Reproductive Success of Eastern Mosquitofish (Gambusia affinis) Exposed to Pulp and Paper Dominated Receiving Streams and Effects of Masculinization Responses

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Mosquitofish (Gambusia) are sexually dimorphic; adult males have an elongation of the anal fin to form a copulatory structure known as a gonopodium. Several studies since the early 1980s have reported elongated anal fins in female mosquitofish exposed to pulp and paper mill discharge, a phenomenon known as masculinization. Although adverse impacts have been suggested in these previous reports, the influence of masculinization on reproductive success has not been assessed for pulp mill effluent-exposed eastern mosquitofish (Gambusia holbrooki or affinis). The current study compared fecundity (number of fry per female at parturition) to an index of masculinization (ratio of anal fin rays 4 to 6). Pregnant females were collected from two effluent-receiving streams in Florida: Rice Creek and the Fenholloway River, over two reproductive seasons (2003 and 2004). Masculinization was consistent between years, with clear effects at the Fenholloway River site, while the response was minimal or nondetectable at the Rice Creek site. Masculinization was not correlated with the production of fry/fecundity at either site. Data suggest differing reproductive seasonal strategies between basins and populations but do not demonstrate any effects of pulp and paper exposure on reproductive success in mosquitofish.

Key words: masculinization, reproductive success, Gambusia, pulp and paper, fish

Introduction

Sublethal effects of paper mill effluent exposure on fish have been a major focus of aquatic environmental health research for the past two decades (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, and decreased egg production (Van der Kraak et al. 1992; Gagnon et al. 1994; Munkittrick et al. 1999; NCASI 2000; Sepulveda et al. 2001, 2003; McMaster 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 have remained largely untested.

Development of male-like secondary sex characteristics in female Gambusia, specifically masculinization of the anal fin into a gonopodial-like structure, has been historically reported in mill effluent-receiving streams (Howell et al. 1980; Drysdale and Bortone 1989; Cody and Bortone 1997; Bortone and Cody 1999; Jenkins et al. 2001; Parks et al. 2001). Although negative impacts on reproduction were initially implied or suggested 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 Gambusia reared in 100% final effluent were not altered from controls (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); however, these observations were not detected in more recent studies (Felder et al. 1998; D’Surney et al. 2000). Recent reports (Noggle 2005; Noggle et al. 2010) have also demonstrated that improvements in processing technologies and reductions in black liquor losses by the paper industry have reduced masculinization responses in Gambusia relative to these initial investigations: (Howell et al. 1980; Drysdale and Bortone 1989; Cody and Bortone 1997).

Gambusia, as members of the live-bearing family Poeciliidae, develop eggs internally and ovulate immediately before parturition of fry (Meffe and Snelson 1989). As nonsuperfetating lecithotrophes, Gambusia 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 the reproductive season occurs across summer months with low to no reproduction in winter months (Constanz 1989). Environmental cues control the beginning and end of the reproductive season: the onset of the reproductive season is triggered by a rise in water temperature, while photoperiod (decreasing day length) controls gonadal recrudescence (Koya and Kamiya 2000;
Koya and Iwase 2004). This complicates the study of the effects of environmental toxicants on fecundity in this species.

A major drawback among existing studies of Gambusia and reproductive function in paper mill effluents is the lack of corresponding anal fin morphology data to evaluate the potential association, if any, between masculinization and reproduction (i.e., fecundity measured by brood size). Moreover, as far as we are aware, no research has been conducted to assess measures of fecundity across populations within a water basin (a measure of fecundity and recruitment) for Gambusia. The objective for the current study was to evaluate fry production in female Gambusia collected from effluent-dominated receiving streams, and evaluate whether masculinization alters reproductive success or function.

**Materials and Methods**

**Mill Characteristics**

The Fenholloway River and Rice Creek mills are very different in wood furnish, processing, and product. The Rice Creek mill, in operation since 1947, is a bleached/unbleached kraft mill that produces kraft and tissue papers. Furnish is 50:50 hardwood:softwood. The Fenholloway River mill, in operation since 1954, is a dissolving kraft mill that produces high grade cellulose. Furnish is 100% softwood. At the time of fish collections, both mills used aeration with microbial degradation as secondary effluent treatment. In addition, Rice Creek had activated sludge. Effluent discharge volumes at the time of collections were 106 million litres per day (mld) (28 million gallons per day [mgd]) into Rice Creek and 163 mld (43 mgd) into the Fenholloway River.

**Site Locations**

Mosquitofish were sampled during 2003 and 2004 downstream from two paper mill effluent-dominant streams in Florida where masculinized females have been previously documented (Fig. 1). The effluent-dominated streams were: the Fenholloway River associated with the Buckeye Technologies, Perry, Fla. mill; and Rice Creek associated with the Georgia Pacific, Palatka, Fla. mill. One in-stream exposed and one unexposed site from each of these two systems were surveyed for fry production.
production in females. Within Rice Creek, mosquitofish were collected from a site 8 km upstream of the effluent discharge (unexposed = RC-Upstream) and from the discharge point (exposed site = RC-Exposed). Within the Fenholloway River, fish were collected as close as possible to the discharge point which was 5 km downstream of the effluent discharge (exposed = FR-Exposed), and at a reference site several km upstream from the mouth of the Econfina River (unexposed = FR-Ref).

Fish Collections

Mosquitofish were collected at each site at one time point in 2003 to validate procedures and produce preliminary results. Fish were collected monthly for four months during 2004 to better assess reproductive success across the primary reproductive season. Dip nets and multiple personnel were used to collect mosquitofish within one day in each system. Sampling concluded at each site when an estimated 75 to 100 adult female *Gambusia* were collected for fry production studies. All *Gambusia* were kept alive in aerated bait buckets and transported back to the laboratory for processing. Water quality parameters were measured at each site at the time of mosquitofish collection: dissolved oxygen, temperature, pH, conductivity, salinity, and turbidity.

In the laboratory, *Gambusia* were sorted into age-sex groups, giving preference to gravid females for the preparation of fry production studies. Fish were handled as little as possible using latex gloves to minimize stress on gravid females. Groups were divided as follows: gravid females (gravid spot and swollen abdomen); nongravid females (lack of or partial gravid spot and slim abdomen); adult males (fully differentiated gonopodium); developing males (elongated gonopodium lacking terminal differentiations); and juveniles (<20 mm standard length and lacking gravid spot and gonopodium). Urogenital papillae were used to confirm gender when necessary using a dissecting microscope. Females were evaluated for masculinization using a standard anal fin index which represents the ratio of length for fin rays 4 and 6 (Fig. 2).

Laboratory Fry Production

Approximately fifty gravid females from each site (total 4 sites) were held for thirty days to monitor fry production for each of the 2003 and 2004 collections. Each female was placed individually in a modified plastic hatchery chamber, purchased from Aquatic Ecosystems (Apopka, Fla.), that included a hinged lid to prevent escape and a 7.6 cm length of artificial green *Cabomba* grass to provide cover for females and fry. Upper portions of the

![Female mosquitofish](image1)

**Female**

![Male mosquitofish](image2)

**Male**

**Fig. 2.** Anal fin morphology for adult *Gambusia*. Females are generally larger in body weight and length than male fish. Male fish have an elongated anal fin, representing an elongation of fin rays 3, 4, and 5, to form a copulatory structure known as a gonopodium. Females normally have no or minimal elongation of these fin rays. An anal fin index, or ratio of length for fin rays 4 and 6, compares the primary elongated fin ray with a fin ray which does not elongate to calculate an index of anal fin elongation or masculinization.
hatchery chamber were available to females, while the lower portion was accessible only by fry via a slotted barrier. Newborn fry instinctively seek escape and protection from the mother due to her cannibalistic instinct. Fish acclimated to a 50:50 pond:well water mix for 24 to 48 hours. After acclimation, hatchery chambers with females were transferred to two 1.2-m by 2.4-m by 15-cm (4' by 8' by 6") shallow tanks receiving a 50:50 filtered pond:well water mix. 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 when detected to be euthanized, counted, and preserved in 10% neutral buffered formalin for assessment of deformities and fry weight. Chambers were rinsed and females were returned to the tank for observation of secondary brood production. Females were fed ad libitum with Tropical Prime flakes (Zeigler Brothers, Gardners, Pa., nutritional composition 45% minimum protein, 9% minimum fat, 4% maximum fibre). Water quality (dissolved oxygen, temperature, pH, conductivity, salinity, incident light, and turbidity) was measured three times a week. Incident light was measured at 15 points evenly spaced throughout each tank. All other water quality measurements were made at a single location in each tank.

Measurements and Statistics

Body weight and standard length were used to calculate condition factor, \( K = \frac{\text{weight}}{\text{length}^3} \times 100 \text{ (g/cm}^3)\), as an indication of overall health used by the aquaculture industry (values of at least 1 are considered healthy, Hile 1936). For fry production studies, increased 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. 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. Interactions by site and month and anal fin index (masculinization) were also analyzed using an analysis of covariance (ANCOVA), and influence of month and anal fin index within each site were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Differences (HSD). Statistical significance was set at \( \alpha = 0.05 \) for all tests. All statistical analyses were conducted using SAS version 9.0.

The length ratio of anal fin rays 4 and 6 was calculated as a sensitive index of anal fin elongation (Angus et al. 2001; Noggle 2005). Fin morphology masculinization was analyzed within sex using one-way ANOVA to test for significant variation by site. Any data failing tests for normality and homogeneity of variance were transformed using ANOVA were analyzed for multiple comparisons using Tukey’s HSD.

Since water parameters were measured repeatedly, water quality (2004) 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 using ANOVA were also analyzed for multiple comparisons using Tukey’s HSD.

Results

Water Quality

In general, most water quality parameters (temperature, conductivity, salinity, and turbidity) were higher at effluent-exposed sites compared with unexposed sites (Table 1). The pH did not differ across sites. Dissolved oxygen was lower at the Fenholloway River exposure site during 2003, but not during 2004. Elevated temperature at effluent-exposed sites may be important for differences in reproductive stage since reproduction is initiated by a rise in temperature (Koya and Kamiya 2000).

In the fry production tanks, conductivity, salinity, and turbidity were low and comparable to the reference and upstream field sites (Table 2). Temperatures were intermediate between exposed and unexposed sites, and homogeneity of variance were transformed using log transformations. Interactions by site and month and anal fin index (masculinization) were also analyzed using an analysis of covariance (ANCOVA), and influence of month and anal fin index within each site were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s Honestly Significant Differences (HSD). Statistical significance was set at \( \alpha = 0.05 \) for all tests. All statistical analyses were conducted using SAS version 9.0.

The length ratio of anal fin rays 4 and 6 was calculated as a sensitive index of anal fin elongation (Angus et al. 2001; Noggle 2005). Fin morphology masculinization was analyzed within sex using one-way ANOVA to test for significant variation by site. Any data failing tests for normality and homogeneity of variance were transformed using ANOVA were analyzed for multiple comparisons using Tukey’s HSD.

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<table>
<thead>
<tr>
<th>Site</th>
<th>Fenholloway River</th>
<th>Rice Creek</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference</td>
<td>Exposed</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>22.8 ± 0.6</td>
<td>28.4 ± 1.0</td>
</tr>
<tr>
<td>Conductivity (µS)</td>
<td>287.4 ± 69.4</td>
<td>2,105 ± 211.3</td>
</tr>
<tr>
<td>Salinity (ppt)</td>
<td>0.2 ± 0.03</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg/L)</td>
<td>4.50 ± 0.27</td>
<td>4.92 ± 1.42</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>1.84 ± 0.27</td>
<td>13.43 ± 1.42</td>
</tr>
<tr>
<td>pH</td>
<td>7.2 ± 0.2</td>
<td>7.3 ± 0.2</td>
</tr>
</tbody>
</table>
averaging closer to exposed sites. Dissolved oxygen was adequate for fish survival (overall average 6.34 mg/L). Incident light was consistent throughout the tanks.

**Gambusia Morphology**

Body size was similar for female *Gambusia* across sites and years. Mean body weight was 0.504 ± 0.058 g, while standard body length was 29.8 ± 0.96 mm. Condition factor was above one for all collections and sites (1.91 ± 0.04), which indicated adequate general health across all sites.

**Fin Morphology**

Anal fin elongation in female *Gambusia* was detected only in the Fenholloway River exposure site in 2003 and 2004 (Tables 2 and 3). The masculinization response was not detected at the Rice Creek exposure site in 2003 or 2004. This absence represents the first time since 1998 (when the response was initially monitored at Rice Creek) that masculinization was not detected. The diminished masculinization response at Rice Creek may have resulted from a cumulative effect of process changes and upgrades at the Georgia Pacific Palatka mill since 1998 (Noggle et al. 2010).

**Fry Production**

A preliminary assessment of reproductive success and fry production for *Gambusia* as a function of mill exposure was conducted in 2003 to develop and validate husbandry techniques. The results for 2003 (Table 3) demonstrated the utility of using hatchery chambers and captive husbandry procedures to assess both reproductive success and fry production for *Gambusia* from natural field sites in the laboratory. Approximately 90% of all females subsequently produced fry under captive conditions, and approximately 18% of all females produced a secondary clutch at a mean interbrood interval of approximately 25 days. Fry weights were similar across sites during 2003 (Table 3) and the incidence of fry anomalies or deformities

| TABLE 2. Water quality parameters measured three times weekly during laboratory fry production of female mosquitoifish collected from field sites in Rice Creek during summer 2003 to 2004 |
|---------------------------------|----------|----------|
| **Mean ± SE**                   | **Range** |          |
| Temperature (°C)                | 25.2 ± 0.24 | 24 - 27  |
| Conductivity (µS)              | 292.6 ± 10.8 | 320 - 335 |
| Salinity (ppt)                 | 0.14 ± 0.01  | 0.1 - 0.2 |
| Dissolved Oxygen (mg/L)        | 6.34 ± 0.27  | 5.1 - 7.8 |
| Turbidity (NTU) *              | 0.77 ± 0.25  | 0.2 - 1.4 |
| pH                              | 8.0 ± 0.06   | 7.5 - 8.8 |
| Incident light (µmol photons/s/m²) * | 12.6 ± 0.1  | 12.1 - 12.9 |

* Measured once a week.
* At 400–700 nm.

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**TABLE 3. Reproductive and morphological characteristics of females (♀) collected from Fenholloway River and Rice Creek and monitored for fry production in 2003**

<table>
<thead>
<tr>
<th></th>
<th>Fenholloway River</th>
<th>Rice Creek</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FR-Ref</td>
<td>FR-Exposed</td>
</tr>
<tr>
<td># ♀ Start</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>Standard Length (mm) *</td>
<td>30.9 ± 0.52</td>
<td>28.2 ± 0.57</td>
</tr>
<tr>
<td>Index of Anal Fin Elongation (Ray 4/Ray 6) *</td>
<td>1.2 ± 0.01</td>
<td>1.5 ± 0.06 *</td>
</tr>
<tr>
<td>% ♀ Parturition  *</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>Total Fry (1st production)</td>
<td>689</td>
<td>531</td>
</tr>
<tr>
<td>1st Adjusted Fecundity (fry/female) *</td>
<td>19.8 ± 2.1</td>
<td>15.1 ± 3.3</td>
</tr>
<tr>
<td>1st Fry Weight (mg) *</td>
<td>8.9 ± 0.21</td>
<td>8.5 ± 0.24</td>
</tr>
<tr>
<td>% ♀ with 2nd Production *</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Total fry (2nd production)</td>
<td>47</td>
<td>129</td>
</tr>
<tr>
<td>2nd Adjusted Fecundity (fry/female) *</td>
<td>19.7 ± 1.6</td>
<td>3.9 ± 0.9 *</td>
</tr>
<tr>
<td>2nd Fry weight (mg) *</td>
<td>9.4 ± 0.25</td>
<td>7.7 ± 0.26</td>
</tr>
<tr>
<td>Median interbrood interval (days)</td>
<td>24</td>
<td>24</td>
</tr>
</tbody>
</table>

* Mean ± SE.
* Referring to primary (1st) production of surviving females.
* Referring to secondary (2nd) production of females that had primary production.
* Significantly different than REF site (p < 0.05) indicating masculinization.
* Significantly different than the unexposed site within basin.
was generally 5% or less across all sites. Observed deformities included edema, skeletal abnormalities (lordosis and scoliosis), or premature abortion of embryos. Site comparisons suggested both a decreased total fry production per site for effluent-exposed sites versus nonexposed sites within both basins (Table 3), as well as a reduced adjusted fecundity (Fig. 3; mean fry per female as a function of body weight) for exposed sites. However, females were collected in a single month during a several month period of the summer reproductive season for Gambusia in Florida. Thus, the study design did not account for potential asynchronous reproductive activity across sites. Additional analysis accounting for such variance was necessary to further verify any effects of mill exposure on Gambusia reproductive success.

To account for asynchrony of Gambusia reproduction across sites, fry production was also evaluated across four months (May, June, July, and August) of the 2004 summer reproductive season for Gambusia collected from the Rice Creek and Fenholloway River basins. Results for 2004 (Table 4) indicated significant differences across months for percent of females producing offspring, total fry produced, and adjusted fecundity for both basins, as well as site differences within each basin. While masculinization was consistently observed for adult females at all summer months from the exposed site within the Fenholloway basin, there was no detected correlation between masculinization as measured by the anal fin index and reproductive success in Gambusia. The percent of total females producing fry, the total number of fry produced, and adjusted fecundity were not associated with anal fin elongation/masculinization. Results for adjusted fecundity across sites and months did, however, indicate significant seasonal differences between the Rice Creek and Fenholloway River basins (Fig. 3), implying potentially differing reproductive strategies rather than masculinization effects. Indeed, the presence of fin elongation was not representative of fecundity, suggesting that anal fin elongation was not predictive of any observed effects on reproduction.

**Discussion and Conclusions**

In general, most adult Gambusia females produced fry regardless of pulp and paper effluent exposure. Primary clutch sizes were similar across sites and within basins, although the number of offspring per female varied widely from a few fry to several dozen. These results were within ranges reported historically for Gambusia (Krumholz 1948; Rosen and Bailey 1963; Hughes 1985; Meffe and Snelson 1989; Specziar 2004).

Although Gambusia are considered nonsuperfetating, a Bahamian species (Gambusia hubbsi) demonstrated superfetation as a possible tool to reduce reproductive costs (Downhower et al. 2002). Superfetating species produce small broods (around 1 to 5 fry) more frequently than nonsuperfetating species, and a shift to this tactic could potentially bias comparison to nonsuperfetating populations. Therefore, this trait was examined for potential alteration by mill effluents by retaining females for the full 30 days even after primary production in 2003. Fifteen to thirty percent of primary producing females also produced a second clutch of comparable size within the established 24 to 28 day interbrood interval for nonsuperfetation in this species. 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 this monitoring and interbrood period.

These studies represent the first examination of actual fry production as a measure of reproductive success in Gambusia exposed to paper mill effluents.

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**Fig. 3.** Summary of results for 2004: adjusted fecundity (mean fry per female) and the percent of total females that successfully produce fry. Rice Creek basin sites have increased fecundity and reduced % females reproducing as compared with the Fenholloway River basin. Data suggest potentially differing reproductive strategies between basins to produce adequate fry for eventual recruitment. Sites within basins do not differ regardless of mill exposure.
Reproductive Success of Masculinized Mosquitofish

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 detected obvious adverse impacts. Further, the current studies are the first to assess masculinization and the relationship to reproductive outcome.

Results indicated that reproductive success of Gambusia is likely not impacted by effluent exposure from modern paper mills. Rather, seasonal differences in fecundities have resulted in the adaptation of different site-specific reproductive strategies. Fenholloway River females displayed reduced fecundity and masculinized anal fins consistently over the observation period but with a greater percent of total females successfully reproducing. In contrast, Rice Creek females were more fecund but with a lower percent of total females successfully reproducing. These data emphasize the need to examine seasonal synchrony between sites and other aspects of reproductive strategies for a population as an integral part of assessments for potential adverse effects of exposure to enable the accurate identification of effects. It is also possible that effluent-exposed females begin the reproductive season earlier than females living in nonexposed sites, perhaps caused by higher water temperature rather than differing reproductive strategies across basins. 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 Gambusia 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 also affect fecundity. For example, increased turbidity at effluent-dominated sites may decrease predation risk of Gambusia, and 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, which may explain observed variation in fecundity between basins in this study. 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). This possibility reinforces the importance of documenting reproduction throughout the reproductive season in studies of this nature.

### Table 4. Reproductive and morphological characteristics of females (♀) collected for fry production from the Fenholloway River and Rice Creek in 2004

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>FENHOLLOWAY RIVER 2004</th>
<th>RICE CREEK 2004</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>May</td>
<td>June</td>
<td>July</td>
</tr>
<tr>
<td># ♀ Start</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard Length (mm)</td>
<td>31.3±0.3</td>
<td>26.7±0.5</td>
<td>28.5±0.4</td>
</tr>
<tr>
<td></td>
<td>1.2±0.01</td>
<td>1.1±0.01</td>
<td>1.1±0.01</td>
</tr>
<tr>
<td>%♀ Parturition</td>
<td>38</td>
<td>66</td>
<td>80</td>
</tr>
<tr>
<td>Total fry (1st Production)</td>
<td>100</td>
<td>92</td>
<td>233</td>
</tr>
<tr>
<td>Adjusted fecundity</td>
<td>3.1±1.0</td>
<td>2.3±0.7</td>
<td>5.2±1.2</td>
</tr>
</tbody>
</table>

*Mean ± SE.

ćReferring to primary (1st) production of all females.

ćSignificantly different than unexposed site indicating masculinization.

ćSignificantly different from unexposed site within month collected.
Decisions on reproductive success based only on fecundity may likely misrepresent actual population patterns; therefore, we recommend documentation of 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 the Fenholloway River may counteract the somewhat lower fecundities than the Rice Creek fish exhibited in these studies. Preliminary relative abundance data (Noggle 2005) indicated a 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 may be negatively influencing fry production.

The biological relevance of using anal fin length as a bioindicator of effects on reproductive success in *Gambusia* was weakened by initial reproductive success studies that could not link the observation with altered fecundity. Differences in fecundity may ultimately reflect an adaptation of reproductive strategy in effluent-exposed fish, as opposed to negative impacts on reproductive success. In addition, considering the reduction and in some cases elimination of anal fin elongation in female *Gambusia* exposed to pulp and paper mill discharge over the past decade (Noggle et al. 2010), mosquitofish masculinization may no longer be a useful marker of adverse effects from exposure to pulp and paper mill effluents.

Future studies would be valuable at the population or fish community level. This study did not account for ecological factors such as predation or eutrophication that may have influenced the observed differences in fecundity. Therefore, we cannot conclude that mosquitofish populations living in effluent-exposed streams are either compromised or enhanced by ecological conditions. However, preliminary relative abundance data for age/sex structure in Noggle (2005) did not suggest adverse population structures at effluent-exposed sites.

**References**


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