Endocrine Disruption in Male Mosquitofish (*Gambusia holbrooki*) Inhabiting Wetlands in Western Australia

Running Title: Endocrine disruption in mosquitofish, West Australia

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Abstract

The use of gonopodial indices as potential indicators of endocrine disruption in the mosquitofish *Gambusia holbrooki* inhabiting south west Australian wetlands was investigated. A minimum of fifty mature males was collected from each of five water-bodies in the Swan Coastal Plain, Western Australia, in order to measure morphological features related to reproduction. A set of morphological measurements were used to derive the following indices: gonopodium length/standard body length, pre-anal length/standard body length, the index of elongation and the percentage of male fish with hooks on the distal end of the gonopodium. Indices of male mosquitofish collected from Jack Finney Lake, located in the Curtin University campus, suggest the presence of endocrine disrupting chemicals (EDCs) in this water-body, while those from Lake Kulinup suggest this is a site of concern. Indices of male fish from the Wagerup wetland, Lake Monger and Loch McNess indicate that fish inhabiting these wetlands are not affected by EDCs. This preliminary study suggests that EDCs may be present in a number of wetlands of the Swan Coastal Plain. Further study using EDC specific markers such as vitellogenin induction in male mosquitofish is required to confirm whether EDCs are present in these water-bodies.

Keywords – biomonitoring, endocrine disruption, morphological indices, *Gambusia*

Introduction

In recent years concern has increased over the effects of anthropogenic chemicals on the endocrine systems of wildlife. The hypothesis that widespread low-level exposure to endocrine disrupting chemicals (EDCs) can potentially affect an organism's development and a population's viability is well documented. Studies have indicated that chemicals with endocrine disrupting properties can alter the development of secondary sexual characteristics and may impair the reproductive functions of fish (Jobling *et al.*, 1998; Batty and Lim, 1999; Doyle and Lim, 2002; Toft *et al.*, 2004; 2005).

The environmental effects and risk posed by EDCs in Western Australia (WA) are poorly understood, due to a paucity of recorded studies. It is recognised that EDCs have the potential to enter WA's environment from local mining and manufacturing processes, sewage discharges, and waste management practices (Rose *et al.*, 2003). A few studies have been completed on the effects of EDCs in WA's marine environment such as imposex in intertidal whelks as related to tributyltin (TBT) exposure (Reitsema *et al.*, 2003). To date, no studies have investigated the presence of EDCs in WA's freshwater environments, although a field study on the east coast of Australia have described altered morphological characteristics in freshwater fish chronically exposed to EDCs (Batty and Lim, 1999).

This preliminary study aimed to investigate the possible presence of EDCs in freshwater wetlands of the Swan Coastal Plain, by comparing morphological characteristics in male mosquitofish (*Gambusia holbrooki)* living in potentially contaminated wetlands with those from a reference lake.

G. holbrooki belong to the family of live bearing freshwater fish, the Poeciliidae (Batty and Lim, 1999). They are native to Southern and Central America and were introduced into Western Australia in the 1930s for mosquito control. Th*e* fish are easily collected in Perth's metropolitan water-bodies due to their wide distribution, large population sizes and, as they are strongly tolerant to a wide range of environmental conditions, and survive in heavily contaminated water-bodies (Morgan *et al.*, 2004).

Male *G. holbrooki* are excellent biological indicators of endocrine disruption, due to the fact that they have distinguishable hormone dependent sexual dimorphism under androgenic or oestrogenic control (Batty and Lim, 1999; Toft *et al.*, 2003). On reaching sexual maturity, the anal fin and spinal column of the male undergoes a series of hormone dependent changes that result in the development of a gonopodium, which is used for the transfer of spermatozeugmata during copulation. There are 10 rays in the anal fin of *G. holbrooki* (Doyle and Lim, 2002). In sexually immature males, the anal fin rays numbered 3 through to 6 are all approximately the same length. However, during the development of the male gonopodium, anal rays 3, 4, and 5 elongate and are modified becoming approximately twice as long as the other rays in the fin. In the female both the anal fin and the body increase in size proportionally, and growth of both continues after reaching sexual maturity (Angus *et al.*, 2001).

The ratio of the length of ray 4 (which is in the centre of the gonopodium) to ray 6 can be considered as an index of maturity, known as the index of elongation (Angus *et al.*, 2001). During sexual maturation hooks and serrae develop on the distal portion of the gonopodium. The hooks and serrae act as holdfast devices during the transfer of spermatozeugmata, and their presence indicates that the gonopodium is fully developed and the fish sexually mature (Doyle and Lim, 2002).

Materials and Methods

Sampling sites

G. holbrooki were randomly sampled at five water bodies located around Perth, Western Australia. These included wetlands located in Alcoa Parklands surrounding bauxite refinery sites at Wagerup and Pinjarra, as well as the metropolitan lakes, Lake Monger, Jack Finney Lake and Loch McNess. The animals were treated humanely according to the Curtin University Animal Ethics Approval number N-53-03.

The Wagerup Alcoa wetland is located on the land surrounding Alcoa's Wagerup Bauxite Refinery approximately 125 km south east of the Perth central business district (CBD) (Figure 1). Alcoa uses the land surrounding the refinery as experimental farmland for cropping and livestock rearing. No drains enter or discharge from this wetland. Its main water sources are rainfall, groundwater seepage and surface water run-off from the surrounding area. The wetland may be contaminated by polycyclic aromatic hydrocarbons (PAH's) associated with the use of farmland vehicles. Excess nutrient inputs may also occur from fertilizers used in farming practices and from livestock waste. These contaminates have the potential to be carried into the wetland with surface water runoff and groundwater seepage.

The Pinjarra Alcoa wetland is situated in farmland surrounding Alcoa's Pinjarra Refinery approximately 90km south east of the Perth CBD. This wetland is known as Lake Kulinup and is an important regional wetland formed from a former clay "borrow" pit at the refinery (Alcoa, n.d.). This wetland is relatively closer to the refinery than the wetland at the Wagerup Refinery, which could increase the chance of contamination from by-products of the refinery. However this is considered unlikely as all process fluids are contained and there is no avenue for contamination via surface water runoff or ground water seepages (Simon Sandover, Alcoa, pers comm.). The lake has the potential to receive excess nutrient inputs with surface water runoff carrying fertilizers that are used in the surrounding farmland areas.

Lake Monger is a large urban water-body with high recreational values located 5 km from the Perth CBD. The lake has been used as a stormwater drainage basin since the early 1900s, being fed by underground springs, groundwater and 23 stormwater drains. It eventually discharges into the Swan River via the Mounts Bay Drain. Lake Monger is surrounded by urban land uses with rapid urbanization causing a decline in the health of this lake. The clearing of riparian vegetation has led to nutrient enrichment in the waterway (Lund and Davis, 2000).

For 59 years, Lake Monger was used as a site for rubbish dumping for the reclamation of shallow swamplands. This practice ceased in 1966, however in some reclaimed areas the rubbish remains 2 to 3 metres below the parkland surfaces. Studies conducted in the early 1990’s found levels of arsenic, zinc copper, lead, phenols and fenitrophion in the water column exceeded recommended levels for aquatic life. PAHs and high levels total phosphorous are also present in the lake (City of Perth, 1992). The main sources of these contaminants include surface water runoff from surrounding urban areas and extensive drainage from surrounding roads including an adjacent freeway. The sandy soils surrounding the lake lack any capacity to retain nutrients (Swan River Trust, 1999) and fertilizers and herbicides used in lawn maintenance contribute to the lake’s contamination

(City of Perth, 1992; Lund and Davis, 2000).

Jack Finney Lake is located on the Curtin University Bentley campus. It is an artificial lake formed by lining the bottom of a hollow with clay. Two drains enter the lake, one from the surrounding parking area and lawn, and the other from an adjacent roadway. The water flowing in from the drains and surface water runoff from its surrounds are suspected to contribute contamination. Consequently, the lake has the potential to receive PAHs via surface water runoff and groundwater seepage. In addition, the lake receives inputs from the surrounding grassed area that is regularly treated with fertilizers and herbicides.

Loch McNess is located in the northeast section of Yanchep National Park, approximately 50 km north of Perth. Compared to other metropolitan wetlands, the conductivity and nutrient levels in Loch McNess are very low, which is attributed to the constant inflow of fresh groundwater and minimal anthropogenic inputs (Gordon *et al.*, 1981), and as such the lake is considered to have high environmental values. For these reasons, Loch McNess was used as the reference site in this study. Yanchep National Park is used for recreational purposes, and fertilizers are used to maintain the lawns, ovals and a golf course in the area surrounding Loch McNess. Surface water runoff and groundwater seepage have the potential to carry these fertilizers into the lake. Nutrients leaching into surrounding ground from underground septic tanks, used in the park since the 1930s, enter the groundwater. However, based on the low recorded nitrogen levels in the lake, the tanks do not appear to have had a significant effect on the water quality of the lake (CALM, 1989).

Sampling and measurement of mosquitofish characteristics

A minimum of fifty male *G. holbrooki* was captured at each site, using a dip net on a rod. Fish were placed in sealed plastic bags immediately upon capture and put on ice, until returned to the laboratory where they were frozen in a –20°C freezer. The fish were thawed before they were sorted by sex and maturity levels. Only mature males from each site were used for measurement. The presences of hooks on the gonopodium was considered an indication of sexual maturity; however males that lacked hooks but had standard lengths equal to or greater than the smallest individual with hooks from the respective site were also measured as they were considered to be of similar age. Sampling occurred in October 2004.

The measurements recorded for each mature male included standard body length, gonopodium to snout length (pre-anal length), 4th ray length (gonopodium length), 6^{th} ray length, and the presence or absence of hooks on the distal portion of the gonopodium (Figure 2). Measurements were taken under a dissecting microscope using a ruler with 0.1mm divisions. The following indices were calculated: (1) the gonopodium length/standard body length ratio, (2) the pre-anal length/standard body length ratio, (3) the 4^{th} ray/6^{th} ray ratio, and (4) percentage of fish with hooks. As no single index can reveal the severity and extent of endocrine disruption, this suite of complementary ratios were used in order to provide a better overview of endocrine disruption effects.

Statistical Analysis

For each index, the data were tested for normality and homoscedasticity. Where these conditions were not met the data was log-transformed. Statistical analysis was undertaken using the statistical package, SPSS 11.5 for Windows. Data for the morphological indices of the gonopodium were analysed using one-way analysis of variance (ANOVA). Where significant differences between sites were found ($p \leq 0.05$), Tukey's Multiple Comparison Test was used to identify differences between the means. The presence/absence of hooks on the gonopodium was tested using the Kruskal-Wallis Test with "site" as the grouping variable. Data are presented as mean ± standard error (SEM).

Results

The mean standard lengths of male fish were significantly different between sites ($p < 0.001$; Table 1). Fish at Jack Finney Lake and Lake Kulinup were significantly shorter than those from the reference site, Loch McNess. The largest mean standard length was recorded at the reference site.

Significant differences occurred in the mean gonopodium lengths of male fish between sites ($p < 0.001$; Table 1). Male fish from Jack Finney Lake and Lake Kulinup had significantly smaller mean gonopodium lengths relative to those from the reference site. However, the largest mean gonopodium length was recorded at the Wagerup wetland.

The mean ratios of gonopodium length/standard body length of the male fish also significantly differed between sites ($p < 0.001$; Figure 2A). Male fish from Jack Finney Lake had a significantly smaller mean ratio than those from the other sample sites except for Lake Kulinup. Male fish captured at the Wagerup site recorded the largest gonopodium length /standard body length ratio.

The mean pre-anal length/standard body length ratio of male fish showed a significant difference between the sites ($p < 0.001$; Figure 2B). Male fish from the Wagerup site had a lower ratio than that of all other sample sites, while the ratios for male fish from Jack Finney Lake were significantly higher than fish from all the other sites.

The mean index of elongation (the ratio of the 4th ray length/6th ray length) of male fish showed a significant difference between the sites ($p < 0.001$; Figure 2C). Male fish from Jack Finney Lake were shown to have a significantly smaller ratio than individuals from all other sample sites, while fish from Lake Monger were found to have the largest ratio.

The percentage of male fish with gonopodial hooks was significantly different between populations of the wetland sites sampled ($p < 0.001$; Figure 2D). All male fish sampled from Loch McNess and Wagerup Alcoa wetlands, had hooks on their gonopodia, the percentage of which were significantly higher than those from the other sites. The percentage of male fish with hooks on their gonopodia from the latter sites (Jack Finney, Lake Kulinup and Lake Monger) differed significantly from one another. Male fish from Jack Finney Lake had the lowest percentage of gonopodia with hooks.

Discussion

The results from this preliminary survey (summarised in Table 2) suggest that endocrine disruption is occurring in some wetlands around Perth, as there were significant differences in the morphological indices between populations inhabiting the selected sample sites. Doyle and Lim (2002) showed significant effects on the gonopodial characteristics (e.g. gonopodium length, 4th to 6th ray ratio) when exposed to varying dilutions of oestradiol under laboratory flow through conditions, which confirms that gonopodial characteristics can be affected by EDCs.

The variable standard lengths recorded between the sites indicate that the fish are exhibiting differential growth patterns. Since all the fish measured were mature males, a shorter standard length might reflect reduced growth related to exposure to contaminants, or may simply reflect the trophic status (i.e. food availability) of the environment in which they were collected. The mean gonopodial length in fish from Jack Finney Lake was significantly shorter than those at the other sites except Lake Kulinup. Batty and Lim (1999) found that a reduced gonopodial length suggests the presence of endocrine disruptors in the water, when they compared male *Gambusia* collected from up and downstream of a sewage plant. In our study however, the standard fish lengths of male fish from Jack Finney and Kulinup Lakes were the shortest recorded; correspondingly the sites that recorded the longest gonopodial lengths also had the largest standard lengths.

In order to remove the confounding effect of age and the size of the fish, the ratio of gonopodium length to standard length was investigated. As the fish are of different sizes, and thus the gonopodium lengths vary, the standard length is used in the ratio to normalise the data. The development of the gonopodium is under androgenic control and normally occurs as the testes begin to produce androgens at the time of sexual maturation. Exposure of male fish to EDCs could potentially interfere with the normal development of this secondary sexual characteristic (Angus *et al.*, 2001). Results indicate that male *G. holbrooki* inhabiting Jack Finney Lake and Lake Kulinup had the lowest gonopodium length to standard length ratios relative to fish inhabiting the other sites, suggesting that EDCs might be present at these sites. The Wagerup wetland had individual males with the highest gonopodium length/standard length ratio, followed by fish from Lake Monger and Loch McNess indicating that sexual development has progressed normally at these three sites.

The pre-anal length/ standard body length ratio relates to the forward translocation of the gonopodium as the male fish matures (Rosa-Molinar *et al.*, 1998), which is essential for its proper function (Rosa-Molinar *et al.*, 1996). This forward translocation can be affected by EDCs while the gonopodium is developing therefore; a large pre-anal length to standard body length ratio indicates possible endocrine disruption. Male fish from Jack Finney Lake were found to have the highest ratio; this was significantly greater than those from all other sites. This again suggests the presence of EDCs in Jack Finney Lake.

As the fish matures the 4^{th} ray elongates while the 6^{th} ray does not, therefore this ratio provides an index of gonopodium elongation related to proper sexual maturation (Angus *et al.*, 2001). The ultimate length of the 4^{th} ray is shorter when fish are exposed to EDCs (Doyle and Lim, 2002) and therefore, a lower ratio suggests endocrine disruption has occurred. It was observed that fish collected at Jack Finney Lake had the lowest 4^{th} ray to 6^{th} ray ratio pointing to a level of endocrine disruption; this concurs with other endpoints such as the shortest gonopodium length and the lowest gonopodium/standard length ratio. Interestingly, male fish in Loch McNess were found to have the next lowest 4^{th} to 6^{th} ray ratio, although it was significantly higher than that in fish from Jack Finney Lake. Fish collected from Loch McNess were not expected to be exposed to EDCs, as it is located in a National Park. The low value of this index at a site which is theoretically pristine calls for chemical analysis of water and sediments for the presence of EDCs. Alternatively, this result might indicate that the 4^{th} to 6^{th} ray ration is too variable between fish populations, to be used as an indicator of endocrine disruption. Male fish from Lake Monger and Wagerup Alcoa wetlands had the highest elongation ratios, supporting the notion that these sites are not affected by EDCs.

Significant differences were found in the presence/absence of anal hooks on the distal end of the gonopodia of the fish sampled from the various sites. This characteristic is an indicator of sexual maturity in males and was found to be lowest at Jack Finney Lake, while hooks were present on the gonopodia of 100% of the fish sampled at the Wagerup Alcoa wetlands and Loch McNess indicating they were all sexually mature males. In other studies, fish that were exposed to high levels of EDCs had a lower proportion of gonopodial with hooks compared with that of unexposed fish of the same age and size (Doyle and Lim, 2002). The low percentage of fish with hooks on their gonopodia at Jack Finney Lake once again suggests that endocrine disruption could be occurring in the *G. holbrooki* inhabiting this site.

In summary, there were significant differences in gonopodial characteristics of male mosquitofish from the sites sampled. Jack Finney Lake located on Curtin University grounds had gonopodial characteristics that indicated the highest level of endocrine disruption compared with that of fish from the other sample sites. This may be due to the input of PAHs from surrounding roads and car parks and from the fertilizers and herbicides used on the surrounding grassland. Added to this, Jack Finney Lake has no form of flushing and does not allow for contaminants to seep out of the lake.

The sites least affected by EDCs appear to be the Wagerup Alcoa Wetland, Lake Monger and Loch McNess. Lake Kulinup represented a site of concern that would require further study to determine the extent of endocrine disruption occurring in the mosquitofish that inhabit the lake. Lake Kulinup is located closer to the refinery than the wetland on the Wagerup Refinery site. The refinery is a possible source of EDCs due to atmospheric fallout; however this would require additional study that incorporated water quality sampling. The lack of any indication of endocrine disruption in Lake Monger was unexpected as this water body is highly impacted by surrounding anthropogenic activities. It is possible that the efforts to revegetate the banks of Lake Monger with riparian vegetation is contributing to filtering excess contaminants or the lake may is polluted with chemicals that do not induce endocrine disruption.

As no single index alone can reliably detect endocrine disruption in the mosqutiofish, it is recommended that a number of indices be used to determine the presence of EDCs in the environment. Coupled with this further studies should include more EDC specific endpoints such as vitellogenin induction in exposed male fish as well as chemical analysis for putatively identified EDCs in water and sediment samples. This will allow better assessment of endocrine disrupting effects of water bodies based on a weight of evidence approach. The age of the fish collected should also be determined by otolith analysis to ensure all the fish sampled are of similar age and maturity level.

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Table 1: Morphological measurements (Mean ± SEM) recorded on male *Gambusia holbrooki* collected from selected wetlands. Within each parameter, groups with similar letters are not statistically different ($p > 0.05$).

Site / Parameter	Loch McNess	Jack Finney Lake	Kulinup	Wagerup	Lake Monger
N	60	79	73	55	62
Standard Length (mm)	21.9 ± 0.2^{b}	20.6 ± 0.2^{a}	20.8 ± 0.2^{a}	21.7 ± 0.2^{b}	21.3 ± 0.2^{ab}
Gonopodium length (mm)	7.0 ± 0.1^{b}	6.2 ± 0.1^{a}	6.4 ± 0.1^{a}	7.2 ± 0.1^{b}	6.9 ± 0.1^{b}

Table 2: Summary of results obtained with the morphological ratios measured on *Gambusia holbrooki* collected from selected wetlands. A '+' indicates that the indices suggests the presence of EDC at this site, while a 'O' indicates that no effects were observed.

Site / Index	Loch McNess	Jack Finney Lake	Kulinup	Wagerup	Lake Monger
Gonopodium length/ Standard body length	O	+	+	O	O
Pre-anal Length/ Standard body length	O	+	+	O	O
Index of Elongation (4^{th} ray length/6^{th} ray length)	O	+	O	O	O
Hook Presence	O	+	+	O	O

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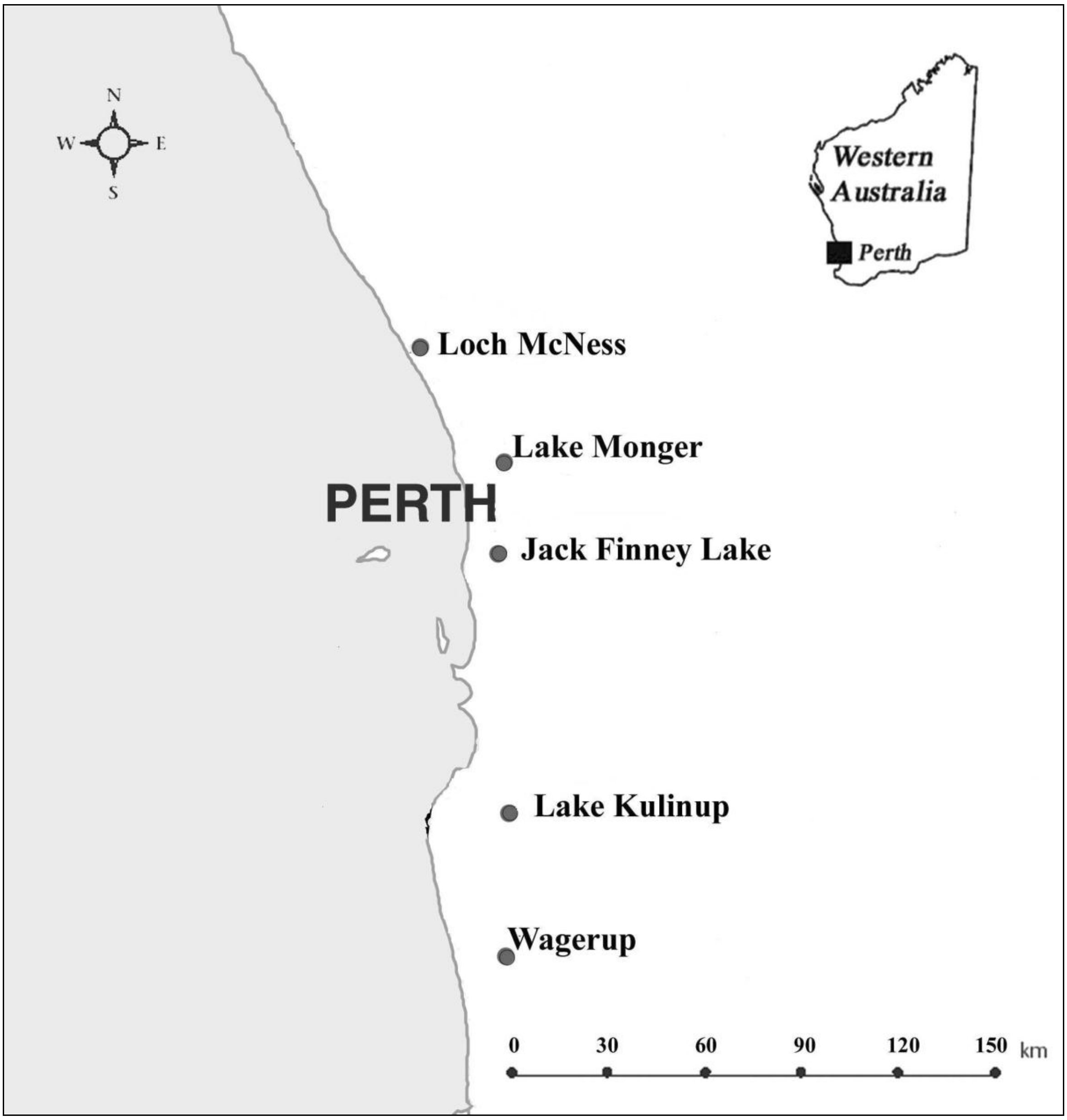
Figure 1. Location of fish sampling sites in the Swan Coastal Plain.

Figure 2. Camera lucida drawings of: (A) the modified anal fin of a sexually mature male *Gambusia holbrooki*. Fin rays 3 to 5 elongate to form the gonopodium, which is used for sperm transfer during copulation; and (B) the distal tip of the gonopodium of a sexually mature male *Gambusia holbrooki.* The presence of the hooks (H) and serrae (S) indicates that the gonopodium is fully developed acting as holdfast mechanisms during sperm transfer (modified from Doyle and Lim, 2002).

Figure 3. Mean indices values (± SEM) for the five wetlands sampled in the Swan Coastal Plain (A) Gonopodium length/standard length ratio (B) Pre-anal length/standard length ratio (C) Index of elongation (D) Hook presence (%). Bars with same letter have no significant site differences ($p \geq 0.05$). N as in Table 1.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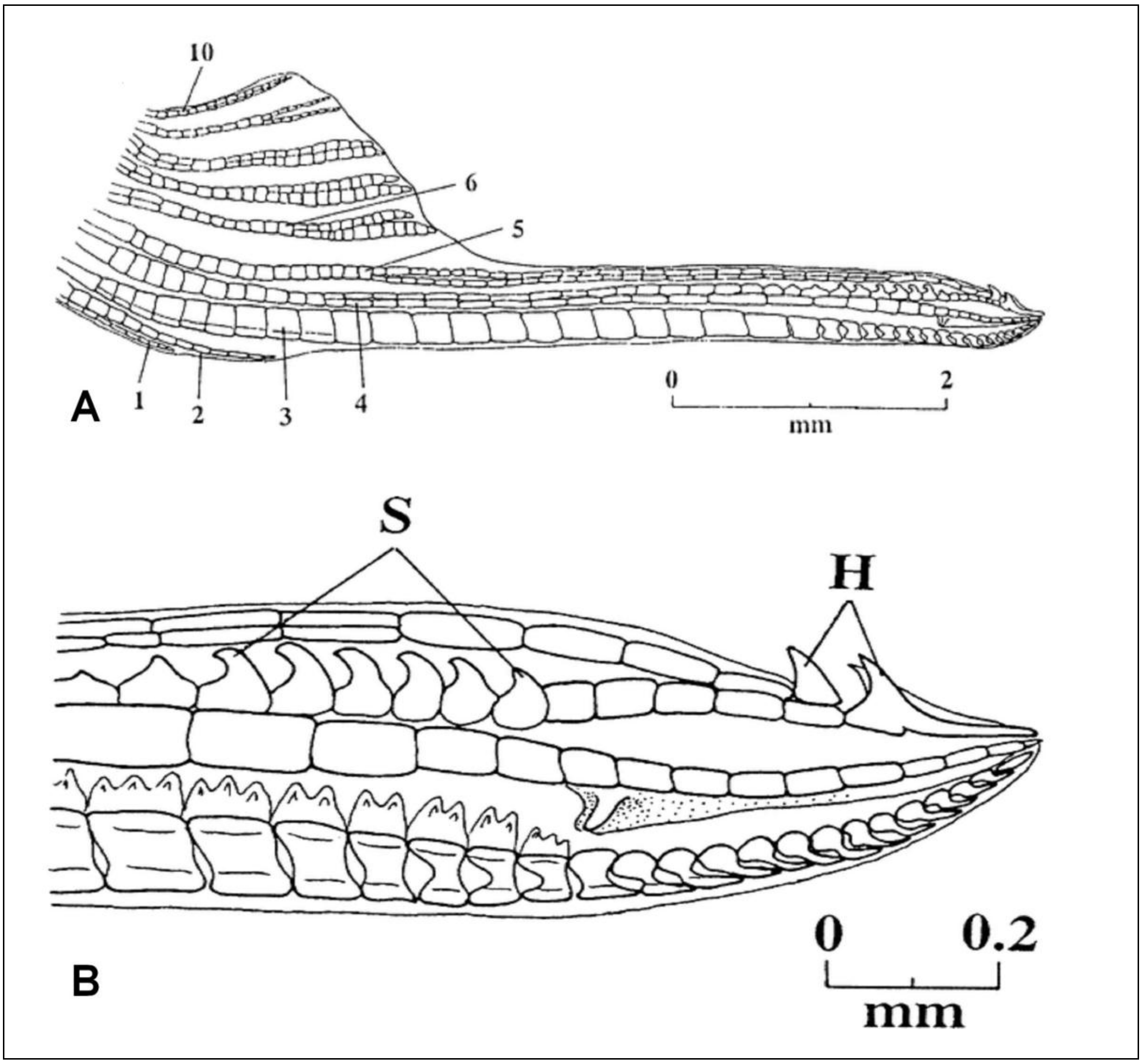
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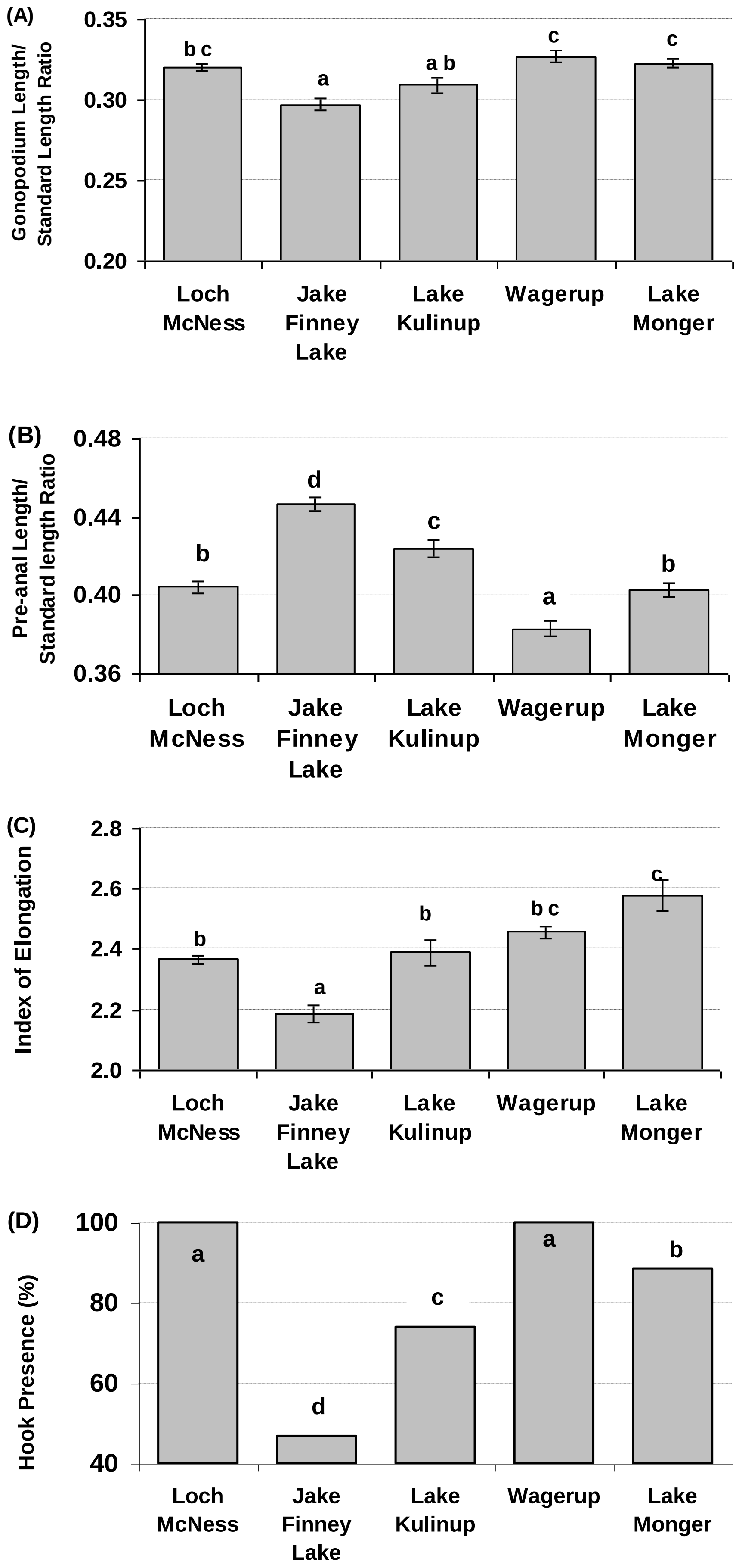
(A)
Gonopodium Length/ Standard Length Ratio
0.35
0.30
0.25
0.20
b c
a
a b
c
c
Loch McNess
Jake Finney Lake
Lake Kulinup
Wagerup
Lake Monger
(B)
Pre-anal Length/ Standard length Ratio
0.48
0.44
0.40
0.36
b
d
c
a
b
Loch McNess
Jake Finney Lake
Lake Kulinup
Wagerup
Lake Monger
(C)
Index of Elongation
2.8
2.6
2.4
2.2
2.0
b
a
b
b c
c
Loch McNess
Jake Finney Lake
Lake Kulinup
Wagerup
Lake Monger
(D)
Hook Presence (%)
100
80
60
40
a
d
c
a
b
Loch McNess
Jake Finney Lake
Lake Kulinup
Wagerup
Lake Monger