Evaluating naturalized populations of western mosquitofish, *Gambusia affinis*, in the Verde River watershed for biomarkers of endocrine disruption

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Abstract

Invertebrate and vertebrate aquatic organisms exhibit a number of responses to xenobiotics, making them excellent bioindicators of environmental contamination. Rivers in the arid Southwest may be particularly vulnerable to chemical pollution as there is limited availability of surface water for dilution effects. Based on a prior study of benthic macroinvertebrate community structure in the Verde River watershed, Arizona, we identified five sites that may represent different pollution loads and a site that receives only spring-fed water. To determine whether these sites may also affect endocrine function in aquatic vertebrates, we used naturalized populations of western mosquitofish (Gambusia affinis) as a bioindicator for estrogenic or androgenic markers of pollution. There were no significant intrasex differences in anal fin lengths among all sites, suggesting that fish raised in these waters did not experience feminization or masculinization during development. However, body length in females and mass in males differed among sites, but these were not related to predicted pollution levels. The lack of differences in anal fin length suggests that there is no overt disruption of androgen activity in these sampled reaches. However, the differences in body size and mass may be related to

complex biotic and abiotic interactions within the watershed. Our results suggest that water in the areas sampled may not affect androgenic activity in these fish. Furthermore, this study illustrates that within a watershed's geographic microscales there are differences in population traits. These differences need to be taken into account when trying to find correlations between pollution and physiological outcomes in natural populations.

Introduction

We study rivers in order to assess whether the water is safe for human health and protect aquatic life (ADEQ, 2008). The Verde River is specifically of interest because it is an important natural resource that provides ecosystem goods and services. Due to the presence of perennial waters, this waterway is a critical habitat for several rare, threatened and endangered species; primary and secondary habitat for multiple wildlife species. It is also a source for municipal water supplies (Phoenix) and agricultural uses, recreation uses and local uses. The Verde River is considered to be a multi-use waterway and surrounding land use – municipal, agricultural, cattle grazing, silviculture, mining and recreational – results in multiple potential point and non-point pollution sources (Black et al., 2005)

Prior macroinvertebrate community analysis suggests impairment (Shroer, 2012, Master's Thesis). Due to limited dilution effects in the arid southwest, it may increase risk of significant exposure to endocrine disrupting compounds (EDCs).

In order to test for EDCs we used mosquitofish (*Gambusia affinis)* are an indicator species in similar studies and are widely distributed and readily available (Chinathamby et al., 2012; Hou et al., 2011; Norris et al., 2011; Xie et al., 2010; Game

et al., 2006; Toft et al., 2003; Batty and Lim, 1999). *G. affinis* exhibit easily discernible changes in gonopodial morphology (anal fin length; Figure 2) in response to estrogenic or androgenic EDCs (Angus et al., 2001) can show masculinization (Howell et al., 1980) or feminization (Angus et al., 2005). We also are in the process of running enzyme-linked immunoabsorbent assays (ELISA) to test for a protein called vitellogenin. Estrogen disruption can be measured in male fish by quantifying levels of the biomarker vitellogenin. Vitellogenin is an egg yolk protein that is present in female fish, but can be produced in males through estrogen receptor activation by EDCs (Kamata et al., 2011). Vitellogenin presence in the blood of males indicates endocrine disruption because it is not present naturally; therefore the levels of vitellogenin in males indicate levels of the amount of EDC exposure (Tolar et al., 2000).

Methods:

- Five Verde River sites (treatment) and one spring-fed pond reference site (Table 1; Figure 2).
- Collocated all fish collections from sites found to have unique or interesting macroinvertebrate communities.
- Fish collected using minnow traps and transported in oxygenated coolers to NAU's BioAnnex.
- MS-222 (0.2% solution) euthanization
- Fish morphometrics:
    - Total length (TL): snout to caudal fin tip
    - Standard length (SL): snout to penduncle tip

- Body Mass (BM)
- Number of Embryos

- Statistical Analysis: Students T-test with Bonferroni correction ($\alpha = 0.0125$).
  - Gonopodial length (GL)
    - Fourth anal fin length to SL ration (R4: SL)
    - Sixth anal fin length to SL ration (R6:SL)
    - Anal fin rays 4th through 6th ration (R4:6)

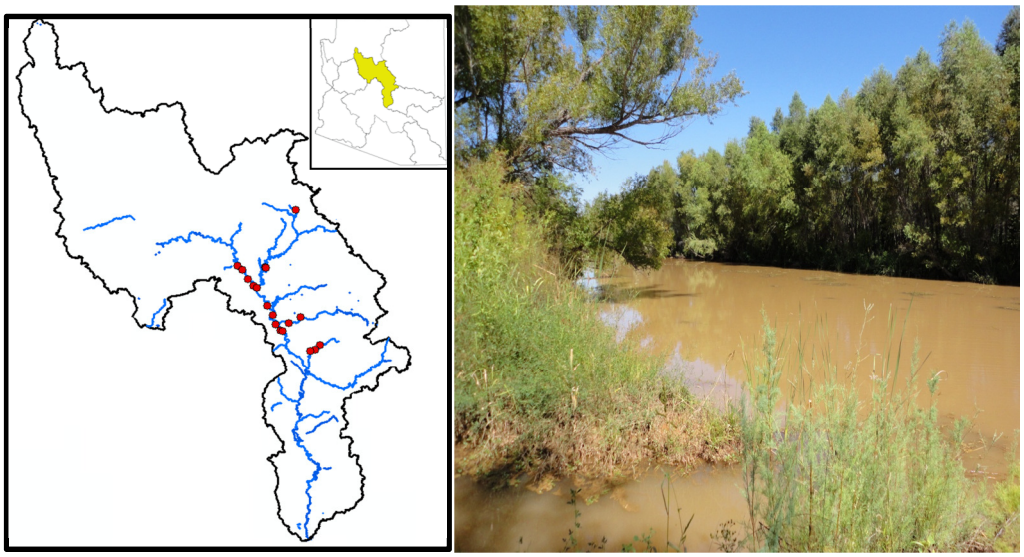

Figure 1. Habitat of the Verde River (left panel) and map of the Verde River Watershed with study sites in red (right panel).

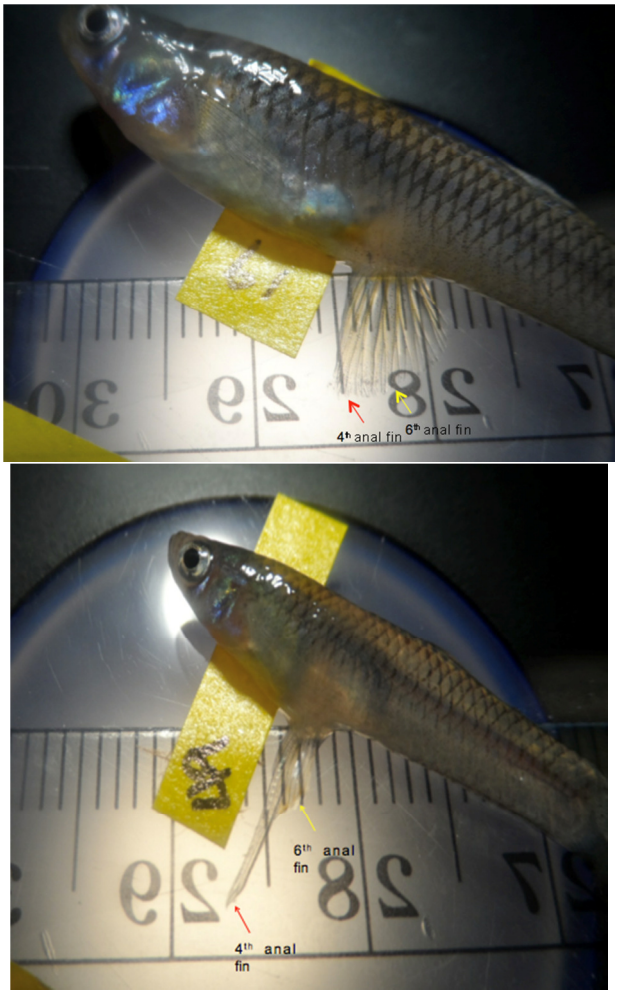

Figure 2. Male (left panel) and Female (right panel) 4th and 6th anal fins, clearly showing elongation of the 6th ray.

Results

- Condition factor (BM/SL) was significantly less (Student's t-test; $p \leq 0.01$) in males from one site (Site 4) compared to the reference site but was not significant for other sites in males or for any sites in females (Fig. 3).
- In females, R4:SL from several sites (Sites 2, 4 and 5) and R6:SL from one site (Site 5) were significantly less (Student's t-test; $p \leq 0.01$) (Fig. 4) from the

reference site; however, no significant differences in R4:SL or R6:SL were seen in males (Fig. 5).

- No significant differences were seen in TL, SL, GL, BM or R4:6 (Fig.6) within or between sites in either sex. Fish from two Verde River sites (Sites 4 and 6) tend to have more embryos, but the differences were not significant when compared between all sites (Fig. 7).
- Although we found differences in body mass and standard length when compared between sexes, within sexes all sites were found to be non-variant across sites (Fig. 8).
- ELISA optimization helped determine the ideal primary and secondary antibody concentration to utilize for vitellogenin identification (Fig. 9).

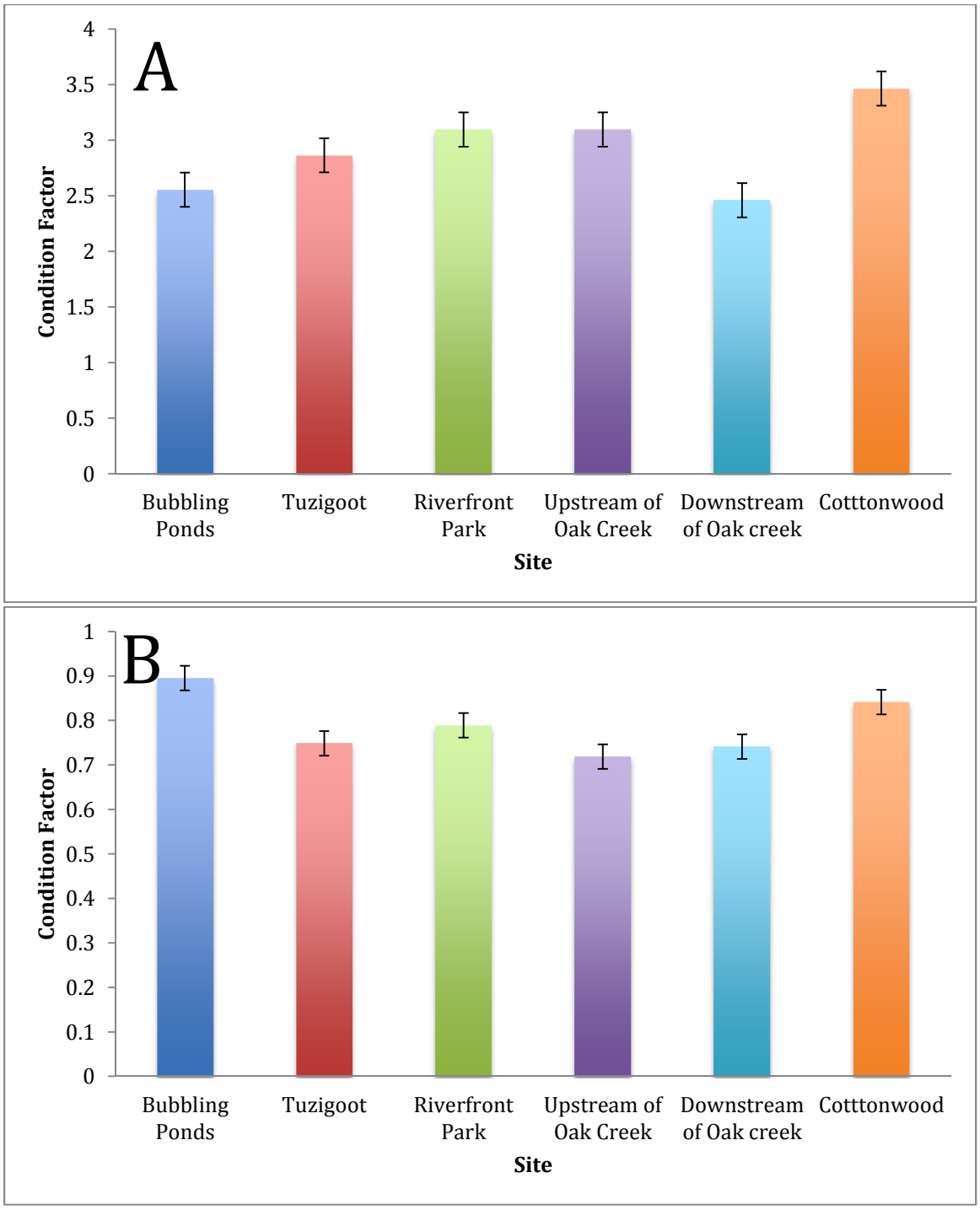

Figure 3. Condition factor (body mass/ standard length) of female (A) and male (B) *G. affinis*

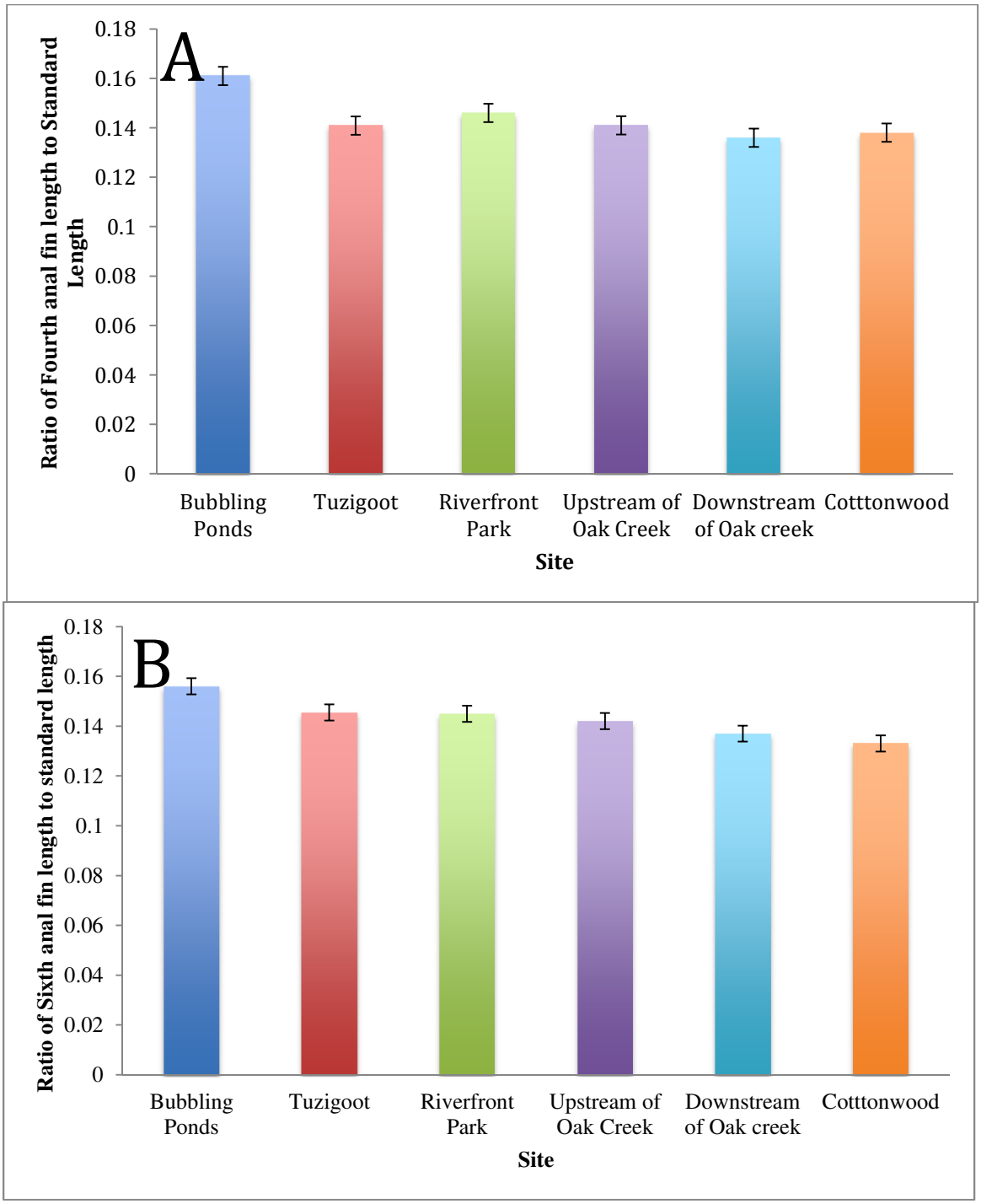

Figure 4. The ratio of fourth (A) and sixth (B) anal fin length to standard length in female *G. affinis*

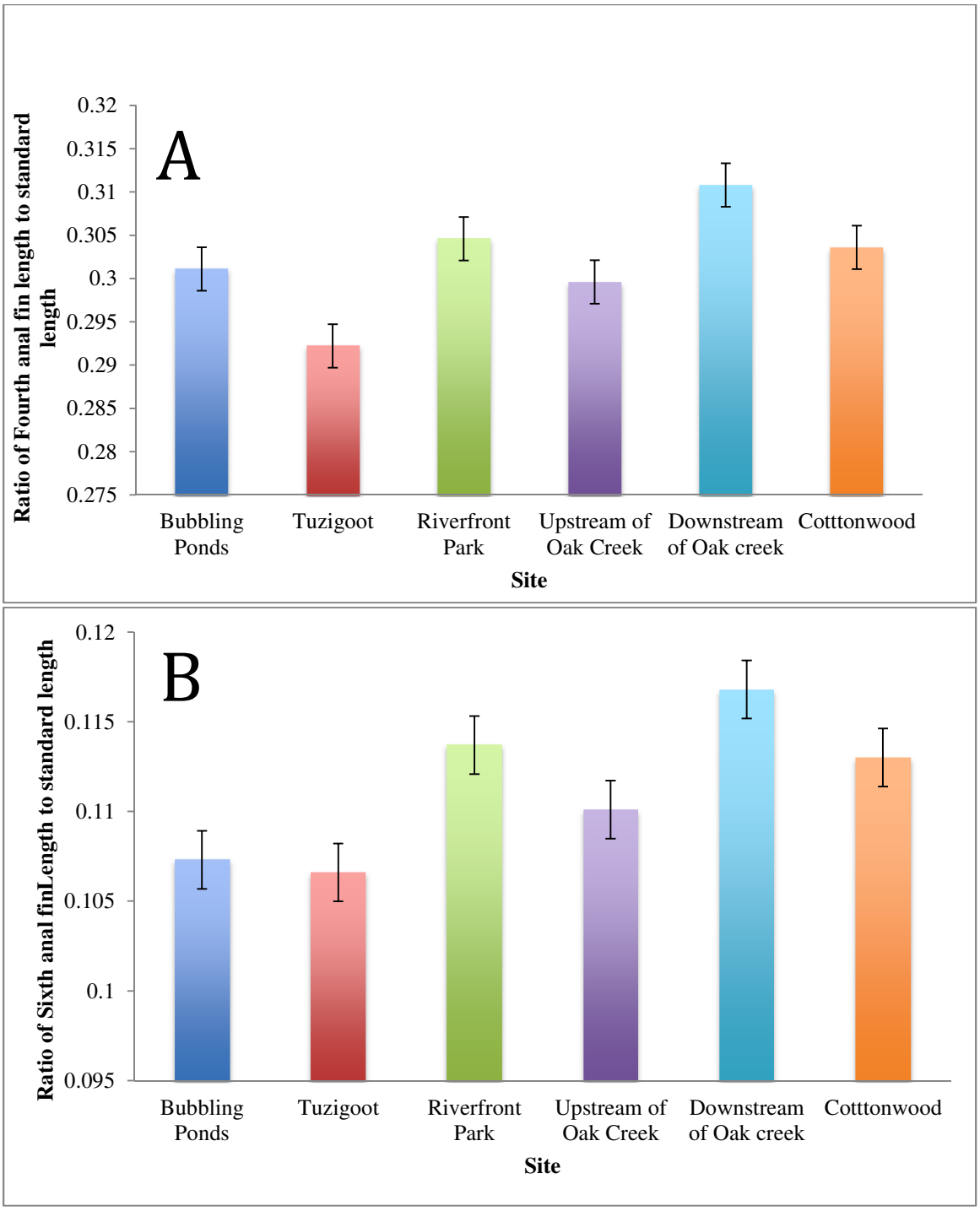

Figure 5. The ratio of fourth (A) and sixth (B) anal fin length to standard length in male *G. affinis*.

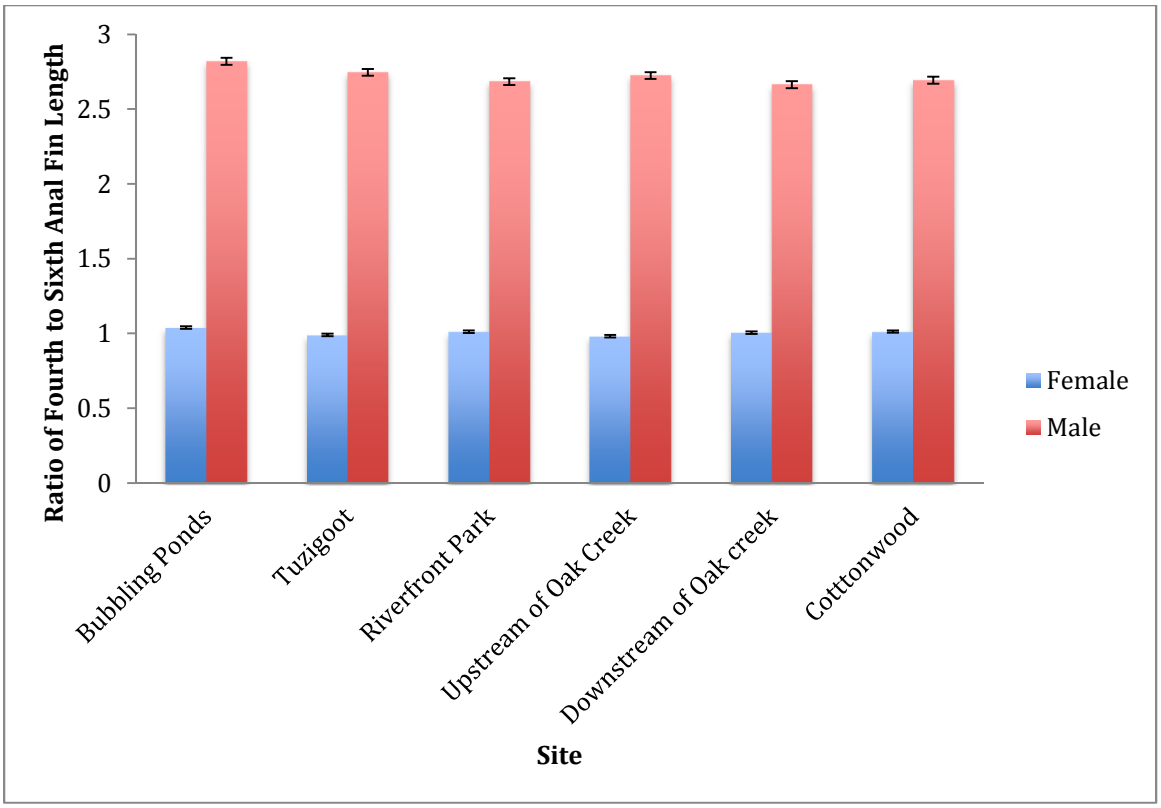

Figure 6. The ratio of fourth to sixth anal fin length of female and male *G. affinis*.

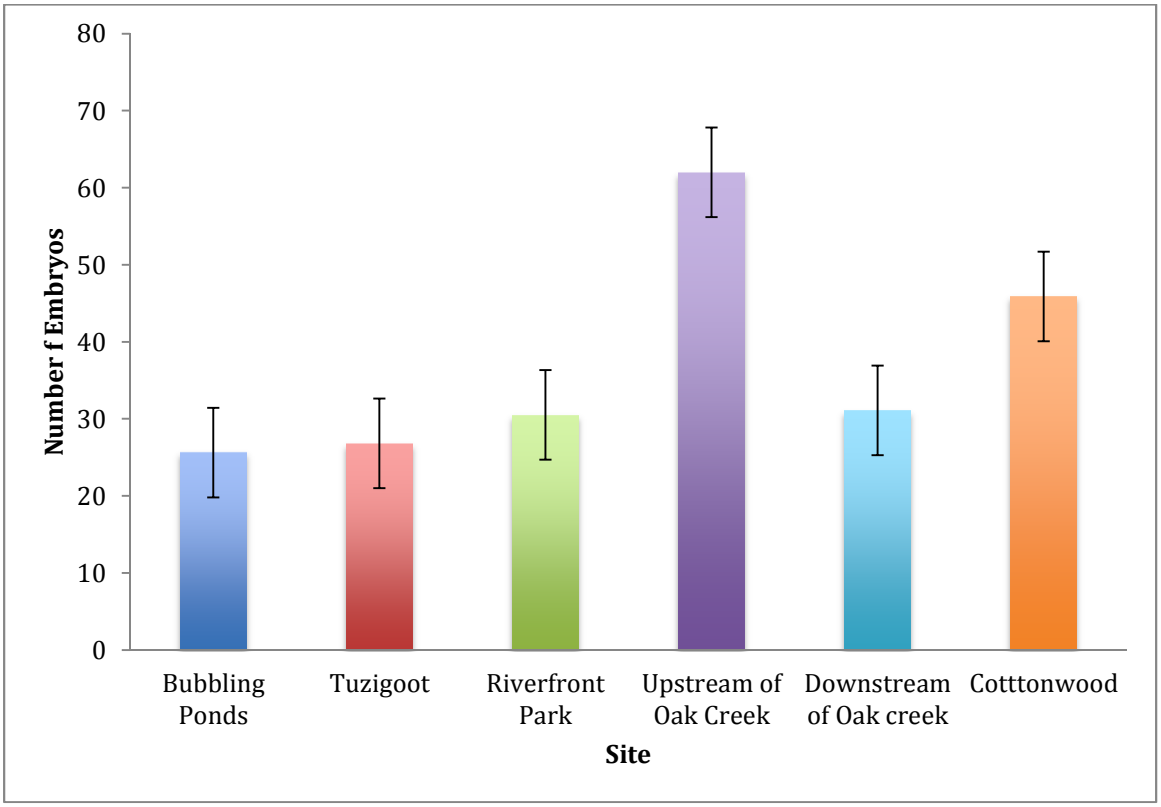

Figure 7. The mean number of embryos per female *G. affinis*.

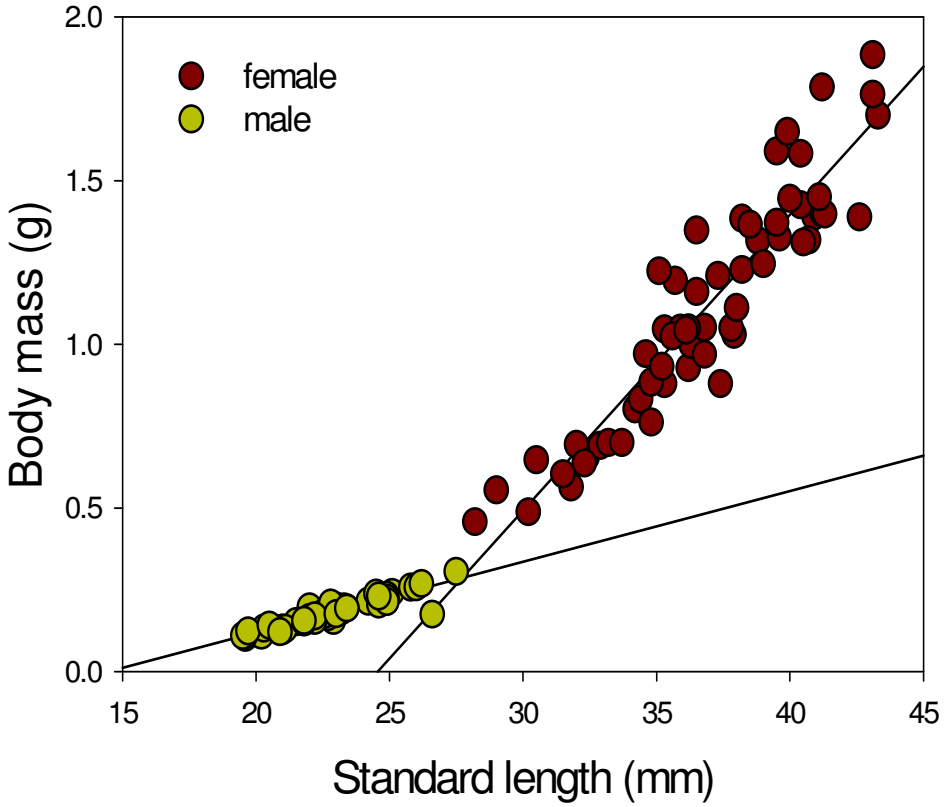

Figure 8. Relationship between body mass and standard length of female and male *G. affinis*.

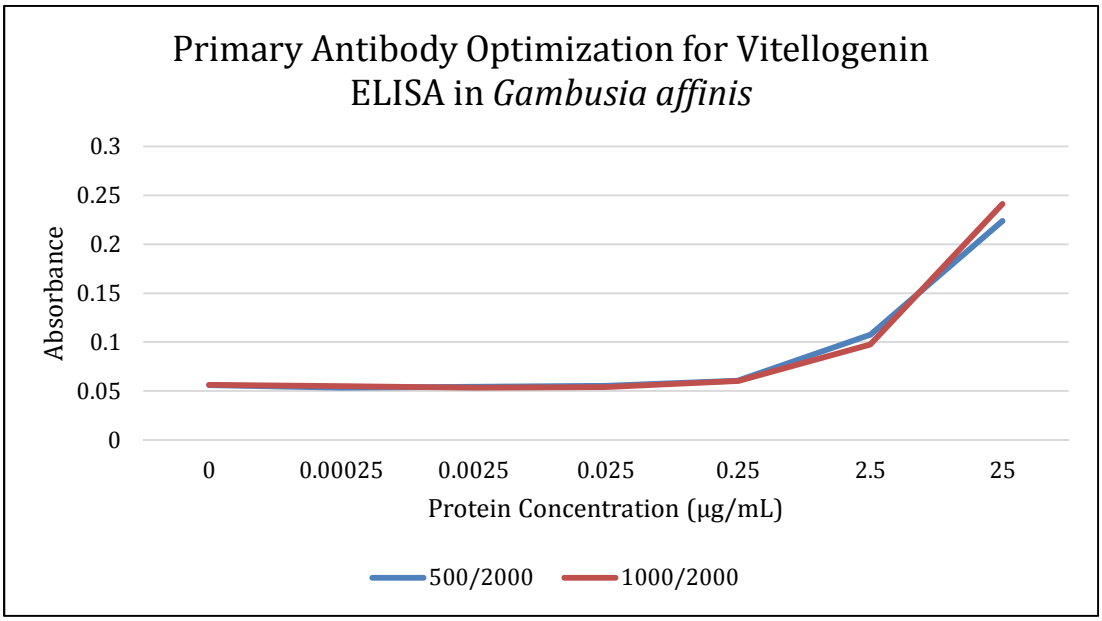

Figure 9. Primary antibody optimization for vitellogenin ELISA

Summary & Discussion

Our morphometric results indicate no overt evidence of estrogen or androgen agonist or antagonist activity that affects mosquitofish development from the reaches sampled in the Upper Verde River, Arizona. Past studies have found that statistically significant differences in gonopodium length between sites indicate alteration of androgen production caused by EDCs (Batty and Lim, 1998). Although significant differences between some treatment and reference sites were observed for condition factor in males and R4:SL and R6:SL in females, we observed no significance in the R4:6 comparison between sexes. Based an earlier study showing that only the ratio for R4:6 between females and males can indicate an androgenic response (Angus et al., 2001), the absence of differences in gonopodium length in this study suggest there is no change in androgen production nor any androgenic compounds in the water. Variation in condition factor may be due to physiology or biotic (e.g., food resource availability, feeding regimes, etc.) or abiotic (e.g., weather, stream flow) interactions within each ecosystem (Marentette et al., 2010).

Significant differences in R4:SL and R6:SL at several sites may be caused by site-specific variability, including physicochemical factors (e.g., temperature; Meffe 1992), social environment (e.g., sex ratios; Bisazza and Marin 1995) and population density (Zulian et al. 2005) affecting general fin growth compared to overall body growth. Adaptive selection in fish populations chronically exposed to contaminants may result in evolution of resistant phenotypes, as observed in Atlantic killifish (*Fundulus heteroclitus*) in severely contaminated habitats (Nacci et al. 2009). Such

exposures may lead to population differences in susceptibility that may make populations resistant to further disruption.

Although our results initially suggest that there may not be overt indications of EDC contamination in these regions of the Verde River watershed, it is possible that evaluating current natural populations for EDC outcomes can be confounded by a combination of resistance related to past selection and phenotypic plasticity to highly variable environmental conditions (Chinathamby et al., 2012). Confirmatory laboratory exposures of fish from unexposed regions were conducted and morphometric indicated nothing significant. ELISA optimization demonstrated the ideal concentration of primary and secondary antibody to use when running further laboratory tests of fish.

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