provided further characterization of seasonal changes in sex steroids.

Introduction

Sublethal effects of pulp and paper mill effluent exposure on fish have been a major focus of aquatic environmental health concerns for over a decade (Sodergren 1991, Servos et al. 1996, Ruoppa et al. 2000, Stuthridge et al. 2003, Borton et al. 2004). Reported effects include induction of liver detoxification systems, alterations in sex steroid concentrations and production/metabolism, reduced gonadal development, decreased egg production, and decreased fry survival (Van der Kraak et al. 1992, Gagnon et al. 1994a, Munkittrick et al. 1999, NCASI 2000a, Sepulveda et al. 2003, Parrott et al. 2004, McMaster et al. 2003). Whether or not these effects represent actual adverse effects in terms of reproductive success or population and community level impacts remains controversial. Equally debatable are potential mechanisms of action, since pulp mill effluents are a complex mixture and composition varies not only among mills but often within a mill.

The discovery of carcinogenic dioxins and furans, especially the polychlorinated congeners like TCDD and TCDF, in fish living downstream of a pulp and paper mill in the 1980s initiated government regulation of the industry to remove elemental chlorine and chlorinated compounds from effluent (Smook 1999). These chlorinated compounds were long considered the primary bioactive agents causing reported sublethal effects in fish. Now that chlorine emissions were virtually removed, a concomitant lack of effects in fish was not observed, although many effects were reduced (Lehtinen 2004, McMaster et al. 2003, and Chapter 3). Process changes implemented to remove chlorine also coincided with reduction in many other effluent components, especially wood extractives.

Therefore, in recent years wood extractives such as resin acids, fatty acids, phytosterols, and lignin have become the focus as potential bioactive effluent components associated with noncarcinogenic sublethal effects in fish.

Phytosterol derivatives have been theoretically associated with fish effects for decades. In the laboratory, androgens were formed by bacterial degradation of phytosterols and absorbed by mosquitofish (Denton et al. 1985, Howell and Denton 1989). One of the originally documented fish effects of pulp and paper mill effluents involved development of male secondary sex characteristics in female mosquitofish (Howell et al. 1980, Drysdale and Bortone 1981, Cody and Bortone 1997, Bortone and Cody 1999, Jenkins et al. 2001, Parks et al. 2001). Initial discovery was in fish from Elevenmile Creek, FL, a low flow minimal dilution stream (Howell et al. 1980), and reports of two other mills in FL that discharge into low flow streams reported equivalent masculinization (Drysdale and Bortone 1981, Cody and Bortone 1997, Bortone and Cody 1999). Yet these studies did not directly compare these systems in one comprehensive study and several processing improvements at all three mills over the past 20 years may have improved effluent quality and changed equivalence of fish response. Other evidence supports a differential response among mills: recent whole-effluent exposure studies in Canada and New Zealand document large differences in masculinization response time, from 3 wks to beyond 6 months (McCarthy et al. 2004, Van den Huevel et al. 2004b). Currently, variation in response at the mills in FL is likely but has not been specifically addressed.

The objective of this study was to determine if FL mills still had equivalent masculinization responses in mosquitofish as previously reported. In light of processing

upgrades and different processing technologies, masculinization was predicted to vary. Also, whole body sex steroids were analyzed by radioimmunoassay (RIA) to evaluate implications of the androgen-induced hypothesis on steroid concentrations and ratios.

Materials and Methods

Mill Characteristics

The three pulp mill effluent receiving systems in FL where masculinized female anal fins have been previously documented were surveyed within a three week period during the reproductively active season in July and August of 2001 (Figure 4-1, Appendix A). Analogous site types were sampled for each system: two reference sites; an upstream site; a site before discharge; the discharge or outfall site; and at least one downstream site. Reference sites included sites within the same basin as exposed sites, and sites within blackwater streams since all receiving streams were naturally tannic. Upstream sites were far enough upstream to prevent effects of backflushing from the effluent discharge, indicated by very low conductivity and low to no concentrations of effluent components (Table 4-1). Predischarge sites comprised exposure to 100% effluent before discharge into the receiving stream, either in final retention ponds (Rice Creek and Elevenmile Creek) or the discharge canal (Fenholloway River). Discharge sites were dominated by effluent (80 to 90%), since dilution rates are low for all three systems. Downstream sites were at least one-third distance from discharge to mouth of the stream, where effluent was more diluted (40 to 50% effluent maximum).

The mills discharging into these systems are very different (Table 1-1, Chapter 1). In general, they differ in furnish, product, and secondary treatment. Two of the three mills are bleached kraft and were subject to EPA's Cluster Rule: the Elevenmile Creek mill has been elemental chlorine free (ECF) since 1995, while the Rice Creek mill

implemented major process improvements (including ECF technology) several months before fish collections in May. The Fenholloway River mill is much different than the other two, using a dissolving kraft pulping process to produce high grain cellulose. Thus it was not subject to many of the Cluster Rule requirements, such as ECF bleaching.

Water Samples

Before fish collection, water quality parameters typically affected by pulp and paper mill effluents were measured at each site: dissolved oxygen, temperature, pH, conductivity, salinity, and turbidity. Single grab water samples were also collected before fish collection to document potential exposure of fish to specific effluent components. Samples were preserved (buffered), and sent to the National Council for Air and Stream Improvement, Inc. (NCASI) for chemical analysis. Water from all sites was analyzed for chlorinated phenolics (12 Cluster Rule compounds plus 16 others), 10 resin acids (including 3 chlorinated), 3 fatty acids, 4 phytosterols, total organic carbon, condensable tannins, and polyphenolics. Additional effluent components were analyzed in 100% whole effluent: metals, nonmetals (such as chloride and fluoride), and neutral semivolatiles. Columbia Analytical Services conducted the chlorophenolic analyses, CH2M Hill conducted the TOC analyses, and the NCASI West Coast Regional Center conducted all other analyses (NCASI 1986, 1997).

Fish Samples

Approximately 200 adult fish per site were collected using dip nets and/or a backpack electroshocker. Fish designated for hormone analysis (20 to 30 each sex per site) were processed on-site. First, fish were euthanized with a terminal waterborne dose of buffered tricaine methanesulfonate (Tricaine-S, Western Chemical Inc., Ferndale, WA, USA), then weighed using a digital scale ($\pm$ 0.001 g) and measured for standard length ($\pm$

0.01 mm) using a pair of digital calipers. Under a dissecting scope, gender was identified using the presence (female) or absence (male) of a urogenital papilla (see Chapter 2 for validation of this sexing technique). Each fish was photographed using a digital camera then placed on ice until transferred to a -80°C freezer for subsequent radioimmunoassay (RIA) of sex steroids. Anal fin images of these fish were measured by computer ($\pm$ 0.01 mm) using trace mode in Sigma Scan Pro © 5.0 from the base of Rays 4 and 6 along the curve of each ray to the tip. Remaining fish were euthanized and preserved in 10% neutral-buffered formalin for histological verification of gender.

Sex Steroids

Whole body primary sex steroids (17β-estradiol and testosterone for this species) were analyzed using a modified RIA method originally developed for serum and plasma samples of common carp, *Cyprinus carpio* (Goodbred *et al* 1997), and since adapted for use in a variety of other aquatic species and tissue media such as plasma of largemouth bass, *Micropterus salmoides* (Gross et al. 2001) and mantle of freshwater invertebrates (Gross et al. 2000). For methods and validation of this assay, see Chapter 2.

Statistics

Body weight and standard length were used to calculate condition factor, K = weight / length^3 x 100 (g/cm^3), as an indication of overall health used by the aquaculture industry (values at least 1 are considered healthy, Hile 1936). The length ratio of anal fin Rays 4 and 6 was calculated as an index of anal fin elongation. Estrogen and testosterone concentrations were used to calculate a ratio indicating masculine hormone profile (E:T < 1) or feminine hormone profile (E:T > 1).

Any data failing tests for normality and homogeneity of variance were transformed using log transformations. Originally arcsine transformation was used for anal fin data

(Noggle et al. 2004), but arcsine is most appropriate for ratio data ranging from 0 to 1 (Anderson and McLean 1974), and both anal fin elongation index and E:T ratio extend beyond 1.0. Also, log transformation was recommended by Angus et al. (2001) as appropriate for anal fin elongation index.

Anal fin morphology and sex steroid data were analyzed within sex using two-way analysis of covariance (ANCOVA) to test for significant variation by site and mill. Site differences within mill were also analyzed by one-way ANOVA, as were differences among mills by site type. Potential effects of size class was revisited and analyzed by one-way ANOVA within site. Significant differences in ANCOVA and ANOVA were analyzed for multiple comparisons using Tukey's HSD. Relationship between anal fin morphology and sex steroids were analyzed overall and by site in two ways: first, by examining Pearson's correlations of the index of anal fin elongation to sex steroid concentrations and ratio, then by t-test for differences in index of anal fin elongation between females with masculine versus feminine E:T ratios. Statistical significance was set at $\alpha < 0.05$ for all tests. All statistical analyses were conducted using SAS © version 9.0.

Results and Discussion

Water Quality

As expected, conductivity, salinity and turbidity were higher at effluent-exposed sites (Table 4-1). Conductivity was highest in 100% effluent from the Fenholloway River (2,321 µS) mill, followed by effluent from Rice Creek (1,916 µS) and Elevenmile Creek (1,660 µS). Temperature in effluent-dominated sites was also elevated compared to some, but not all, unexposed sites. Dissolved oxygen remained high enough to support fish at most sites in Rice Creek and Elevenmile Creek (> 4 mg/L), with the exception of

the predischarge site for Rice Creek (2.14 mg/L). The Fenholloway River was poorly oxygenated along the entire length sampled regardless of effluent exposure: the predischarge site had more suitable dissolved oxygen levels to support fish. The fact that mosquitofish were present in this low oxygen environment demonstrates their tolerance to extreme environmental conditions. Finally, pH was elevated in effluent-exposed sites compared to upstream sites; some references [REF2 for Rice Creek and REF1 for Fenholloway River] had similar pH. Notably, pH was very acidic at the upstream and second reference Fenholloway River sites [U(5) and REF2], likely reflecting the tannic, and blackwater nature of these systems.

Water Chemistry

Chemical analyses of single grab samples from the water column at fish collection sites distinguished the 100% final effluent before discharge site (highest chemical concentrations) from the discharge site (intermediary concentrations) and unexposed sites (lowest concentrations) (Table 4-2). Chlorinated compounds (chlorinated phenolics, chlorinated resin acids) were nondetectable or at the lower calibration limits in all three systems (data not shown). Ions and heavy metals were within normal, acceptable ranges for pulp mill effluents: mg/L for ions (sodium, calcium, fluoride, chloride, etc.) and µg/L for metals (24 measured from aluminum to mercury to zinc). Neutral semivolatiles were at nondetectable concentrations in Rice Creek effluent samples; nondetectable concentrations in Elevenmile Creek effluent samples except 9.6 µg/L dichlorodimethyl and 1.5 µg/L 2,3,4,5-tetramethylcyclopentenoneulfone; and 9 of 14 analytes (such as cyclopentophenones, acetophenone and camphor) averaged 7.6 µg/L and ranged 1.6 to 31 µg/L in Fenholloway River effluent samples. Essentially these latter data reveal the more

odiferous nature of Fenholloway River effluent as opposed to an indication of increase effluent exposure.

Reference and upstream sites had little to no measurable effluent components (Table 4-2). Wood extractives (e.g. resin and fatty acids, phytosterols, polyphenolics (measuring tannin/lignin content), condensable tannins, and total organic carbon) decreased at downstream sites compared to predischarge sites for all systems (Table 4-2), as expected with dilution. In accordance with conductivity measurements, most effluent components analyzed had an among mill trend of highest concentration in Fenholloway River effluent, intermediary concentration in Rice Creek effluent, and lowest levels in Elevenmile Creek effluent (Table 4-2).

Body Size and Condition

No effect of effluent exposure on body size and condition, in terms of site, was detected for males or females across all three systems surveyed (Table 4-3). Rather, variability in length and weight was associated with unexposed sites and demonstrated the importance of multiple reference streams for these more variable endpoints compared to female anal fin elongation (Chapters 2 and 3 and discussion below). Condition factor was sufficient for both sexes (> 1) and indicated all fish were in good general health.

Males

Male body size was not affected by effluent-exposed site for any of the three systems, especially considering the variation detected among unexposed sites (Table 4-3). For the Rice Creek system, smaller males occurred at the second reference site [REF3] and the predischarge site [PRE-DIS]. However, male condition was > 1 indicating adequate general health for all sites (statistically greater at the upstream site although probably not biologically significant). Males collected from the Elevenmile Creek and

Fenholloway River systems were of equal body length across all sites, but body weight and condition factor were lowest at the second reference site [both REF2] compared to predischarge and discharge sites [PRE-DIS and DIS for both]. Condition factors were all above one and healthy for these males. Overall, male body size was not affected by effluent.

Females

Variation in body size related to reference site was detected in females among all three systems, as opposed to effect related to effluent exposed sites (Table 4-3). In the Rice Creek system, the largest females (length and weight) were collected before discharge into Rice Creek [PRE-DIS]. The shortest females were collected from the outfall and first downstream sites [DIS and D(1)]. However, condition factor was good (> 1) across all sites with the only statistical differences observed between the second reference site [REF3] and the first reference site [REF2] as well as the discharge site [DIS]. At the Fenholloway River system, significant variation in body size occurred for the unexposed sites but no significant effects associated with exposed site were observed. Condition factor indicated good general health (> 1) across all sites. Females from the Elevenmile Creek system were of equal length and weight across sites, and the only statistical difference in condition factor was observed at the second reference site [REF2] compared to the other sites. As for the other systems, condition factor was consistently < 1.

Anal Fin Morphology

Female, and not male, anal fin morphology was significantly affected by effluent exposed site for all three systems (Figure 4-2). Precocious maturation in males was not supported, and natural variation as opposed to effluent-associated variation was detected.

Female anal fin elongation was reduced compared to historical reports indicating terminal differentiations; overall elongation resembled a developing male gonopodium as opposed to a mature male gonopodium. Using water chemistry data as a mark of potential exposure (Table 4-2), the greatest extent of elongation coincided with highest concentrations of wood extractives (Fenholloway River); intermediary elongation with medium amounts of extractives (Rice Creek); and the smallest response with the lowest concentrations of extractives (Elevenmile Creek). Examining female anal fin elongation by size class did not consistently support the concept of a sensitive (adult) life stage; rather, differential or dynamic exposure was supported by these data. (Dynamic exposure refers to variable concentrations of effluent components over time, dependent on factors such as tree species for furnish, within plant processing spills, rainfall/dilution, and bacterial degradation.) However genetic differences among these three mosquitofish populations can not be ruled out as an explanation for different responses by size class across mills.

Males

Males did not appear influenced by effluent-exposed site in the 2001 collections (Figure 4-2B). No significant differences existed among males in the Rice Creek or Fenholloway River systems. At the Elevenmile Creek system, gonopodia were longer at 100% whole effluent and outfall sites [PRE-DIS and DIS] compared to one reference site [REF2] but not compared to the other unexposed sites [REF1 and U(1)]. Site and mill did not covary, but among mills Rice Creek males had significantly longer gonopodia at the discharge site [D] and the upstream site [U] compared to the other two mills. Variation was high and statistically significant among the three mills' reference sites as

well. Again, this is evidence for a natural variability in this response or environmental stressors on males as opposed to abnormalities in effluent exposed males.

Males were also analyzed by size class within each system to address precocious maturation. Three size classes were identified (5 to 10 males per group): < 20 mm; 20 to 24.99 mm; and 25 to 29.99 mm. If precocious maturation was occurring, males in the smallest size class would exhibit abnormally long gonopodia similar in elongation to males in the larger size classes. Analysis by size class alone at Rice Creek and Fenholloway River field sites found significantly smaller gonopodia in males from the < 20 mm class (2.37 $\pm$ 0.05) compared to the 20 to 24.99 mm class (2.52 $\pm$ 0.06), as expected under normal conditions. At Elevenmile Creek sites, the 25 to 29.99 mm class had a larger average (2.72 $\pm$ 0.09) than the two smaller classes (2.07 $\pm$ 0.10 and 2.02 $\pm$ 0.11, respectively) which did not differ from each other. Direct analysis of standard length to gonopodial length (Ray 6) did not reveal any site-specific differences within mill (data not shown). Therefore, in this study there was no evidence for precocious maturation in males living in effluent-receiving streams.

Females

For all three systems, anal fin elongation in masculinized females never approached the male gonopodium in length or terminal differentiation. In terms of length, altered females from exposed sites averaged an index of 1.5, while normal males and normal females from unexposed sites averaged indices of 2.5 and 1.1, respectively. No terminal structures (hooks, serrae, or blade) were observed on the tip of any altered female, in contrast to historical collections of mosquitofish at these same sites (Howell et al. 1980, Drysdale and Bortone 1981, Cody and Bortone 1997). This lack of terminal

differentiation indicated a reduction in response since process modifications have been implemented over the years.

Anal fin elongation in female mosquitofish was detected at all three systems (Figure 4-2A). At Rice Creek, elongation was significantly different between reference sites [R1 and R2] and effluent-exposed sites [P-D, D, D1 for R1 and only P-D to R2], but not between upstream [U] and exposed sites. Females from effluent-exposed sites along the Fenholloway River had significant anal fin elongation compared to nonexposed sites. Interestingly, elongation was greatest at the farthest downstream site. Site fidelity in this species and the large distance between the discharge and this site (~12 km) mean flushing of fish from upstream was not likely, thus lending more support to an additional factor(s) in the receiving stream responsible for the observed response. Elevenmile Creek females also displayed anal fin elongation when data was log transformed as suggested by Angus et al. (2001). Females from the predischarge and discharge sites [P-D and D] had significantly longer anal fin elongation than females from the upstream site [U].

Interaction of site and mill significantly covaried. Among mills, Fenholloway River had the greatest degree of elongation for all effluent-exposed sites, Rice Creek was in between the other two before and at the discharge [P-D and D], and Elevenmile Creek consistently had the least degree of elongation among exposed sites. This pattern mirrored that of wood extractives (Table 4-2); therefore concentrations of these compounds may be useful in predicting degree of response (or vice versa).

Effects of size class as an estimation of age was re-examined for these collections, since 2000 collections at Rice Creek (Chapter 2) indicated differences among size classes at the discharge site. In 2001, size class differences were again detected at the discharge

site: the smallest size class (20 to 24.99 mm) had longer anal fin elongation than the other classes (25 to 29.99 mm and 30+ mm). This result was slightly different than what was observed in fall 2000 data, when females in the middle two size classes (25 to 29.99 mm and 30 to 34.99 mm) had significantly longer anal fins compared to the largest size class (35 to 39.99 mm). Also different than 2000 data, in 2001 significantly longer elongations (0.1 mm longer) were detected in the smallest size class (20 to 24.99 mm) versus the largest (30+ mm) at the first reference and upstream sites [REF2 and U(8)]. These data combined with the overall anal fin elongation differences among sites described above for Rice Creek supports a background incidence of elongation in female mosquitofish and decreases the apparent specificity of this trait for use as a bioindicator of pulp and paper mill effluents.

Size class analysis at the other two systems produced different results. Anal fin elongation did not vary by size class within sites at Fenholloway River, while size class was significantly influential at the predischarge site only at Elevenmile Creek. In this case, the largest class (30+ mm) had significantly longer anal fin elongation compared to the smallest class (20 to 24.99 mm). Taken as a whole, these data on size class differences among mills do not support the idea of sensitive life stages among adult females; rather, they indicate a dynamic exposure and/or the possibility of genetic differences among populations. The Rice Creek data also revealed a natural variation of the masculinization response at unexposed sites which had not been demonstrated so overtly in previous collections (Chapters 2 and 3).

Sex Steroids

Seasonal differences among systems were indicated by concentrations of individual steroids for both sexes. Additional environmental factors influencing steroid levels

besides effluent exposure were also implied by different response patterns among effluent exposed sites. 17β-estradiol was elevated in males from Rice Creek and Fenholloway River, and the E:T ratio was estrogen-biased for a small number of these males, providing the first preliminary evidence for an estrogenic effect of pulp mill effluent on this species. Weak evidence of increased testosterone in females from Rice Creek and Fenholloway River was apparent, and boosted by an increased frequency of masculinized steroid ratios for these sites. Based upon steroid data among these mills, pulp mill effluents may result in estrogenic action on males and androgenic action on females at the physiological level. Perhaps more strongly, these data also stress the need for seasonal characterization of hormone levels in this species to allow for a more conclusive interpretation.

Males

17β-estradiol in males was significantly elevated at the first downstream site for both Rice Creek and Fenholloway River compared to all other sites (Figure 4-3A). Males living upstream of effluent discharge in Elevenmile Creek also had significantly higher 17β-estradiol concentrations compared to all exposed sites [P-D, D, D1]. In light of data from 2000 and 2002 for Rice Creek (Chapters 2 and 3), these contrasting differences may represent different seasonal stages among these fish populations. Values ranged from less than 100 pg/g similar to 2000 data, averaged around 500 pg/g similar to 2002 data, but ranged from 1000 to 2000 pg/g 17β-estradiol for Rice Creek and Fenholloway River males (the highest recorded up to that point). Combined with the knowledge of (potential) exposure differences (Table 4-2), one may speculate elevated estrogen in males as a potential effect of effluent exposure in addition to the seasonal variation and dynamic exposure already implied.

Similar to previous data on Rice Creek males (Chapters 2 and 3), individual testosterone concentrations varied greatly among nonexposed sites (Figure 4-3B). Statistically significant depression in testosterone at Fenholloway River and Elevenmile Creek depended upon which unexposed site was used as reference, precluding strong conclusions about effects of effluent exposure on concentrations of testosterone in males.

The vast majority of E:T ratios in male mosquitofish (95%) were normal and testosterone-biased with the exception of less than 5% of males that had estrogen-biased ratios. Half of these hormonally feminized males were from the downstream site on Fenholloway River [D1] where the average E:T ratio was 1.03$\pm$ 0.14. One to two were from the other two effluent-exposed sites of the Fenholloway system; one from an effluent-exposed site of the Elevenmile Creek system; two each from effluent-exposed sites; and one from a reference site of the Rice Creek system. Ratios other than at the downstream Fenholloway site were within the tenths range (mean of 0.10 to 0.51 and standard error (se) of 0.01 to 0.09), similar to 2002 Rice Creek males as opposed to 2000 males. Considering the water chemistry data and the 17β-estradiol absolute concentrations it is possible the feminized ratio at downstream Fenholloway is an indication of an estrogenic effect on males. Since the ratio was normal before discharge and at the discharge of the Fenholloway River, additional environmental factors influencing this response (e.g. differential bacterial degradation of phytosterols) are supported by these data.

Females

17β-estradiol concentrations in female mosquitofish were not influenced by effluent exposure in summer 2001. Concentrations were within ranges reported for Rice

Creek in 2000 and 2002 (Chapters 2 and 3), even the one significantly elevated peak at the upstream Fenholloway River site compared to all other sites for that system (see Figure 4-4A). Site and mill did not significantly covary, i.e. there was no interaction between the two variables. 17β-estradiol varied among mills at two sites including the upstream site; therefore these differences are likely attributed to differences in habitat and seasonality among regions as opposed to effluent-related differences.

Weak evidence for elevated testosterone existed for females at Elevenmile Creek and Fenholloway River (Figure 4-4B). No significant differences in testosterone concentrations were detected in females from Rice Creek, despite the peak at the discharge site (which had the largest variation of all sites). Concentrations were within ranges reported for Rice Creek in 2000 and 2002 (Chapters 2 and 3). Site and mill did not covary for this hormone in females, although significant mill differences were detected for all site types. Testosterone was statistically elevated at Fenholloway River sites compared to the other two systems for both exposed and nonexposed sites. Similar to 17β-estradiol in females, these differences may reflect seasonality as opposed to effluent-related effects.

Average E:T ratios were all above one and implied normal feminine hormonal profiles, although variation was high (average 10 $\pm$ 2.8 standard error). Plotting the frequency of masculine versus feminine hormonal profiles for females revealed unique patterns for each system and a higher occurrence of skewed profiles than males (Figure 4-5). Rice Creek females had significantly different ratios among sites ($\chi^2 = 27.95$, df = 6, $p < 0.05$) with a skew toward masculinized profiles at the effluent-exposed sites. However, frequency was not as high as for 2002 females (Chapter 3), perhaps caused by

frequent mill shutdowns as Cluster Rule process changes were being implemented that season (pers. obs.). Masculinized hormone profiles were more common in the Fenholloway River system at both effluent exposed and unexposed sites ($\chi^2 = 14.31$, df = 5, $p < 0.05$), even the pristine Econfina River [R1 and R2]. Skewed steroid ratios at the reference sites for this system are very important and imply the E:T ratio can be altered by environmental factors completely separate from pulp and paper mill effluents. In contrast to the other two systems, masculinized steroid ratios in females from Elevenmile Creek system occurred at very low levels (in one or two females per site) and incidence was not significantly different among sites ($\chi^2 = 0.8064$, df = 5, $p > 0.05$). Overall, frequency of masculinized hormone profiles in effluent exposed fish resembled the stronger trend among mills for anal fin elongation and was supported by relative concentrations of effluent components.

Anal Fin Elongation and Sex Steroids

Despite associations between these two biomarkers and effluent exposed sites in females, females with elongated anal fins were just as likely to have a masculinized hormonal profile as a normal female profile. Lack of demonstrated effect on anal fins in males and the low occurrence of feminized hormonal profiles essentially precluded comparison of these endpoints in males.

Males

Because of the low occurrence of feminized hormonal profiles for males (< 5%), statistical analysis was not robust when this group was compared to the dominant, normal masculinized profile. In the Fenholloway River and Elevenmile Creek systems, no correlation existed for the male index of anal fin elongation to estrogen, testosterone, or the E:T ratio ($r^2 < 0.1$ and $p > 0.05$ for correlations). For Rice Creek males, statistical

significance ($p<0.05$) was attained between the index and 17β-estradiol and testosterone separately but not as a ratio: correlation coefficients were low and indicated a weak negative correlation ($r^2 = -0.182$ and $r^2 = -0.255$, respectively). T-tests between masculinized and feminized hormone profiles of males were equally inconclusive because of small sample sizes for hormonally feminized males. At this point, the lack of demonstrated effect on anal fin morphology coupled with the low occurrence of feminized hormone profiles in males does not suggest a relationship between these biomarkers.

Females

Although the frequency of masculinized hormone profiles in effluent exposed fish resembled the stronger trend among mills for anal fin elongation, statistical comparison of these two biomarkers in these same fish did not reveal any relationship. This result is consistent with results obtained in the survey of mosquitofish before and after process changes at the Rice Creek system (Chapter 3). No correlation existed for the index of anal fin elongation to estrogen, testosterone, or the E:T ratio ($r^2 < 0.5$ and $p > 0.05$). Across all sites and within sites average elongation did not differ between females with masculine and feminine hormone ratios (Figure 4-5). Therefore, females with normal feminine hormone profiles were just as likely to have masculinized anal fins as females with masculine hormone profiles. This does not rule out alterations in sex steroid ratios contributing to elongation of the anal fin, since sex steroids were measured after onset of elongation. Seasonal changes in sex steroids, but not anal fin elongation, support this point (Chapter 2). However, presence of the altered hormonal profile cannot be used to

predict occurrence of anal fin elongation in individual females living in effluent-receiving streams, based upon these data.

Conclusions

Mosquitofish responded differentially to pulp and paper mill effluent exposure at three mills in FL, contrary to historical reports of similar responses in females at the mills investigated. With the added support of water chemistry data, anal fin and sex steroid biomarkers in females displayed a graded androgenic response among mills: the greatest response at Fenholloway River, an intermediary response at Rice Creek, and the lowest response at Elevenmile Creek. Males do not appear affected by effluent exposure in terms of anal fin morphology, while a weak estrogenic response was indicated by changes in the E:T ratio. These gender-specific endocrine disruptive effects have also been reported for fathead minnows (*Pimaphales promelas*) under controlled exposure to Canadian pulp mill effluents (Parrott and Wood 2002, Parrott et al. 2003, 2004). Whether or not these effects lead to adverse impacts on reproductive success and concomitant impacts on higher levels of biological organization remains to be seen (Chapter 6).

Several limitations to this data set make these conclusions tentative and expose the need for basic biological data in toxicological studies. The inherent natural variation of these endpoints, especially sex steroids, within unexposed sites reduces the specificity of response for pulp mill effluents. Further complicating interpretation is the lack of baseline data on seasonality of steroid levels in this species. Without such a benchmark, a formal conclusion relating observed effects to pulp mill effluents remains elusive. A third major limitation was the single time point water sampling for chemical analysis. Although an improvement over the studies reported in Chapters 2 and 3, these samples

represent only a snapshot of continually changing effluent concentrations and composition. For example, one such factor that may change effluent concentrations over time is precipitation, especially for these low flow systems (Appendix A). Fish from Elevenmile Creek and Fenholloway River were under drought conditions the year of collection, while Rice Creek fish experienced normal yearly rainfall. If a dynamic exposure scenario proves crucial to interpretation of effects (see Chapter 3 for a more detailed discussion), documenting chemical exposure has to occur over time.

Despite these limitations, the mosquitofish remains promising as a bioindicator species of pulp mill effluents. Differences in responses among mills coincided with effluent components meaning this species could potentially be used to document varying effluent quality. However, contribution of additional environmental factors is supported by the increased response detected further downstream and not at sites of highest effluent concentration within a mill. A threshold response is also supported by these data, and the apparent disparity between anal fin morphology and sex steroid response may be quite useful to determine the threshold in future research. Controlled whole effluent exposures are necessary to adequately document this potential threshold (see Chapter 5). Recent work in Canada and New Zealand has shown wide variation in induction time for female anal fin elongation that does not correlate with phytosterols concentrations in effluent (McCarthy et al. 2004). This complexity supports the contribution of additional factors within receiving streams which requires further investigation. If the phytosterol bacterial degradation hypothesis is correct, the additional factor may be a change in bacterial communities that transform phytosterols into bioactive compounds at varying rates and efficiencies. While the mechanism behind observed responses requires more extensive

and well-coordinated research, effects-based monitoring using species such as mosquitofish may be the best solution in the meantime.

Table 4-1. Water quality parameters of field collection sites associated with three effluent-receiving streams in Florida the summer of 2001.

Site Type[a]	R1	R2	U	P-D	D	D1	D2
Rice Creek	REF2	REF3	U(8)	PRE-DIS	DIS	D(1)	D(3)
Temperature (°C)	24.5	25.7	24.1	30.2	27.7	27.6	30.4
Conductivity (µS)	143.3	330.7	227	1916	1580	1417	1185
Salinity (ppt)	0.1	0.2	0.1	1.0	0.8	0.7	0.6
Dissolved Oxygen (mg/L)	5.49	5.76	5.71	2.14	5.69	13.3	6.45
Turbidity (ntu)	2.02	4.29	15.3	13.5	20.4	12.7	5.76
pH	7.7	6.6	6.4	7.8	7.6	7.6	7.5
Fenholloway River	REF1	REF2	U(5)	PRE-DIS	DIS	D(12)	NC[b]
Temperature (°C)	23.7	27.6	26.6	31.6	27.8	27.1	NA[c]
Conductivity (µS)	225	67.5	83.6	2321	1158	1336	NA
Salinity (ppt)	0.1	0.0	0.0	1.0	0.3	0.7	NA
Dissolved Oxygen (mg/L)	5.20	4.71	2.22	4.48	3.27	2.35	NA
Turbidity (ntu)	5.73	2.92	1.1	39.4	19.8	15.3	NA
pH	7.8	4.5	3.9	7.4	7.1	7.5	NA
Elevenmile Creek	REF1	REF2	U(1)	PRE-DIS	DIS	D(5)	NC[b]
Temperature (°C)	24.3	24.0	25.9	0.3	28.3	26.3	NA[a]
Conductivity (µS)	50.3	32.8	72.7	1660	1135	432.5	NA
Salinity (ppt)	0.0	0.0	0.0	0.8	0.6	0.2	NA
Dissolved Oxygen (mg/L)	5.46	7.71	7.74	4.09	5.09	6.13	NA
Turbidity (ntu)	7.87	3.33	7.46	22.6	49.8	15.5	NA
pH	5.9	6.2	6.2	8	7.8	7.1	NA

[a]R# = first or second reference; U=upstream; P-D=predischarge retention pond or canal; D = discharge or outfall; D#= first or second downstream

[b]*NC* = not collected for this system

Table 4-2. Concentration of selected effluent components in single grab water samples from field collection sites associated with three effluent-receiving streams in Florida the summer of 2001.

Site Type[a]	R1	R2	U	P-D	D
Rice Creek	*REF2*	*REF3*	*U(8)*	*PRE-DIS*	*DIS*
Total RAFA[a] (µg/L)	0	0	15	100	18
Campesterol (µg/L)	ND[c]	ND	ND	1.2	ND
Stigmasterol (µg/L)	ND	ND	ND	4.6	1.7
Stigmastanol (µg/L)	ND	ND	ND	8.4	1.8
β-sitosterol (µg/L)	ND	ND	ND	28.2	4.8
TOC[b] (mg/L)	4.1	10.4	18	69.7	24.9
polyphenolics (mg/L)	1.6	3.2	2.6	25	6.0
condensable tannins (mg/L)	0.6	1.0	0.7	3.5	1.2
Fenholloway River	*REF1*	*REF2*	*U(5)*	*PRE-DIS*	*DIS*
Total RAFA[a] (µg/L)	4	5	6	326	102
Campesterol (µg/L)	ND	ND	ND	3.4	1.8
Stigmasterol (µg/L)	ND	ND	ND	10.1	7.9
Stigmastanol (µg/L)	ND	ND	ND	14.3	7.6
β-sitosterol (µg/L)	ND	ND	ND	70.6	40.8
TOC[b] (mg/L)	73.2	55.9	107.7	164.7	83.5
polyphenolics (mg/L)	12	8.1	17	39	24
condensable tannins (mg/L)	5.1	4.0	2.5	9.5	5.1
Elevenmile Creek	*REF1*	*REF2*	*U(1)*	*PRE-DIS*	*DIS*
Total RAFA[a] (µg/L)	3	9	3	5	2
Campesterol (µg/L)	ND	ND	ND	ND	ND
Stigmasterol (µg/L)	ND	ND	ND	2.6	ND
Stigmastanol (µg/L)	ND	ND	ND	4.2	1.5
β-sitosterol (µg/L)	ND	ND	0.8	2.5	ND
TOC[b] (mg/L)	18	ND	ND	31.8	24.9
polyphenolics (mg/L)	1.5	0.2	1.3	5.6	2.3
condensable tannins (mg/L)	0.7	0.6	0.7	5.6	0.9

[a]RAFA = resin acids and fatty acids
[b]TOC = total organic carbon
[c]ND = nondetectable

Table 4-3. Body size parameters (ave ± se) for mosquitofish collected from three effluent-receiving streams in Florida the summer of 2001. Significant differences ($p < 0.05$) are noted by site within each system.

Site type[a]	R1	R2	U	P-D	D	D1	D2
Rice Creek	REF2	REF3	U(8)	PRE-DIS	DIS	D(1)	D(3)
♂ Sample Size	30	20	28	21	21	24	24
♂ Body Weight (g)	0.235+0.021[d]	0.148+0.013	0.198+0.016	0.155+0.012	0.167+0.010	0.177+0.017	0.172+0.015
♂ Standard Length (mm)	22.72+0.62	19.75+0.57e	22.49+0.61	19.68+0.54e	20.69+0.46	21.24+0.61	20.97+0.56
♂ Condition Factor (g/cm3)	1.87+0.04	1.83+0.06	2.64+0.11f	1.97+0.04	1.87+0.06	1.73+0.03	1.76+0.03
♀ Sample Size	53	55	55	50	34	51	55
♀ Body Weight (g)	0.489+0.042	0.473+0.030	0.508+0.035	0.639+0.062g	0.336+0.028	0.444+0.045	0.531+0.048
♀ Standard Length (mm)	27.65+0.68	26.90+0.47	28.00+0.52	29.45+0.90g	24.74+0.61h	26.58+0.63h	27.83+0.75
♀ Condition Factor (g/cm3)	2.02+0.04	2.28+0.04i	2.13+0.04	2.19+0.04	2.09+0.11	2.13+0.09	2.10+0.03
Fenholloway River	REF1	REF2	U(5)	PRE-DIS	DIS	D(12)	NCb
♂ Sample Size	20	17	20	24	23	20	NAc
♂ Body Weight (g)	0.152+0.012	0.127+0.012j	0.146+0.007	0.1.94+0.019	0.192+0.019	0.148+0.010	NA
♂ Standard Length (mm)	19.63+0.53	19.74+0.49	19.79+0.27	21.32+0.64	21.09+0.63	19.53+0.45	NA
♂ Condition Factor (g/cm3)	1.94+0.07	1.59+0.06f	1.87+0.06	1.88+0.04	1.93+0.08	1.93+0.04	NA
♀ Sample Size	44	46	36	55	46	55	NA
♀ Body Weight (g)	0.347+0.037	0.450+0.046	0.200+0.016f	0.445+0.032	0.364+0.022	0.441+0.024	NA
♀ Standard Length (mm)	24.18+0.55k	26.96+0.83	21.62+0.56l	26.51+0.57	25.29+0.51	26.66+0.48	NA
♀ Condition Factor (g/cm3)	2.32+0.23	1.97+0.03m	1.89+0.05n	2.25+0.05	2.15+0.05	2.22+0.03	NA
Elevenmile Creek	REF1	REF2	U(1)	PRE-DIS	DIS	D(5)	NC[a]
♂ Sample Size	20	20	23	23	24	23	NA
♂ Body Weight (g)	0.150+0.008	0.144+0.008o	0.199+0.019	0.205+0.013	0.193+0.014	0.187+0.014	NA
♂ Standard Length (mm)	19.57+0.34	20.15+0.34f	20.76+0.59	21.16+0.50	21.18+0.50	21.06+0.47	NA
♂ Condition Factor (g/cm3)	1.98+0.04	1.74+0.04	2.08+0.04	2.14+0.05	1.97+0.04	1.93+0.05	NA
♀ Sample Size	55	42	55	54	55	55	NA
♀ Body Weight (g)	0.539+0.037	0.431+0.044	0.505+0.036	0.506+0.043	0.461+0.026	0.435+0.028	NA
♀ Standard Length (mm)	27.69+0.60	26.82+0.77	27.37+0.54	26.96+0.75	26.86+0.49	26.16+0.55	NA
♀ Condition Factor (g/cm3)	2.39+0.09	1.98+0.04f	2.27+0.03	2.28+0.03	2.25+0.02	2.27+0.03	NA

[a]R# = first or second reference; U = upstream; P-D = predischarge retention pond or canal; D = discharge or outfall; D# = first or second downstream

[b]NC = not collected

[c]NA = not available statistically differs from:

[d]REF1, PRE-DIS, DIS

[e]REF2 and U(8)

[f]all sites

[g]DIS and D(1)

[h]U(8), PRE-DIS, D(3)

[i]REF2 and DIS

[j]PRE-DIS and DIS

[k]REF2 and D(5)

[l]all sites except REF1

[m]PRE-DIS and D(12)

[n]all sites except REF2

[o]DIS

Figure 4-1. Maps of field sites. A) Relative location of the stream systems in Florida. B) Rice Creek sites. C) Fenholloway River sites. D) Elevenmile Creek sites. Symbols distinguish sites exposed to effluent: circles = unexposed sites and triangles = exposed sites. Site abbreviations denote upstream (U) or downstream (D) of discharge, followed by approximate distance (km) from discharge in parentheses. PRE-DIS indicates site before discharge into the creek; DIS denotes site at discharge into creek; and REF indicates reference site, followed by identifying number.

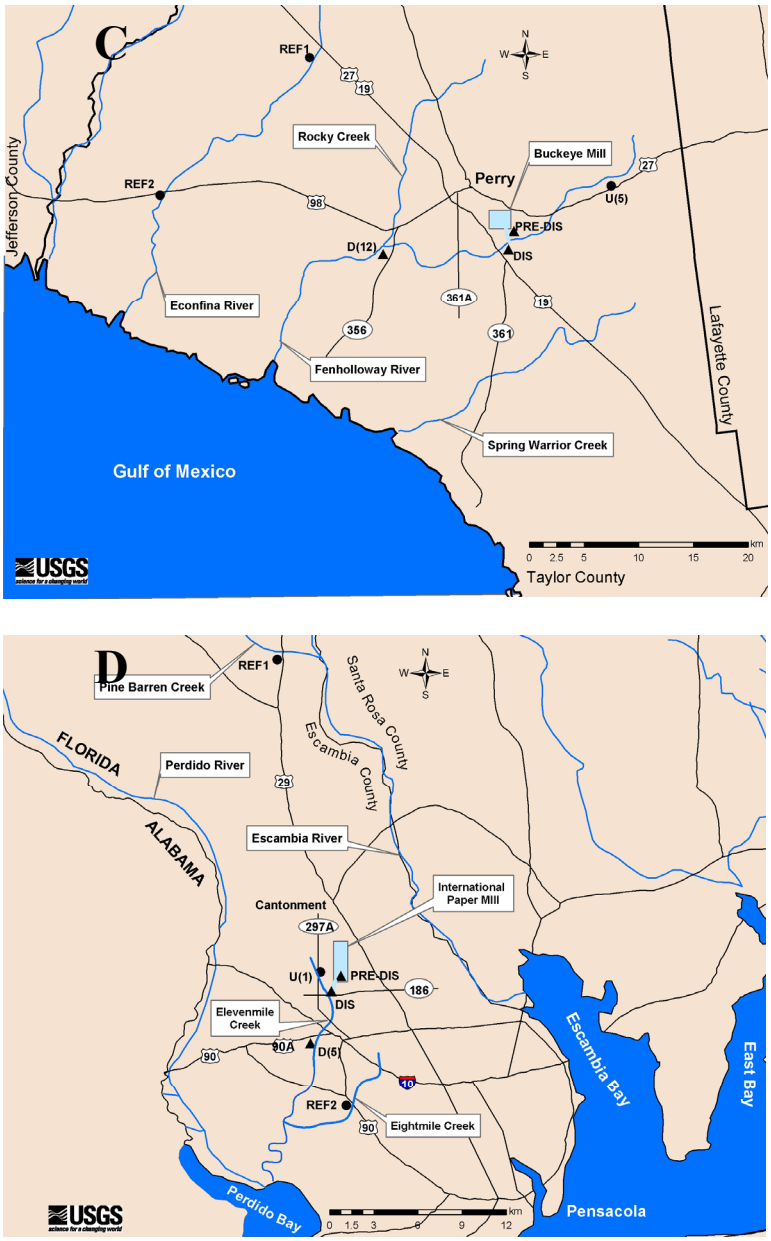


Figure 4-1. Continued

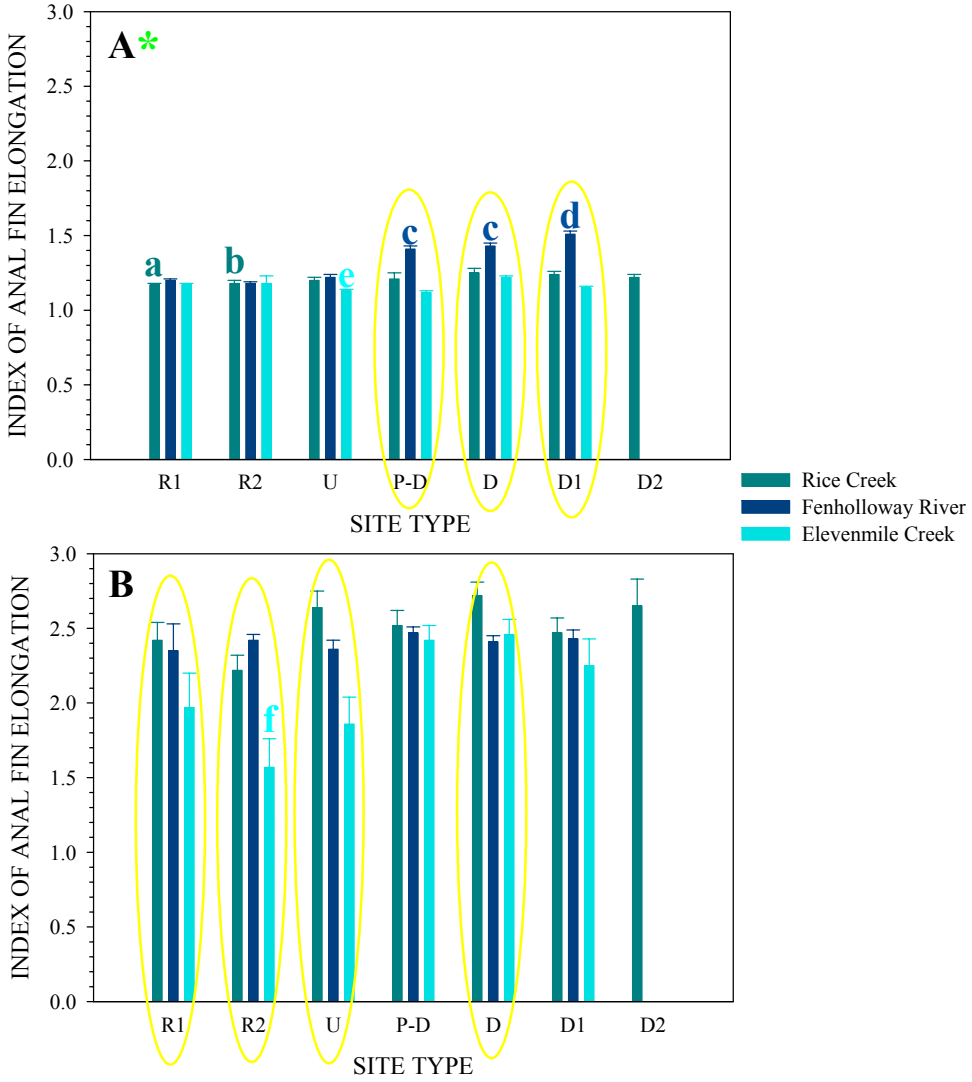


Figure 4-2. Index of anal fin elongation (tracings of Ray 4 / Ray 6, computer-aided measurement of fresh fish) for mosquitofish collected in summer 2001 from three effluent-receiving systems in Florida. A) Females. B) Males. Letters indicate significant differences by site within system ($p<0.05$): "a" denotes differences to P-D, D and D1 (Rice Creek system); "b" denotes differences to P-D (Rice Creek system); "c" denotes differences to nonlettered sites (Fenholloway River system); "d" denotes differences to all other sites (Fenholloway River system); "e" denotes differences to P-D and D (Elevenmile Creek system); "f" denotes differences to P-D and D (Elevenmile Creek system). Yellow circles signify statistically significant differences among mills ($p < 0.05$). Green asterisk marks interaction between site and mill for females only.

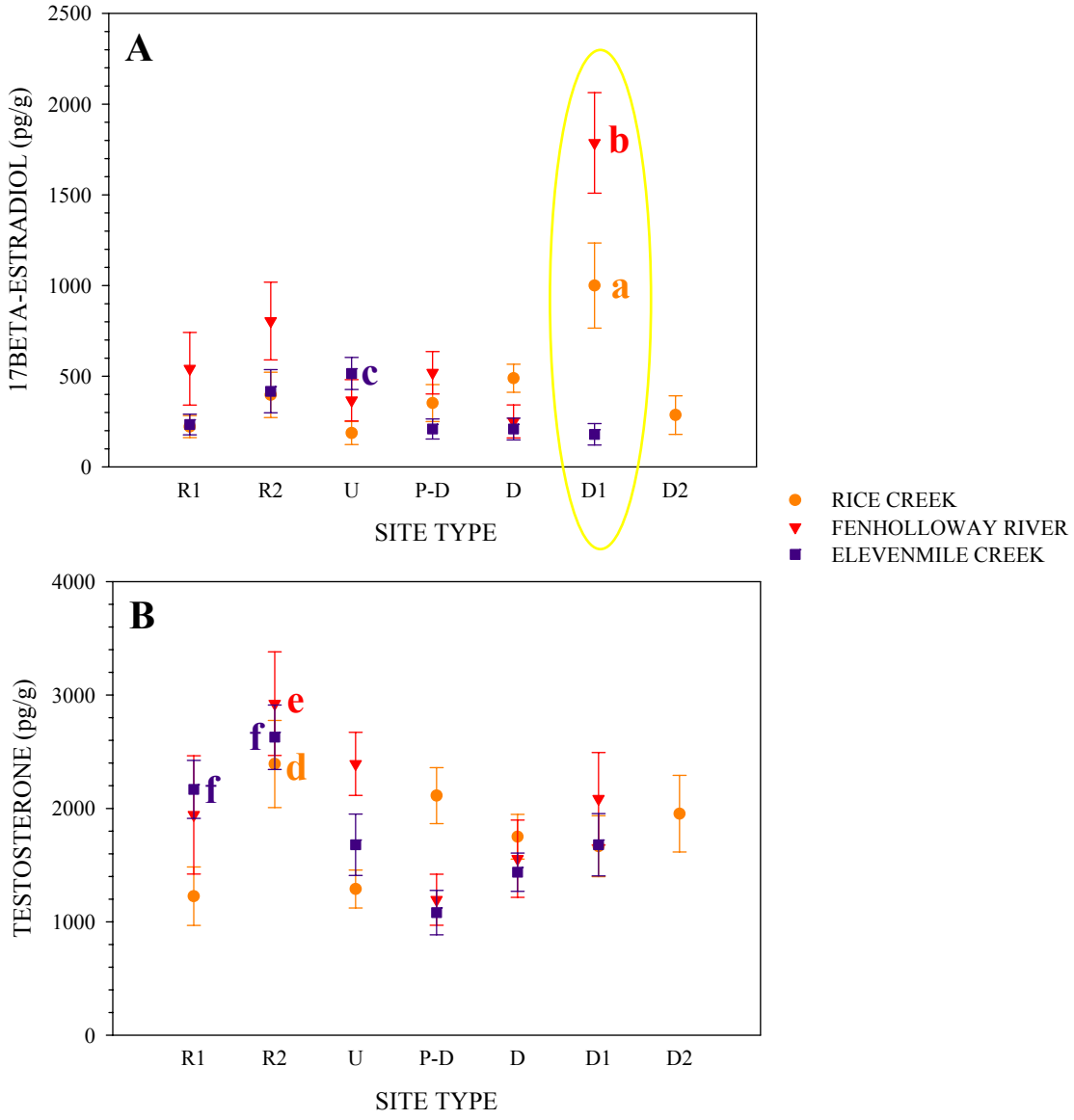


Figure 4-3. Whole body sex steroids (ave + se) for male mosquitofish collected from three effluent-receiving streams in Florida the summer of 2001. A) 17β-estradiol. B) Testosterone. Letters indicate significant differences by site within mill ($p < 0.05$): "a" and "b" denote differences to all other sites; "c" denotes differences to P-D, D, D1; "d" denotes differences to R1 which is also different to U; "e" denotes differences to P-D; "f" denotes differences to P-D (and R2 also different to D). Yellow circle indicates significant differences among mills at that site. Site type and mill did not covary for either hormone.

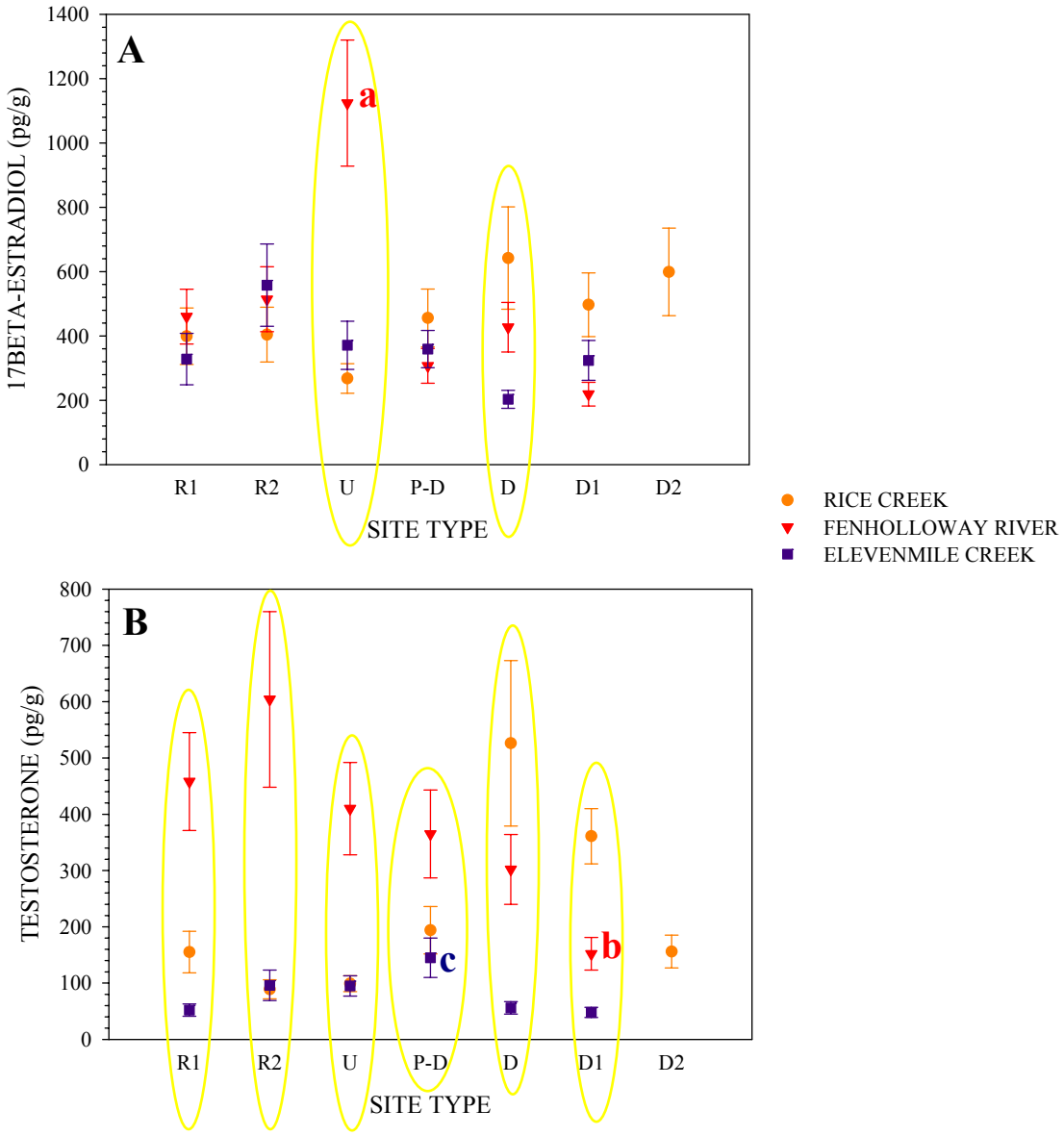


Figure 4-4. Whole body sex steroids (ave ± se) for female mosquitofish collected from three effluent-receiving streams in Florida the summer of 2001. A) 17β-estradiol. B) Testosterone. Letters indicate significant differences by site within mill ($p < 0.05$): "a" denotes differences to nonlettered sites; "b" denotes differences to R2; "c" denotes differences to R1, D, and D1. Yellow circles indicate significant differences among mills at that site. Site type and mill did not covary for either hormone.

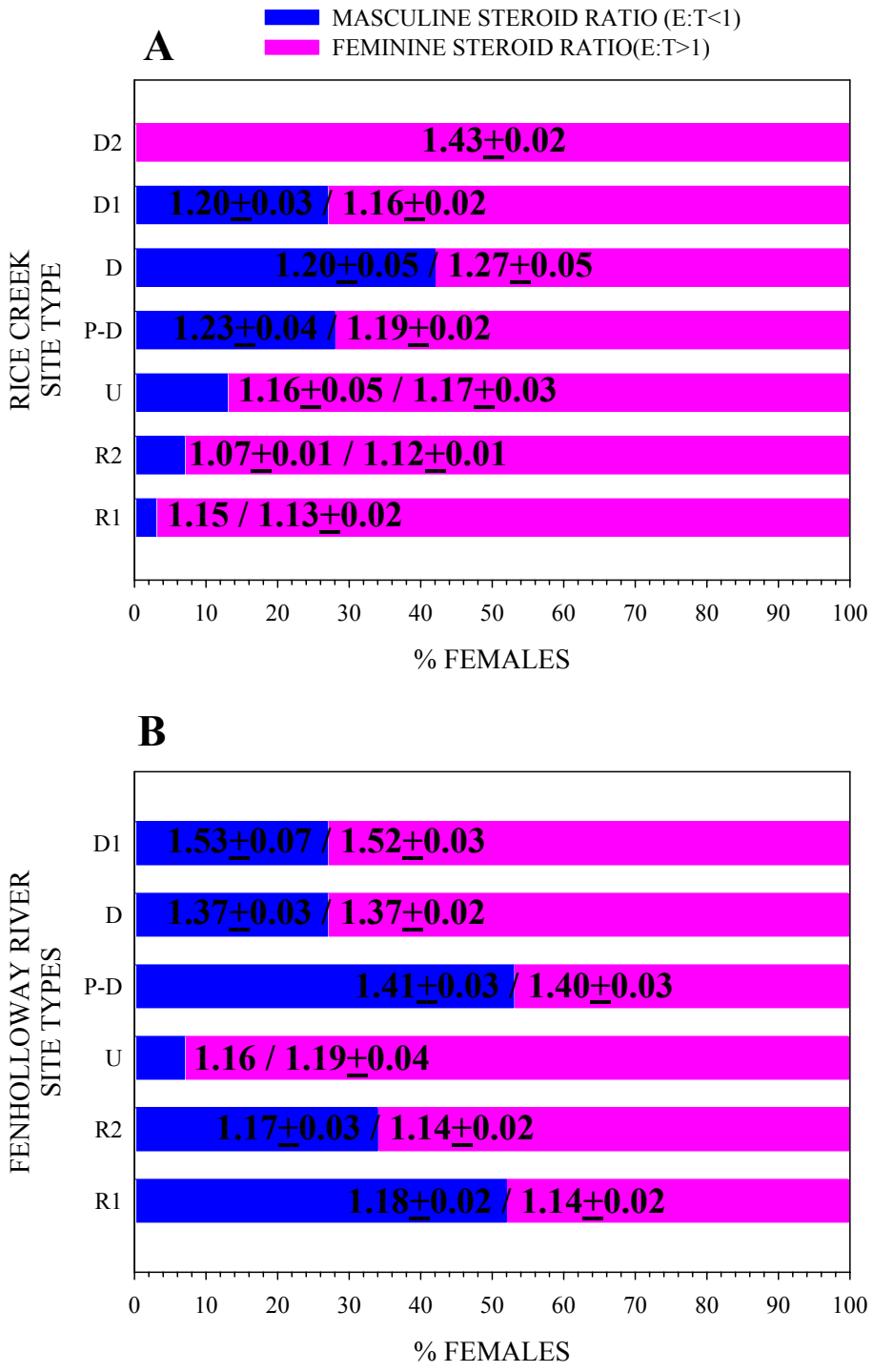


Figure 4-5. Percentage of female mosquitofish with masculine and feminine sex steroid ratios collected from three effluent-receiving streams in Florida the summer of 2001. Index of anal fin elongation (ave ± se) is given for fish in each of these groups by site (no significant differences at $p < 0.05$). A) Rice Creek ($\chi^2 = 27.95$, df = 6, $p < 0.05$). B) Fenholloway River ($\chi^2 = 14.31$, df = 5, $p < 0.05$). C) Elevenmile Creek ($\chi^2 = 0.8064$, df = 5, $p < 0.05$).

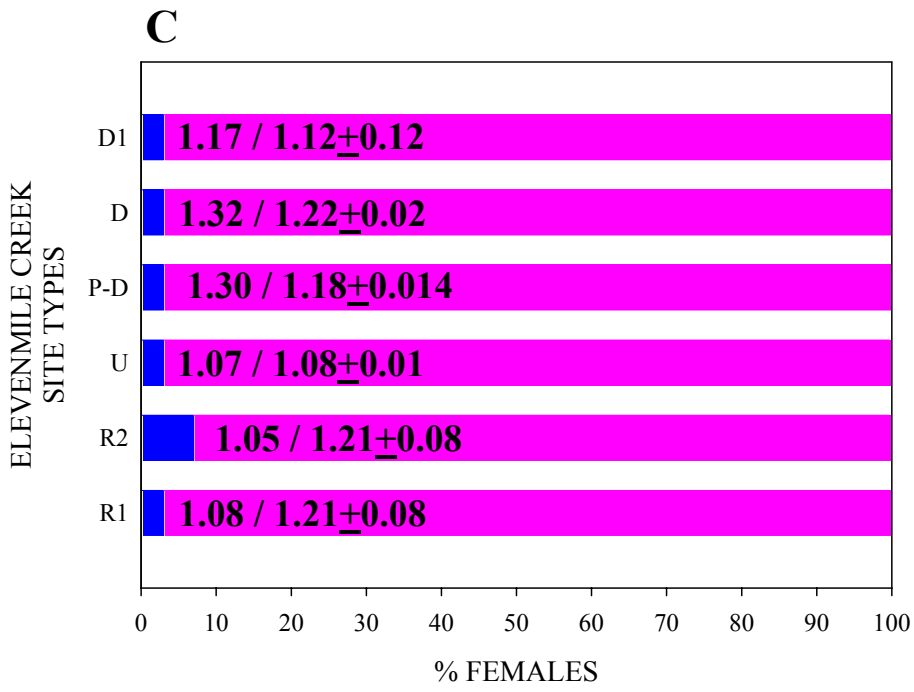


Figure 4-5. Continued

CHAPTER 5
DIFFERENTIAL INDUCTION OF EFFECTS IN MOSQUITOFISH EXPOSED TO BLEACHED KRAFT MILL EFFLUENT

Masculinization, or development of male secondary sex characteristics in female mosquitofish, has been detected in pulp and paper mill effluent-receiving streams for decades. While laboratory exposure to bacterially-degraded phytosterols, the hypothesized mechanism, has replicated masculinization of females, controlled exposure to whole effluent dilutions has produced inconsistent masculinization results. This study compared and contrasted short-term (4 week) whole effluent exposures in flow-through tanks (0, 10, 20, 40, and 80% effluent dilutions) against *in* situ exposure of caged fish at field sites (upstream, before discharge, and discharge sites). While the anal fin Ray 4 to Ray 6 length ratio was not affected in either sex, whole body sex steroid alterations suggested either dynamic exposure and/or multiple modes of action along the reproductive-endocrine axis. Specifically, males expressed feminized estrogen to testosterone (E:T) ratios and females expressed masculinized ratios at midpoint sampling of field-exposed fish followed by partial or complete recovery of normal profiles at final sampling. Skewed steroid ratios did not manifest in tank-exposed fish until final sampling. Hormonal effects were detected at effluent dilutions within yearly instream effluent concentrations (about 60%). Full characterization of seasonality in hormones; intensive documentation of exposure; and the study of additional environmental factors such as bacterial degradation of effluent components into bioactive compounds would greatly improve strength of results.

Introduction

Masculinized mosquitofish collected in pulp and paper mill effluent receiving streams have been documented in Florida for over twenty years (Howell et al. 1980, Drysdale and Bortone 1981, Cody and Bortone 1997, Bortone and Cody 1999, Jenkins et al. 2001, Parks et al. 2001, Chapters 2 through 4). Masculinization refers to development of male secondary sex characteristics in females. Mosquitofish are sexually dimorphic: males develop a gender-specific copulatory organ, or gonopodium, as part of final maturation. The gonopodium is an extension and differentiation of Rays 3, 4, and 5 of the anal fin (Turner 1941a). Based upon observations of female exposure to androgens (Turner 1941b, 1942a,b) and bacterial degradation of phytosterols into androgens (Marcheck et al. 1972), an androgen-mediated mechanism has been hypothesized to explain this phenomenon. Laboratory exposure to bacterially-degraded phytosterol preparations confirmed the potential of this hypothesis (Denton et al. 1985, Howell and Denton 1989), yet few studies have demonstrated response under controlled exposure to whole effluents.

Controlled exposure to whole effluent dilutions has produced inconsistent masculinization results. Initially in support of field collections, static renewal exposure of newborn mosquitofish to water collected 3.6 km downstream from Elevenmile Creek induced elongated anal fins (measured as anal fin length) in females upon maturity (Drysdale and Bortone 1989). While my study research was being conducted, researchers in Canada and New Zealand were also studying masculinization of adult female mosquitofish using controlled (mainly static renewal, one flow-through) exposures to 15%, 70% or 100% effluent (McCarthy et al. 2004). Four of seven pulp mill effluents induced masculinization (all static renewal). Among these four pulp mill

effluents, two induced elongation relatively quickly (within 3 weeks), while the other two required 24 weeks of exposure. No association between induction and type of mill or concentration of β-sitosterol was established. Apparently, duration of exposure required to produce effect varies widely. However, three important caveats exist for these studies:

- Other than Elevenmile Creek, comparative field studies were not conducted to determine if effects existed in wild mosquitofish exposed to these effluents.
- All but one exposure required holding and transport of effluent back to the exposure system, with unknown consequences to effluent composition.
- Also noteworthy, masculinization was measured qualitatively, as either presence/absence or staged using categories established by Howell and Denton (1989).

One of these controlled exposures detected differences in masculinization due to effluent treatment and filtration (Ellis et al. 2003). Secondary treatment of effluent reduced gonopodial development by 25%, yet masculinization remained significantly greater than controls. Filtration of treated effluent, removing many organic extractives adsorbing to particulates, also removed the response.

This exposure was repeated two years later, after treatment system maintenance was improved as indicated by gradual reduction in total suspended solids. Using the more specific Ray 4 to 6 length ratio, masculinization was not induced (van den Huevel et al. 2004b). Since exposure duration remained the same (3 weeks), it was unknown if the effect was entirely removed or if time to manifestation was extended. Regardless, these experiments strongly correlate masculinization with adsorbable organic effluent components, such as low molecular weight wood extractives.

The objectives of this study were: to assess the masculinization response (for secondary sex characters and steroids) in mosquitofish under controlled and *in situ* field exposure to effluents from Rice Creek and Fenholloway River; to examine induction of

responses under controlled conditions before and after major process changes at the Rice Creek mill; and to evaluate associations between concentrations of wood extractives in effluent and response in mosquitofish.

Materials and Methods

Controlled and *in situ* field exposure of mosquitofish to whole effluent dilutions was attempted on three separate occasions, with limited success during the second attempt. Thus stated objectives were restricted to assessing the masculinization response, for secondary sex characters and steroids, in mosquitofish under controlled and *in situ* field exposure to effluents from Rice Creek; and to evaluating associations between concentrations of wood extractives in effluent and responses in mosquitofish. Controlled tank exposures at the Rice Creek mill, using the facility described below, were tested in summer 2000. High fish mortality that was caused by several factors, including very high densities (over 1,000 fish per 1,500 L tank), precluded analysis. Tank exposures were modified and repeated concomitantly with field exposures at Rice Creek field sites in the summer of 2002. This study was completed with partial fish loss and is the focus of this chapter. As part of the fry production study in 2003 (Chapter 6), *in situ* field exposures at Rice Creek and Fenholloway River field sites were initiated but terminated prematurely because of several complications such as very acidic pH at the upstream Fenholloway site (averaging 3.5) and severe flooding that overflowed cages and made them inaccessible. Thus a large amount of effort yielded comparatively little data, and redesign of exposure scenarios is highly recommended.

Mill Characteristics and Exposure Scenarios

Mosquitofish were exposed to bleached/unbleached pulp and paper effluent from the Georgia-Pacific mill in Palatka, Florida, USA (Chapter 1, Table 1-1). The mill

discharges into Rice Creek, a tributary of the St. Johns River. Rice Creek is a low-flow stream, so dilution factor for effluent is low until it reaches the Saint Johns River. Major process changes to comply with US EPA's Cluster Rule were implemented in May of 2001, and a reduction in effects on mosquitofish was observed (Chapter 3).

Controlled whole effluent exposure of mosquitofish was conducted using a unique onsite tank facility near the head of the discharge canal (Figure 5-1). This facility was constructed primarily for flow-through exposure of largemouth bass in 1998 (Sepúlveda 2000, Quinn 2004) and has also been used for exposure of freshwater mussels (T.S. Gross, unpublished data). Water from a nearby well was pumped to a series of three 30 kL aboveground pools for filtration and removal of sulfates and metals, and then pumped to a head tank. Simultaneously, 100% final effluent pulled midstream from the head of the canal was pumped to an adjacent head tank. Gravity was used to bring water from both lines down to aerated 1,500 L round tanks, and flow meters were used to control output from each line into the five sets of tanks (duplicates were connected). Adjustment of flow meters (15 L per minute total per tank) enabled effluent concentrations of 0, 10, 20, 40, and 80%. These concentrations were used for two reasons: 1) environmental relevance, since effluent accounts for an average of 60% flow upon discharge into Rice Creek and drops to < 10% at the St. Johns River; and 2) interspecies comparison, since largemouth bass were also exposed to these concentrations.

Mosquitofish were also caged and submerged *in situ* at field sites surveyed regularly by our laboratory. Four sites were selected for field exposure, two each of exposed and unexposed sites (Figure 5-2). Large 120 cm by 60 cm by 60 cm net cages

(3 mm mesh) were framed with PVC pipe and fitted with PVC-reinforced screen lids. These same cages (without lids) housed fish in the tank exposures to provide equal densities of fish. Artificial cover (three to four green cheerleading pompons secured to top corners of cage) was provided to reduce aggression (a problem in the 2000 tank exposures). *In situ* cages were floated underneath shade along vegetated banks (within 1 m from the bank). Targeted depths within the cage were approximately 30 to 40 cm, while distance between bottom of cages and stream sediments varied from 0 to approximately 60 cm (depending on tidal influence and rainfall). Cages were anchored to nearby trees and/or metal stakes.

Exposures lasted for 4 weeks, based upon the length of time reported for mature gonopodial induction in females (2 to 3 weeks) exposed to bacterially degraded phytosterols (Denton et al. 1985, Howell and Denton 1989). Approximately five thousand eastern mosquitofish were donated by Watts Aquatics (Lilith, FL) and transported to the US Geological Survey, Florida Integrated Science Center, Center for Aquatic Resource Studies, Gainesville, FL, for acclimation to captivity in 1500 L round tanks for one month. Artificial feed (Tetramin Flakes, Zeigler, Gardner, PA) used throughout the study was introduced at this time. Two to three hundred fish (estimated by weight at ~250 g/m^3) were randomly assigned to each exposure group and transported to the mill in Palatka. Exposures commenced in midMarch 2002 on a staggered schedule over a ten day period, two to three treatments or sites added every other day. Water quality parameters (dissolved oxygen, temperature, pH, conductivity, salinity, and turbidity) and cages were monitored and fish were fed three times a week in the mornings throughout exposure.

Water Samples

Single grab water samples were collected before fish sampling at weeks zero, two, and four to document potential exposure of fish to specific effluent components. Samples were preserved (buffered) and refrigerated for analysis. However, malfunction of the refrigerator caused spoilage and rendered the first two sets of samples entirely useless. Water samples for field exposures at week four were the only salvageable samples and were analyzed for phytosterol content by the University of Florida Department of Environmental Engineering's analytical chemistry core.

Fortunately, NCASI was conducting fathead minnow (*Pimaphales promelas*) exposures at the same time and provided chemical analysis data of 100% whole effluent measured for their study. Effluent was analyzed for chlorinated phenolics (12 Cluster Rule compounds plus 16 others), 10 resin acids (including 3 chlorinated), 3 fatty acids, 4 phytosterols, total organic carbon (TOC), condensable tannins, and polyphenolics. Additional effluent components were analyzed in 100% whole effluent: metals, nonmetals (such as chloride and fluoride), and neutral semivolatiles. Columbia Analytical Services conducted the chlorophenolic analyses, CH2M Hill conducted the TOC analyses, and the NCASI West Coast Regional Center conducted all other analyses.

Morphological Endpoints

For each sampling week, 25 fish of each sex per treatment/site were targeted; however, low densities in the absence of obvious fish mortality at week two of sampling reduced this number to 15 fish (5 males and 10 females per treatment/site). First, fish were euthanized with a terminal dose of buffered tricaine methanesulfonate (Tricaine-S, Western Chemical Inc., Ferndale, WA, USA), then weighed using a digital scale ($\pm$ 0.001 g) and measured for standard length ($\pm$ 0.01 mm) using a pair of digital calipers. Under a

dissecting scope, gender was identified using the presence (female) or absence (male) of a urogenital papilla (Chapter 2). Each fish was photographed using a digital camera then placed on ice until transferred to -80°C freezer for subsequent radioimmunoassay (RIA) of sex steroids. Anal fin images of these fish were measured by computer (±0.01 mm) using trace mode in Sigma Scan Pro © 5.0 from the base of Rays 4 and 6 along the curve of each ray to the tip.

Hormonal Endpoints

Whole body primary sex steroids (17β-estradiol and testosterone for this species) were analyzed using a modified RIA method originally developed for serum and plasma samples of common carp, *Cyprinus carpio* (Goodbred *et al* 1997), and since adapted for use in a variety of other aquatic species and tissue media such as plasma of largemouth bass, *Micropterus salmoides* (Gross et al. 2001) and mantle of freshwater invertebrates (Gross et al. 2000). Chapter 2 provides detailed methods and validation of this assay.

Statistics

Body weight and standard length were used to calculate condition factor, $K = \text{weight} / \text{length}^3 \times 100$ (g/cm^3), as an indication of overall health used by the aquaculture industry (values of at least 1 are considered healthy, Hile 1936). The length ratio of anal fin Rays 4 and 6 was calculated as an index of anal fin elongation. Estrogen and testosterone concentrations were used to calculate a ratio indicating masculine hormone profile (E:T < 1) or feminine hormone profile (E:T > 1).

Anal fin morphology and sex steroid data were analyzed within sex using two-way analysis of covariance (ANCOVA) to test for significant variation by treatment/site and week. Any data failing tests for normality and homogeneity of variance were log-transformed. Treatment or site differences within week were also analyzed by

one-way ANOVA, as were differences by week. Significant differences in ANCOVA and ANOVA were analyzed for multiple comparisons using Tukey's HSD for field exposures and Dunnett's for tank exposures. Relationship between anal fin morphology and sex steroids were analyzed overall and by treatment/site in two ways: first, by examining Pearson's correlations of the index of anal fin elongation to sex steroid concentrations and ratio, then by t-test for differences in index of anal fin elongation between females with masculine versus feminine E:T ratios. Statistical significance was set at $\alpha < 0.05$ for all tests. All statistical analyses were conducted using SAS © version 9.0.

Results and Discussion

All exposures continued to completion except for the field exposures at the reference site [REF2]. Just before the midpoint sampling for this site, cages were vandalized and destroyed.

Water Quality

Conductivity, salinity, turbidity and pH were significantly elevated in 40% and 80% effluent treatments (Table 5-1). Dissolved oxygen was adequate for fish survival (> 4mg/L) and equivalent among treatments because of aeration of tanks. For field exposures, water temperature, conductivity, salinity, dissolved oxygen, and turbidity significantly differed among all sites (Table 5-2). Temperature, conductivity, salinity, and turbidity were elevated at effluent exposed sites, while dissolved oxygen was very low before discharge (PRE-DIS < 2 mg/L) and exceptionally high at the outfall (DIS > 10 mg/L). The pH differed significantly between exposed sites [PRE-DIS and DIS] but neither was different compared to the upstream site [U(8)].

Water Chemistry

Salvaged water samples from week four field exposures were analyzed for phytosterol content. Campesterol, stigmastanol, and stigmasterol were all below detection limits (< 12 µg/L). β-sitosterol was also below detection limit for the upstream site. β-sitosterol was highest at the outfall site [DIS 45 µg/L] and somewhat lower before discharge [PRE-DIS 32 µg/L]. More sensitive analysis by NCASI confirmed higher concentrations of β-sitosterol than the other phytosterols in 100% final effluent (Table 5-3).

Examining these compounds over the duration of field and tank exposures provided compelling support for dynamic exposure of mosquitofish (Figure 5-3). (Dynamic exposure refers to variable concentrations of effluent components over time, dependent on factors such as tree species for furnish, within plant processing spills, rainfall/dilution, and bacterial degradation.) In general effluent components decreased during exposures: the most dramatic drop was in resin acids, while phytosterols were more stable (Figure 5-3 and Table 5-3). Dilutions of these values could be roughly extrapolated to assess potential exposure of tank fish; however, additional environmental factors, such as rainfall and bacterial communities, may be influencing cage-exposed fish. These exposures began during a period of low rainfall in March, continued under average precipitation through April and ended at low rainfall in May (Appendix A). Therefore field exposure of caged fish may have been highest in March, lowest in April but then increased again in May, perhaps to concentrations intermediate between March and April. Without continuous monitoring, these concentrations are approximate and

consider rainfall only. Additional factors (bacterial activity) may have created even greater flux.

Body Size and Condition

Exposures had no impact on body size and condition for males. Females grew significantly longer under *in situ* field exposures (since females, and not males, grow continuously throughout life this result was not surprising). However, tank-exposed females did not grow suggesting increased food availability in field cages, beyond the Tetramin flakes provided three times a week, may have caused growth in field-exposed females.

Males

There were no significant differences by week or among treatments in length, weight, or condition factor of males exposed to whole effluent dilutions at the tank facility (Table 5-4). All males remained healthy (CF > 1) for the duration of exposure. Slight variations in body size occurred by week and by site in caged males exposed *in situ* at field sites, although these two factors did not covary (Table 5-5). Standard length was slightly elevated for males at week two but not week four. Also, overall condition factor was higher for males from the discharge site [DIS] compared to the other two sites [U(8) and PRE-DIS], although they were all above one indicating adequate general health.

Females

There were no significant differences by week or among treatments in length, weight, or condition factor of females exposed to whole effluent dilutions at the tank facility (Table 5-4). All females remained healthy (CF > 1) for the duration of exposure. In contrast, females caged at field sites showed increased growth (Table 5-5). Since

females grow continuously throughout life, making body length approximate to age (Meffe and Snelson 1989), female growth by week was expected. However, given an absence of growth among tank exposed females, these data suggest field-exposed females may have had increased food consumption. Tank-exposed fish only received Tetramin flakes as a food source, while field-exposed fish potentially accessed any planktonic or plant particles passing through the cages.

Anal Fin Morphology

Neither sex responded to treatment in tanks or the field in terms of anal fin length. While this result was expected for males, it was unexpected for females. The only plausible conclusion for this lack of induction was the short duration of exposure which was based upon results published elsewhere after laboratory exposure of mosquitofish to degraded effluent components and not whole effluent dilutions (Denton et al. 1985, Howell and Denton 1989).

Males

Male mosquitofish exposed to whole effluent dilutions in tanks did not vary in gonopodial length regardless of exposure dose or duration (Figure 5-4B). Variation in gonopodial length was greater than for females. Similarly, caged males in field sites displayed no changes in gonopodial length, and the only site-related difference was between the upstream and discharge sites [U(8) and DIS]. Gonopodia of males from the discharge site were shorter, although this was not significantly different than control (week zero) males. Overall, effluent exposure did not affect male anal fin morphology in terms of length (2.5 ± 0.08). These findings support previously reported surveys of male mosquitofish collected from effluent-exposed Florida streams (Chapters 2 through 4).

Females

Similar to males, female anal fin elongation was not significantly different by week or treatment/site (Figure 5-4A). Average index of anal fin elongation (Ray 4 to Ray 6) was 1.14 $\pm$ 0.02. These values are in accordance with field data for females at unexposed sites (Chapters 2 through 4). Also, reported Ray 4 to Ray 6 length ratios for unexposed females in the 11keto-testosterone study by Angus et al. (2001) averaged just above 1 (estimated 1.1 from Figure 5-4A). In contrast to these exposures, wild female mosquitofish collected not only downstream of effluent discharge [DIS, D(1), D(3)] but also before discharge [PRE-DIS] displayed masculinized anal fin elongations (1.25$\pm$0.03 Chapters 2 through 4). Thus induction of response was expected but not supported.

Exposure research by McCarthy et al. (2004) indicates duration of exposure could explain this lack of induction. They found time to induction varied dramatically by mill effluent (from 3 to 24 weeks). Also possible but not probable, the mosquitofish population used for these exposures were somehow resistant or acclimated. To date, this has never been documented in eastern or western mosquitofish. Fish were farm-raised in manmade ponds separated from any known point sources of pollution, but these fish were not directly tested for prior chemical exposure. Another alternative relates to sensitive life stage. For many other fish species, such as rainbow trout, *Oncorhynchus mykiss* (van den Huevel et al. 2002), increased sensitivity to effluent exposure occurs during critical windows of development, growth, and/or maturation. Laboratory exposures of mosquitofish to bacterially degraded phytosterols (Denton et al. 1985, Howell and Denton 1989), whole effluent exposures (Ellis et al. 2003, McCarthy et al. 2004, van den Huevel et al. 2004b), and lack of consistent size/age class related effects in wild-caught

fish (Chapter 2) indicate mosquitofish females probably lack any particularly sensitive life stages relative to anal fin elongation.

Sex Steroids

In contrast to anal fin morphology, sex steroids for both males and females were influenced by effluent exposure. Hormones were altered (mostly depressed except 17β-estradiol in males) by effluent exposure and later appeared to recover among *in situ* field-exposed fish. Tank-exposed fish seemed to lag behind responses in field-exposed fish, especially when E:T ratios were examined. Males seemed more sensitive than females regarding skewed sex hormone ratios. Feminized hormone ratios (in males) and masculinized ratios (in females) were induced within two weeks in field-exposed fish with recovery or near recovery to normal ratios by the end of four weeks. Skewed ratios were not significantly induced below the highest effluent concentration in tank-exposed fish until four weeks. Very likely dynamic exposure of mosquitofish to bioactive effluent components (or degradation products) explains these results, although influence of toxicant(s) at different stages of steroid biosynthesis, transport, action, and degradation may also play a role.

Males

Before exposures, male sex steroids were normal and testosterone-biased at an E:T ratio of 3:1 (Figure 5-5). Slight variation in 17β-estradiol levels was detected at 20% effluent (Figure 5-5A), but other than that no differences in this hormone were detected from tank exposures by week and/or treatment. An exceptionally large peak in 17β-estradiol was detected in *in situ* field-exposed males at the predischarge site [PRE-DIS] at week two, with apparent recovery at week four to levels similar to males

from the other field sites. This recovery of normal steroid levels suggests either an acclimation response or a significant change in exposure. Unfortunately the latter cannot be directly deduced from available chemical data, although a decrease in exposure is indicated (Figure 5-3). The rise in 17β-estradiol agrees with field collection data (Chapter 4), although significant elevation occurred at the discharge site and not at the site before discharge.

Testosterone concentrations did not vary among treatments at week two, but by week four they had dropped significantly at 0% and 40% effluent (Figure 5-5B). Further, testosterone was significantly lower at 40% and 80% effluent compared to 0% for week four. The decrease at 0% over time implies a seasonal change in testosterone, but does not explain the significant decreases at the highest effluent concentrations. Testosterone in field-exposed males displayed a completely different pattern. Testosterone was depressed at the outfall for week two samples compared to males exposed upstream [U(8)]. By week four testosterone was significantly increased at both exposed field sites [PRE-DIS and DIS]. One possible explanation for this increased testosterone at week four is differential exposure to androgenic compounds between weeks and between whole effluent dilutions and instream field dilutions. Tentative evidence for dynamic exposure in the sex steroid response of wild-caught males (Chapters 3 and 4) lends credence to this conclusion. However, several alternatives influencing the endocrine system also exist.

Negative feedback loops are common in endocrine systems of fish and higher vertebrates (Van Der Kraak et al. 1998, Jalabert et al. 2000, Lister and Van Der Kraak 2001). Elevated estrogen or testosterone levels relay signals to the pituitary to decrease

production of trophic hormones that are responsible for initiating biosynthesis. Elevated 17β-estradiol may be initiating a feedback loop to bring these two hormones back into balance.

Another point of regulation potentially affecting these steroid levels is aromatase, the monooxygenase enzyme responsible for converting androgens into estrogens. Orlando et al. (2002) predicted inhibited activity of this enzyme in female mosquitofish collected from the Fenholloway River may cause elevated androgens that in turn produce masculinized anal fins in females. Contrary to their hypothesis, aromatase activity was actually elevated in both brain and ovarian tissue of females collected downstream of effluent discharge in the Fenholloway. Elevated aromatase activity may explain results observed in males, leading to elevated 17β-estradiol. In support, E:T ratios were estrogen-biased by week two in males caged in both effluent-exposed field sites [PRE-DIS and DIS] and in 80% effluent in tanks (Figure 5-6). By week four, field-exposed males demonstrated recovery back to normal testosterone-biased ratios, while even more tank-exposed males displayed feminized hormone ratios at 20%, 40% and 80%. Effects on ratios of tank-exposed males were due more to a drop in testosterone than a rise in 17β-estradiol, and this differential response within the same sex may allude to different chemicals affecting the hypothalamo-pituitary-gonad axis or possibly different components of this axis being affected. Since the difference in effect was between tank- versus field-exposed fish, the hypothesis of dynamic exposure may be more plausible at this point.

Females

Significant elevation in 17β-estradiol occurred in control fish by week two (0% effluent) with recovery to pre-exposure levels by week four (Figure 5-7A). These changes are an important reminder about increasingly obvious seasonal effects on sex steroids in this species. Through time, significant decreases in 17β-estradiol occurred in females from 40% and 80% exposure groups at week four, although the only significant difference among treatments at week four was at 40%.

Similar to testosterone patterns between tank- and field-exposed males, 17β-estradiol in females from field exposures demonstrated an opposite response. Large variation differences among sites were not detected, but there was a significant increase in 17β-estradiol by the end of exposure. Potentially the females exposed in tanks may have lagged behind in response to depressed estrogen levels compared to field-exposed females. Such a "lag time" in response between the two groups may explain testosterone data in males as well, and suggests an environmental factor in the natural system produced a more rapid response. It would be important and informative to extend tank exposures and increase sample sizes to determine if dominant hormones for each sex eventually catch up to observed responses in field-exposed fish.

Testosterone concentrations in females were not significantly different among treatments in the tank exposures, nor was there a significant difference through time (Figure 5-7B). Testosterone in field-exposed females resembled the pattern for 17β-estradiol (and testosterone in field-exposed males), with a decrease in testosterone at the discharge site relative to upstream caged fish by week two followed by an increase at week four. These shifts become clearer when relative amounts of E:T are examined.

Figure 5-8 depicts percentages of females with normal estrogen-biased sex steroid ratios and masculinized, testosterone-biased ratios. *In situ* field-exposed females at week two had significantly different distribution of ratios than predicted by chance, with effluent-exposed females displaying a slightly greater tendency to have masculinized profiles. By the end of exposure (week four), the difference disappeared and steroid ratios were mostly estrogen-biased and indistinguishable from females exposed to the upstream unexposed site. Tank-exposed females did not have significantly masculinized hormone ratios until week four, and the response was greater than that observed for field-exposed females at week two.

What could be causing a delayed reaction in tank exposed fish when they are most likely exposed to greater concentrations of effluent? And why do caged fish at the predischarge site respond more similar to fish at the outfall site as opposed to highest concentrations in tanks? Because we used fish from the same aquaculture facility, genetic differences are an unlikely explanation for the differential response. Further, the fact that tank-exposed fish eventually respond with similarly biased hormone profiles suggests a similar cause (although not conclusive). Assuming a similar cause, probable factors that vary between tank and field exposures include chemical composition, food sources, and bacterial communities.

There were actually three exposure systems that varied among each other: exposures in tanks to 100% whole effluent dilutions; field exposures before discharge in retention ponds; and field exposures at the discharge point. All three were essentially flow-through systems in terms of water, but water parameters were literally and theoretically different. Field cages hovered over and around the soft muck of sediments

built up at field sites over time while tanks accumulated sediments from relatively recent effluent flow only. This difference, coupled with the probability of field-exposed fish to prey on wild food, indicates greater potential for bioaccumulation and biomagnification to influence responses in field-exposed fish compared to tank-exposed fish. The retention ponds before discharge [PRE-DIS] were statistically different from the discharge site [DIS] in terms of water quality parameters such as pH and dissolved oxygen (Table 5-3). Further, dissolved oxygen in tanks was in between values at field sites, and pH was highest in tanks. Factors such as pH and dissolved oxygen differentially affect bacterial survival (Dick et al. 1998), thereby influencing which species thrive where. Conductivity, salinity, and turbidity were comparable among exposures, and readings at both effluent-exposed field sites fell within 40–100% effluent for tank exposures (turbidity perhaps the most important for light penetration).

The field sites also differed chemically. Phytosterol concentrations were estimated to be higher at the discharge site (see water chemistry results above). Quinn (2004) detected an abundance of nonionic surfactant degradation products (nonylphenol and octylphenol) at the outfall [DIS] where a liquid oxygen injection system is also located (explaining extremely high dissolved oxygen levels around 10 mg/L). Nonylphenol is considered a weak estrogen in terms of estrogen receptor binding and mRNA expression (Nimrod and Benson 1996), and has been implicated as a bioactive agent in pulp and paper mill effluents (Lee and Peart 1999). Thus the environmental conditions among these three systems were likely very different and suggest variable exposure among exposure treatments and sites, in addition to the dynamic exposure (Figure 5-3 and Table 5-3).

Anal Fin Elongation and Sex Steroids

Barring alteration in anal fin morphology for either sex, any relationship between anal fin elongation and sex steroids could not be analyzed for this exposure study. Since changes in sex steroids were observed, the most that can be concluded is a sensitivity difference, alluded to by previous field work (Chapters 3 and 4): sex steroids were emphatically more sensitive than anal fins in terms of exposure. However, the usual caveats apply for these hormones as a biomarker (normal seasonal fluctuations and actual adverse impacts of altered sex steroids are unknown).

Conclusions

In summary, controlled whole effluent exposure in tanks and *in situ* exposure in the field differentially affected anal fin morphology and sex steroids in mosquitofish. Male anal fins were not affected by exposure, as expected based upon field surveys. However, anal fin elongation in females has been well-documented for this mill and exposures failed to induce an analogous response. This lack of induction may have been caused by insufficient exposure duration (4 weeks). On the other hand, sex steroids for both sexes were more sensitive to effluent exposure, and males may be more sensitive for this endpoint based upon steroid ratios. Response patterns differed between tank- and field-exposed fish, and between fish from effluent-exposed field sites. These differences indicated either variation in exposure and/or multiple mechanisms affecting steroid production, transport, action and metabolism.

Aspects of biosynthesis (aromatase) and direct action of hormones (androgenic compounds) were already discussed under steroid hormone results in males as examples of the various endocrine pathways that may be affected by exposure to pulp and paper mill effluents. Several more have been documented both *in vitro* and *in vivo* for fish

species. For example, fish from Jackfish Bay in Ontario displayed reduced ovarian steroidogenic capacity that was possibly linked to reduced cholesterol availability (cholesterol is the starting compound for lipophilic steroid hormone production of estrogens, progestins, androgens, and glucocorticoids) (Van Der Kraak et al. 1998 and McMaster et al. 2003). Alternatively, pulp mill effluents were shown to bind sex hormone binding globulin (SHBG), a carrier protein for sex steroids in the bloodstream, potentially causing displacement and thus more rapid clearance of endogenous steroids (Hewitt et al. 2000). Degradation and excretion of sex steroids in and of itself may also be affected. For example, the conjugating detoxification enzyme UDP-glucoronosyl transferase has been both inhibited and induced by exposure to whole effluents or effluent components such as resin acids (Oikari et al. 1983, Förlin et al. 1985, Andersson et al. 1988b, Lindstrom-Seppa and Oikari 1988, Lindstrom-Seppa and Oikari 1989). Increased metabolic clearance would depress endogenous steroid levels, while decreased clearance would build them up. None of these mechanistic studies have been performed on mosquitofish and would require characterization before evaluating which, if any, are responsible for observed effects on sex steroids.

Although exposure of mosquitofish to bleached/unbleached kraft mill effluents failed to induce changes in anal fin morphology, different patterns of the more sensitive response in sex steroids support continued development of mosquitofish as a bioindicator species. The link between whole body steroid levels and anal fin morphology remains unclear. The action and effective concentration of steroids or steroid mimics causing anal fin elongation may be independent of whole body steroid levels. Indeed, systemic or peripheral action of sex steroids on anal fin morphology has not been conclusively

distinguished. At this point, without any evidence of direct adverse effect of these biomarkers on mosquitofish (Chapter 6), they could be used as tiered responses indicating varying levels of exposure. Before such application, two points require further investigation: 1) typical seasonality of sex steroid concentrations and ratios throughout the year (ideally this would be examined at more than one type of reference site and characterized in the laboratory); and 2) thorough analysis of actual or direct chemical exposure to these fish, and documentation of potentially important environmental factors such as bacterial communities and biomagnification, should be executed in concert with repeat (and perhaps modified) exposures in the field and in tank dilutions. Despite the limited amount of data returned for the large amount of effort expended, this exposure study was invaluable when contrasted against observed effects on this species in wild field collections.

Table 5-1. Water quality parameters (ave ± se) measured three times weekly (n = 13 total) during four week tank exposures of mosquitofish to bleached/unbleached kraft mill effluent in summer 2002.

Effluent Concentration	0%	10%	20%	40%	80%
Temperature (°C)	21.7±0.4	22.6±1.0	21.8±0.4	22.6±0.5	23.3±0.4
Conductivity (µS)	390.7±19.86	552.7±17.7	466.7±20.82	1380±120[a]	2228±33[a]
Salinity (ppt)	0.2±0.008	0.3±0.01	0.2±0.01	0.7±0.06[a]	1.2±0.02[a]
Dissolved Oxygen (mg/L)	8.59±0.49	8.10±0.45	8.30±0.46	7.42±0.43	6.97±0.38[a]
Turbidity (ntu)	0.60±0.25	2.35±0.21	1.43±0.27	10.9±1.1[a]	18.1±0.7[a]
pH	8.4±0.04	8.3±0.03	8.3±0.03	8.2±0.02[a]	8.2±0.03[a]

[a]significantly different from control (0% treatment)

Table 5-2. Water quality parameters (ave ± se) measured three times weekly (n = 12 total) during caged exposures of mosquitofish to field sites in Rice Creek during summer 2002.

Site	U(8)	PRE-DIS	DIS
Temperature (°C)[a]	21.8±0.3	27.7±0.5	26.1±0.3
Conductivity (µS)[a]	288.9±15.70	2245±14.05	1878±103.4
Salinity (ppt)[a]	0.1±0.01	1.1±0.01	1.0±0.03
Dissolved Oxygen (mg/L)[a]	6.29±0.16	1.74±0.20	10.15±0.77
Turbidity (ntu)[a]	3.96±0.51	33.8±14.9	13.2±0.53
pH[b]	7.4±0.1	8.0±0.03	7.8±0.02

[a]significantly different among all three sites
[b]significantly different between PRE-DIS and U(8)

Table 5-3. Concentrations of selected effluent components in 100% final effluent sampled weekly midJanuary to midMay in 2002 (n = 10, data courtesy NCASI).

	MAX	MEAN	MIN	SE
Total RAFA[a] (µg/L)	11.8	8.4	6.5	0.6
Campesterol (µg/L)	12	5.9	ND[c]	1.4
Stigmasterol (µg/L)	3.3	2.0	1.2	0.2
Stigmastanol (µg/L)	7.8	5.6	4.3	0.4
β-sitosterol (µg/L)	71	47	33	3.8
TOC[b] (mg/L)	1767.2	804.6	96.3	180.7
polyphenolics (mg/L)	38.2	33.5	27.6	1.2
condensable tannins (mg/L)	6.6	5.1	2.5	0.4

[a]RAFA = resin acids and fatty acids
[b]TOC = total organic carbon
[c]ND = nondetectable

Table 5-4. Body size parameters (ave ± se) for mosquitofish exposed to bleached/unbleached pulp mill effluents via whole effluent dilutions for four weeks in summer 2002.

Effluent Concentration	0%	10%	20%	40%	80%
Day 0					
♂ Sample Size	25	NA[a]	NA	NA	NA
♂ Body Weight (g)	0.162±0.09	NA	NA	NA	NA
♂ Standard Length (mm)	22.11±0.37	NA	NA	NA	NA
♂ Condition Factor (g/cm^3)	1.47±0.03	NA	NA	NA	NA
♀ Sample Size	25	NA	NA	NA	NA
♀ Body Weight (g)	0.460±0.039	NA	NA	NA	NA
♀ Standard Length (mm)	28.62±0.716	NA	NA	NA	NA
♀ Condition Factor (g/cm^3)	1.83±0.05	NA	NA	NA	NA
Week 2					
♂ Sample Size	4	4	5	5	4
♂ Body Weight (g)	0.213±0.021	0.253±0.029	0.200±0.020	0.235±0.006	0.201±0.030
♂ Standard Length (mm)	24.23±0.82	24.57±0.95	24.56±0.75	24.88±0.46	24.60±1.36
♂ Condition Factor (g/cm^3)	1.49±0.10	1.68±0.06	1.34±0.10	1.54±0.09	1.32±0.03
♀ Sample Size	11	10	10	10	10
♀ Body Weight (g)	0.390±0.037	0.423±0.056	0.512±0.061	0.498±0.060	0.567±0.043
♀ Standard Length (mm)	28.59±0.86	28.71±1.01	29.94±0.80	30.16±1.00	31.15±0.64
♀ Condition Factor (g/cm^3)	1.64±0.09	1.70±0.08	1.83±0.09	1.74±0.06	1.84±0.07
Week 4					
♂ Sample Size	10	10	10	10	10
♂ Body Weight (g)	0.205±0.018	0.236±0.028	0.178±0.014	0.174±0.010	0.207±0.024
♂ Standard Length (mm)	23.41±0.50	24.53±0.62	23.60±0.87	22.29±0.39	23.00±0.68
♂ Condition Factor (g/cm^3)	1.57±0.10	1.55±0.07	1.38±0.09	1.56±0.05	1.65±0.09
♀ Sample Size	10	10	10	10	10
♀ Body Weight (g)	0.642±0.108	0.557±0.044	0.503±0.101	0.505±0.020	0.554±0.045
♀ Standard Length (mm)	31.88±1.25	30.74±0.76	29.52±1.26	30.72±0.40	31.35±0.68
♀ Condition Factor (g/cm^3)	1.84±0.11	1.89±0.06	1.78±0.10	1.74±0.04	1.77±0.06

[a]NA = not applicable

Table 5-5. Body size parameters (ave ± se) for mosquitofish caged in Rice Creek field sites for four weeks in summer 2002.

Effluent Concentration	Control	U(8)	PRE-DIS	DIS
Day 0				
♂ Sample Size	25	NA[a]	NA	NA
♂ Body Weight (g)	0.162±0.09	NA	NA	NA
♂ Standard Length (mm)	22.11±0.37	NA	NA	NA
♂ Condition Factor (g/cm^3)	1.47±0.03	NA	NA	NA
♀ Sample Size	25	NA	NA	NA
♀ Body Weight (g)	0.460±0.039[d]	NA	NA	NA
♀ Standard Length (mm)	28.62±0.716[d]	NA	NA	NA
♀ Condition Factor (g/cm^3)	1.83±0.05	NA	NA	NA
Week 2				
♂ Sample Size	NA	4	4	4
♂ Body Weight (g)	NA	0.144±0.018	0.180±0.028	0.207±0.040
♂ Standard Length (mm)	NA	22.83±0.50	23.15±1.17	24.16±1.07[b]
♂ Condition Factor (g/cm^3)	NA	1.19±0.06	1.42±0.04	1.43±0.09[c]
♀ Sample Size	NA	10	10	10
♀ Body Weight (g)	NA	0.783±0.084	0.609±0.088	0.539±0.048
♀ Standard Length (mm)	NA	34.10±0.99	33.09±1.09	30.66±0.90
♀ Condition Factor (g/cm^3)	NA	1.91±0.05	1.58±0.07	1.81±0.06
Week 4				
♂ Sample Size	NA	10	10	9
♂ Body Weight (g)	NA	0.221±0.053	0.161±0.011	0.204±0.012
♂ Standard Length (mm)	NA	25.07±1.05	22.85±0.61	23.56±0.49
♂ Condition Factor (g/cm^3)	NA	1.36±0.10	1.34±0.03	1.54±0.02[c]
♀ Sample Size	NA	10	10	9
♀ Body Weight (g)	NA	0.625±0.076	0.541±0.04	0.616±0.034
♀ Standard Length (mm)	NA	32.85±0.84	31.99±0.66	33.55±0.74
♀ Condition Factor (g/cm^3)	NA	1.70±0.12	1.63±0.06	1.63±0.06

[a]NA = not applicable
[b]statistically different than week 0 ($p < 0.05$)
[c]statistically different (over all 4 weeks) to other sites ($p < 0.05$)
[d]statistically different than week 2 and week 4 ($p < 0.05$)

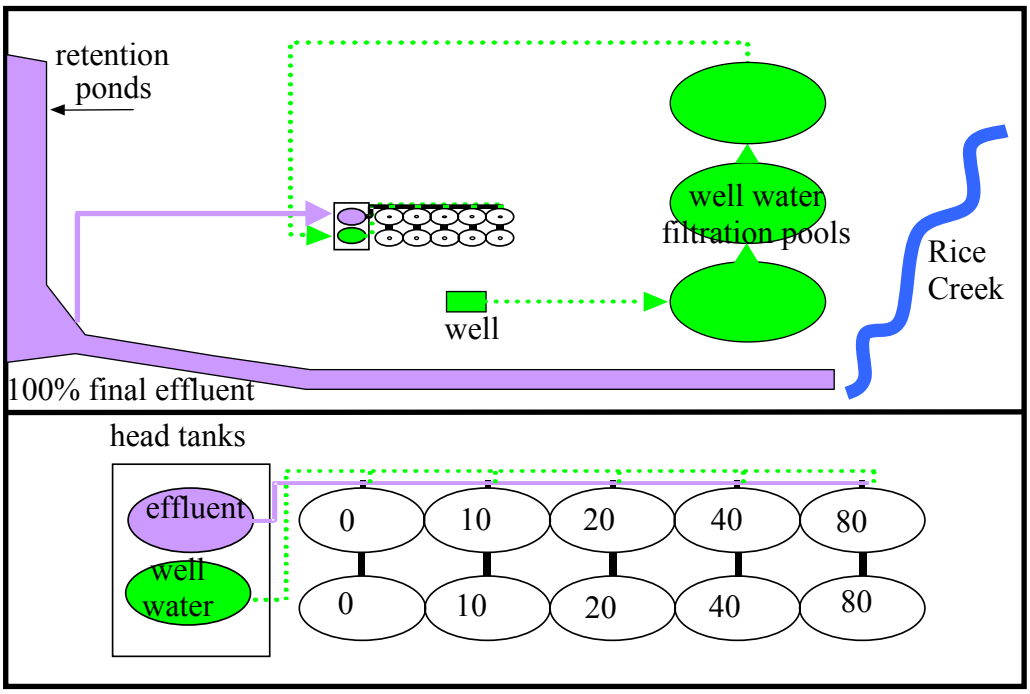


Figure 5-1. Diagram of tank facility for flow-through whole effluent exposure of mosquitofish in summer 2002 at Georgia-Pacific's Palatka, FL operation.

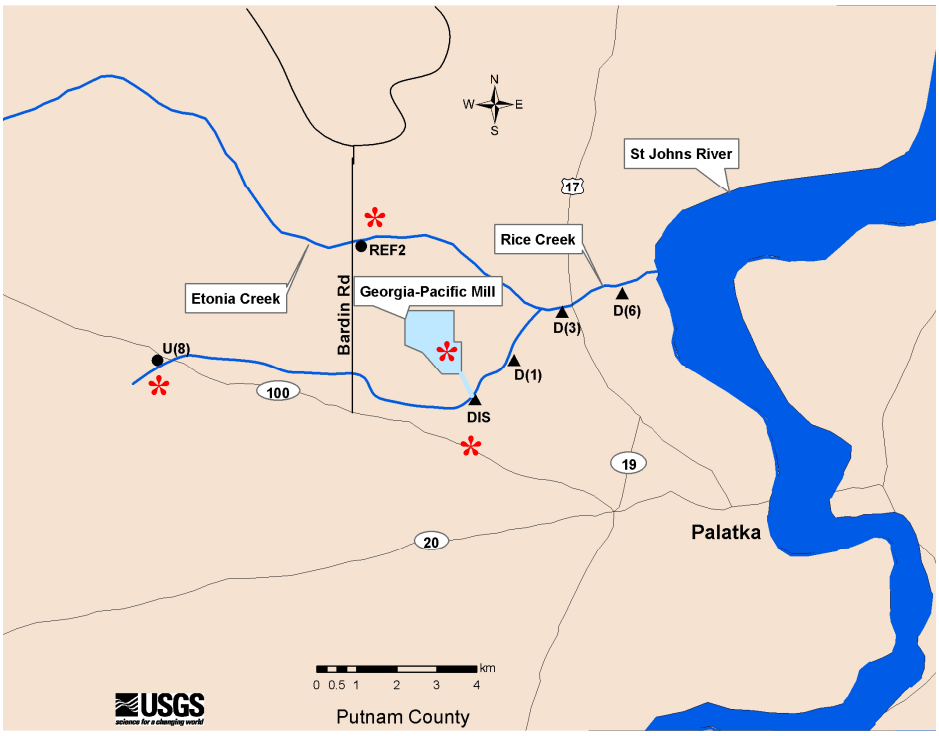


Figure 5-2. Map of cage locations for *in situ* field exposures at Rice Creek, FL, in 2002 (red asterisks). Other field sites routinely surveyed for mosquitofish masculinization are also indicated. Site symbols distinguish sites exposed to effluent: circles = unexposed and triangles = exposed. Site abbreviations denote upstream (U) or downstream (D) of discharge, followed by approximate distance (km) from discharge in parentheses; PRE-DIS indicates site before discharge into the creek; DIS denotes site at discharge into creek; REF indicates reference site, followed by identifying number.

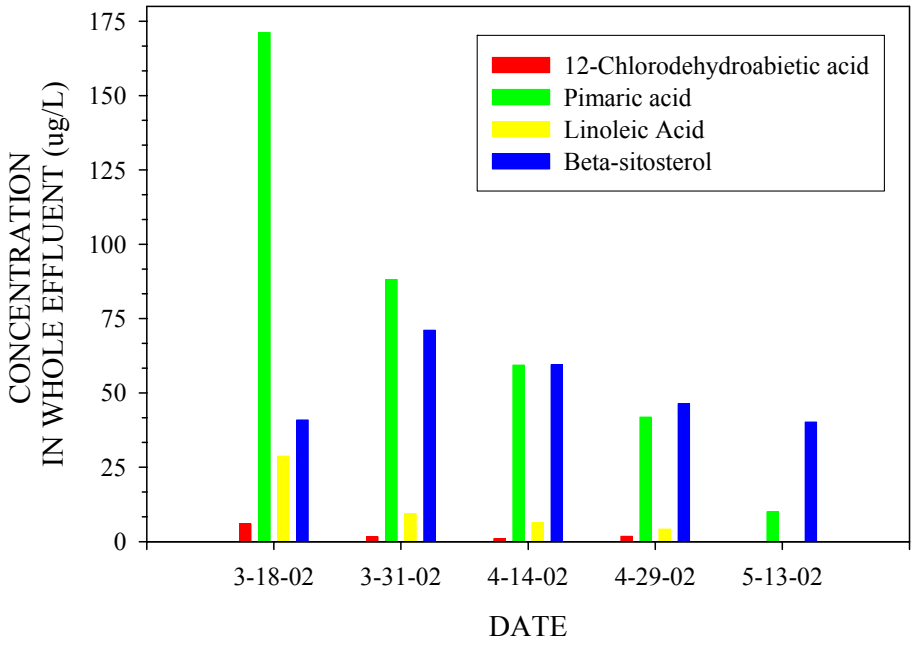


Figure 5-3. Concentrations of selected wood extractives in 100% final effluent from the Rice Creek mill during tank and field exposures of mosquitofish. 12-chlorodehydroabietic acid is a chlorinated resin acid, pimaric acid is a resin acid, linoleic acid is a fatty acid, and beta-sitosterol is a phytosterol.

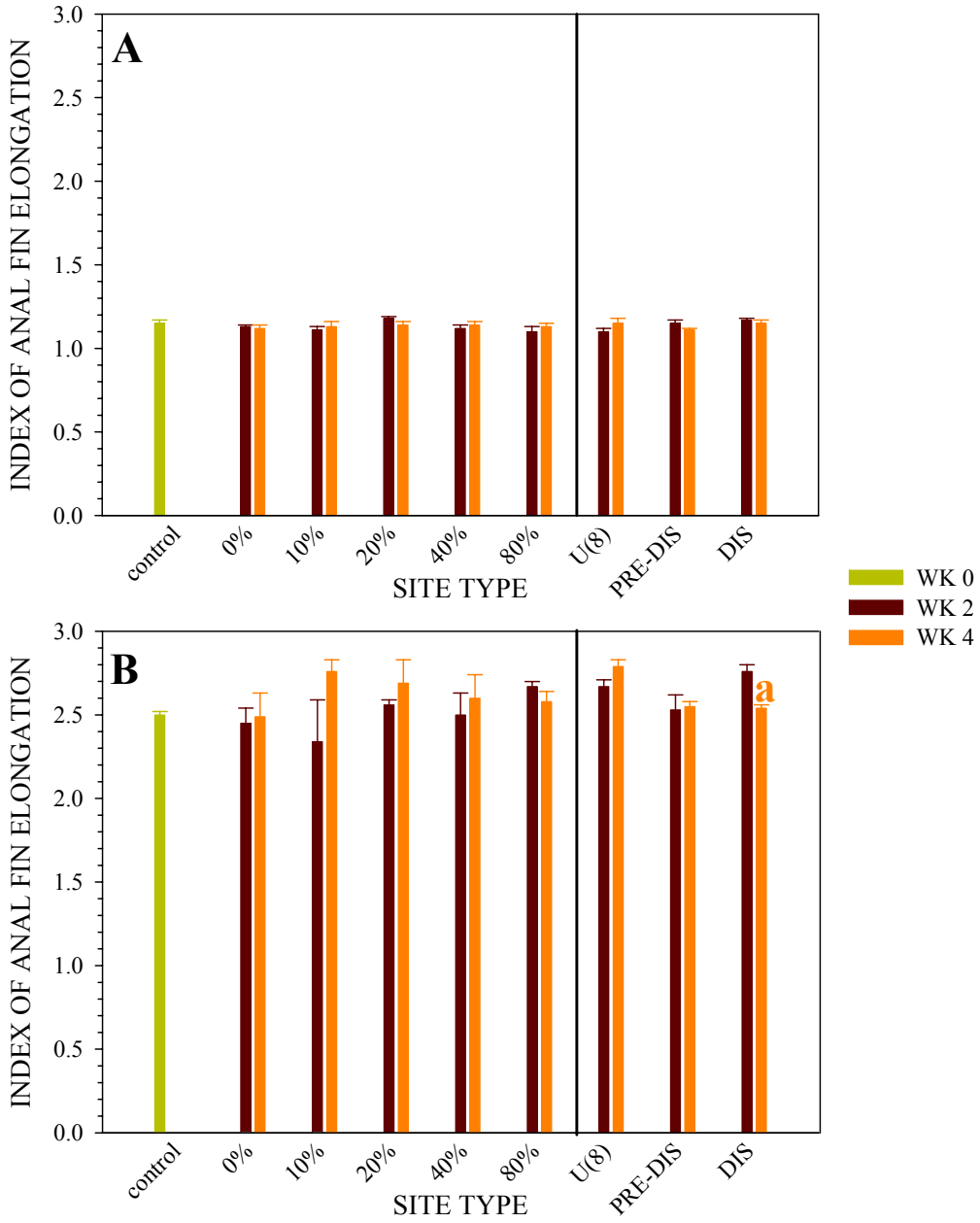


Figure 5-4. Index of anal fin elongation (length ratio of Ray 4 to Ray 6) for mosquitofish exposed to bleached/unbleached pulp mill effluents via whole effluent dilutions or onsite caged exposures for four weeks in summer 2002. A) Females. B) Males. Solid black line separates tank from *in situ* field exposures. Letter "a" indicates significant differences by site ($p < 0.05$).

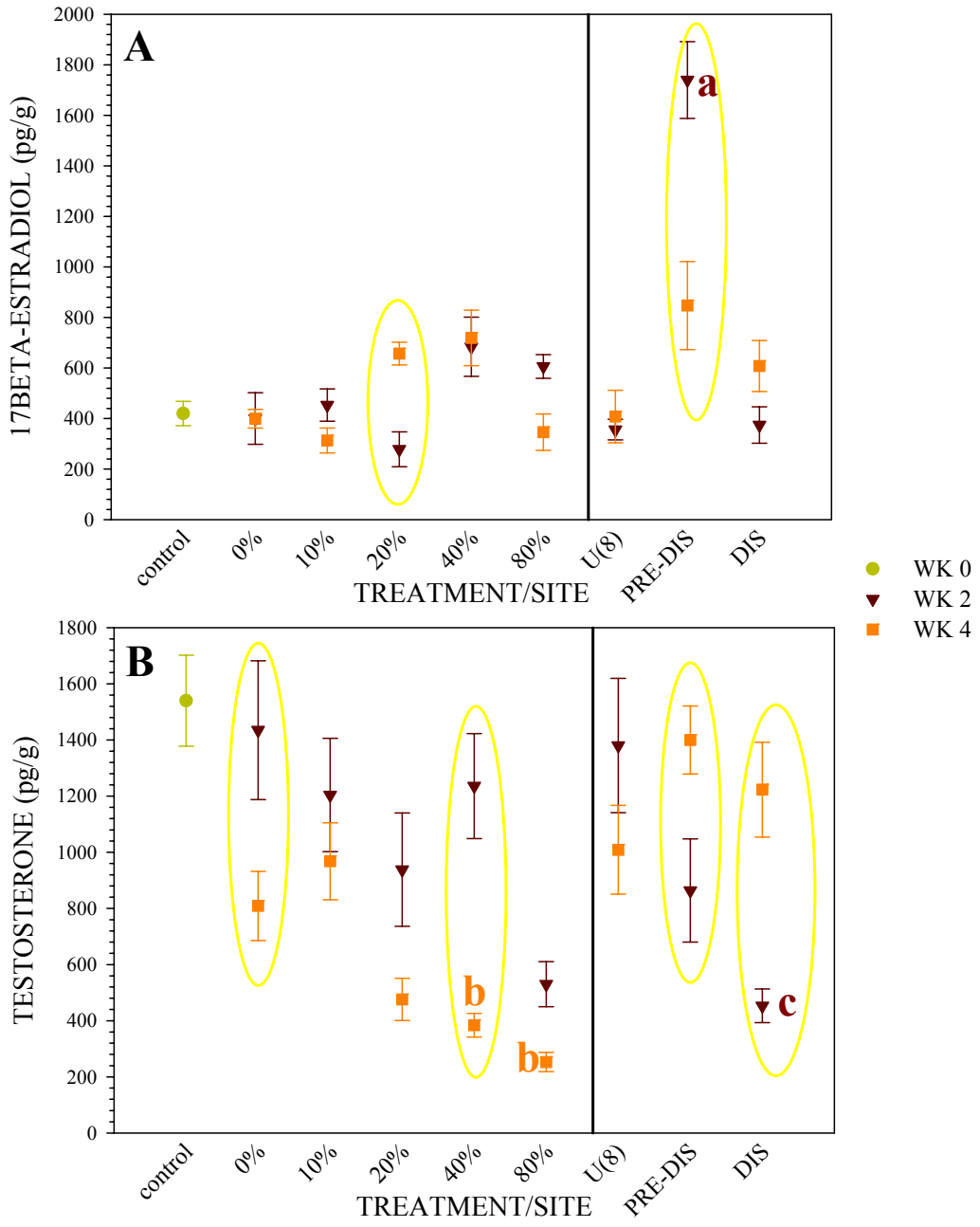


Figure 5-5. Whole body sex steroids (ave ± se) for male mosquitofish exposed to bleached/unbleached pulp mill effluents via whole effluent dilutions or onsite caged exposures for four weeks in summer 2002. A) 17β-estradiol. B) Testosterone. Solid black line separates tank from *in situ* field exposures. Letters indicate significant differences by treatment or site within week ($p < 0.05$): "a" denotes differences to PRE-DIS and DIS; "b" denotes differences to all other sites; "c" denotes differences to U(8). Yellow circles indicate significant differences by week. Treatment or site and week did not covary for either hormone.

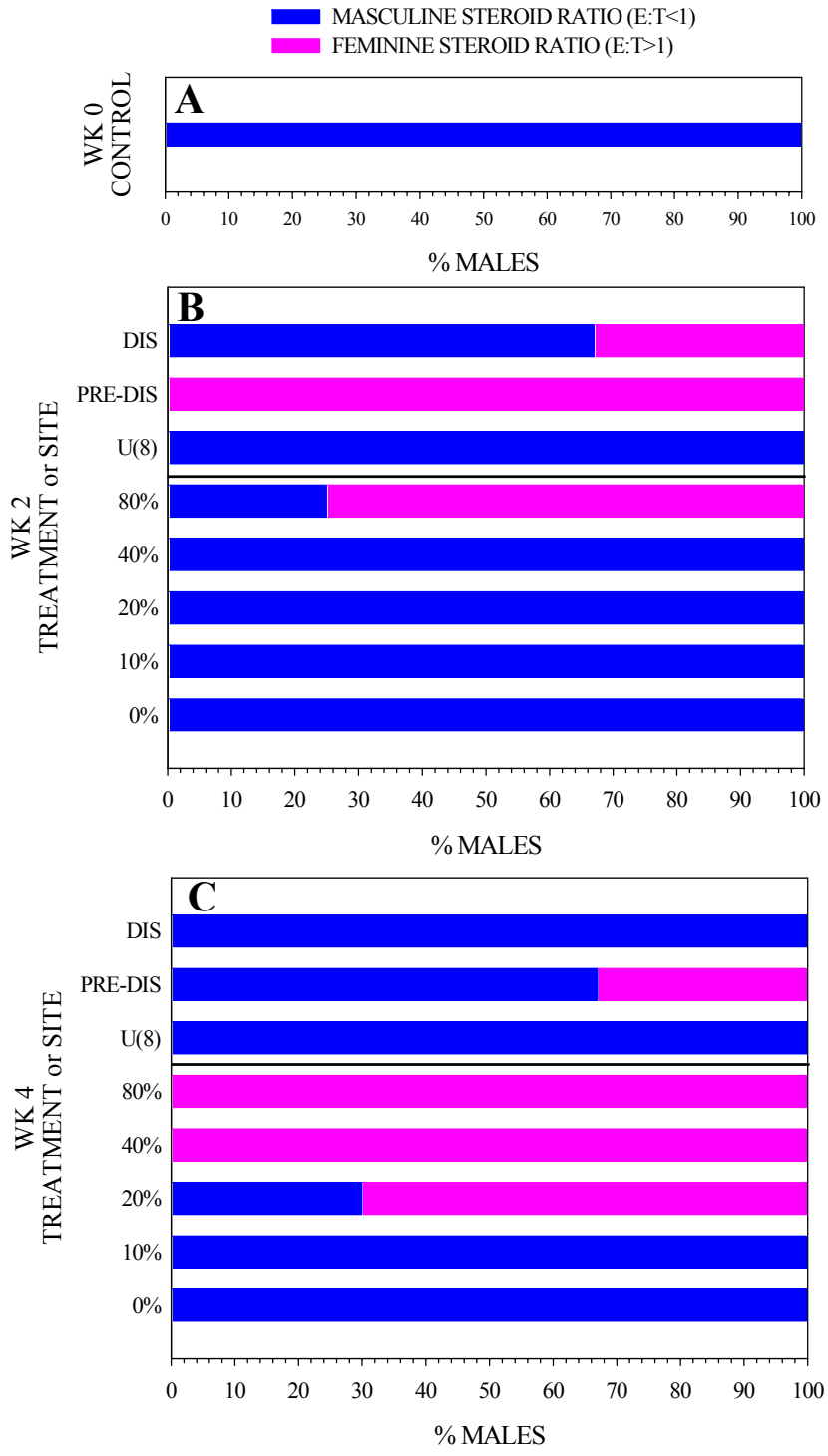


Figure 5-6. Percentage of male mosquitofish with masculine and feminine sex steroid ratios exposed to bleached/unbleached pulp mill effluents via whole effluent dilutions or *in situ* field exposures for four weeks in summer 2002. A) Control males (week 0 sample). B) Midpoint sampling (week 2). C) Final sampling (week 4).

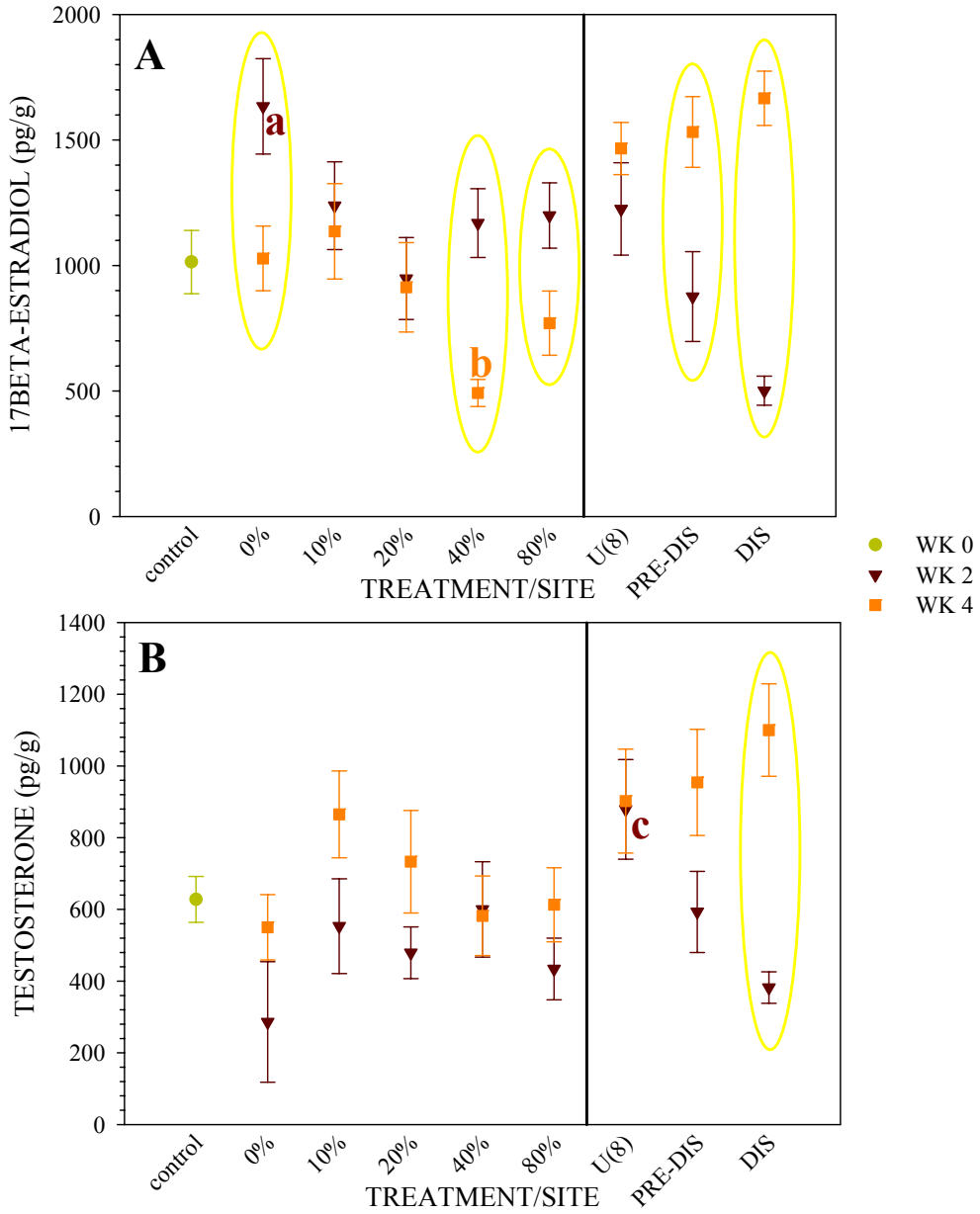


Figure 5-7. Whole body sex steroids (ave ± se) for female mosquitofish exposed to bleached/unbleached pulp mill effluents via whole effluent dilutions or onsite caged exposures for four weeks in summer 2002. A) 17β-estradiol. B) Testosterone. Solid black line separates tank from *in situ* field exposures. Letters indicate significant differences by treatment or site within week ($p < 0.05$): "a" denotes differences to 20%; "b" denotes differences to 0%; "c" denotes differences to DIS. Site type and mill did not covary for either hormone.

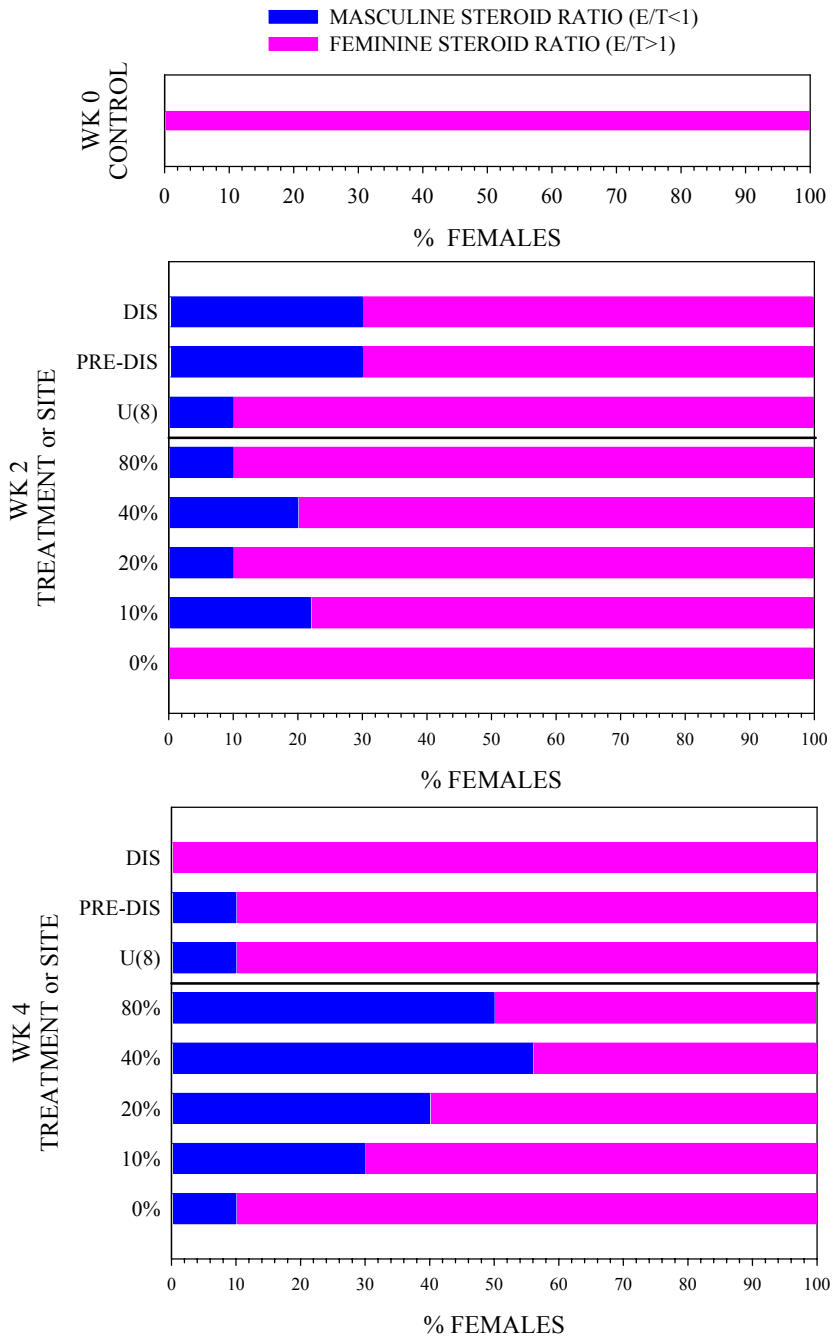


Figure 5-8. Percentage of female mosquitofish with masculine and feminine sex steroid ratios exposed to bleached/unbleached pulp mill effluents via whole effluent dilutions or *in situ* field exposures for four weeks in summer 2002. A) Control females (week 0 sample). B) Midpoint sampling (week 2). C) Final sampling (week 4).

CHAPTER 6
INVESTIGATION OF REPRODUCTIVE SUCCESS IN MOSQUITOFISH LIVING IN PULP AND PAPER MILL EFFLUENT DOMINATED SYSTEMS

The impact of female mosquitofish masculinization or development of male secondary sex characteristics on reproductive success has not been directly addressed despite speculation of negative impacts. Histologically, masculinized females are normal, and limited brood size data (separate from masculinization studies) are conflicting but tend toward a lack of effect. The current study evaluated fry production and masculinization in females collected from two effluent-receiving streams in Florida for two reproductive seasons. In addition, population structure and abundance was tentatively evaluated the first season and masculinization and sex steroids were measured in adults. Potential exposure was documented by analysis of wood extractives in water samples from field sites. Morphological masculinization was consistent between years for one mill while the response was only detected the second year of study at the other mill. Sex steroid alterations measured the first year were weakly affected relative to measurements in previous studies (Chapters 2 through 5). Neither of these biomarkers could be associated with fry production or population structure differences. Fecundity appeared reduced in the first year's single collection for both effluent-exposed sites but population structures implied mosquitofish at effluent-exposed sites may have started reproducing sooner than at unexposed sites. The second year of fry production studies over several months affirmed different reproductive patterns in females among sites and through the season. Furthermore, overall fecundities were higher in females from one

exposed site relative to respective references. Rather than negatively impacting fecundity, pulp and paper mill effluent exposure may be stimulating modified reproductive strategies in mosquitofish influenced by changes in environmental and ecological factors as opposed to chemical exposure.

Introduction

Sublethal effects of pulp and paper mill effluent exposure on fish have been a major focus of aquatic environmental health concerns for over a decade (Sodergren 1991, Servos et al. 1996, Ruoppa et al. 2000, Stuthridge et al. 2003, Borton et al. 2004). Reported effects include induction of liver detoxification systems, alterations in sex steroid concentrations and production/metabolism, reduced gonadal development, decreased egg production and decreased fry survival (Van der Kraak et al. 1992, Gagnon et al. 1994a, Munkittrick et al. 1999, NCASI 2000a, McMaster et al. 2003, Sepúlveda et al. 2003, Parrott et al. 2004). Whether or not these effects represent actual adverse effects in terms of reproductive success or population and community level impacts remains controversial.

Development of male-like secondary sex characteristics in female mosquitofish, specifically masculinization of the anal fin into a gonopodial-like structure, has been reported in pulp mill effluent-receiving streams for decades Howell et al. 1980, Drysdale and Bortone 1981, Cody and Bortone 1997, Bortone and Cody 1999, Jenkins et al. 2001, Parks et al. 2001, Chapters 2 through 4). Although impacts on reproduction were initially implied by this phenomenon, normal ovaries lacking any testicular tissue were consistently reported in masculinized females (Howell et al. 1980, Hunsinger et al. 1988, Ellis et al. 2003, McCarthy et al. 2004). In addition, sex ratios of mosquitofish reared in 100% final effluent were not altered (McCarthy et al. 2004). Decreased potential

fecundity, measured as brood size of developing embryos, was reported in early preliminary work (Rosa-Molinar and Williams 1984), but has not been detected in more recent studies (Felder et al. 1998, D'Surney et al. 2000). With the drastic improvements in processing technologies by the pulp and paper industry, masculinization has been reduced relative to initial reports (Howell et al. 1980, Drysdale and Bortone 1981, Cody and Bortone 1997, Chapters 3 and 4) and it is possible reproduction was previously impacted but may no longer be impaired.

Mosquitofish, as members of the livebearing family Poeciliidae, develop eggs internally and ovulate immediately before parturition of fry (Meffe and Snelson 1989). As nonsuperfetating lecithotrophes, mosquitofish develop a single brood at a time and exhibit yolk-loading of eggs similar to egg-laying (oviparous) species without maternal investment during embryological development (Turner 1937). Compared to egg-laying species, reproduction is asynchronous and reproductive season occurs through summer months with low to no reproduction in winter months (Constanz 1989). Environmental cues control the beginning and end of the reproductive season: onset of reproductive season is triggered by a rise in water temperature while photoperiod (decreasing daylength) signals gonadal recrudescence (Koya and Kamiya 2000, Koya and Iwase 2004).

A major drawback among existing studies of mosquitofish reproduction in pulp and paper mill effluents is the lack of corresponding anal fin morphology data to evaluate potential association of masculinization and reproduction. Also, these studies represent potential effects on reproductive success as opposed to actual fecundity or production of fry. No work has been done to assess potential impacts of effluent exposure on

populations of mosquitofish in reference to reproductive level effects. The primary objective for the current study was to evaluate fry production in female mosquitofish collected from effluent receiving streams and relate this endpoint to masculinization of the anal fin. Preliminary investigation of population structures and abundance at effluent-exposed sites was a secondary objective to provide perspective on fry production studies. These collections were also evaluated for anal fin morphology and sex steroids in adult fish.

Materials and Methods

Mill Characteristics

Two pulp and paper mill effluent receiving systems in Florida where masculinized female anal fins have been previously documented were surveyed for mosquitofish reproduction studies in the summers of 2003 and 2004 (Figure 6-1 and Appendix A). The Fenholloway River was the focus of an abbreviated population survey in 2003, while one instream exposed and one unexposed site from each of the two systems were surveyed to assess fry production in females (once in 2003, and over a four month period in 2004).

The Fenholloway River and Rice Creek mills are very different in furnish, processing and product (Chapter 1, Table 1-1). The Rice Creek mil is a bleached/unbleached kraft mill therefore subject to EPA's Cluster Rule. The Fenholloway River mill uses a dissolving kraft pulping process to produce high grain cellulose and is regulated under completely different guidelines.

Water Samples

Before fish collection, water quality parameters typically affected by pulp and paper mill effluents were measured at each site: dissolved oxygen, temperature, pH,

conductivity, salinity, and turbidity. Single grab, unfiltered surface water samples (1 L) were also collected before fish collection to document potential exposure of fish to specific effluent components. Samples were preserved (buffered), and sent to the National Council for Air and Stream Improvement, Inc. (NCASI) for chemical analysis by GC/MS. Water from all sites was analyzed for 10 resin acids (including 3 chlorinated), 3 fatty acids, 4 phytosterols, total organic carbon (TOC), condensable tannins, and polyphenolics. Chlorinated phenolics (12 Cluster Rule compounds plus 16 others) were analyzed in 100% final effluent for the first two field collections in 2003, and then discontinued since there were nondetectable levels across sites. The exception to these findings was a small peak of 2-chlorosyringaldehyde (3.0 µg/L) in the Fenholloway mill effluent at the first sampling. Columbia Analytical Services conducted the chlorophenolic analyses, CH2M Hill conducted the TOC analyses, and the NCASI West Coast Regional Center conducted all other analyses (NCASI 1986 1997).

Population Survey

As a preliminary investigation into population structure and relative abundance of mosquitofish inhabiting effluent-receiving systems, field collection sites were surveyed systematically in May 2003 using dip nets along shallow vegetated banks. Three to five observers were spaced a minimum of 10 meters apart throughout sampling and number of sweeps, estimated time, and estimated area sampled were monitored and recorded by an independent observer. Sampling concluded when an estimated 75 to 100 adult female mosquitofish were collected for fry production studies. All mosquitofish were kept alive in aerated bait buckets and transported back to the laboratory for processing.

Morphology

Back at the laboratory, mosquitofish were sorted into age-sex groups, giving preference to gravid females for preparation of fry production studies. Fish were handled as little as possible and wearing latex gloves to minimize stress on gravid females. Groups were divided as follows: gravid females (anal spot and swollen abdomen); nongravid females (lack of or partial anal spot and slim abdomen); adult males (fully differentiated gonopodium); developing males (elongated gonopodium lacking terminal differentiations); and juveniles (< 20 mm standard length and lacking anal spot and gonopodium). Urogenital papillae were only used to distinguish gender in fish difficult to sex, as manipulation under a dissecting scope places more stress on gravid females.

Fish designated for hormone analysis (20 to 30 adult males and females per site) were processed the day of collection. First, fish were euthanized with a terminal dose of buffered tricaine methanesulfonate (Tricaine-S, Western Chemical Inc., Ferndale, WA, USA), then weighed using a digital scale ($\pm$ 0.001 g) and measured for standard length ($\pm$ 0.01 mm) using a pair of digital calipers. Under a dissecting scope, gender was reaffirmed using the presence (female) or absence (male) of a urogenital papilla (see Chapter 2 for validation of this sexing technique). Each fish was photographed using a digital camera then placed on ice until transferred to –80°C freezer for subsequent radioimmunoassay (RIA) of sex steroids. Anal fin images of these fish were measured by computer ($\pm$ 0.01 mm) using trace mode in Sigma Scan Pro © 5.0 from the base of Rays 4 and 6 along the curve of each ray to the tip. Remaining fish were euthanized and preserved in 10% neutral-buffered formalin for determining population structure.

Sex Steroids

Whole body primary sex steroids (17β-estradiol and testosterone) were analyzed using a modified RIA method originally developed for serum and plasma samples of common carp, *Cyprinus carpio* (Goodbred *et al* 1997), and since then adapted for use in a variety of other aquatic species and tissue media such as plasma of largemouth bass, *Micropterus salmoides* (Gross et al. 2001) and mantle of freshwater invertebrates (Gross et al. 2000). For methods and validation of this assay, see Chapter 2.

Fry Production

Fifty gravid females from one exposed and unexposed site per system (total 4 sites) were held for thirty days to monitor fry production. Each female was placed individually in a modified plastic hatchery chamber purchased from Aquatic Ecosystems (Apopka, FL) that included hinged lids to prevent escape and 3" of artificial green Cabomba to provide cover for females and fry. Upper portions of the hatchery chamber were available to females while the lower portion was accessible only by fry. Newborn fry instinctively seek escape and protection from the mother and her cannibalistic instinct. Fish in hatchery chambers were initially held in 2' round tanks with site water and acclimated to 50:50 pond:well water mix for 24 to 48 hours by gradual drip of lab water and graded pH shifts of 0.1 per hour and no more than 1 unit per day. After acclimation, females were transferred to two 4' by 8' by 6" shallow tanks receiving 50:50 filtered pond:well water mix from a head tank. Chambers were randomized with respect to location in tanks, and 100 chambers filled each tank allowing for up to 200 chambers total. Full spectrum lighting was set on a 14:10 hour light:dark schedule to simulate increased photoperiod and keep females in reproductive mode.

Chambers were monitored daily for fry production. Fry were removed immediately then euthanized, counted and preserved in 10% neutral buffered formalin for assessment of deformities and fry weight. Chambers were rinsed and females returned to the tank for observation of secondary brood production. Initially, females were fed daily *ad libitum* with Tropical Prime flakes (Zeigler Brothers, Gardners, PA, nutritional composition 45% min protein, 9% min fat, 4% max fiber). Feeding was later reduced to every other day because of a water mold infestation (Saproligniosis of Class Oomycetes) attributed to overfeeding. Water mold required treatment with an overnight static salt immersion at 3 ppt. Water quality (dissolved oxygen, temperature, pH, conductivity, salinity; incident light and turbidity once a week) was measured three times a week and shallow tanks were cleaned.

In 2004, this experiment was repeated monthly from May to August to assess fry production throughout the reproductive season. A final collection in September was cancelled because of hurricane conditions. The only differences between years were: females were euthanized once fry were produced in 2004, and fry deformities and weights were not measured.

Statistics

As part of the population survey in 2003, body weight and standard length were used to calculate condition factor, $K = \text{weight} / \text{length}^3 \times 100$ (g/cm^3), as an indication of overall health used by the aquaculture industry (values at least 1 are considered healthy, Hile 1936). The length ratio of anal fin Rays 4 and 6 was calculated as an index of anal fin elongation. Estrogen and testosterone concentrations were used to calculate a ratio indicating masculine hormone profile ($E:T < 1$) or feminine hormone profile ($E:T > 1$). For fry production studies, body length has been shown to positively affect brood size

(Krumholz 1948, Hughes 1985, Meffe and Snelson 1989), so the number of fry produced by each female was divided by her standard length for statistical analysis.

Differences in population structure among sites were analyzed by χ^2 test for independence. Relative abundance of mosquitofish was estimated by calculating catch per unit effort (CPUE) as number of fish per sweep. Anal fin morphology and sex steroid data of adult fish from these collections were analyzed within sex using one-way analysis of variance (ANOVA) to test for significant variation by site (t-test for Rice Creek fish). Any data failing tests for normality and homogeneity of variance were transformed using log transformations. Significant differences in the ANOVA tests were analyzed for multiple comparisons using Tukey's HSD. Relationship between anal fin morphology and sex steroids were analyzed by t-test for differences in index of anal fin elongation between females with masculine versus feminine estrogen to testosterone (E:T) ratios.

Since water parameters were measured repeatedly, water quality (2004 data) and chemistry (2003 and 2004) were analyzed using one-way ANOVA to test for significant variation by site (t-test for Rice Creek samples). Significant differences in the ANOVA tests were analyzed for multiple comparisons using Tukey's HSD.

Fry production data were analyzed between sites using t-tests within systems. Any data failing tests for normality and homogeneity of variance were transformed using log transformations. Differences in numbers of live versus dead fry and deformities were analyzed by χ^2 test for independence. For the 2004 data, interaction by site and month was also analyzed using an analysis of covariance (ANCOVA), and influence of month within each site was analyzed by one-way ANOVA followed by Tukey's HSD.

Statistical significance was set at $\alpha = 0.05$ for all tests. All statistical analyses were conducted using SAS © version 9.0.

Results and Discussion

Water Quality

In general and as expected, most water quality parameters were higher at effluent-exposed sites compared to unexposed sites (Table 6-1 for 2003 and Table 6-3 for 2004). Elevated temperature at effluent-exposed sites may be important, since reproduction is initiated by a rise in temperature (Koya and Kamiya 2000). In 2004, dissolved oxygen was low at the effluent-exposed Fenholloway site (<1 mg/L), but elevated at the Rice Creek discharge site (due to oxygen injection system mentioned in Chapter 5).

Conductivity, salinity and turbidity for fry production tanks were low and comparable to reference and upstream field sites (Table 6-2 for 2003 and Table 6-4 for 2004). Temperatures were intermediate between exposed and unexposed sites, averaging closer to exposed sites. Dissolved oxygen was adequate for fish survival (overall average 6.34 mg/L). Incident light varied from 60 to 120 Fc with greatest light directly beneath fixtures; light intensities may affect productivity of female mosquitofish when comparing indoor to outdoor lighting (W.K. Bradley pers. comm.), therefore this may have affected fry numbers. Randomization of hatchery chambers in the shallow tanks accounted for this bias.

Overall pH was high (average 8.0): measurements were most similar to the Rice Creek discharge site [DIS], and in May 2004 pH was significantly higher than other months. Although fish were acclimated to water conditions in the laboratory, the large jump in pH during May 2004 (especially for unexposed fish) may have caused stress and

subsequent higher mortality than observed for other months. In support, large shifts to acidic pH were observed to induce 100% mortality in caged fish during attempted exposures at the highly acidic upstream Fenholloway River site [U(5)] regardless of acclimation to the low pH.

Water Chemistry

In general, effluent components were at higher concentrations in Fenholloway River compared to Rice Creek, although resin acid concentrations were comparable across sites during 2003 (Table 6-5 for 2003 and Table 6-6 2004). Water sampling coincided with fish sampling for the 2003 population survey and 2004 female collections, and additional water samples were measured before and after the population survey and after female collection in 2003 (as part of the aborted caged exposures mentioned in Chapter 5). Total resin acids, 3 of 4 phytosterols, TOC, and polyphenolics (lignin content) statistically distinguished effluent-exposed from nonexposed sites. Of the phytosterols, only campesterol was different for Fenholloway River exposure sites in 2003; otherwise this compound was low to nondetect. Analyses of phytosterol concentrations were problematic for the Rice Creek discharge site both years and the Fenholloway River sites in 2004 in terms of low and variable surrogate recoveries (failing quality control); therefore these values must be considered estimates. Fatty acids and condensable tannins were not consistently distinguishable among sites for 2003, while condensable tannins were higher in exposed sites for 2004.

Examining concentrations over time revealed the dynamic (potential) exposure of mosquitofish to pulp and paper mill effluent components. Fish were collected for the 2003 population survey under relatively stable concentrations in Fenholloway River, while concentrations in Rice Creek were dropping dramatically (Figure 6-2). When

females were collected for fry production in July, dehydroabietic acid (DHAA) was at its peak for Fenholloway River and was equivalent to May concentrations for Rice Creek. Comparison to monthly precipitation for each region (Appendix A) does not match perfectly (weekly precipitation data would have been a better comparison). Population collections were made in May, during a month of average rainfall for both systems. At Rice Creek, June peaked with flooding, and females were collected for fry production in July when rainfall was low. At Fenholloway River, June began flood conditions of the rainy season, and females were collected for fry production studies in July when rainfall remained elevated. The apparent disparity between precipitation and effluent concentrations serves as another demonstration of multiple factors influencing effluent exposure, in this case perhaps including changes from mill output.

DHAA concentrations over three of the four fish collections in 2004 revealed stable exposure in fish from Rice Creek and a large drop in exposure at Fenholloway River (Figure 6-3). Concentrations of DHAA in June and July at Fenholloway were double concentrations in 2003. Again, monthly precipitation data did not match these patterns consistently (remembering rainfall data is a monthly average while chemistry data are single time points for these months). Rainfall was consistently high for the Rice Creek system over collections because of several intense storms, whereas precipitation at Fenholloway River was high in June, low in July, then high again in August.

Population Survey

Preliminary investigation of relative population abundance (CPUE) and age-sex structure showed mosquitofish populations at instream effluent-exposed sites were equally, if not more prolific than populations at unexposed sites. For example, median CPUE was highest at the first Fenholloway River downstream site [D(5)] (Figure 6-4),

and juvenile production was greatest instream and closest to discharge for both systems (Figure 6-5). In contrast, relative abundance appeared lower at the discharge point for Rice Creek [DIS] versus the upstream site [U(8)]. Abundances probably reflect ease of sampling related to water level and precipitation just before or during days of collection, rather than actual density of mosquitofish. The discharge site at Rice Creek was especially flooded the day of collection, although monthly rainfall was average, and fish were spread out across shallow swamp backwaters as opposed to concentrated near the banks of the creek. Intermittent rain showers during fish collection at the Fenholloway River reference site [REF2] made it very difficult to see fish to even scoop. Therefore CPUE data may reflect an active sampling bias instead of or in addition to actual differences in abundance. Further, these data sets were not large enough for formal statistical analysis such as nonparametric tests on CPUE data or estimation and modeling. At the least, these data demonstrated mosquitofish populations were not severely impacted by effluent exposure which is not surprising given their tolerance for environmental extremes such as high salinity and low dissolved oxygen (Meffe and Snelson 1989, Nordlie 2000).

A more salient point out of these preliminary data is the apparently different stages in reproductive season among sites (Figure 6-5). Practically 100% of females from upstream sites were nongravid and most females from the Fenholloway River reference site were also not gravid. Internal gross examination of gonads of formalin-preserved fish confirmed these designations based upon anal spots and body shape (data not shown). In contrast, females closest to effluent outfall in both systems were mostly gravid; and approximately 50% of females collected further downstream and before

discharge in Fenholloway and females collected before discharge into Fenholloway were gravid.

Since gravid females had been collected earlier in the season in previous years at unexposed sites (March and April 2000), this lack of gravid females at unexposed sites was surprising and precluded continuation into fry production studies. Juveniles were present at these sites, indicating females had already been pregnant earlier in the season, so either an environmental factor paused the reproductive season (which has not been documented) or these fish were more synchronous in reproductive strategy than previously believed. Evidence exists for two reproductively active female populations in mosquitofish: overwintering females and young-of-year females (Hughes 1985, Haynes and Cashner 1995, Fernandez-Delgado and Rossomano 1997). Overwintering females tend to produce larger clutches earlier in the season and eventually die out. Young-of-year females produce smaller clutches of fry, and these females may or may not live to become overwintering females the following year. Possibly the unexposed populations were in between major production by overwintering females and young-of-year initial fry production, whereas exposed populations already had young-of-year females reproducing. Higher temperatures at effluent-exposed sites may trigger onset of reproduction earlier than at unexposed sites. This would explain the greater proportion of juveniles at instream effluent-exposed sites as well. Therefore, comparing fry production at the same time point, as performed in 2003, may not be an accurate reflection of reproductive success in the population. Instead, different stages of the reproductive season may have been compared. This is one of the main reasons

females were examined on a monthly basis in 2004, to provide a more accurate picture of reproductive stages among sites.

The other aspect examined by this portion of the study was adult sex ratios. Normal sex ratios are debatable but range from approximately 1:1 to strongly female-biased (Krumholz 1948, Meffe and Snelson 1989). A variety of ecological factors such as predation and habitat preferences can alter sex ratios (Casterlin and Reynolds 1977, Britton and Moser 1982) therefore alterations in the field must be interpreted with caution. Normal sex ratios (1:1 to female-biased) were detected at both Rice Creek sites and at the upstream, predischarge, and furthest downstream sites in Fenholloway River (Figure 6-5). However, male-dominated sex ratios (approximately 1:2) were observed at the Fenholloway reference site and the first downstream site. Overall, neither effluent-exposed site nor potential exposure documented by water chemistry can be associated with these apparent alterations in sex ratio.

Body Size

Body size was not impacted by effluent-exposed site for both systems in fish collected for the population survey in summer 2003 (Table 6-7). Condition factor was above one and indicated adequate general health across all sites, regardless of any (erroneous) statistical differences by site within the Fenholloway River system. Compared to the upstream site at the Fenholloway River [U(5)] but not the reference site [REF2], females from effluent-exposed sites were larger. No significant differences were detected between upstream and discharge sites in Rice Creek for either sex.

Anal fin morphology

Anal fin elongation in female mosquitofish was detected for the Fenholloway River system only in adult mosquitofish collected for the population survey in summer 2003

(Figure 6-6). No other statistically significant impacts were observed for both systems. This collection represents the first statistically defined lack of morphological masculinization in Rice Creek female mosquitofish, which was diminished since major process improvements in May 2001 (Chapters 3 and 4). Anal fin elongation in Fenholloway River females was similar to elongation observed in 2001 (Chapter 4). These results may imply a threshold concentration of bioactive component(s) lying somewhere between Fenholloway River and Rice Creek measurements in water samples (Table 6-5). Alternatively, unknown contributions of environmental factors may have varied in the Rice Creek system for the 2003 collection.

Sex steroids

Sex steroids displayed high variation, similar to previous field collections (Chapters 2 through 5). The strongest effect by effluent-exposed site occurred in males for the Fenholloway River system (Figure 6-7). 17β-estradiol was low in females from the upstream Fenholloway River site [U(5)] compared to all other sites, and there was no difference between Rice Creek sites. Testosterone was elevated in females before discharge into Fenholloway River compared to the reference site [REF2] but not to the upstream site [U(5)]. Testosterone was also elevated in effluent-exposed females compared to upstream females in Rice Creek. Males collected at the first downstream site in Fenholloway River [D(5)] had significantly higher 17β-estradiol concentrations compared to both unexposed sites and before discharge [REF2, U(5), PRE-DIS]. Males from Rice Creek had similar sex steroid concentrations between sites.

Sex steroid ratios were most altered in males from Fenholloway River (Figure 6-8): Twenty-two percent of males from the first downstream site [D(5)] and 56% of males

from the second downstream site [D(12)] had feminized ratios. Average ratios were 1.10 $\pm$ 0.09 for D(5) and 0.93 $\pm$ 0.28 for D(12). This response is similar to the response observed in 2001 collections (Chapter 4), when about half the males (47%) at the second downstream Fenholloway River site [D(12)] had feminized ratios (1.03 $\pm$ 0.14). In contrast to 2001 data for Fenholloway, skewed steroid ratios were nearly absent in females (Figure 6-7A). Sex steroid ratios were generally normal for females in both systems: 96% of females had normal feminized ratios and the average ratio was above one for all sites. Among females with masculinized ratios, most were from the discharge site in Rice Creek [DIS], at a frequency less than 2002 field data (Chapter 3) and in accordance with 2001 field data and 2002 caged data (Chapters 4 and 5). This variation in effect on ratios in females, all after major process changes, provides yet another piece of support for dynamic exposure of fish.

Anal Fin Elongation and Sex Steroids

Sex steroids and anal fin elongation were each altered in different groups of adult mosquitofish in the 2003 population survey. Females from Fenholloway River were the only group to display significant effect of effluent-exposed site on anal fin morphology (Figure 6-6), while males from Fenholloway and females from Rice Creek were the only groups to display alterations in sex steroid ratios (Figure 6-8). These results provided even stronger evidence against the predictive value of whole body sex steroids as a biomarker for anal fin elongation. Comparing the index of anal fin elongation between hormonally masculine and feminine groups, the only statistical difference was observed at Rice Creek discharge [DIS] where normal feminized females had a greater index than masculinized females. Of course, overall females from this site did not have significant anal fin elongation so the latter point is essentially moot. As discussed in Chapter 3, this

does not rule out alterations in sex steroid ratios contributing to elongation of the anal fin, since sex steroids were measured post-elongation (in Fenholloway females). However, presence of the physiological alteration cannot be used to predict occurrence of anal fin elongation in individual females living in effluent-receiving streams, based upon these data.

Fry Production

Fry production varied between years, with a general reduction in 2004. Initial 2003 production appeared consistently reduced in association with effluent-exposed sites for both systems, whereas 2004 production over 4 months was again reduced in Fenholloway River females but elevated in Rice Creek females relative to unexposed sites. However, fecundity between unexposed sites from each system varied equally to fecundity within each system, implying different reproductive strategies. Different patterns of anal fin elongation than those for fecundity suggested anal fin elongation was not predictive of observed effects on reproduction.

Summer 2003

Despite mortality and potential stress from a water mold infestation, most females produced fry (Table 6-8). In general, primary clutch sizes averaged around 15 to 20 fry and varied widely from one or two fry to several dozen. These results were within ranges reported for mosquitofish (Krumholz 1948, Rosen and Bailey 1963, Hughes 1985, Meffe and Snelson 1989, Specziar 2004).

Although mosquitofish are considered nonsuperfetating, a Bahaman mosquitofish species (*Gambusia hubbsi*) demonstrated superfetation as a possible reduction in reproductive costs (Downhower et al. 2002). Superfetating species produce small broods (around 1 to 5 fry) more frequently than nonsuperfetating species, and a shift in this tactic

could potentially bias comparison to nonsuperfetating populations. Therefore this trait was examined for potential alteration by pulp and paper mill effluents by retaining females for the full 30 days even after primary production. Fifteen to thirty percent of primary producing females also produced a second clutch of comparable size (for all but Fenholloway River females producing a smaller secondary clutch) within the established 24 to 28 day interbrood interval for nonsuperfetation in this species (Table 6-8 and Figure 6-10A). Thus, effluent exposure did not alter this reproductive strategy. Further, sperm storage by female mosquitofish was reaffirmed since females were not exposed to males during the monitoring period.

Females differed morphologically between systems and sites in 2003. Females from effluent-exposed sites were smaller than females from unexposed sites (Table 6-8), and body length correlated with raw fecundity, or clutch size ($r^2 = 0.553$, $p < 0.05$). Larger females tend to have larger clutches (Krumholz 1948, Hughes 1985, Meffe and Snelson 1989), therefore clutch size was divided by standard length for each female before statistical analysis. The index of anal fin elongation (length of Ray 4 to Ray 6) was statistically greater at the Fenholloway effluent-exposed site but not at the Rice Creek discharge site, in agreement with data from the population survey (Figure 6-6A).

Fry viability was high based upon few stillborn/dead fry, low rates of deformity and little change in fry weight. Most fry were born live (Figure 6-9A) and there were no significant differences in numbers of live versus dead fry. Deformity rates were very low, usually less than 10% per clutch (Figure 6-9B). Relative to viviparous species that lay thousands of eggs (e.g. largemouth bass), mosquitofish invest much more energy into smaller clutches (Constanz 1989) and thus an extremely low rate of deformity was

expected. Deformities were either edema, skeletal abnormalities (lordosis and scoliosis) or premature abortion of embryos. The statistically significant peak in deformities at the Rice Creek discharge site was caused by one entire clutch (n = 8) aborted prematurely; no other females displayed abortive behavior. Individual fry weight was estimated by weighing clutches and dividing by clutch size. For the first ten clutches measured, individual fry were also weighed to validate the estimation. Individual weight was consistently underestimated by 0.1 mg and final weights were corrected as such. Fry weight was not affected by effluent-exposed site for primary production in Fenholloway females and secondary production in Rice Creek females, but was significantly depressed in secondary Fenholloway production and primary Rice Creek production (Figure 6-10B). This inconsistent result probably reflects weight differences due to clutch size as opposed to effluent-related effects: the few especially large clutches (n > 50) had much smaller fry as a constraint on body size of the female.

Lower adjusted fecundities occurred at effluent-exposed sites for both stream systems (Figure 6-10A). (Since fecundity was analyzed in relation to standard length, data are presented as adjusted fecundity by multiplying each clutch size to standard length ratio by the average standard length for the female's collection site.) However, the likelihood of different reproductive stages among sites (Figure 6-5) precluded definitive conclusion based upon these data and demonstrated the need for monitoring fry production throughout the reproductive season.

Summer 2004

Fry production in 2004 was lower than the previous year in terms of number of females producing young and overall fecundities (Table 6-9 and Table 6-10, Figure 6-11). Female mortality rates were similar, and fungal growth was noted on several

females in 2004 although not to the extent of outbreak or infestation requiring treatment in 2003. Females from unexposed sites were not as reproductively active as those from effluent-exposed sites in the May collection, indicated by lower rates of parturition (only two females from upstream Rice Creek produced clutches). Again, this suggests females living in effluent begin reproducing earlier than females in unexposed sites. Standard length was more similar in females between sites than in 2003, and the pattern of body length changes throughout months reinforced the hypothesis of two distinct groups of females reproducing: larger, overwintering females producing fry initially, then young-of-year females beginning to produce at smaller sizes.

Anal fin elongation was present in females from effluent-exposed sites for both systems and varied among months (Table 6-9 and Table 6-10, Fenholloway River and Rice Creek, respectively). Statistically significant monthly changes in elongation may support the concept of two reproductive classes of mosquitofish females in populations, and/or differential exposures. The reappearance of masculinized anal fins in females from Rice Creek demonstrated this effect was not entirely eliminated, as implied by 2003 data. Potential exposure was different between years: resin and fatty acids were substantially lower in 2004, estimated phytosterols were equivalent, and TOC, polyphenolics and condensable tannins were higher in 2004. Rainfall was higher and more stable in 2004 than 2003 (Appendix A). Without knowing specific bioactive compounds and fluctuation of effluent concentrations, linking reappearance of this effect to exposure differences is difficult. Resin and fatty acids may not be the bioactive components since they were actually higher in 2003 when anal fin elongation was absent, and the increase in TOC, lignin and tannin derivatives might support these compounds as

bioactive agents. At the minimum, bioactive compounds remain present in Rice Creek effluent-exposed sites, perhaps teetering on the threshold of the masculinization response.

From the perspective of degraded phytosterols, the unknown contribution of variable bacterial communities may have been influential since concentrations of phytosterols were similar. Yet quantification of these compounds was difficult and may not accurately reflect potential exposure. Regarding bacterial contributions, a separate study of β-sitosterol degradation in April 2004, examining water samples from field sites just before fish collections began, determined microorganisms capable of degradation were present in both effluent-receiving systems and reference sites (Quinn 2004). Degradation half-life was fastest for Rice Creek water sampled at the discharge (22 to 24 days); slightly longer and more variable for Fenholloway River water (24 to 29 days); and slowest at reference sites (32 to 41 days). This study provided strong evidence that bacterial communities differed between effluent-receiving systems, and that degradation can occur in unexposed systems. Thus the supposition of variable bacterial degradation rates of similar phytosterol concentrations is plausible.

Fry vitality was high again in 2004, based upon percentages of fry born live (data not shown). Percentages were statistically lower at the Fenholloway downstream site June through August (Fisher's Exact test), averaging 95% versus 99 to 100% at the reference site. Percentages were not different between sites at Rice Creek and averaged 99% for the upstream site and 98% for the discharge site. Biological relevance of differences for Fenholloway River is probably low, since most females produced 100% live fry and dead fry across all sites occurred as single members of a clutch in most cases as opposed to the total death of a clutch.

Since standard length positively correlated with raw fecundity overall ($r^2 = 0.434$ and $p < 0.050$; females from upstream Rice Creek did not demonstrate a positive correlation dragging the overall correlation down slightly) fecundity was adjusted by length (Figure 6-10). Site and month significantly covaried for the Fenholloway system but not for the Rice Creek system. Different patterns of fecundity were evident for Fenholloway, while Rice Creek females displayed similar patterns across months. At the reference site for Fenholloway River, fecundity was relatively low for three of the four months with a large spike in July. May and August fecundities were greater at the effluent-exposed downstream Fenholloway site across months and between sites, but overall fecundity was statistically reduced at the effluent-exposed site. In contrast, both Rice Creek sites peaked in fecundity later in the summer (August), and fecundity was increased relative to upstream in June at the effluent-exposed site. Overall fecundity at Rice Creek was higher in effluent-exposed fish. Thus the reduced fecundity observed in 2003 was reflected in 2004 sampling of the Fenholloway River but the opposite was detected in Rice Creek, supporting the need for long-term observation of fry production over the reproductive season. In general these differences were related to reduced fecundities between years at both unexposed sites and the Fenholloway downstream site, while fecundity remained stable at the Rice Creek discharge site (Figure 6-10 and Figure 6-11). Overall these data suggested differences in reproductive output over the reproductive season and from year to year, as opposed to effluent-exposure, influenced fry production in female mosquitofish.

Anal Fin Elongation and Fry Production

For both years of fry production, overall fecundity (fry divided by female standard length) did not correlate with anal fin elongation (Ray 4 to Ray 6 length ratio);

$r^2 = 0.0609$ for 2003 and $r^2 = -0.0498$ for 2004 with both $p > 0.05$. Examining fecundity by site for both years and by month for 2004 did not reveal any statistically significant correlations either, with Pearson's correlation coefficient ranging from –0.344 to 0.204 and $p>0.05$. Furthermore, females with the greatest degree of anal fin elongation ≥ 1.3 (all but 5 from effluent-exposed sites) produced fry in comparable numbers to all other females (t- test, $p < 0.05$). Therefore, anal fin elongation did not influence fecundity of female mosquitofish.

Conclusions

These studies represent the first examination of actual fry production as a measure of reproductive success in mosquitofish exposed to pulp and paper mill effluents. Previous investigations of reproduction in this species under effluent exposure were confined to brood size of developing embryos or histological evaluation of gonads, neither of which conclusively detected adverse impacts. Further, the current studies are the first to assess masculinization in addition to measures of reproduction.

Taking these data as a whole, reproductive success of mosquitofish is likely not impacted by pulp and paper mill effluent exposure. Rather, seasonal differences in fecundities suggested mosquitofish may have adapted different site-specific reproductive strategies. Examining data by system, Fenholloway River females displayed reduced fecundity and masculinized anal fins for both years. In contrast, Rice Creek females were less fecund in 2003 data, when masculinized anal fins were absent, but more fecund across months in 2004 when elongated anal fins returned. Combined with the overall lack of correlation between fecundity and anal fin elongation, anal fin elongation cannot be definitively tied to alterations in reproduction. Examining fecundity over time revealed site-specific patterns of production, and age/sex structure in the population

survey suggested effluent-exposed females may begin the reproductive season earlier than females living in nonexposed sites, perhaps caused by higher water temperatures. Increased temperature strongly triggers onset of the reproductive season (Koya and Kamiya 2000, Koya and Iwase 2004), and has been associated with an overall increase in reproductive output (Vondracek et al. 1988).

Differences in seasonal reproductive patterns have been described for mosquitofish populations living in unexposed conditions under the influence of different predation and food availability (Vondracek et al. 1988, Downhower et al. 2000). Further, Downhower et al. (2000) detected rapid phenotypic adjustment or plasticity in reproductive strategies for populations introduced to predator-free habitats in less than 20 years. Therefore, ecological differences among sites caused by long-term effluent dominance could influence fecundity. For example, increased turbidity at effluent-dominated sites may decrease predation risk of mosquitofish; eutrophication of effluent-receiving systems may increase food availability as well. The combined effect of these types of ecological factors likely alters reproductive investments and strategies and may explain observed variation in fecundities. Variation in fecundity over the 2004 reproductive season within a site also supports the concept of two separate reproducing populations, overwintering and young-of-year females (Hughes 1985, Haynes and Cashner 1995, Fernández-Delgado and Rossomanno 1997), stressing the importance of documenting reproduction throughout the reproductive season.

Studies evaluating effects of other contaminants on mosquitofish fry production suggested reproductive tolerance of this species to exposure. Experimental exposure of adult mosquitofish to sublethal concentrations of the nonionic surfactant *Genapol*

OXD-080 (used as an agricultural pesticide) did not affect fry survival (Cabral et al. 1999). Field collection of mosquitofish living in coal ash settling basins did not have altered fecundities or fry viability despite elevated concentrations of metals in both females and fry (Staub et al. 2004). The latter point suggested maternal transfer of contaminants from females to fry, which was also detected after dietary exposure of female mosquitofish to 4-nonylphenol (Thibaut et al. 2002), a weakly estrogenic microbial degradation product of industrial nonionic surfactants that has been detected in Rice Creek (Nimrod and Benson 1996, Quinn 2004).

In contrast to above reports, water-borne exposure to 4-nonylphenol disrupted normal gonadal differentiation in maturing mosquitofish at highest test concentrations (50 µg/L) with a skew toward females, and lower test concentrations (0.5 and 5.0 µg/L) were associated with partially-developed gonopodium in fish with atrophied gonads (Drèze et al. 2000). Gender of these latter fish was unclear, but this work insinuates estrogenic compounds detected in Rice Creek receiving waters could potentially contribute to effects on anal fin morphology and reproduction. Since a low level but persistent estrogen bias in male sex steroid ratios was observed throughout our study, further examination of estrogenic effects of effluent components on mosquitofish is warranted.

Before making a definitive conclusion about reproductive success based on fecundity, the picture needs to be widened even further to document onset and cessation of reproduction across sites. Ideally, population-level studies investigating energetic investments in reproduction would be included to address potential variation in reproductive strategies at effluent-exposed sites. It is possible an earlier onset of

reproduction in effluent-exposed fish at Fenholloway River may counteract the lower fecundities observed in these studies. Preliminary relative abundance data indicated greatly increased density at the downstream Fenholloway site in early summer 2003 (May) so an earlier onset of reproduction is tentatively supported. Also, the overall reduced fecundities between years suggested additional environmental factors were negatively influencing fry production. Long term microcosm experiments studying population structure and recruitment dynamics using the tank facility at Rice Creek (Chapter 4) would be very useful in determining these types of differential reproductive strategies, and females could be subsampled periodically for fry production studies that could mimic experimental predation on populations.

Bioindicator potential for mosquitofish was weakened by initial reproductive success studies that could not link anal fin elongation with altered fecundity. Differences in fecundity may ultimately reflect adaptation of reproductive strategy in effluent-exposed fish, as opposed to negative impacts on reproductive success. Thus, any alterations in anal fin morphology and sex steroids could serve as biomarkers indicating exposure rather than adverse effect.

Table 6-1. Water quality parameters of Fenholloway River and Rice Creek population survey sites during summer 2003.

	Fenholloway River					Rice Creek	
Site	REF2	U(5)	PRE-DIS	D(5)	D(12)	U(8)	DIS
Water Temperature (°C)	21.4	20.8	31.0	27.9	24.7	22.0	26.7
Conductivity (µS)	278	80.5	2,229	1,820	1257	289	1,862
Salinity (ppt)	0.1	0.0	1.1	0.9	0.6	0.1	0.9
Turbidity (ntu)	0.62	1.32	12.0	8.18	5.79	1.03	26.5
pH	7.1	4.9	7.6	7.2	7.2	7.3	7.9

Table 6-2. Water quality parameters (ave ± se) measured three times weekly (n = 16 total) during laboratory fry production of female mosquitofish collected from field sites in Rice Creek during summer 2003.

	Fry Production Tanks
Temperature (°C)	26.1±0.12
Conductivity (µS)	310.7±1.8
Salinity (ppt)	0.1±0.02
Dissolved Oxygen (mg/L)	6.29±0.16
Turbidity (ntu)[a]	1.38±0.19
pH	8.0±0.04
Incident Light (Fc)	83.4±0.9

[a]measured once a week

Table 6-3. Water quality parameters (ave ± se) at field sites where female mosquitofish were collected for fry production studies over 4 months in summer 2004. All parameters were statistically different between exposed and unexposed sites within each system.

	Fenholloway River		Rice Creek	
Site	REF2	D(5)	U(8)	DIS
Temperature (°C)	23.1±0.7	28.8±1.1	23.2±1.5	27.1±1.5
Conductivity (µS)	290.1±70.7	2,085±216.3	185.8±30.3	1,780±78.8
Salinity (ppt)	0.2±0.02	1.1±0.1	0.1±0.03	0.9±0.03
Dissolved Oxygen (mg/L)	4.50±0.27	0.92±0.7	7.20±0.49	8.59±1.03
Turbidity (ntu)	2.04±0.19	13.98±1.46	7.67±2.91	27.2±0.95
pH[a]	7.3±0.1	7.2±0.2	7.1±0.4	7.9±0.1

[a]pH statistically different at Rice Creek only

Table 6-4. Water quality parameters (ave + se) measured three times weekly (n = 16 total) during laboratory fry production of female mosquitofish collected from field sites in Rice Creek and Fenholloway River during summer 2004.

Month	May	June	July	August
Temperature (°C)	25.3±0.3	25.4±0.4	24.7±0.1	24.4±0.3
Conductivity (µS)	238.7±4.1[a]	260.9±12.5[a]	329.4±5.2	323.5±8.4
Salinity (ppt)	0.1±0.0[a]	0.1±0.01[a]	0.2±0.02	0.2±0.02
Dissolved Oxygen (mg/L)	6.76±0.17[b]	5.29±0.32	5.67±0.15	7.67±0.02
Turbidity (ntu)	1.01±0.20[c]	0.79±0.23	0.43±0.02	0.26±0.11
pH	8.6±0.06[d]	7.9±0.01[c]	7.7±0.04	7.7±0.02

[a]significantly different from July and August ($p < 0.05$)
[b]significantly different from June and July ($p < 0.05$)
[c]significantly different from August ($p < 0.05$)
[d]significantly different from all other months ($p < 0.05$)

Table 6-5. Concentrations of selected effluent components (ave ± se) in single grab water samples from field sites where female mosquitofish were collected in Rice Creek and Fenholloway River during summer 2003. Statistical significance ($p < 0.05$) is given by system.

System	Fenholloway River					Rice Creek	
Site	REF2 (n = 3)	U(5) (n = 5)	PRE-DIS (n = 3)	D(5) (n = 7)	D(12) (n = 3)	U(8) (n = 7)	DIS (n = 7)
Total Resin Acids (µg/L)	ND[j]	1.29±1.29	125.46±6.94[b]	78.11±22.83[b]	57.34±4.21	0.20±0.13	98.01±24.44[k]
Total Fatty Acids (µg/L)	2.68±2.68	10.19±6.16	1.50±0.75	2.45±0.41	1.72±1.16	6.01±1.93	8.33±2.65
Campesterol (µg/L)[a]	ND	ND	6.18±0.75[c]	3.12±0.86[d]	3.45±0.78[b]	ND	0.68±0.18
Stigmasterol (µg/L)[a]	0.27±0.27	1.39±0.15	13.26±2.44[c]	6.51±1.76[d]	7.26±1.58[e]	ND	2.36±0.28*
Stigmastanol (µg/L)[a]	ND	ND	14.13±0.75[f]	7.19±1.83[d]	8.30±1.49[b]	ND	2.72±0.32*
β-sitosterol (µg/L)[a]	1.07±0.36	1.75±0.26	106.6±12.2[c]	54.58±14.15[d]	63.1±1.49[b]	0.19±0.0.13	13.97±2.30*
Total Organic Carbon (mg/L)	41.0±8.03	109.1±14.8	154.7±8.6[f]	99.8±6.8[g]	85.2±0.8[h]	31.2±8.8	62.9±2.6*
Polyphenolics (mg/L)	6.98±1.33	12.5±1.6	42.9±0.3.7[f]	25.9±2.6[d]	17.1±2.8[g]	4.9±1.2	21.4±2.9*
Condensable Tannins (mg/L)	1.49±0.14	3.71±0.16	7.76±0.34[i]	5.02±0.37[e]	3.67±0.29	2.05±0.91	3.11±0.57

[a]phytosterol values for Rice Creek discharge site [DIS] failed quality control tests therefore are estimates
[b]different from REF2 and U(5)
[c]different from REF2, U(5), D(5)
[d]different from REF2, U(5), PRE-DIS
[e]different from REF2
[f]different from rest
[g]different from REF2, PRE-DIS
[h]different from U(5), PRE-DIS
[i]different from REF2, U(5), D(12)
[j]ND = nondetectable
[k]different from U(8)

Table 6-6. Concentrations of selected effluent components (ave ± se) in single grab water samples from field sites where female mosquitofish were collected in Rice Creek and Fenholloway River during summer 2004 for fry production studies. Statistical significance ($p < 0.05$) is given by system.

System	Fenholloway River		Rice Creek	
Site	REF2 (n = 3)	D(5) (n = 3)	U(8) (n = 3)	DIS (n = 3)
Total Resin Acids (µg/L)	0.34±0.34	367.36±109.16[d]	0.30±0.30	44.97±6.79*
Total Fatty Acids (µg/L)	0.26±0.26	3.62±2.46	2.74±2.18	1.24±1.24
Campesterol (µg/L)[a]	ND[b]	2.07±1.11	ND	ND
Stigmasterol (µg/L)[a]	ND	5.75±1.85*	ND	3.11±0.34*
Stigmastanol (µg/L)[a]	ND	5.71±1.38*	ND	3.56±0.39*
β-sitosterol (µg/L)[a]	2.82±2.82	50.20±13.73*	0.46±0.46	14.53±0.79*
Total organic carbon (mg/L)[c]	35.0±19.1	139.0±4.4*	44.8±15.1	91.0±15.7
Polyphenolics (mg/L)	4.20±2.12	35.83±5.38*	3.29±1.85	27.23±0.69*
Condensable Tannins (mg/L)	1.36±0.77	8.03±0.25*	2.00±0.81	6.23±0.58*

[a]phytosterol values for both Fenholloway River sites [REF2 and D(5)] and Rice Creek discharge site [DIS] failed quality control tests therefore are estimates
[b]ND = nondetectable
[c]TOC values for August were above maximum temperature required for quality control and thus are only estimates
[d]different from reference [REF2 or U(8)]

Table 6-7. Body size parameters (ave + se) for mosquitofish collected for population survey of Fenholloway River and Rice Creek in May 2003. Significant differences ($p < 0.05$) are noted by site within each system.

	Fenholloway River					Rice Creek	
Site	REF2	U(5)	PRE-DIS	D(5)	D(12)	U(8)	DIS
♂ Sample Size	10	10	10	10	10	10	10
♂ Body Weight (g)	0.211±0.017	0.168±0.022	0.167±0.015	0.236±0.015	0.230±0.026	0.233±0.022	0.205±0.016
♂ Standard Length (mm)	22.74±0.83	22.13±0.95	22.22±0.60	23.37±0.47	22.43±0.63	23.60±0.65	21.99±0.56
♂ Condition Factor (g/cm^3)	1.82±0.16	1.66±0.04	1.48±0.05[a]	1.85±0.10	1.99±0.16	1.73±0.05	1.90±0.07
♀ Sample Size	23	24	24	25	25	25	13
♀ Body Weight (g)	0.531±0.035	0.335±0.030[b]	0.589±0.057	0.754.±0.106	0.628±0.049	0.450±0.026	0.447±0.067
♀ Standard Length (mm)	31.02±0.72	28.17±0.86	30.88±0.91	31.88±1.21[c]	30.45±0.69	28.42±0.56	27.78±1.36
♀ Condition Factor (g/cm^3)	1.74±0.04	1.45±0.04[b]	1.87±0.05[c]	2.12±0.04[d]	2.14±0.05[d]	1.92±0.03	1.91±0.04

[a]statistically differs from REF2
[b]statistically differs from rest
[c]statistically differs from U(5)
[d]statistically differs from non"d" lettered sites

Table 6-8. Reproductive and morphological characteristics of females collected from Fenholloway River and Rice Creek and monitored for fry production in 2003.

	Fenholloway River		Rice Creek	
Site	REF3	D(5)	U(8)	DIS
# ♀ Start	51	51	52	29
% ♀ Mortality	37%	27%	22%	10%
# ♀ End	32	37	41	26
% ♀ Parturition[a]	91%	92%	100%	85%
Total Fry (1° production)	689	531	787	197
1° Clutch Size Range	4–62	1102	265	147
% ♀ with 2° Production[b]	14%	30%	18%	14%
Total Fry (2° production)	47	129	104	27
2° Clutch Size Range	420	629	825	515
Median Interbrood Interval (days)	24	24	25	27
Standard Length (mm)[c]	30.86±0.52	28.21±0.57[d]	31.80±0.61	28.25±0.99[d]
Index of Anal Fin Elongation (Ray 4/Ray 6)[c]	1.17±0.01	1.54±0.06[d]	1.16±0.01	1.19±0.01

[a]referring to primary production of surviving females
[r]eferring to secondary production of females that had primary production
[c]ave±se
[d]significantly different than unexposed site ($p < 0.05$)

Table 6-9. Reproductive and morphological characteristics of females collected for fry production from Fenholloway River in 2004. Significant differences in morphological variables are noted by site or month, and both covaried by site and month ($p < 0.05$).

	Fenholloway River							
Site	REF2				D(5)			
	May 2004	June 2004	July 2004	Aug 2004	May 2004	June 2004	July 2004	Aug 2004
# ♀ Start	50	50	50	42	50	50	50	49
% ♀ Mortality	32%	18%	4%	24%	6%	12%	20%	24%
# ♀ End	34	41	48	32	47	44	40	37
%♀ Parturition[a]	38%	66%	80%	67%	74%	68%	66%	67%
Total Fry (1° Production)	100	92	233	135	264	174	221	241
Clutch Size Range	18	7	16	13	20	15	28	20
Standard Length (mm)[b]	31.31±0.31[d]	26.74±0.46[d]	28.53±0.36	28.24±0.45	30.07±0.39[ce]	28.76±0.75[e]	24.70±0.60[c]	24.34±0.43[c]
Index of Anal Fin Elongation (Ray 4/Ray 6)[b]	1.17±0.01[d]	1.11±0.01	1.12±0.01	1.12±0.01	1.51±0.03[c]	1.57±0.04[c]	1.51±0.04[c]	1.36±0.03[cd]

[a]referring to primary production of all females
[b]ave ± se
[c]significantly different than unexposed site within month
[d]significantly different than rest of months within site
[e]significantly different than July and August within site

Table 6-10. Reproductive and morphological characteristics of females collected for fry production from Rice Creek in 2004. Significant differences are noted by site or month, and index of anal fin elongation only covaried by site and month ($p < 0.05$).

	Rice Creek							
Site	U(8)				DIS			
	May 2004	June 2004	July 2004	Aug 2004	May 2004	June 2004	July 2004	Aug 2004
# ♀ Start	50	49	50	35	50	50	34	49
% ♀ Mortality	30%	12%	22%	37%	14%	4%	24%	29%
# ♀ End	35	43	39	22	43	48	26	35
%♀ Parturition[a]	4%	67%	68%	57%	28%	74%	62%	71%
Total Fry (1° Production)	20	155	627	217	74	141	141	541
Clutch Size Range	19	16	78	62	12	21	19	41
Standard Length (mm)[b]	28.55±0.43[e]	30.21±0.50[e]	30.00±0.72[d]	29.35±0.96[d]	28.35±0.42	28.57±0.51[c]	29.62±0.62[c]	31.89±0.54[c,d]
Index of Anal Fin Elongation (Ray 4/Ray 6)[b]	1.16±0.01	1.16±0.01	1.13±0.01	1.14±0.02	1.24±0.05	1.21±0.01[c]	1.21±0.02[c]	1.27±0.01[c]

[a]referring to primary production of all females
[b]ave ± se
[c]significantly different than unexposed site within month
[d]significantly different than rest of months within site
[e]significantly different than July and August within site

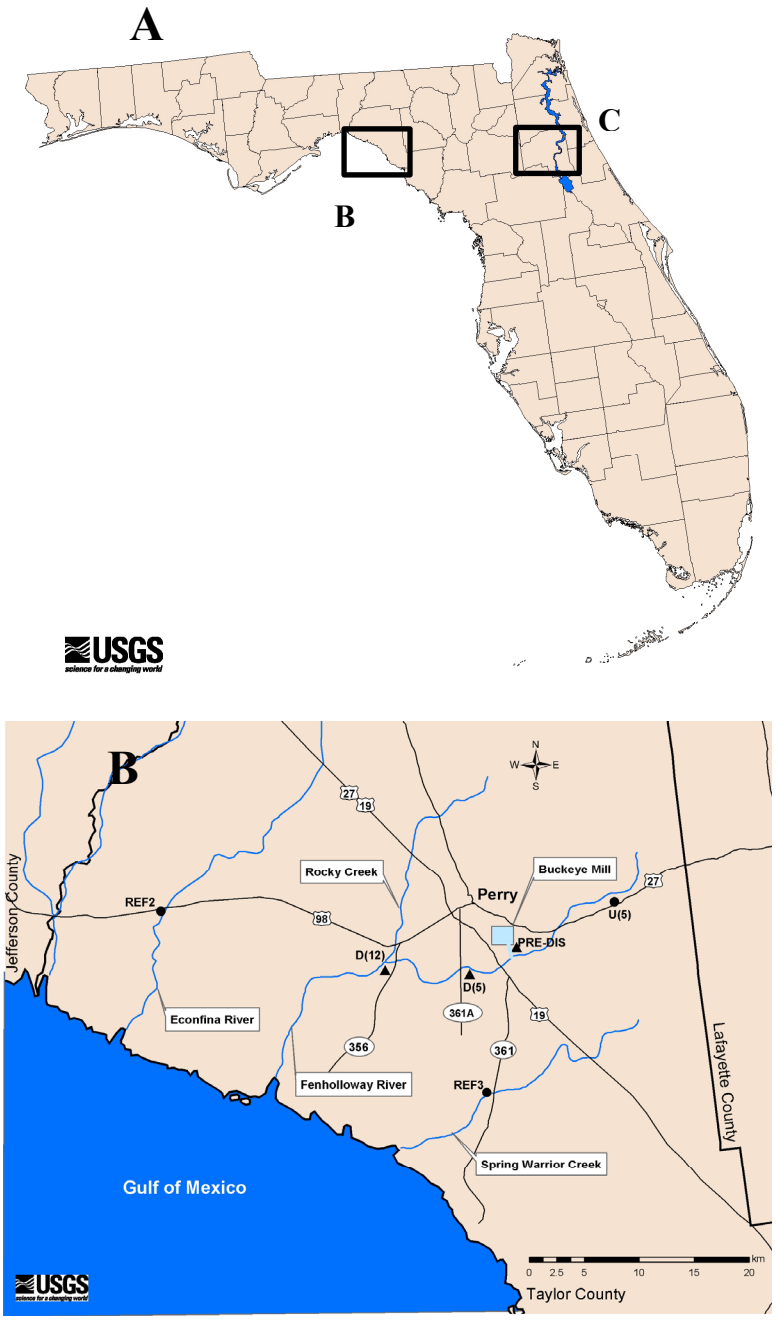


Figure 6-1. Maps of field sites. A) Relative location of the stream systems in Florida. B) Fenholloway River sites. C) Rice Creek sites. Site symbols distinguish effluent exposure: circles = unexposed and triangles = exposed. Site abbreviations denote upstream (U) or downstream (D) of discharge, followed by approximate distance (km) from discharge in parentheses. PRE-DIS indicates site before discharge into the creek; DIS denotes site at discharge into creek; and REF indicates reference site, followed by identifying number.

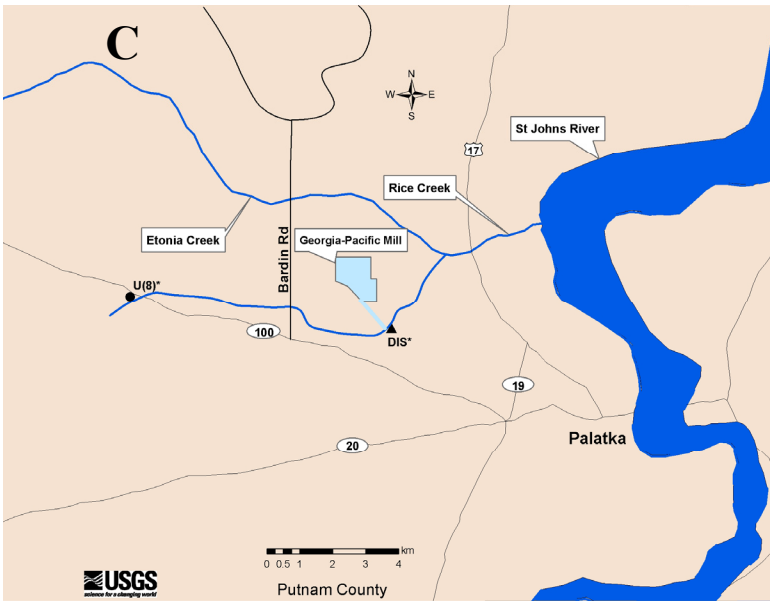


Figure 6-1. Continued

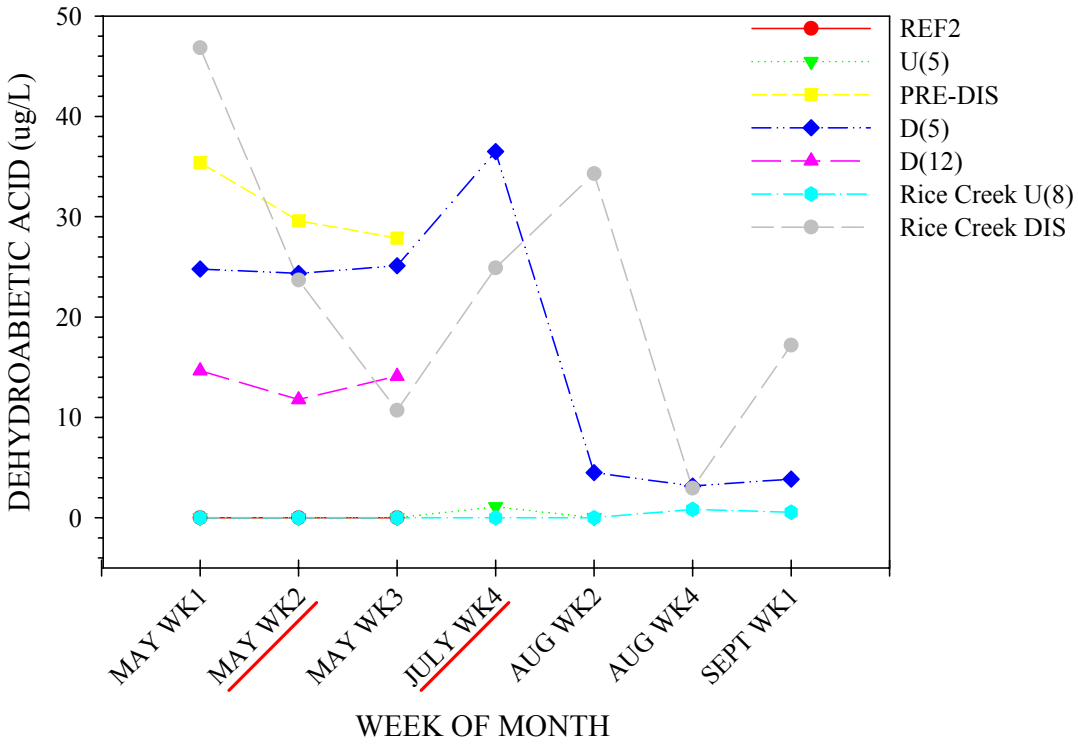


Figure 6-2. Representative changes in resin acid concentrations during summer 2003 at Fenholloway River and Rice Creek field sites where mosquitofish were collected. Values at unexposed sites were nondetect (at or below 2 ug/L) and may not be visible underneath Rice Creek U(8) symbols. Red underline indicates fish collection for population survey (May) and fry production (July).

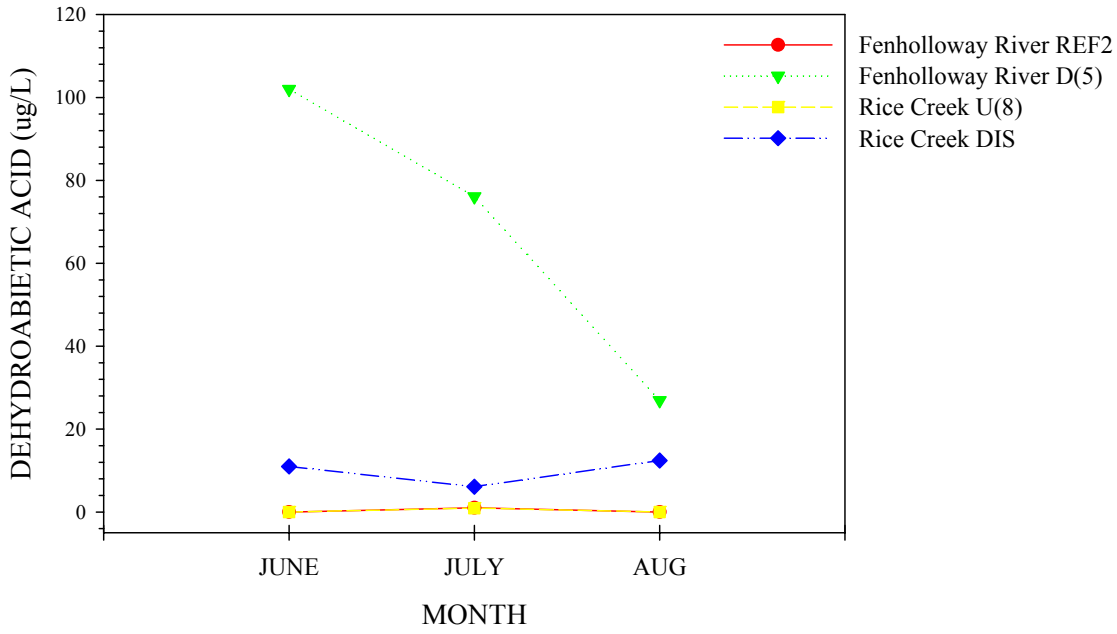


Figure 6-3. Representative changes in resin acid concentrations during summer 2004 at Fenholloway River and Rice Creek field sites were mosquitofish were collected at the same time. Values at Fenholloway River reference site [REF2] were nondetect (at or below 1.0 ug/L) and may not be visible underneath Rice Creek U(8) symbols.

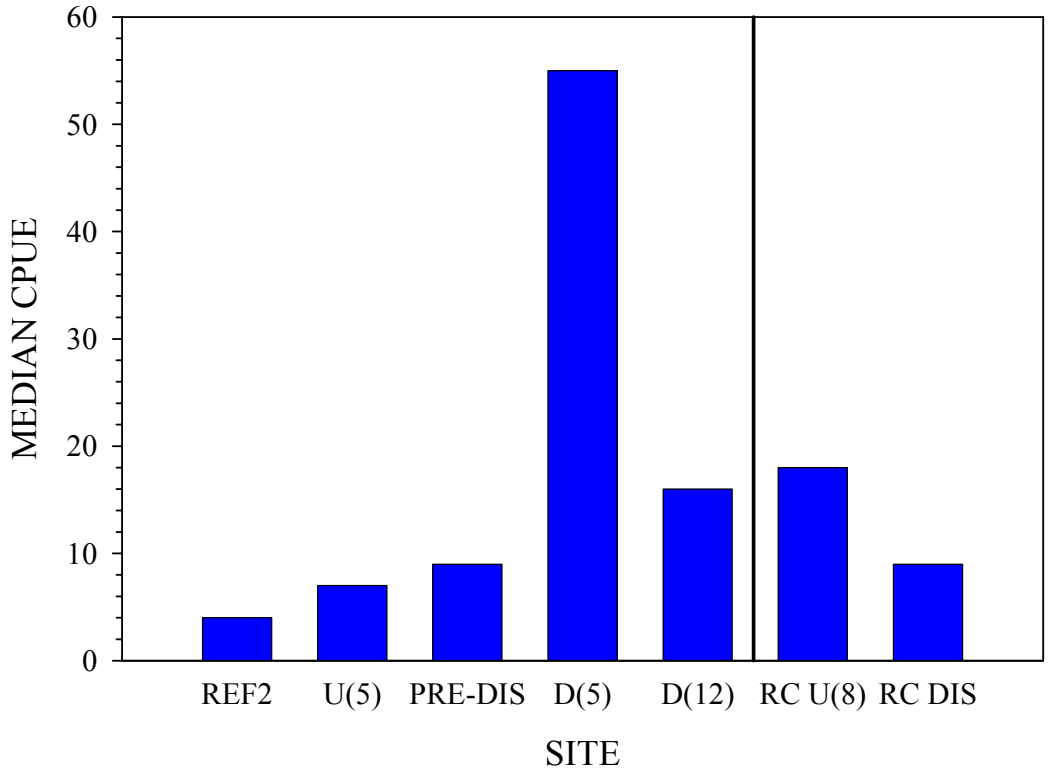


Figure 6-4. Estimated relative abundances of mosquitofish for Fenholloway River and Rice Creek (RC) sites. Solid black line separates the two systems. Abundances were calculated as median catch per unit effort (CPUE) based upon number of fish per 10 sweeps by observer (3 to 5 observers per site).

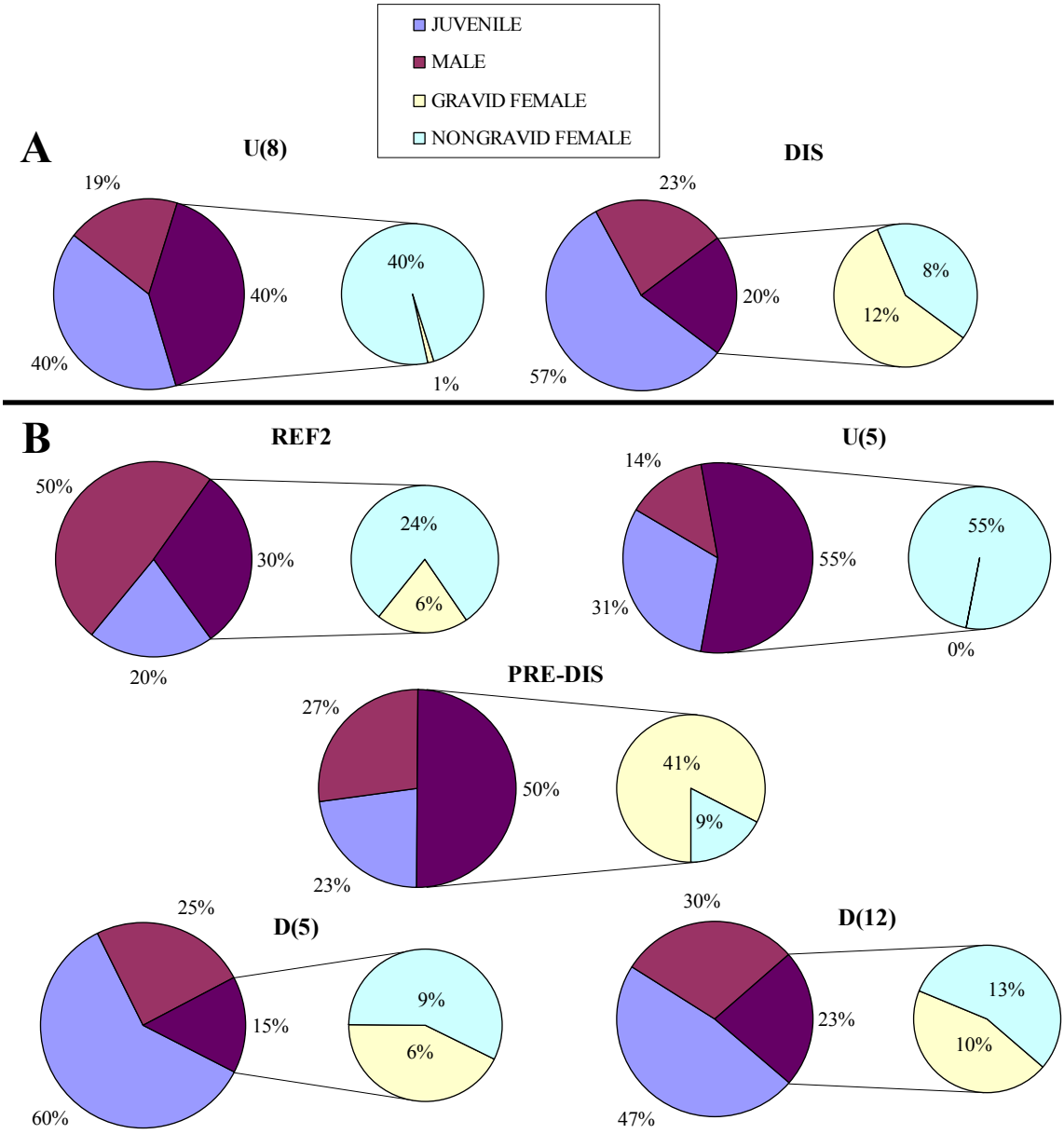


Figure 6-5. Estimated age and sex structure of mosquitofish populations living near pulp and paper mill effluent discharge. A) Rice Creek. Distributions were significantly different (χ^2=34.03, df=3, p<0.05 Fisher's Exact Test). B) Fenholloway River. Distributions were significantly different (χ^2=200.2, df=12, p<0.05 Chi Square Test for Independence). Site abbreviation descriptions are given in Figure 6-1.

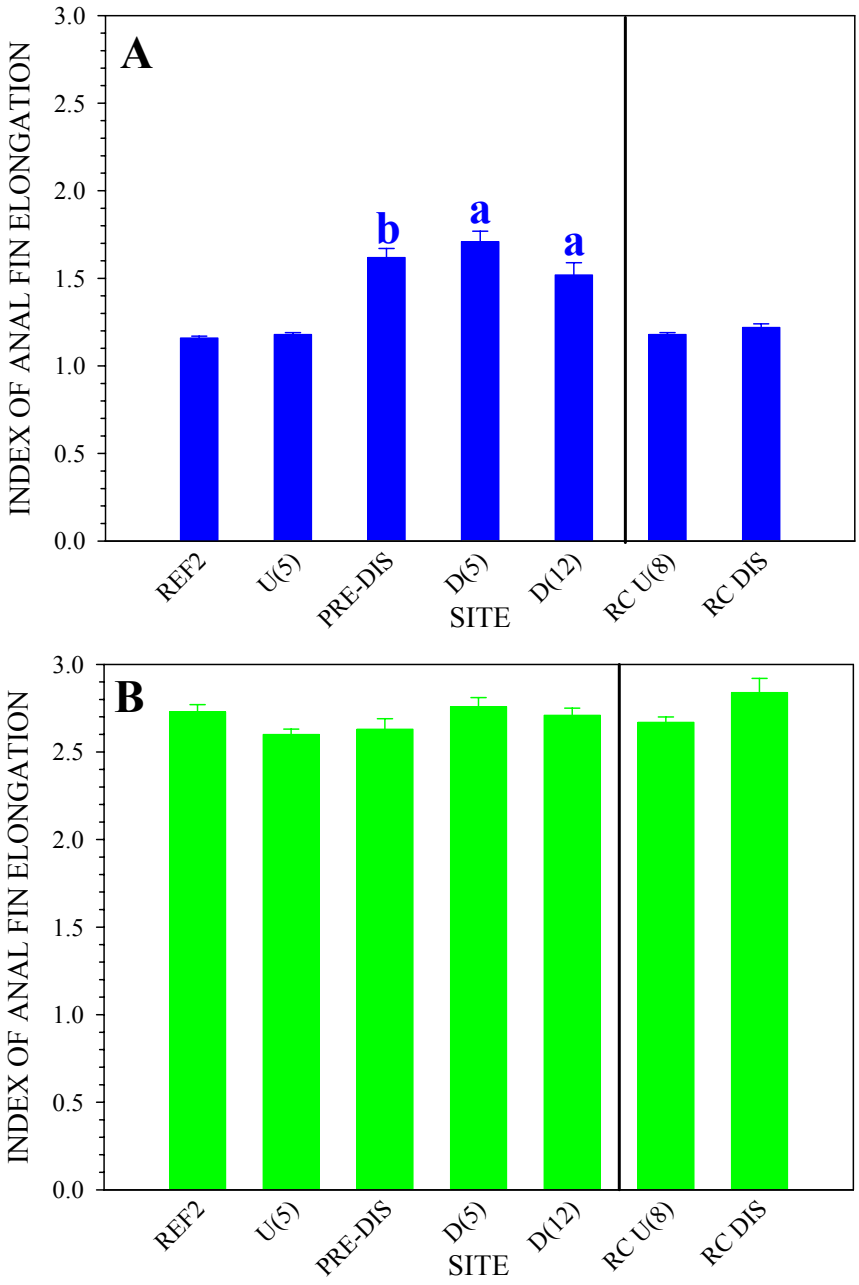


Figure 6-6. Index of anal fin elongation (tracings of Ray 4 / Ray 6, computer-aided measurement of fresh fish) for mosquitofish collected in summer 2003 from Fenholloway River and Rice Creek (RC). Stream systems are divided by a solid black line. A) Females. B) Males. Letters indicate significant differences by site within system ($p < 0.05$): "a" denotes differences to all sites but PRE-DIS (Fenholloway River system); "b" denotes differences to REF2 and U(5) (Fenholloway River system).

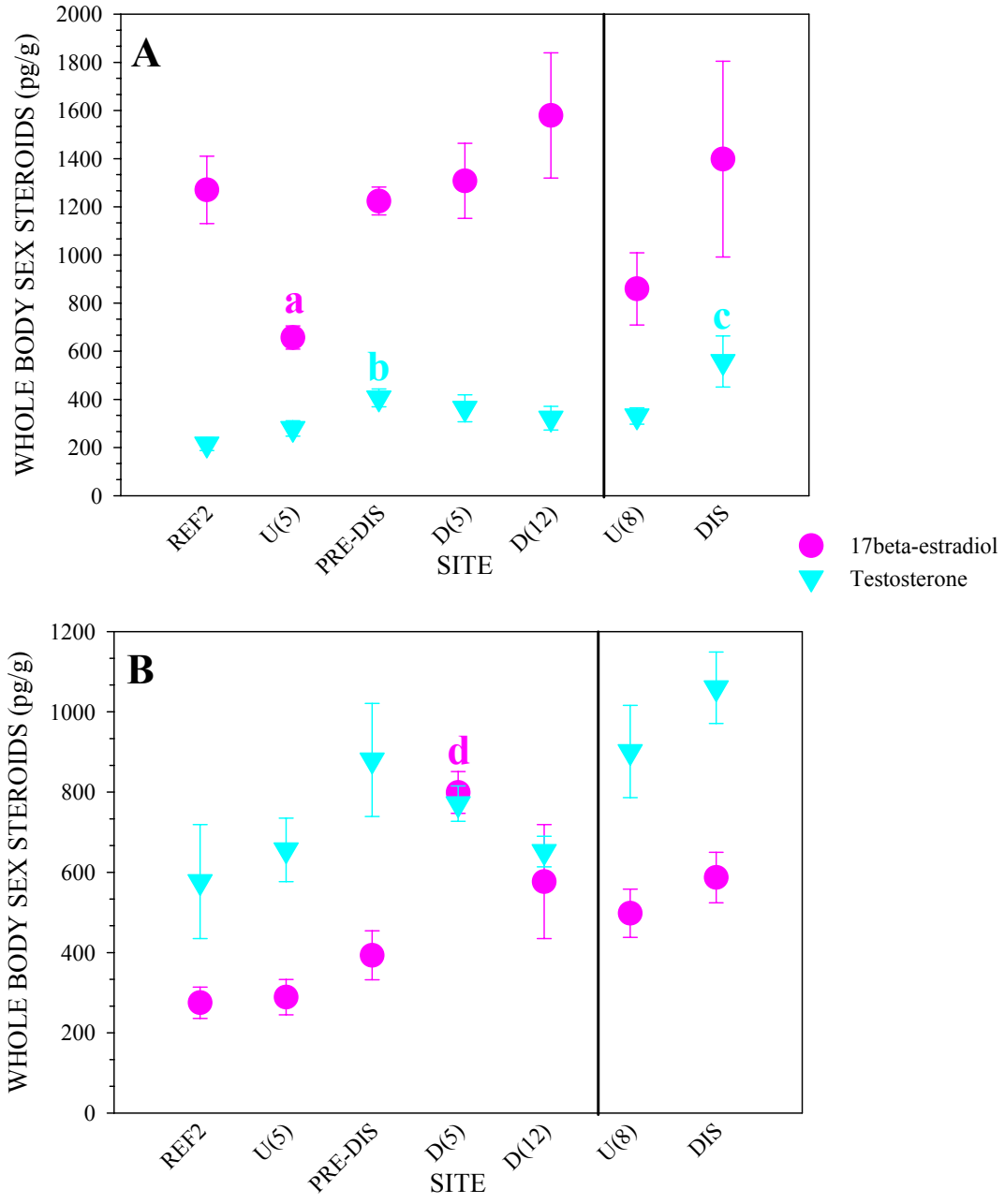


Figure 6-7. Whole body sex steroids (ave ± se) for mosquitofish collected in summer 2003 from Fenholloway River and Rice Creek. Stream systems divided by solid black line. A) Females. B) Males. Letters indicate significant differences by site within mill ($p < 0.05$): "a" denotes differences to all other sites; "b" denotes differences to REF2; "c" denotes differences to U(8); "d" denotes differences to REF2, U(5), PRE-DIS.

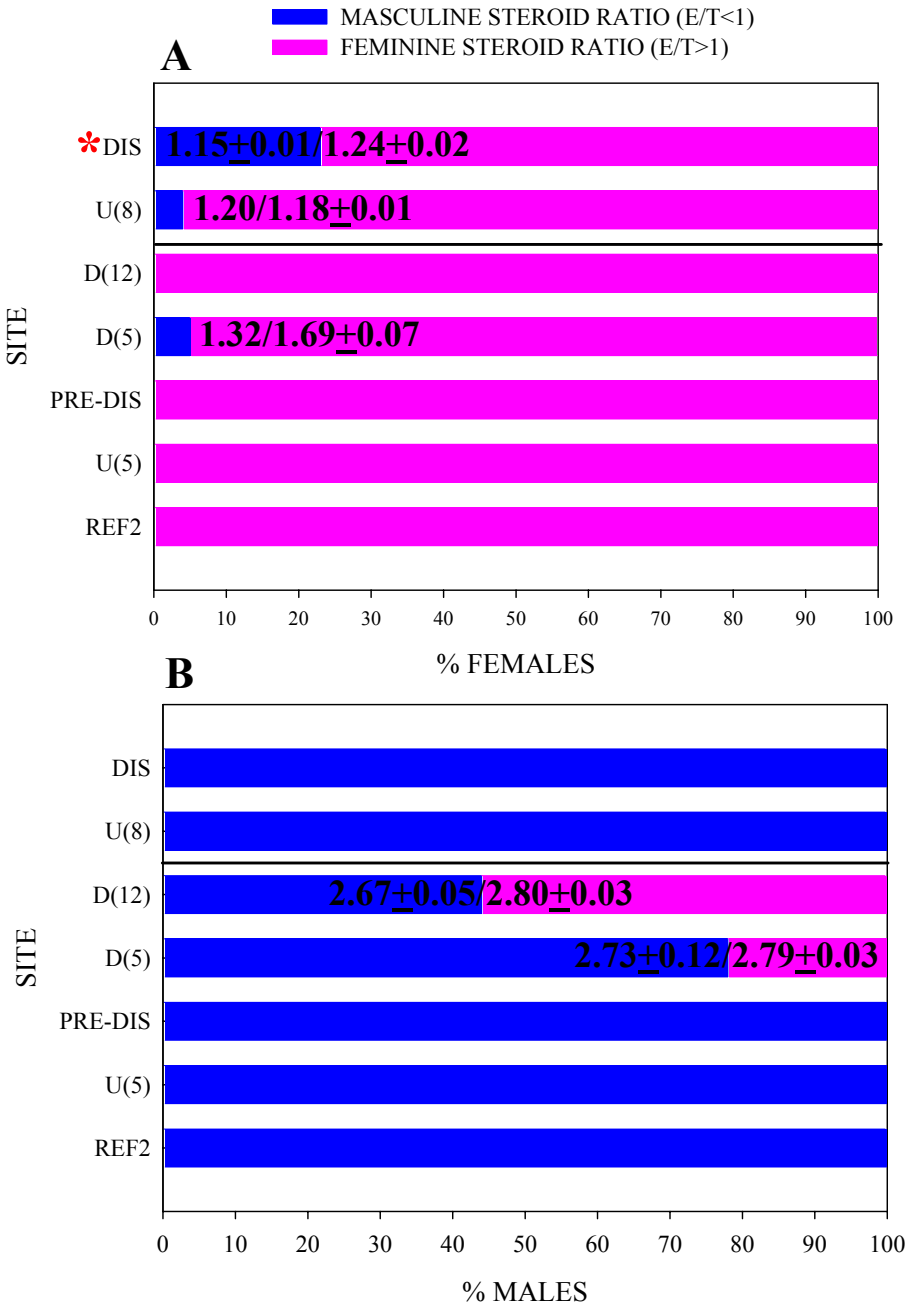


Figure 6-8. Percentage of mosquitofish with masculine and feminine sex steroid ratios collected in summer 2003 from Fenholloway River and Rice Creek (systems divided by solid black line). A) Females. Frequency of ratios was statistically significant for Rice Creek (Fisher's Exact test, $p < 0.05$) and Fenholloway River ($\chi^2 = 20.20$, df = 4, $p < 0.05$). B) Males. Frequency of ratios was statistically significant for Fenholloway River ($\chi^2 = 182.5$, df = 4, $p < 0.05$). Index of anal fin elongation (ave $\pm$ se) is given for fish with both masculine and feminine ratios by site. Red asterisk indicates significant differences in elongation at $p < 0.05$ between ratios.

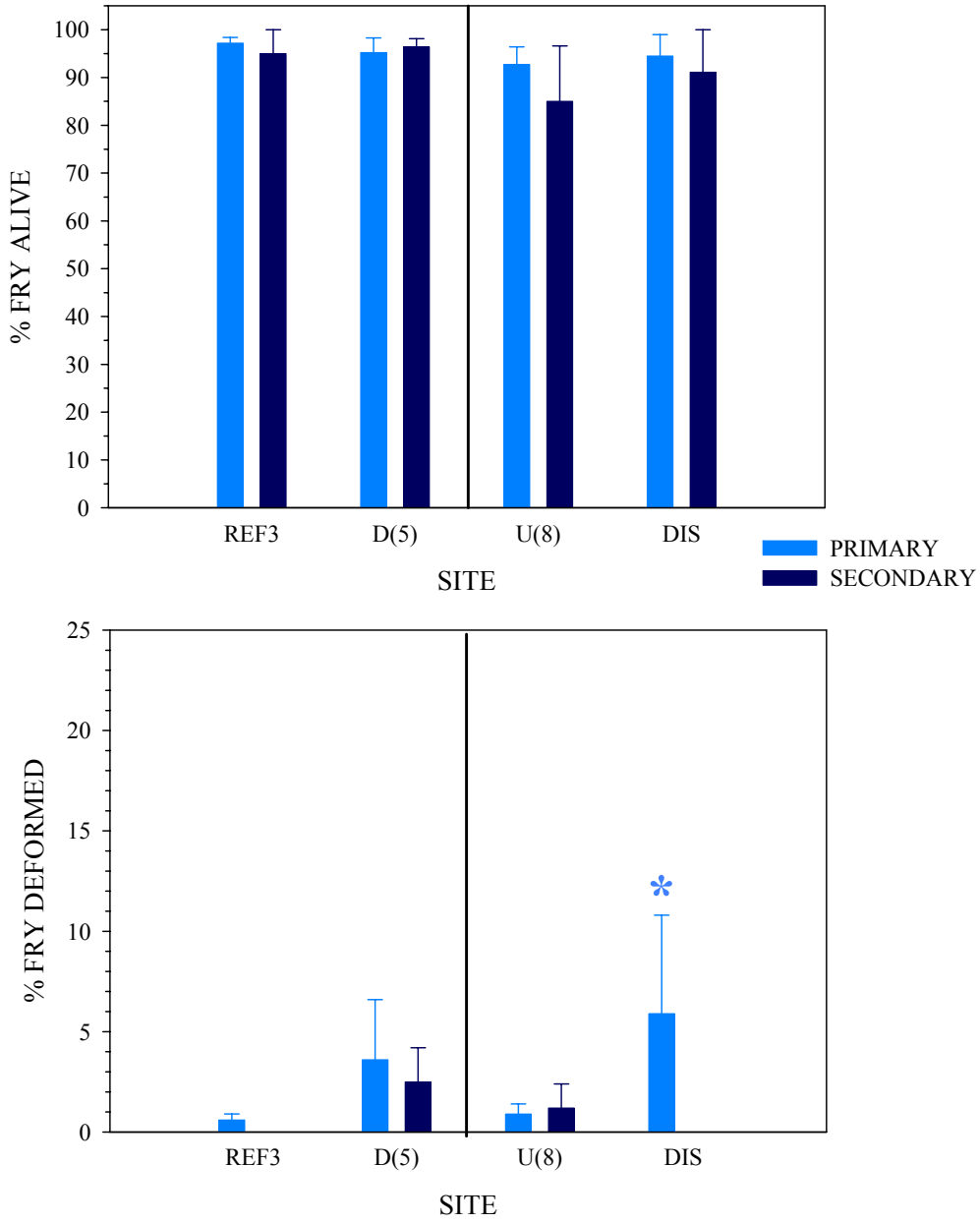


Figure 6-9. Viability of primary and secondary clutches produced by females collected from Fenholloway River and Rice Creek in 2003. A) Average percentages (+se) of live fry per female within one day of parturition. B) Average percentages (+se) of deformed fry per female. Field sites are divided by a solid black line (Fenholloway River sites on the left and Rice Creek on the right). Missing bars mean there were zero deformed fry. Light blue asterisk indicates statistical difference from primary production at upstream Rice Creek site (for total fry deformed versus normal within each site, Fisher's Exact test, $p < 0.05$).

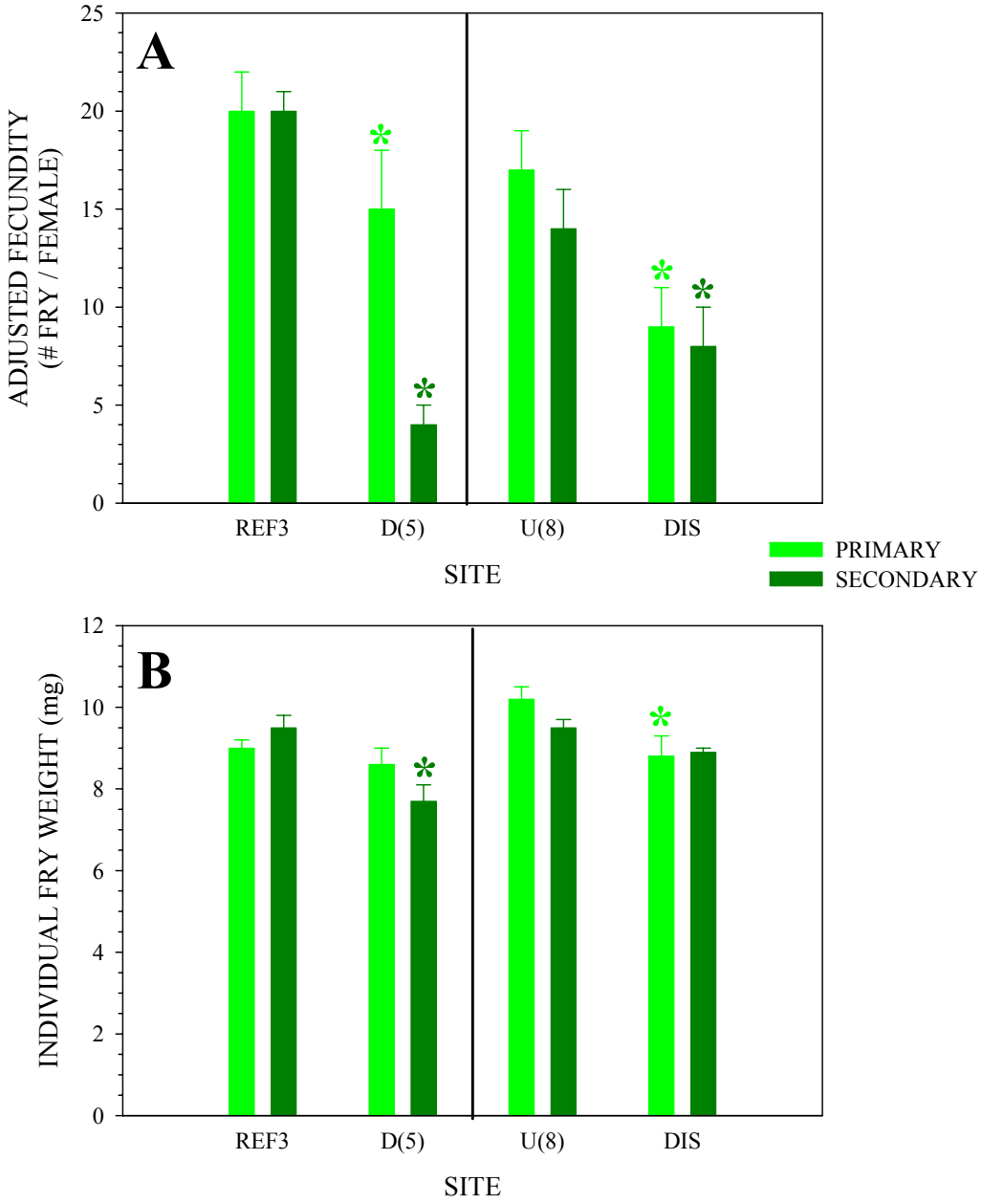


Figure 6-10. Fecundity and individual fry weight of primary and secondary clutches produced by female mosquitofish collected from Fenholloway River and Rice Creek in summer 2003. A) Adjusted average fecundity (+se) or number of fry corrected for standard length of individual females. B) Average individual fry weight (+se) calculated by weighing clutches and dividing by raw fecundity. Field sites are divided by a solid black line (Fenholloway River sites on the left and Rice Creek on the right). Asterisks are color coded within primary and secondary production and indicate significant differences to unexposed site within each system ($p < 0.05$).

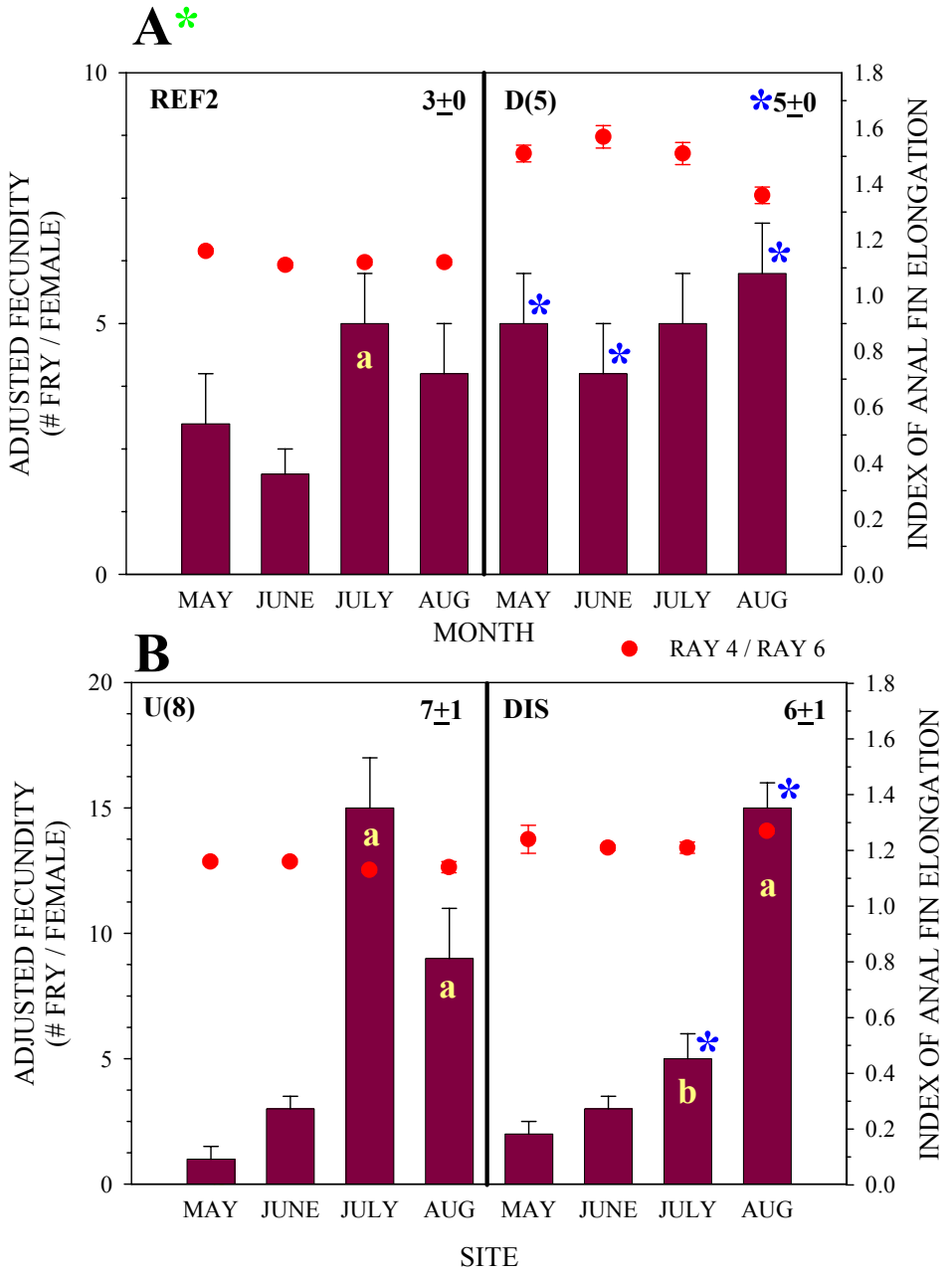


Figure 6-11. Adjusted fecundity of primary clutches produced by female mosquitofish collected monthly in 2004. A) Fenholloway River. B) Rice Creek. Solid black line separates sites within each system. Solid red circles indicate index of anal fin elongation (ave ± se) for females producing fry. Site symbols are given in upper left-hand corners, while average (± se) adjusted fecundity for sites are given in upper right-hand corners. Significant differences by month within a site are denoted by letters: "a" is different than May and June; and "b" is different than May. Blue asterisks indicate significant differences between sites within a month (above bars) or for the season (next to overall average) for each system. Green asterisk indicates significant interaction or covariance by site and month.

CHAPTER 7
EVALUATION OF MOSQUITOFISH AS A BIOINDICATOR OF PULP AND PAPER MILL EFFLUENT EXPOSURE

The goal of my study was to evaluate whether sublethal effects in mosquitofish can be reliably used to indicate adverse impacts of pulp and paper mill effluents. This chapter summarizes conclusions form previous research chapters, and then evaluates this research in light of specific aims and suitability of mosquitofish as a bioindicator species. In addition, mosquitofish are compared to other fish models that respond to pulp and paper mill effluents, and future studies are proposed that would resolve inconclusive aspects and address questions revealed by this work.

My research has contributed several key points of knowledge about mosquitofish exposed to pulp and paper mill effluents. Anal fin morphology was only affected in females, not males. There was no apparent precocious maturation among males exposed to mill effluents. Masculinization of the female anal fin is sensitive to differences in effluent composition: relative concentrations of wood extractives reflected degree of anal fin elongation. Also, the concept of a threshold response was supported by an ephemeral occurrence of elongation in females from Rice Creek. Masculinization was not seasonally affected, as previously reported, indicating this endpoint may serve as a biomarker of past (chronic) exposure or at a potentially sensitive life stage. In contrast, sex steroids may serve as a biomarker of current or recent exposure, especially considering whole effluent exposure results. Both sexes responded hormonally, providing strong evidence for multiple endocrine-mediated mechanisms not just an

androgen-mediated response. Neither sex steroids nor fry production were related to anal fin elongation in females, indicating masculinization is not predictive of current physiology or ultimately reproductive output.

Summary

Gender identification techniques, anal fin morphological measurements and whole body sex steroid analyses were suitably validated from a methodological viewpoint to support use of mosquitofish as a bioindicator of pulp and paper mill effluents. Seasonal changes in sex steroids, but not alterations in female anal fin morphology, suggested sex steroids may be a more sensitive and labile biomarker of differential effluent exposure, while female anal fin elongation may be a more static and historical biomarker. Across all studies, effects in males were restricted to changes in sex steroids, mainly elevated 17β-estradiol, accompanied by a shift to estrogen-biased steroid ratios. Precocious maturation of males was not supported by these studies. Female sex steroids generally responded with a masculinized steroid profile caused by increased testosterone. Analysis of female anal fin morphology by size class as an estimation of age did not consistently reveal a more sensitive adult life stage; however, juvenile versus adult exposure was never addressed and could potentially influence extent of elongation.

Extensive field work in receiving streams of one mill throughout process changes and among several mills indicated reductions in wood extractives in pulp mill effluents is associated with reduced, but not entirely eliminated, masculinization of the female anal fin. Masculinized hormone profiles in females and feminized hormone profiles in males were consistently detected for all but one mill where masculinization of the female anal fin was the least. Skewed steroid ratios at reference sites for the system with greatest

degree of morphological masculinization was very important and implied the estrogen to testosterone ratio can be altered by environmental factors completely separate from pulp and paper mill effluents. Equally important, fish collected before discharge into receiving streams (i.e., retention ponds) did not always respond to the degree of fish collected in the receiving stream. In general, reference site selection was an important aspect of determining statistically significant responses, and comparison between unexposed sites demonstrated inherent natural variability of biomarkers. No clear relationship existed between anal fin elongation and whole body primary sex steroid concentrations or ratios, although this conclusion does not mean development of anal fin elongation in females is independent of sex steroid concentrations. It does mean sex steroids are not predictive of anal fin elongation.

Controlled whole effluent exposure in tanks and *in situ* field exposure in cages did not induce female anal fin elongation, while sex steroids in both genders were differentially altered between tanks versus cages. Masculinization of the anal fin was likely not induced because of abbreviated exposure; but the unexplored possibility of sensitive life stage(s) could also explain this lack of induction. Skewed sex steroid ratios were induced sooner in cages than tanks, and steroids had recovered in caged fish by the end of the study. However, compared to field collections, steroid responses under controlled exposure were more pronounced. This study reinforced the idea of dynamic exposure dependent on environmental factor(s) in addition to effluent exposure.

Finally, reproductive success was investigated over two years in conjunction with masculinization biomarkers. Fry production of wild-caught female mosquitofish was evaluated at one time point the first year and over several months the second year. A

preliminary population survey was also conducted the first year. Morphological masculinization was consistent between years for one mill while the response was only detected the second year of study at the other mill. Sex steroid alterations were weakly affected relative to measurements in previous studies. Neither of these biomarkers could be associated with fry production or population structure differences. Initially, fry production appeared decreased at effluent-exposed sites but population structures implied mosquitofish at effluent-exposed sites may have started reproducing sooner than fish at unexposed sites. Fry production over several months the following year affirmed different reproductive patterns in females among sites and throughout the year. Further, overall fecundities were higher in females from one exposed site and lower from the other relative to respective references. Rather than negatively impacting fecundity, pulp and paper mill effluent exposure may be stimulating distinctive reproductive strategies in mosquitofish influenced by changes in environmental and ecological factors as opposed to chemical exposure.

Specific Aims Revisited

Explicitly stated objectives for my study were divided into two specific aims with associated hypotheses. Within each specific aim, three subaims were identified and studies developed for each. Broadly speaking, these aims were ambitious and reality dictated focus on one main aspect, variation in masculinization response in the field.

The first specific aim, to determine the effects of improved mill technology on masculinization of female mosquitofish, was satisfied for two of three subaims associated with this field work. Because of complications with induction studies, field studies could neither be supported nor refuted by more controlled exposure. Through field studies, the hypothesis that reduction in brown side effluent components (i.e., wood extractives such

as phytosterols and resin acids) will reduce anal fin elongation and hormonal alteration in female mosquitofish was supported. An important limitation to this work was the increasingly apparent seasonality of hormone concentrations that remained uncharacterized. Variations in response, potential exposure, and environmental factors (such as precipitation) suggested a scenario of dynamic exposure to pulp mill effluents.

The second specific aim, to evaluate the reproductive success of mosquitofish exposed to pulp and paper mill effluents, and associated subaims were too broad for practical purposes and efforts were subsequently narrowed down toward fry production studies. Rather than provide a conclusive answer to the hypothesis that exposure to pulp and paper mill effluents will not impair reproductive success of mosquitofish, this research posed questions about adaptation of reproductive strategies under effluent exposure. Similar to complications experienced with induction studies for masculinization in the first specific aim, controlled (caged) exposures designed to evaluate fry production were not completed. Thus these data require controlled exposures for more support. Nonetheless, these studies did not demonstrate a relationship between fecundity and masculinization.

Overall, the proposed work was skewed toward field work and away from whole effluent exposures. Controlled exposure to effluent components in the laboratory was avoided in favor of more relevant whole effluent exposures, since mechanistic-based questions were not directly addressed. However, difficulties with cages used for *in situ* field exposures created a bias in available data. This bias meant conclusions could be influenced by uncontrollable factors inherent in field work such as potential population

differences and unknown actual exposures. The ideal of paired field and laboratory work was reinforced by this inadvertent shortcoming.

Bioindicator Criteria Revisited

Based upon contributions of my study, mosquitofish are not adversely affected by pulp and paper mill effluents. Therefore, at this point mosquitofish biomarkers may be used to indicate exposure but not effect. While responses were detected in both anal fin morphology and whole body sex steroids, a relationship between these parameters could not be established. Method variability was acceptable but natural variability was high for sex steroids, therefore detection of changes due to effluent exposure were obscured especially in light of uncharacterized seasonal effects. Masculinization of the anal fin was sensitive to differences caused by changing effluent compositions, but the unique nature of the systems studied (low-flow, effluent dominated streams) questions applicability to more average effluent-receiving systems. Finally, impacts on reproduction were not consistent, and fecundity differences may actually reflect different reproductive strategies among sites.

Whole effluent exposures by this laboratory and other researchers (Ellis et al. 2003, McCarthy et al. 2004, van den Huevel et al. 2004b) have not consistently supported observations in the field relative to anal fin masculinization. This is another point that precludes implementation of mosquitofish as a bioindicator. Current exposures were based on the premise that masculinization occurs in adult females as indicated by laboratory exposure to bacterially degraded effluent components (Denton et al. 1985, Howell and Denton 1989). Exposure throughout maturation may reveal juveniles are a more sensitive life stage.

Pending such exposures and further analysis of seasonality in hormones, anal fin morphology and sex steroids could potentially be used on a mill-specific level as biomarkers of exposure. Candidate mills would include effluent-dominated receiving streams and receiving streams in developing countries lacking modern processing technologies. Sex steroids could serve as a marker of recent exposure, while anal fin morphology could serve as a more static marker of previous, longer-term exposure.

A final point about utility of mosquitofish as a bioindicator is the biological significance of observed effects. Higher level impacts aside, what extent of anal fin elongation is biologically relevant? Statistical differences continue to be detected, yet the degree of masculinization has lessened considerably from initial reports of fully formed gonopodia (Howell et al. 1980, Bortone and Drysdale 1989, Cody and Bortone 1992). Under dietary exposure to 11keto-testosterone, female Ray 4 to Ray 6 length ratios ranged from 1.35 to 1.50 at 20 to 100 μg/g feed (Angus et al. 2001), which is at least 1 mm less than normal male ratios that averaged 2.5. Therefore biologically significant differences based upon exposed females would be a smaller magnitude change than differences based upon normal male gonopodial length. This debate ironically centers upon quantification of the masculinization response, which was previously assumed to be a more appropriate and accurate measure of masculinization than categorically or nominally scored responses in previous studies. Yet quantifying the response may have led to erroneous conclusions. Thus, biological significance may be retained in future studies by blending the two types of measurements and scoring females by absence and presence/extent of elongation, then measuring ray lengths for each of these groups.

My study addresses applicability and relevance within the species, but applicability to other species remains to be studied. Mosquitofish presence and/or abundance may be negatively associated with presence/abundance of other native small fish species and serve as a bioindicator of adverse effects on fish communities. Behavioral research on interactions among introduced mosquitofish and native small fish species in Australia and Spain concluded mosquitofish deleteriously compete with native species (Rincon et al. 2002, Warburton and Madden 2003). Possible mechanisms included predation on early life stages, increased aggression, and reduced feeding rates. Impacts of mosquitofish on fish communities in pulp and paper mill effluent receiving systems are unknown. Such research would have to carefully separate species interactions from environmental constraints that may negatively impact other fish living in effluent. Thus this last major bioindicator criterion, applicability, requires further investigation before mosquitofish can be confidently accepted or rejected as a bioindicator of pulp and paper mill effluents.

Other Model Fish Species

What are the differences between mosquitofish responses to pulp and paper mill effluents and responses in other fish species? First of all, mosquitofish are unique in their reproductive mode. They are ovoviviparous/livebearers, while other species studied (such as fathead minnows, *Pimephales promelas*, and largemouth bass, *Micropterus salmoides*) are oviparous/egg-layers. Mosquitofish are better suited as models for maternal transfer of contaminants in this respect, since maternal nourishment (specifically facultative matrotrophy or conditional maternal provisioning of developing embryos) was recently demonstrated for mosquitofish (Marsh-Matthews et al. 2001, Demarais 2003). Thus mosquitofish more accurately reflect placental nourishment of

human fetuses than egg-laying fish species and may extrapolate to humans more effectively.

Similarly, circulating sex steroids in mosquitofish may be more similar to humans than other fishes. Testosterone, and not 11-ketotestosterone, is the dominant active androgen in mosquitofish (Borg 1994, Chapter 2). Although progesterone was not measured in my study, research on ovoviviparous and viviparous elasmobranches showed progesterone patterns were analogous to humans with a rise in progesterone at the periovulatory period that was sustained by pregnancy (Koob and Callard 1999).

Research has been conducted on egg-laying fish species exposed to effluent from Rice Creek, affording more direct comparison of mosquitofish to other fish models. Largemouth bass, exposed for 56 days in the same tank facility used for mosquitofish, responded with reduced circulating sex steroids and gonad size in adults at 20 to 40% effluent dilution and decreased fry growth and survival at 10% dilution and greater (Sepúlveda 2000, Sepúlveda et al. 2001, Sepúlveda et al. 2003). After EPA Cluster Rule process changes, effects on sex steroids and gonad size did not manifest until 40% effluent dilution or greater (Noggle et al. 2004b). (Fry production has not been assessed since process changes.) In contrast, my study showed effects on mosquitofish sex steroids at 10% or greater effluent dilution after process changes (Chapter 5), although anal fin elongation was not induced (Chapter 5) and fry production in wild-caught females was not affected (Chapter 6). Similar to largemouth bass, fathead minnow research conducted before process changes showed reproductive-level effects (decreased egg production) at slightly higher effluent concentrations (23% or greater) (NCASI

2000a). Masculinization in fathead minnows was not observed either before (NCASI 2000a) or after (DL Borton, pers. comm..) processing improvements.

Relative to these data, mosquitofish are the most suitable model of the three fish species to examine masculinization effects. Hormonally, they may be more sensitive than bass and minnows. However, bass may be the most sensitive in terms of adverse reproductive effects, followed by the minnows. Mosquitofish, on the other hand, may be responding with a shift in reproductive strategy as opposed to direct adverse impact. If the mosquitofish responses can be adequately referenced to reproductive impacts in bass and minnows, then mosquitofish may indeed become a suitable bioindicator of adverse effect.

Future Work

Six aspects of mosquitofish responses to pulp and paper mill effluents require further investigation: seasonality; dynamic exposure; population-level responses; fish community assessments; mechanism of anal fin elongation; and adaptation to exposure.

A major drawback to many toxicology studies is insufficient knowledge of background normal responses (Van Der Kraak et al. 1998). My study implied seasonality of mosquitofish hormones, and warrants a full characterization of hormone profiles at least monthly throughout the year in a reference site and under laboratory conditions. Progesterone should be included in this characterization to determine as well. Related characterization of fecundity (fry production) throughout the entire reproductive season needs to be conducted as well for multiple reference sites and effluent-exposed sites. Admittedly, such a project would be very labor intensive but would allow better interpretation of current reproductive success studies.

A scenario of dynamic exposure was implied by my study. Concentrations of effluent components in water samples and precipitation levels in surveyed regions varied widely, but additional environmental factors such as bacterial communities may be influential and crucial to actual exposures. These aspects need to be addressed then linked back to actual exposures and effects in fish. For example, bacterial communities could be surveyed over several weeks in the field at receiving streams and before discharge in conjunction with chemical analysis of effluent, water, and sediment samples. Based upon these results, laboratory studies could be designed to evaluate degradation products of observed effluent components by different bacterial species. Finally, observed degradation products could be examined back in the field in water, sediment, and fish samples to provide insight into actual exposures and effects. Since bacterial degradation products are a key component to the current hypothesis of mosquitofish masculinization, this type of study should be a high priority.

Population-level work in microcosms would refine analysis of adverse impacts on mosquitofish. The flow-through tank facility at the Rice Creek mill would facilitate such studies, allowing controlled examination of changes in population structure and juvenile recruitment over time. This work could also be compared to fry production studies conducted on females collected from field sites and provide further insight into variations in reproductive strategies in response to effluent exposure.

To address potential adverse impacts of mosquitofish on fish communities, community health assessments of receiving streams, such as the index of biologic integrity, could be performed (see Karr and Chu 1999 for an explanation of this metric and Adams et al. 1992 for a description of fish community assessment in a pulp and

paper mill effluent-receiving stream). Coupled with laboratory studies of interactions among fish species from field sites, these data would provide the most insight into applicability and relevance of mosquitofish as a bioindicator of pulp and paper mill effluent exposure.

Research on the mechanism of anal fin development may refine the search for bioactive effluent components by allowing identification of genes induced by exposure to specific components. Most research on fin growth and development has been conducted on the zebrafish, *Danio rerio* (Johnson and Bennett 1999). Much of this work involved regeneration studies on adults (which is appropriate for investigation of mosquitofish anal fin elongation), although the genetic control of anal fin regeneration specifically has not been fully characterized. Studies on livebearers themselves have identified at least two genes that may be involved in anal fin elongation in mosquitofish. Swordtail research showed development of male secondary sex characteristics in anal and caudal fins was linked to upregulation of *msxC* expression (Zauner et al. 2003). Further, this regulation was different than regulation of fin development in zebrafish. Most recently, research on gonopodial development of maturing western mosquitofish (*Gambusia affinis*) showed androgen-dependent fin elongation was associated with sonic hedgehog (*Shh*) expression in the distal ray epithelium (Ogino et al. 2004). Two isoforms of the androgen receptor, ARα and ARβ, were identified and predominantly expressed in distal regions of elongating anal fin rays. Therefore, anal fin elongation appears locally stimulated by exogenous androgens at the fin itself. This latter research on genetic control of anal fin elongation may prove invaluable in mosquitofish populations where the masculinization response is in question (i.e., Rice Creek and Elevenmile Creek).

Finally, potential adaptation and tolerance of mosquitofish in pulp and paper mill effluents remains to be addressed. Another small fish species, the killifish or mummichog (*Fundulus heteroclitus*), was evaluated for sensitivity to laboratory exposure to potent dioxin-like compounds specifically IUPAC PCB No. 126 and 3-methylcholanthrene (Nacci et al. 1999). Killifish from a Superfund site heavily contaminated with PCBs were more tolerant to these exposures than fish from nearby reference populations: survival was greater and EROD activity was lower. Similarly, mosquitofish collected from sites with high concentrations of pesticides showed inherited tolerance to organic contaminants (Andreason 1985). Although survival and EROD activity may not be relevant to adaptation and tolerance of mosquitofish to pulp and paper mill effluents, my study implies reproductive strategies are adapted (Chapter 6). For example, Meffe and Snelson (1993) showed energy allocation during reproduction had large interindividual variation under laboratory study of mosquitofish collected from a site that was probably contaminated (on the US Department of Energy's Savannah River Site). These results also supported the potential for facultative matrotrophy in mosquitofish which was demonstrated more conclusively by direct experimental visualization of maternal transfer (Marsh-Matthews et al. 2001, Demarais and Oldis 2003). Thus, facultative matrotrophy could be one way mosquitofish adapt reproductive strategies to environmental conditions.

APPENDIX A
FIELD SITES

This appendix contains information about locations for all field sites associated with my study (Tables A-1, A-2, and A-3). (Maps for these sites can be found in Chapters 2 through 6.) In addition, monthly rainfall data by region are represented in Figure A-1. These quality-controlled data were obtained from the National Oceanic Atmospheric Administration's National Climatic Data Center in Asheville, NC, USA (2005a,b,c). Since all three receiving systems are low-flow streams they are theoretically vulnerable to flooding with higher dilution of effluent. Conversely, periods of drought may concentrate effluent. Thus, precipitation adds yet another element of complexity to exposure of wild fish to pulp mill effluents.

In Figure A-1A, Rice Creek fish were living under drought conditions in 1999 and up to initial collections in March and April 2000. (Females live 1 to 2 years, while males usually live no more than one year in the wild (Meffe and Snelson 1989). Total yearly rainfall for 1999 was 40.90 inches versus a normal average of 50.42 inches, and by the end of 2000 the yearly total (49.28 inches) was back up to normal levels. In 2001, precipitation was similar to historical levels (52.93 inches total in 2001) with a large flood peak just after July and August collections in September. Tank and *in situ* field exposures began during a period of low rainfall in March 2002 and continued under average precipitation in April. Flooding occurred after exposures in June; fish were sampled early June before any of the major storms. Precipitation during 2003 was uncharacteristically cyclic with regular periods of flooding, the greatest in March.

Population collections were made in May, during a month of average rainfall. June peaked again with flooding, and then females were collected for fry production in July when rainfall was low. *In situ* field exposures occurred in August, and flooding during this month contributed to premature termination of exposures. Finally, the most recent collections for fry production studies were monthly from May to August 2004. Rainfall remained cyclic up to May, when monthly average was normal, but the rest of collections occurred in higher than normal precipitation from several intense storms that released 2.3 to 3.0 inches per storm.

Initial Fenholloway River collections were conducted in August 2001. The region was under drought conditions, with yearly total precipitation of 41.37 inches compared to normal average of 58.15 inches. No collections were made in 2002, when rainfall was slightly higher than normal at 62.12 inches. Intense flooding characterized the region in 2003, with abnormal highs in March and October and a higher than normal rainy season June to August. Total yearly precipitation was highest for all years of collection at 81.73 inches. Population collections were made in May, during a month of average rainfall. June began flood conditions of the rainy season, and females were collected for fry production studies in July when rainfall remained elevated. Caged exposures occurred in August, and flooding during this month contributed to premature termination of exposures. Most recent collections for fry production studies were monthly from May to August 2004. Precipitation data is complete through September and indicates a normal nine month total at 50.30 inches compared to a historical nine month total of 48.59 inches. However, rainfall cycled between lower than average rainfall in May and July versus elevated rainfall in June and August.

The third system, Elevenmile Creek, was surveyed in August 2001. Fish collection coincided with normal precipitation for the rainy season. Preceding collection, rainfall was lower than normal with the exception of high rainfall in March. Total yearly precipitation indicates drought conditions at 47.53 inches versus a normal of 62.25 inches.

Table A-1. Latitude, longitude, and descriptions for mosquitofish collection sites in Rice Creek.

Rice Creek[a]	Latitude	Longitude	Description
REF1[b]	N29°36.422’	W081°36.309’	Upstream of Rice Creek at Blackbird Point in Saint Johns River
REF2[b]	N29°43.020’	W081°43.534’	Etonia Creek (at Bardin Rd), tributary of Rice Creek
REF3[b]	N29°52.461’	W082°22.030’	Santa Fe River at Hwy 121 boat ramp near confluence of New River
U(8)[c]	N29°41.233’	W081°44.510’	Upstream of discharge at S.R. 100 in Rice Creek
PRE-DIS[d]	N29°41.155’	W081°42.050’	Retention Pond 4, before discharge into Rice Creek
DIS[e]	N29°40.730’	W081°41.648’	Discharge point at first aerator into Rice Creek
D(1)[f]	N29°41.324’	W081°40.914’	Downstream of discharge at second aerator in Rice Creek
D(6)[f]	N29°41.971’	W081°39.960’	Downstream of discharge at SR 17 bridge in Rice Creek

[a]Map of Elevenmile Creek in Chapter 4
[b]REF indicates reference site, followed by identifying number
[c]U denotes upstream of discharge, followed by approximate distance (km) from discharge in parentheses
[d]PRE-DIS indicates site before discharge into the creek
[e]DIS denotes site at discharge into creek
[f]D denotes downstream of discharge, followed by approximate distance (km) from discharge in parentheses

Table A-2. Latitude, longitude, and descriptions for mosquitofish collection sites in Fenholloway River.

Fenholloway River[a]	Latitude	Longitude	Description
REF1[b]	N30°15.079’	W083°42.026’	Econfina River at US 19 crossing
REF2[b]	N30°08.556’	W083°51.944’	Econfina River at US 98 boat ramp
U(5)[c]	N30°06.177’	W083°26.369’	Upstream of discharge at US 27 in Fenholloway River
PRE-DIS[d]	N30°03.942’	W083°33.275’	Canal leading from retention ponds to Fenholloway River
DIS[e]	N30°04.069’	W083°33.326’	Discharge point at old railroad crossing in Fenholloway River
D(12)[f]	N30°04.540’	W083°39.769’	Downstream of discharge at Hampton Springs Bridge in Fenholloway River

[a]Maps of Fenholloway River in Chapters 4 and 6
[b]REF indicates reference site, followed by identifying number
[c]U denotes upstream of discharge, followed by approximate distance (km) from discharge in parentheses
[d]PRE-DIS indicates site before discharge into the creek
[e]DIS denotes site at discharge into creek
[f]D denotes downstream of discharge, followed by approximate distance (km) from discharge in parentheses

Table A-3. Latitude, longitude, and descriptions for mosquitofish collection sites in Elevenmile Creek.

Elevenmile Creek[a]	Latitude	Longitude	Description
REF1[b]	N30°46.553’	W087°20.326’	Pine Barren Creek, tributary of Escambia River
REF2[b]	N30°29.605’	W087°19.494’	Eightmile Creek, tributary of Elevenmile Creek
U(1)[c]	N30°34.986’	W087°19.705’	Upstream of discharge at US 297A in a headwater tributary of Elevenmile Creek
PRE-DIS[d]	N30°34.730’	W087°19.223’	Retention pond before discharge into Elevenmile Creek
DIS[e]	N30°34.432’	W087°19.329’	Discharge point at Kingsfield RD in Elevenmile Creek
D(5)[f]	N30°32.075’	W087°20.587’	Downstream of discharge at Ninemile RD in Elevenmile Creek

[a]Map of Elevenmile Creek in Chapter 4
[b]REF indicates reference site, followed by identifying number
[c]U denotes upstream of discharge, followed by approximate distance (km) from discharge in parentheses
[d]PRE-DIS indicates site before discharge into the creek
[e]DIS denotes site at discharge into creek
[f]D denotes downstream of discharge, followed by approximate distance (km) from discharge in parentheses

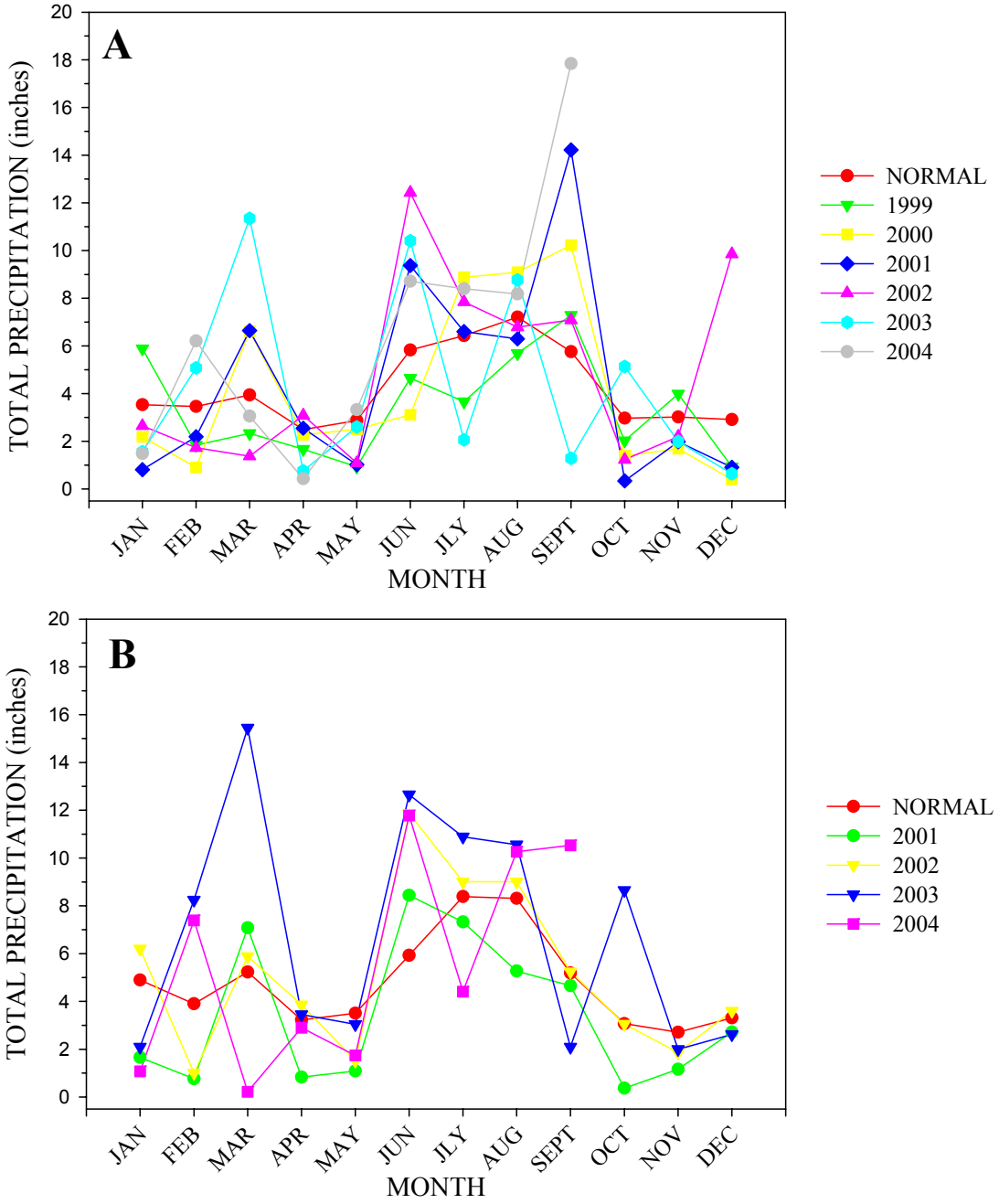


Figure A-1. Total monthly precipitation for Florida regions where mosquitofish were collected from pulp and paper mill effluent-receiving systems (National Climactic Data Center, Asheville, NC). A) Palatka region 1999 to 2004, Rice Creek. B) Perry region 2001 to 2004, Fenholloway River. C) Pensacola region 2001, Elevenmile Creek. "Normal" indicates historical average precipitation.

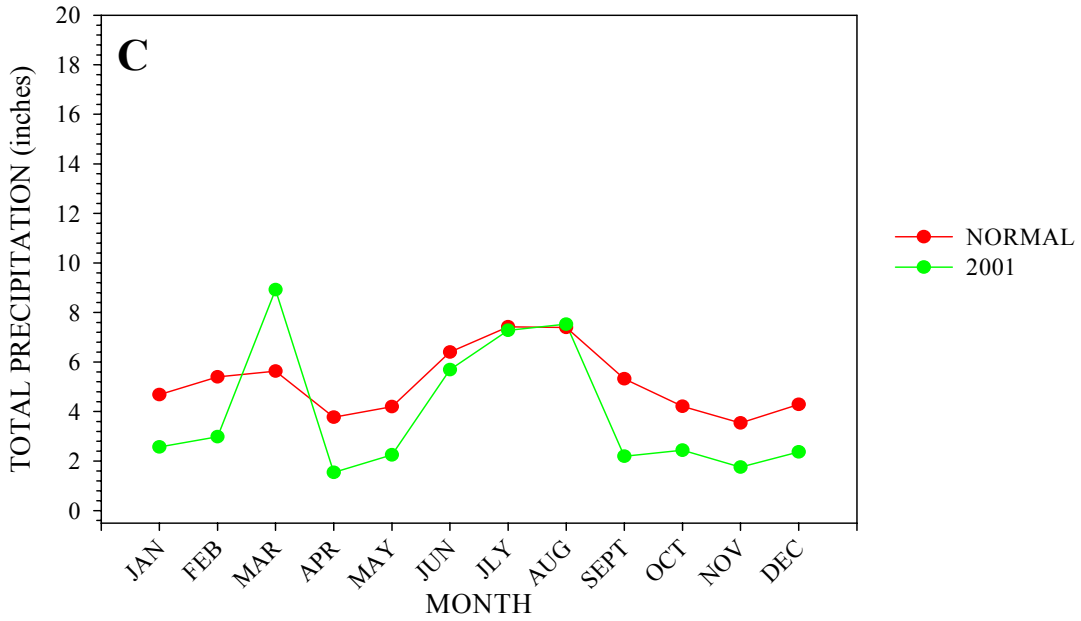


Figure A-1. Continued

APPENDIX B
SEX STEROID RADIOIMMUNOASSAY PROTOCOLS

This appendix details the working protocols used to perform radioimmunoassay of whole body sex steroids in mosquitofish. Validation statistics for this assay are provided in Chapter 2.

Digestion

- Add KOH (30%) to each fish at a rate of 3 times the body weight (volume) in individual vials. For example, add 1.2 mL 30% KOH to 0.4 g tissue. (Make sure the lid is on every cryobox before boiling; since caps will pop off of vials otherwise.)
- Boil samples in cryoboxes for 20 minutes in a shaking water bath at 80 to 100°C.
- Vortex cryoboxes for 1 minute.
- Chill cryoboxes for 5 minutes on ice.
- Remove 50 µL from each vial (4 times for 2 hormones in duplicate) and aliquot in large glass tubes for ether extraction.
- 50 µL samples in large glass tubes can be stored in a -10°C freezer before extraction; any remaining samples should be placed in a -80°C freezer for long-term storage.

Extraction

- Thaw 50 µL samples.
- Turn on the vortex evaporator (Labonco); fill the ether trap with a thin layer of methanol and dry ice, and then seal the trap.
- Fill an aliquot bottle with diethyl ether, calibrate for 4 mL, and remove bubbles from aliquoter.
- Fill a shallow tray with methanol and dry ice.
- Once samples have thawed, squirt each large glass tube with 4 mL diethyl ether

- After filling a rack, vortex the rack for 1 minute.
- Place the rack in the dry ice/methanol tray and allow every sample to precipitate into a white pellet (up to 3 or 4 minutes) Replenish the dry ice to keep the precipitation temperature low enough.
- Pour the supernatant into smaller glass tubes.
- Place small tubes containing supernatant in the evaporator.
- Turn on the vacuum and then the vortex and evaporate samples for 10 to 15 minutes. (All the ether does not need to evaporate during the first extraction.)
- Add another 4 mL diethyl ether, and then precipitate and evaporate samples a second time. However, this time make sure all the ether has evaporated.
- Evaporated samples can be stored in a -10°C freezer for up to a week.

Radioimmunoassay

- Remove evaporated samples in small glass tubes from -10°C freezer.
- Transfer small tubes to new white racks: 60 tubes to two racks for each hormone, leaving first and last columns for controls; 2 of each sample to a rack set, loaded top to bottom vertically; 11 new tubes at first and last columns; leaving an opening at the second to bottom space. Four racks can hold up to 100 samples at a time.
- Prepare the standards (8 exponential dilutions from 1-1,000 pg per hormone). Vortex each standard upon first use. Pipette 50 µL of each standard to corresponding new tubes at first and last columns of each rack set (3^{rd}-10^{th} tubes from top). Increase accuracy by using a new pipette tip for each sample. Load the weakest to strongest dilution from top to bottom. Save the standards until radioactivity has been added, then dispose them down the sink.
- Add 200 µL PBSGA buffer to each dilution standard. Add 100 µL PBSGA buffer to the total count tubes (TC) and nonspecific binding tubes (NSB) (the 4 corners of rack set). Add 250 µL PBSGA buffer to NSB (to bring total volume of PBSGA to 530 µL), maximum binding tubes ($B_{0)}$, and TC (first 2 and last tubes along first and last columns). Finally, add 250 µL PBSGA buffer to all tissue samples.
- Prepare the antibody for each hormone (Ab). Vortex Ab upon first use. Add 100 µL Ab to all tubes but TC and NSB (4 corners).
- Prepare radioactive-labeled hormones (marked with tritium). Carefully stir upon first use. Carefully add 100 µL radioactive hormone to all tubes.
- Incubate samples in a refrigerator for 24 to 48 hours.

- Prepare charcoal dextran and vortex for several minutes upon first use. Add 250 µL water to TC (at bottom 2 corners) and separate TC tubes from the rack set. Add 250 µL charcoal dextran to all other tubes within 5 to 7 minutes.

- Shake the rack set a few times, and then load and balance sample tubes in the centrifuge (Beckman J-6). Set the centrifuge at 3 rpm and spin for 10 minutes.

- Mix 4 mL scintillation cocktail with 400 µL of each sample and place mixture in a scintillation vial. Load vials into scintillation racks, and then load racks into the liquid scintillation counter (Packard Tricarb 1600).

- Dispose of waste appropriately.

APPENDIX C
POSTER AND PLATFORM PRESENTATIONS OF DISSERTATION RESEARCH

Noggle JJ, Bradley WK, Smith JT, Gross TS. Platform presentation, "Relationship between changing effluent quality and reproductive effects in Eastern gambusia." 24th annual meeting of the Society of Environmental Toxicology & Chemistry, Austin, TX, November 9-13, 2003.

Noggle JJ, Smith JT, Ruessler DS, Quinn BP, Holm SE, Sepulveda MS, Gross TS. Platform presentation, "Paper mill process modifications reduce biological effects on largemouth bass and Eastern gambusia." 5th International Conference on Fate and Effects of Pulp and Paper Mill Effluents, Seattle, WA, June 1-4, 2003.

Noggle JJ, Bradley WK, Borton DL, Smith JT, Gross TS. Platform presentation, "Comparison of anal fin morphology & hormone status in gambusia among Florida pulp & paper mills." 5th International Conference on Fate and Effects of Pulp and Paper Mill Effluents, Seattle, WA, June 1-4, 2003.

Noggle JJ, Bradley WK, Borton DL, Smith JT, Gross TS. Poster presentation, "Comparison of anal fin morphology in female gambusia among three Florida pulp and paper mills." 23rd annual meeting of the Society of Environmental Toxicology & Chemistry, Salt Lake City, UT, November 16-20, 2002.

Noggle JJ. Invited platform presentation, "Gambusia and pulp & paper mill effluents." Georgia-Pacific Corp. 2002 Environmental Conference, Atlanta, GA, September 16 & 17, 2002.

Noggle JJ, Ruessler DS, Sepulveda MS, Holm SE, Gross TS. Poster presentation, "Responses of Eastern mosquitofish to papermill effluent exposure." 22nd annual meeting of the Society of Environmental Toxicology & Chemistry, Baltimore, MD, November 11-15, 2001.

Noggle JJ, Bradley WK, Ruessler DS, Sepulveda MS, Gross TS. Poster presentation, "Considerations in mosquitofish and papermill effluent studies: analysis of methodology." 22nd annual meeting of the Society of Environmental Toxicology & Chemistry, Baltimore, MD, November 11-15, 2001.

Noggle J, Ruessler D, Sepulveda M, Holm S, Gross T. Poster presentation, "Effects of papermill effluent on secondary sex characteristics in mosquitofish." 40th annual meeting of the Society of Toxicology, San Francisco, CA, March 25-29, 2001.

Noggle JJ, Gross TS. Platform presentation, "Effects of paper mill effluents on secondary sex characteristics in mosquitofish." 4th annual meeting of the Southern Conference of Researchers in Aquatic Diseases, Gainesville, FL, February 10-12, 2001. 2nd place Best Student Presentation.

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BIOGRAPHICAL SKETCH

Jessica Joy Noggle was born north of Cincinnati in Middletown, Ohio, on September 5th, 1978. Her primary education was at public schools in the Lakota Local School District, also north of Cincinnati, and she graduated in the Top 25 from Lakota High School with an Honors Diploma in 1996. Jessica came to the University of Florida that same year on a National Merit Scholarship with an interest in wildlife veterinary medicine. Graduating Summa Cum Laude in December 1999 with her Bachelor of Science in Zoology and a minor in Wildlife Ecology and Conservation, her focus had shifted to interdisciplinary research. In January 2000, she began working as a research technician in the Ecotoxicology Program at the United States Geological Survey, Florida Integrated Science Center, Center for Aquatic Resource Studies in Gainesville, FL. Her main projects evaluated effects of pulp and paper mill effluents on aquatic wildlife, including mosquitofish. Within the year, Jessica began graduate studies at the University of Florida, becoming a Ph.D. candidate in the Department of Physiological Sciences, College of Veterinary Medicine in 2002. She received her Ph.D. with a specialization in Toxicology in the spring of 2005.