

## *Understanding the effects of temperature on sex ratio in a sexually dimorphic fish species*

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### **Abstract**

The proportion of males to females in any given population is expected to be in equilibrium around 1:1. However, there are cases in nature of extremely skewed sex ratios. Several studies have suggested temperature to be associated with these skewed sex ratios. Here, I examined the relationship between sex ratio and temperature in the globally invasive mosquitofish. Mosquitofish exhibit sexual dimorphism, with females sometimes doubling males in body size. Following predictions from the temperature-size rule, smaller body sizes are expected at warmer temperatures. I therefore predict that hotter temperatures lead to an increase in the fraction of males. To test this prediction, I collected mosquitofish from geothermal ponds and streams in California and New Zealand. I examined the relationship between sex ratio and temperature. As predicted, I found that hotter sites had a greater proportion of males. My results show that temperature may be an explanation for skewed sex ratios in species showing sexual size dimorphism, such as mosquitofish. Results also suggest that increasing temperatures may cause sex ratios to change in nature, with potentially important ecological and evolutionary consequences.

### **Introduction**

In nature, the sex ratio of a population is predicted to be in equilibrium around 1:1. Fisher (1930) was the first to explain how natural selection favors equal sex ratios. Essentially, if one sex is underrepresented in the population, then there is a higher fitness associated with producing that sex. This frequency dependent selection will eventually return the population sex ratio to equilibrium (Fisher 1930, Basolo 1994). However, there are cases where sex ratio variation

occurs and does not return to equilibrium. Explanations for these skewed sex ratios range from local mate competition (Hamilton 1967), to condition-dependent sex allocation (Trivers and Willard 1973), to differential mortality of males and females (Britton and Moser 1982, Arendt 2014) which could be caused by temperature, and to species exhibiting environmental sex determination (Charnov and Bull 1989). Additionally, the explanation for why population sex ratio should be at unity does not explain sex ratio variation over spatial scales within a population. With all these explanations, there are clearly a variety of potential drivers of sex ratio variation, which may contribute to the widespread sex ratio skew in nature.

Invasive species have been known to have skewed sex ratios (Gutowsky and Fox 2011, Devin et al. 2004, Xu et al. 2016, and Fryxell et al. 2015) with possibly unique causes; invasive species are in environments in which they did not evolve, so expression of sex as a phenotype might be different, differential adult mortality of the sexes due to high intraspecific competition in previously uninhabited communities. There may even be a fitness advantage in newly invaded communities to have a female biased sex ratio, increasing reproductive capacity and the success of the invasion. However, these causes are all theorized but empirically untested. Understanding the drivers behind sex ratio variation in invasive species is important because their effects on invaded communities can depend on sex ratio. Skewed sex ratios in invasive species have been shown to affect the rate of range expansion (Miller and Inouye 2013) by selecting for increased boldness traits (Gutowsky and Fox 2011) which have been shown to increase foraging activity, magnifying their negative impacts (Pintor et al. 2009). Additionally, skewed sex ratios have been shown to affect population growth (Thresher et al. 2013), and community properties such as consumer abundance, producer abundance, and productivity (Fryxell et al. 2015). We may also

be able increase our control over biological invasions by further skewing sex ratios, triggering an allee effect (Tobin et al. 2011).

Mosquitofish (*Gambusia holbrooki* and *G. affinis*) are an example of an invasive species with extreme sex ratio variation across space and time (e.g. from over 90% male (Britton and Moser 1982, Specziár 2004, Alcaraz and García-Berthou 2007) to over 90% female (Haynes and Cashner 1995, Fryxell 2014 unpublished data, Vargas and Sostoa 1996)). Yet, despite our thorough understanding of its consequences, we poorly understand the drivers of such variation (Fryxell et al. 2015).

Mosquitofish are one of the world's 100 worst invasive species (Lowe et al. 2000) due to their global distribution, extreme densities, and noxious ecological effects. Their abilities to control mosquito populations led to their global transplantation throughout the 20th century (Pyke 2005). The broad physiological tolerances and aggressive nature of mosquitofish make them a dominant competitor in new environments (Pyke 2005). Through competition and predation, they have had severe impacts on native species across their invaded range, including, for example, on Green and Golden bell frogs in Australia (Morgan and Buttemer 1996), on Blue panchax killifish in Bangkok, Thailand (Myers 1965), and on the Plains topminnow in Nebraska (Haas 2005). Their effects on prey communities can cascade down food webs, fundamentally changing ecosystem characteristics like temperature, light penetration, pH, and nutrient concentrations in invaded locations (Hurlbert et al. 1972). Like many other species in the family *Poeciliidae*, mosquitofish exhibit sexual size dimorphism, with females sometimes doubling males in body size (Krumholz 1948), because males stop growing once reaching maturity. These differences in body size and energetic demands (due to postmaturation growth requirements) cause females to have much stronger competitive impacts (Henkanaththegedara & Stockwe ll

2013, 2014) and cascading ecological impacts (Fryxell et al. 2015). This marked sexual dimorphism makes sex ratio an important mediator of these destructive ecological effects.

Temperature's physiological effects are known to vary with body size (e.g. metabolic rate, lifespan (Gillooly et al. 2001, Angilletta et al. 2004)), due to cost differences in fitness between small and large individuals (Stillwell and Fox 2007). What's more, Atkinson's (1994) *temperature-size rule* states that increasing environmental temperature favors smaller body size. It is thus likely that this rule is expressed in sexually dimorphic species, such as the mosquitofish.

Prior studies have even suggested temperature is a principle driver of sex ratio variation across mosquitofish populations. Although with many suggesting female-biased populations are expected at higher temperatures (Hildebrand 1927, Johnson 1976, Winkler 1995, Krumholz 1948) and few suggesting the opposite (Zulian et al. 1995) there has yet to be a study directly targeting the effect of temperature on sex ratio. Here, following Atkinson's (1994) *temperature-size rule*, I predict that the relative number of smaller bodied male mosquitofish should increase with temperature.

Variation in sex ratio bias over spatial and temporal scales (Reichard et al. 2014, Pettersson et al. 2004) highlights the need for adequate sample sizes to prevent sampling bias. To clarify, one must obtain a representative sample of a population to accurately calculate the sex ratio. Imagine a population where all the male individuals occupy the northern range, all the females occupy the southern range, and they meet once a year to mate. The population sex ratio could be 1:1 but if you sampled in the north only, your results would be biased.

Predictions for the effect of temperature on sex ratio depend on spatial temperature variation. In certain environments organisms have the option to move freely between different thermal zones. To address this issue, we categorized thermal environments based on their degree of spatial temperature variation. Closed sites are geothermal ponds which exhibit no spatial variation. Open sites are characterized by having a spatial thermal gradient. For example, geothermal streams have high water temperatures at their source decreasing in temperature as they flow downstream; this type of thermal environment is relatively constant throughout the year and allows fish to move freely among temperatures. We believe fish in open habitats will spend most of their time in their preferred temperature zone.

Here, we surveyed populations of the western mosquitofish (*Gambusia affinis*) in 20 geothermal locations. We used sites in California, USA and New Zealand to increase the relevance of this study. Using these systems of varying temperature, we were able to see if temperature has an effect on sex ratio.

## **Methods**

We studied temperatures effects on sex ratio across 20 geothermal sites in California, USA and New Zealand. Water temperatures across the sites ranged from 18.7°C to 37.3°C. Historically, all sites were fishless habitats before recent mosquitofish introductions. Mosquitofish are considered invasive across all sites. We classified sites as closed if there was no spatial temperature variation and sites as open if a spatial thermal temperature gradient existed. First, we collected data on population sex ratio and site temperature to compare across all sites, then we collected sex ratio and temperature data within populations at open sites and compare across similar sites.

### Inter-site sampling

Upon arriving at a site, we would first take abiotic data measurements. This was done before the substrate was disturbed from fish sampling. We used an Oakton pH meter and a YSI Proplus for pH, temperature, conductivity, dissolved oxygen content, and salinity. After data collection, we then used seine and hand nets to collect fish samples. To ensure unbiased collections, we constantly moved the net to new locations, sampled the entire water column, and all habitat types. After sampling we then euthanized the fish using MS-222. Fish were either stored on ice and frozen ( $-18^{\circ}\text{C}$ ) for later analysis or were stored in ethanol.

### Intra-site sampling

We studied within site sex ratio in a geothermal area called Wild Willy's Hot Spring (WW), 14 miles east of Mammoth Lakes, CA, USA. WW has many small creeks originating from independent spring heads ( $>40^{\circ}\text{C}$ ) spread across a gentle sloping hillside. Water temperature gradients are formed as the hot spring water flows downstream to ambient temperatures. Mosquitofish live in most of these creeks. At the time of sampling, Summer 2016, creeks from independent springs did not converge at a confluence, although this does happen at other times of the year. To understand how mosquitofish populations differed in sex ratio above and below the stressful temperature of  $\sim 36^{\circ}\text{C}$  we selected five focal spring creeks. This temperature represents the approximate temperature threshold above which mosquitofish become severely stressed (Pyke 2005, Zulian et al. 1995). We used a paired design, sampling above and below the  $36^{\circ}\text{C}$  point in each stream to control for any inter-creek confounding factors, and we also measured a variety of characteristics to quantify these differences.

At each site, we established an upstream and downstream reach that matched certain criteria. In upstream reaches, mosquitofish had to be present and the water temperature 10m from

the springhead must be  $> 36^{\circ}\text{C}$ . In downstream reaches, the creek could not “marsh out” for 10m downstream of  $36^{\circ}\text{C}$ . Each reach was 10m long with upstream reaches always starting at the spring heads, and downstream reaches always starting at  $36^{\circ}\text{C}$ . The reaches were not always contiguous. We installed seines simultaneously across the upstream and downstream ends of each reach to prevent fish passage. Once seines were successfully installed, we used an Oakton pH meter and a YSI Proplus for pH, temperature, conductivity, dissolved oxygen content, and salinity. For river morphology, we measured stream depth and width at the top, middle, and bottom of the reach. We determined surface flow rate (m/s) by placing a ping pong ball at the top of the reach and recorded its travel time to the end of the reach, giving the ball a light flick if it became stuck in an eddy. This data collection protocol was repeated in all upstream and downstream reaches at all sites. We collected all abiotic data before fish sampling before the silty substrate was disturbed during sampling.

Sampling was always done downstream to upstream to minimize anthropogenic disturbance. Mosquitofish collection was done at all sites and the downstream reach was always sampled first. We quantified collection effort by measuring the total number of person-seconds (p-s) spent collecting. To prevent sampling bias during timed collections, we tried to equalize sampling effort (p-s) the entire length of the reach. Search times ranged from 1.25-5 minutes, longer search times correlated with lower fish density. We used hand nets to catch as many fish as possible within the given reach. Because mosquitofish tended to group at the downstream seine during sampling, we did not collect these fish until the collection time was over; we then pulled up the seine and collected all fish on the seine. All fish were euthanized with MS-222 and stored on ice while sampling continued. They then were kept on ice for approximately 24 hours while driving back to Santa Cruz, CA and placed in a freezer ( $-18^{\circ}\text{C}$ ) for later analysis.

### Analysis

In the lab, adult males were identified by the presence (male) or absence (non-male) of a gonopodium (modified anal fin) (Britton and Moser 1982, Geiser 1924, and Hildebrand 1927). Non-male fish (lacking gonopodium) were considered adult females if they were longer than the average male length per location. All non-males shorter than the average male length were considered juveniles. To use the data we collected in WW across all sites, we used a single mean temperature from each site, combining upper and lower temperatures.

We then calculated sex ratio as the proportion of males to females (M:F) and performed linear regressions with temperature across sites. We analyzed sex ratio versus temperature for closed and open sites separately. For sites with multiple samples, we used the mean sex ratio and temperature. For within site comparisons, we plotted the sex ratio from upstream and downstream population samples against temperature and performed a one sample t-test on the differences between upper and lower reaches.

## **Results**

### Inter-site results

Combining our data from California (CA) and New Zealand (NZ), we see an increase in males with increasing temperature (Fig. 1). In NZ, the number of males increases fastest with temperature (Fig. 1). Compared with CA sites, which have a fairly constant sex ratio around 0.6 males:females (M:F)(Fig. 1).

After categorizing sites as open or closed we also found an increase in the number of males with temperature (Fig. 2). However, comparing the differences between closed and open CA sites, we see temperature having a stronger effect in closed sites (Fig. 2). In open CA sites, there is barely an increase in males and the sex ratio is almost constant around 0.6 M:F (Fig. 2).



There was only one closed NZ site, so no relationship was found. However, for open NZ sites we see an increase in the number of males with temperature (Fig. 2).

### Intra-site results

Comparing sex ratio vs temperature within sites we see a decrease in M:F sex ratio in all sites except for WW3 which shows a slight increase towards 1:1 (Fig. 3). WW2 stays relatively constant around 0.85 M:F (Fig. 3). Upper reaches are always hotter than downstream reaches (Table. 1). Dissolved oxygen (DO) is generally higher in lower reaches except for WW3 and WW5, which decrease in DO (Table. 1). Catch per unit effort (CPUE) is always higher in lower reaches except in WW3 and WW5 (Table. 1) WW2 is the only site to show a decrease in salinity (ppt) downstream (Table. 1), all other sites stay relatively constant. Flow rate (m/s) generally increases as water flows downstream, however, in WW2 the flow rate is negligible (Table. 1). WW5 shows the highest increase in flowrate (Table. 1).

## **Discussion**

Consistent with our hypothesis and the *temperature-size rule*, we found higher proportions of males at higher temperatures across locations (fig. 1). Additionally, open and closed sites shared the correlation (fig. 2). However, when looking within open populations we found higher proportions of females in higher temperature zones (fig. 3). Even though the population sex ratios matched our prediction, our results from within populations does not.

Following the *temperature-size rule* (Atkinson 1994) we hypothesized that temperature may be the cause of skewed sex ratios in sexually dimorphic species. Using the invasive and sexually dimorphic mosquitofish, we tested our hypothesis across twenty geothermal locations and within five. Our hypothesis was supported across all sites, however, within populations we see the opposite expectation. One explanation is because of spatial sex ratio variation.

Mosquitofish occupying open sites may independently sort themselves to their preferred temperature within their habitat. These results support several other studies, which notice that there are higher proportions of females in higher temperature water (Hildebrand 1927, Johnson 1976, Winkler 1995, Krumholz 1948). What we don't understand is why this pattern is observed, as it goes directly against the *temperature-size* rule. One possible explanation is that there may be fitness advantages for giving birth in extreme temperatures such as decreased conspecific predation pressure and decreased food competition. At birth mosquitofish are at risk of cannibalism (Dionne 1985), by spending time in extreme temperatures babies may be able to escape this predation. With low food availability in geothermal springs (33°C - 42°C (Hayford et al. 1995)) and associated increase in cannibalism (Dionne 1985) it is possible that giving birth in high temperatures increases the probability of surviving.

The stronger relationship found in NZ may be due to smaller sample sizes (5 sites with lots of variation) when compared to CA sites (15 sites). Additionally, the overall trend in CA site population sex ratios, which is relatively constant, is likely due to major spatial and temporal temperature variation between sites.

This study shows that within a population there can be spatial variation in sex ratio. However, it lacks the aspect of time and it is important to understand how a population's sex ratio varies temporally (Reichard et al. 2014, Pettersson et al. 2004) to determine its ecological impacts. Our findings further suggest that temperature could explain skewed sex ratios in species exhibiting sexual size dimorphism, such as mosquitofish. Sex ratios in nature may change in result of increasing temperatures, with potentially important ecological and evolutionary consequences.

Understanding what drives sex ratio variation in invasive species is also of interest. It will allow us to better predict invasive population dynamics over time (Miller and Inouye 2013, Thresher et al. 2013) and resulting ecological effects (Fryxell et al. 2015). Additionally, this knowledge may be used to further skew invasive sex ratios to the point where an allee effect occurs (Tobin et al. 2011).

Climate change is increasing temperatures across the global range of mosquitofish, and is even causing their range to expand due to their warm water preference. Our results suggest that increasing temperatures will result in an increase in proportion of male mosquitofish. Since males are known to have weaker competitive impacts (Henkanaththegedara & Stockwell 2013, 2014) and resulting weaker ecological impacts on zooplankton and phytoplankton abundances, productivity, pH, and temperature (Fryxell et al. 2015); it is possible that the negative impacts of mosquitofish will lessen with climate change. However, to predict how a single population's impact will change over space and time, we must further study sex ratio variation within populations.

There are countless studies on direct effects of temp on ecological interactions (O'Connor 2009, Hoekman 2010, Jiang & Morin 2004, Hofstetter et al. 2007) but there are no studies about temperature affecting sex ratio in sexually dimorphic species. Using the sexually dimorphic mosquitofish, we showed that mosquitofish sex ratios become increasingly male with increasing temperature across populations. We believe there may be consistent patterns in other sexually dimorphic species. The results of these recommended studies will provide important information on how to predict population dynamics and resulting ecological interactions in the face of climate change.

**Acknowledgments**

This study could not have been completed without the generous funding from the Kenneth S. Norris Center for Natural History, thank you. I would also like to thank the freshwater ecosystems lab at the University of Auckland for providing me essential sex ratio data from New Zealand to broaden the scope of this research. And finally, I want to thank my professor, Eric Palkovacs and PhD candidate, David Fryxell from the University of California Santa Cruz for their continued guidance and support through this research.

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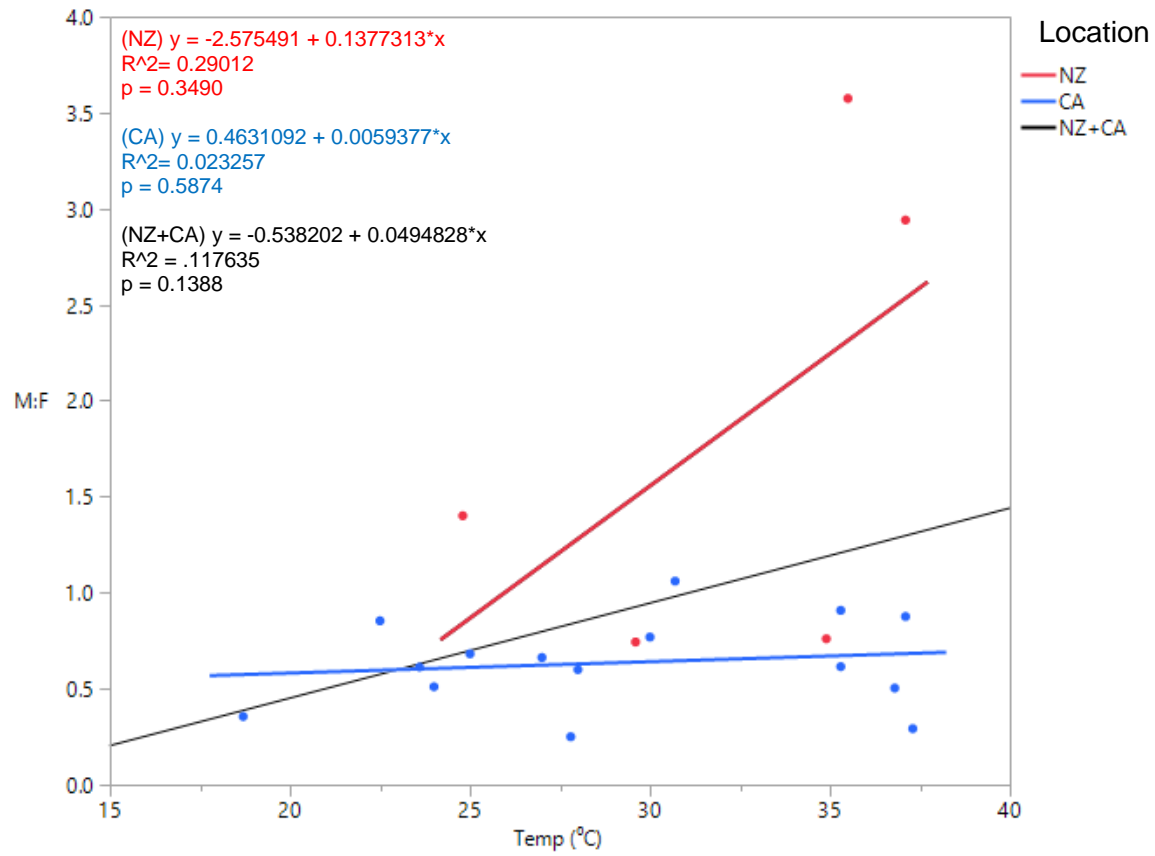
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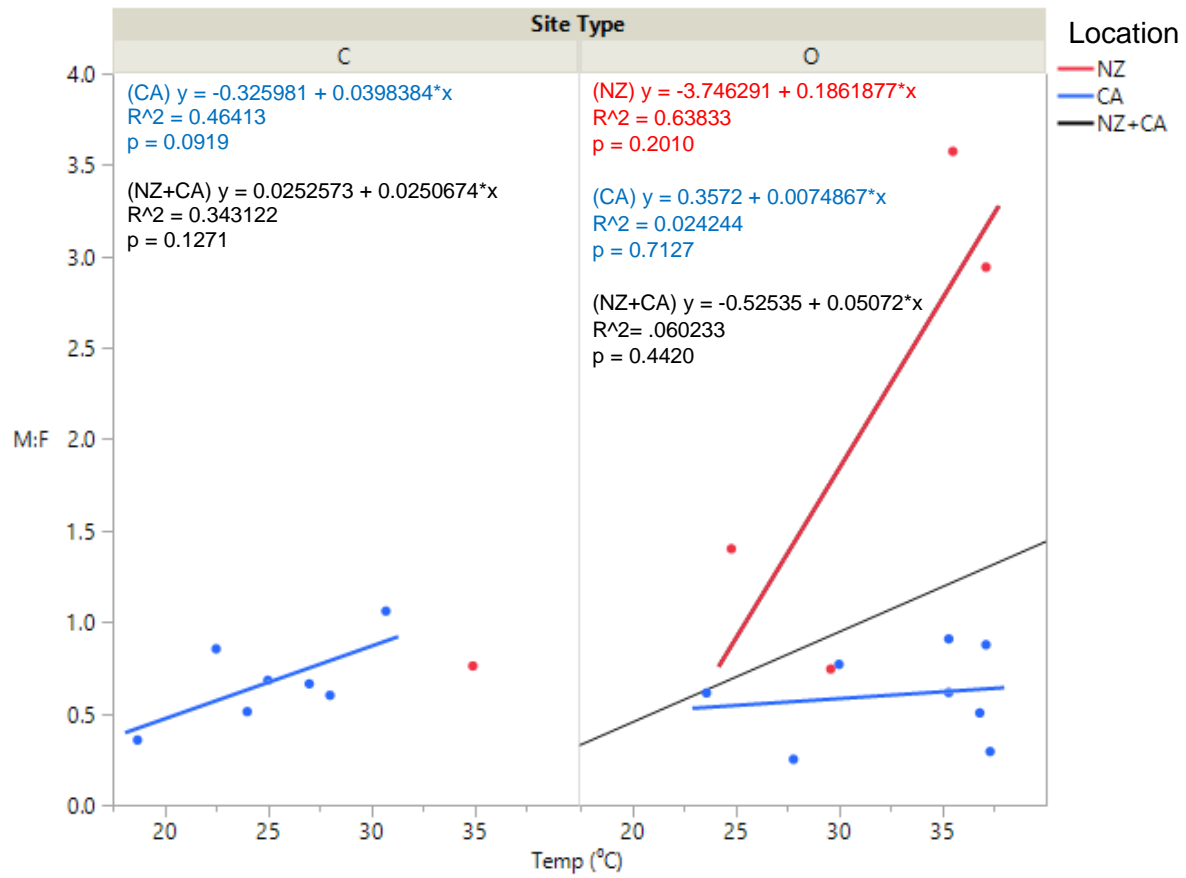
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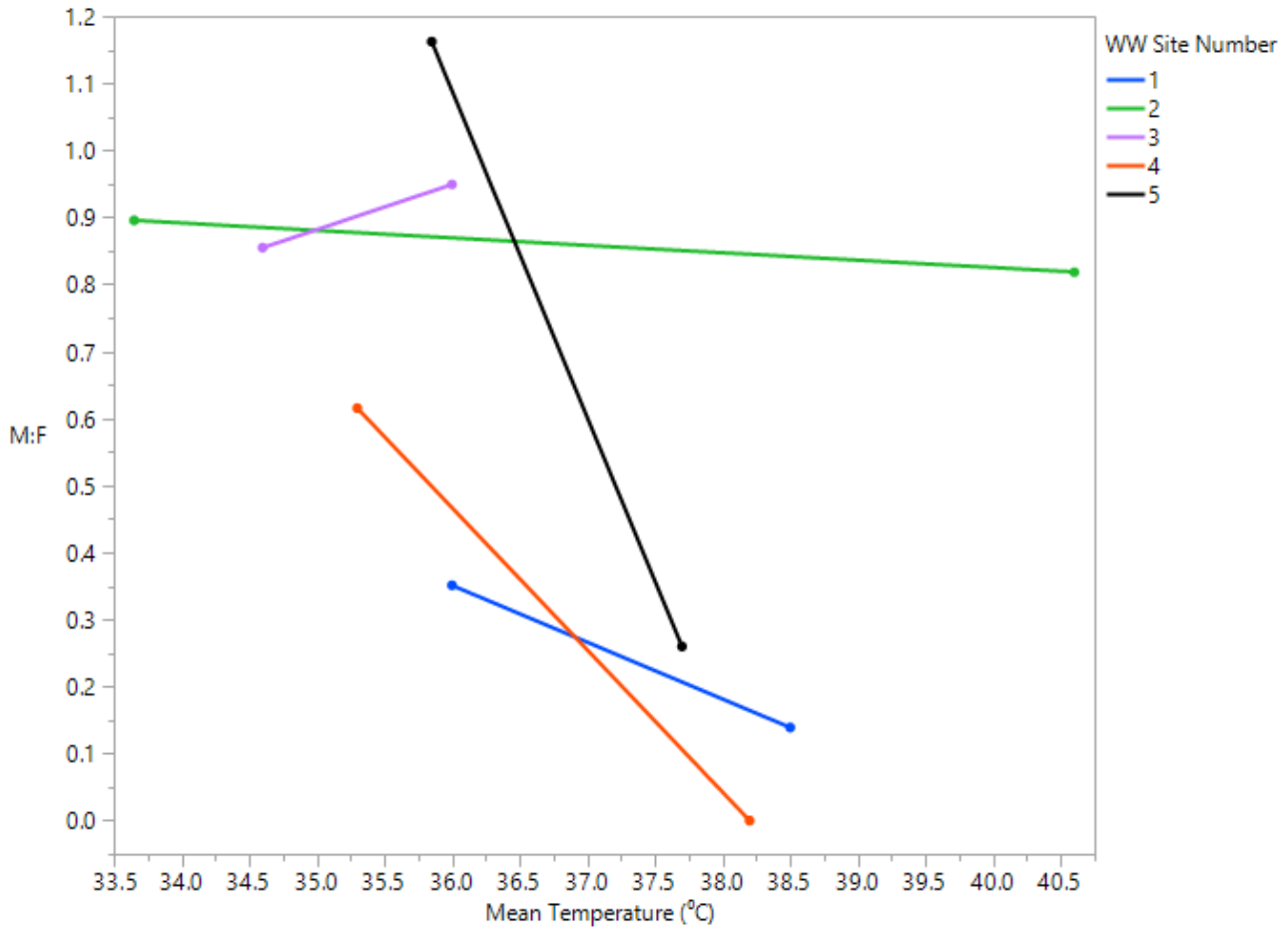
## Figures



**Figure 1:** Bivariate analysis of temperature (°C) versus sex ratio (M:F) from all samples. NZ = New Zealand, CA = California.



**Figure 2:** Bivariate analysis of temperature (°C) versus sex ratio (M:F) in closed and open sites. NZ = New Zealand, CA = California.



**Figure 3:** Wild Willy's (WW) within site comparisons of sex ratio (M:F) versus temperature (°C). All starting points are the downstream reaches and all ending points are the upstream reaches (higher temperatures upstream). T test:  $p=0.1337$

**Table 1:** Wild Willy's (WW) abiotic characteristics. Catch per unit effort (CPUE) is the number of fish caught per second. Dissolved oxygen = DO, Specific conductance = SPC

WW	Reach	Temp(°C)	M:F	pH	DO (mg/L)	CPUE	Salinity (ppt)	SPC (mS/cm)	Flow Rate (m/s)
1	upper	38.5	0.139	7.0	2.86	0.81	0.8	1.713	0.1333
1	lower	36.0	0.351	7.2	3.99	1.33	0.9	1.749	0.1887
2	upper	40.6	0.818	7.1	2.83	0.66	1.6	1.946	negligible
2	lower	33.7	0.896	7.3	8.63	3.91	1.0	1.982	negligible
3	upper	36.0	0.949	7.1	6.60	2.65	1.0	1.936	negligible
3	lower	34.6	0.855	7.2	6.26	2.52	1.0	1.928	0.1887
4	upper	38.2	0.000	7.0	2.67	0.02	0.8	1.708	0.2000
4	lower	35.3	0.615	6.9	5.05	0.32	0.8	1.696	0.2273
5	upper	37.7	0.260	6.9	6.62	1.12	0.8	1.712	0.34
5	lower	35.9	1.162	6.9	5.12	0.83	0.9	1.714	0.4762