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The effect of different feeding regimes on recent nutritional and growth measurements in juvenile California Killifish (Fundulus parvipinnis)

Emily Parks
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A thesis submitted in partial satisfaction of the

requirements for the degree of

Master of Science in Environmental and Ocean Sciences

by

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2022

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DEDICATION

To my supportive friends, boyfriend and family.

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ABSTRACT

Gaining a deeper understanding of in-situ growth approximations for juvenile fishes is one way to understand how food consumption may affect fish growth. If variations in growth rate are strongly mirrored by past food status, then the condition (degree of well-being) of fish can be potentially used as a reference for relative prey availability. Furthermore, confirming that there is a positive relationship between food availability and fish growth rate is a critical first step to deepen our understanding of growth rate variation as well as examining if growth can be a proxy for habitat quality.

The California killifish (*Fundulus parvipinnis*) is an ideal test subject due to both their abundance and high site fidelity in Southern California marshes. This fish links intertidal and subtidal habitats by feeding on the vegetated marsh surface at high tide and returning to deeper marsh channels and seagrass beds at low tide. Tidal flooding of shallow creek channels allows this species to migrate into the vegetated marsh and forage for invertebrates. Thus, the California killifish is important in the estuarine food web, serving as a vector to transfer energy and nutrients off the marsh surface, and as a food source for higher trophic level species.

This controlled laboratory study aimed to validate daily otolith growth increments of the California killifish (*Fundulus parvipinnis)* and examine the reliability of three common growth measures: Fulton's condition factor (Fulton's *K*), RNA:DNA index, and recent otolith growth increments. Fulton's condition factor is a morphometric condition index using length and weight measurements

to provide a direct measurement of growth. Quantification of nucleic acids in white muscle tissue characterized by the RNA:DNA index is another common indirect method for estimating growth rates and nutritional condition in marine organisms. Lastly, otolith increment analysis is an indirect method providing a permanent record of age and past growth rates. Using two different feeding levels in this study allowed closer comparison of how effectively these three measures of nutritional condition and growth can convey past feeding habits.

Fulton's *K* values were strongly representative of past feeding rations after a two-week period, and food deprivation led to a considerable decrease in this index every week. RNA:DNA index values also showed significant differences between the feeding treatments. In contrast, there was weak evidence that recent otolith growth (avg. width of last 3 otolith increments) can relay food condition and the variable average daily otolith increment measurements were less responsive to feeding conditions. From our results, it appears that food consumption is reflected in daily otolith increments by a little over a one-week delay and the response to food was more clearly shown in normalized average otolith increment values. In addition, we recommend caution in the interpretation of otolith increments due to the variability of both the increment size and error introduced by the process of viewing otoliths. Ultimately, gaining perspective on how these growth indices change with respect to food rations will provide a more reliable interpretation of the health of California killifish populations, and potentially improve our assessment in how wetlands are functioning by viewing how well the fish populations are supported.

CHAPTER 1: INTRODUCTION

1.1 Introduction

1.1.1 Southern California Wetlands

Wetlands provide numerous ecosystem services to our society. These include: (1) serving as biodiversity hotspots for many estuarine organisms (Guareschi *et al.*, 2015), (2) storing >50% of the total global soil carbon (Schlesinger and Bernhardt, 2013), (3) producing food security and ecotourism opportunities (Engelhardt and Ritchie, 2001), and (4) aiding in nutrient removal to prevent eutrophication (Ryther and Dunstan, 1971), among many others. Unfortunately, wetlands are also among the most threatened habitats on earth, with approximately 54-57% of global wetland areas lost over the past 100 years due to development (Zedler and Kercher, 2005), and those that remain are often negatively impacted by other anthropogenic stresses such as, sea level rise and invasive species (Elliot *et al.*, 2019).

Southern California wetlands in particular have experienced immense habitat loss (>90%) and fragmentation by human activities, leading to numerous species threatened with extinction (Zedler, 1996). Degradation of these natural wetlands reduces the number of ecosystem benefits and has led to new construction of artificial wetlands to help mitigate the damaged habitat (Levrel *et al.*, 2017; Vymazal, 2011). Creating artificial wetlands that will be functionally equivalent to Southern California wetlands is difficult due to the high interannual variability of inundations and wetland-stream connectivity, such as the unique

flooding scheme that creates an influx of nutrients for wetland-dependent organisms (Anderson & Mitsch, 2006; Chen *et al.,* 2017; Zedler, 1983). Without these systematic flooding events of the shallow creek channels then species would not be able to undergo tidal migration to access more food at the marsh surface, like the California killifish (*Fundulus parvipinnis*). A deeper understanding of fish health would be useful insight of marsh function because it integrates the effects of abiotic and biotic factors acting on the species.

1.1.2 California killifish (*Fundulus parvipinnis*)

Biological indicators (also termed "indicator species" or "biomonitor") are organisms that can be easily monitored and whose status detects or reliably predicts the changes occurring in the ecosystem (Landres, 1988; Bartell, 2006; Burger, 2006). Biological indicators are typically chosen due to their sensitivity to environmental changes being assessed, ecological importance, ease of measurement, and local abundance (Siddig *et al.,* 2016). The strategy relies on the premise that cumulative effects of environmental factors will be reflected in the diversity, abundance, reproductive success, or growth in certain populations (Cairns and Pratt, 1993; Bartell, 2006). These indicator species can range from plants to animals and even microorganisms to detect changes in the natural environment. Therefore, monitoring single population dynamics in various habitats provides a relatively cost-effective as well as reliable tool to enhance ecosystem management and to study community dynamics.

The California killifish, *Fundulus parvipinnis,* could be a prime bioindicator species due both to their high abundance and ecologically important role in the food web of estuarine habitats throughout the Pacific coast of North America (Kwak and Zedler, 1997). Despite being one of the most abundant intertidal salt marsh fishes within their distribution from Morro Bay, California to Bahía Magdalena, Baja California, Mexico, there have been relatively few published studies on this species. This non-migratory species spawns in the intertidal marsh habitats during spring high tides at night from April to June (Bernardi and Talley, 2000), and largely utilizes vegetated marsh surfaces for feeding (West and Zedler, 2000). Based on past research, California killifish have limited interchanges between coastal habitats and high site fidelity within estuaries (Bernardi and Talley, 2000; Robison, 2021). Furthermore, this species' life history is heavily reliant on the productivity from salt marsh habitat and on the access to the resources provided by mixed, semi-diurnal tides (Rickets *et al.*, 1985) that flood these shallow marsh channels. California killifish are also an important food source for higher trophic level species (Kneib, 1997; Talley, 2000; McMahon *et al.,* 2005), making them an important vector for transferring energy and nutrients off the marsh surface.

Previous work on the importance of marsh access to estuarine fishes revealed that the California killifish individuals that had access to the vegetated marsh consumed six times as much food in comparison to the ones who were restricted to deeper creeks (West and Zedler, 2000). Southern California wetlands flood irregularly, so high tide events are crucial to increase access to a variety of

prey sources at the marsh surface and a decrease in predation risk for some residents. Limited vegetated access may make this region more profitable due to the buildup of food resources between the reduced foraging opportunities during low tide (West and Zedler, 2000). Despite the energy cost to forage on the upper marsh surface during high tides, killifish benefited from marsh access in comparison to those restricted to subtidal channels by consuming more food on a daily basis and by maximizing growth early on after hatching (Madon *et al.*, 2001*).* Therefore, investigating how precisely food rations affect indirect and direct growth estimates in killifish populations will provide more answers as to which indicator would be best to estimate past food availability.

1.1.3 Otolith Increment Analysis

One way to examine fish growth history is through the visualization and interpretation of their otoliths ("earstones"). Otoliths, primarily constructed of organic matrix fibers and carbonate crystal depositions, have been commonly used to provide reliable evidence for age and growth rates in teleost fish (Radtke and Dean, 1982; Radtke, 1987; Campana *et al.*, 1997; Morales-Nin 2000). Daily periodicity of the internal micro-increments is represented by alternating opaque and translucent concentric zones that are mainly dependent on endogenous circadian endocrine rhythms and photoperiod (Radtke and Dean, 1982; Campana and Neilson, 1985). Poor feeding conditions slow somatic growth which can cause narrow and indistinguishable otolith increments (Geffen, 1982). Based on the assumption that otolith growth mirrors somatic growth, studies have shown

environmental factors influence otolith growth and increment structure (e.g., temperature and fish size; Mosegaard *et al.,* 1988).

Since otolith increment widths are frequently correlated with daily somatic growth rates, analyzing otolith microstructure is one of the most used methods to create daily reconstruction of growth rates and age determination for fishes (Baumann *et al.,* 2003; Brown *et al.,* 2004; Leonarduzzi *et al.,* 2010). This method relies on two main assumptions: periodicity of otolith increment formation is daily, and there is a strong correlation between otolith increment lengths and fish size (Baumann *et al.,* 2005). Prior to interpreting otolith growth patterns to infer environmental conditions and other life history traits, daily deposition of otolith rings must first be validated. One common method used for age validation is to immerse fish in fluorochrome labeling dyes to produce distinct marks in these calcified structures. Since the relationship between otolith increment deposition and somatic growth may be species-specific, this experiment will help determine whether the otolith increments are deposited daily and if otolith growth can be used as a proxy for somatic growth.

1.1.4 Fulton's Condition Factor

Fulton's condition factor (known as Fulton's *K*) is a common, non-lethal morphometric condition index used by fishery biologists that involves mass and length to quantify the state of well-being ("plumpness") of a fish (Dutta, 1994; Suthers, 1998).

Fulton's $K = W/L^3 \times 100$ (1)

where W is the weight (g) and L is the total length (cm).

Fulton's *K* assumes that heavier fish at a given length are in better condition and has been used to successfully appraise composition of a fish's body, such as lipid content in salmonids (Sutton *et al.,* 2000; Pangle & Sutton, 2005). Growth rates of individual fishes tend to slow down after the onset of sexual maturity, because more energy is diverted to the formation of reproductive tissues, leading to smaller changes in Fulton's *K* with advancing age (Dutta, 1994). A food deprivation study on European sprat juveniles (*Sprattus sprattus*) has shown Fulton's *K* rapidly decrease after 4 days, yet this approximation explained the least amount of variability in sprat growth rate compared to otolith-based and nucleic acid-based indices (Peck *et al.,* 2015). This morphometric index can also relay information about diet characteristics, for example higher Fulton's *K* in flatfish were associated with certain prey groups, polychaetes and copepods (De. Raedemaecker *et al.,* 2012).

1.1.5 RNA:DNA Index

The ratio of ribonucleic acid to deoxyribonucleic acid (RNA:DNA index or R/D) is a reliable biochemical indicator for physiological and recent nutritional status of aquatic organisms (Holm-Hansen *et al.,* 1968). This nucleic acid derived index have been applied successfully in various organisms including microbial communities (Dortch *et al.,* 1983), invertebrates (Wagner *et al.,* 1998; Chícharo *et al.,* 2001), and fish (Buckley, 1984; Clemmesen, 1994; Rooker *et al.,* 1997) to

describe the synthetic capacity of the cell, which is usually correlated with nutritional status. Under the assumption that DNA is relatively constant in diploid cells and ribosomal RNA fluctuates depending on the capacity for protein synthesis, this indirect measurement has been shown to reflect short-term environmental fluctuations better than Fulton's *K* in juvenile flatfish (Folkvord *et al.,* 1995; Clemmesen, 1996; De Raedemaecker *et al.,* 2012). One study evaluating the effect of starvation on the RNA:DNA ratios in *Sciaenops ocellatus* over 5-day starvation periods showed a continuous decrease in RNA:DNA ratios as expected and revealed that as age increased the relative decline was reduced (Rooker and Holt, 1996).

However, there are a few parameters that may alter interpretation of this biochemical indicator. First, there is a temperature effect on RNA:DNA index, as the thermal environment can affect metabolic rate (including protein synthesis). Fishes living in higher temperatures often have higher growth rates per RNA:DNA index value (Islam *et al.,* 2006). Second, younger fish divert more protein synthesis toward somatic growth and so the RNA:DNA index in relation to growth changes depending on the life stage of the fish (Peck *et al.,* 2003; Caldarone, 2005). Third, different spectrofluorometric protocols can lead to varying RNA:DNA index values and so Caldarone *et al.,* 2006 recommended calculating a ratio of the slopes derived from the standard curves (m_{DNA}/m_{RNA}) used to determine the nucleic acid concentrations to better facilitate comparison of these values between laboratories. However, being capable of measuring recent conditions (i.e., 1-3 days, Buckley *et al.,* 1999) is one aspect that makes

RNA:DNA index advantageous relative to other measures of growth that require the input from past feeding history and energy utilization for an individual's life span (Foley *et al.*, 2016).

1.2 Research Objectives

The objective of this study is to evaluate how the amount of food affect recent growth proxies and otolith growth in California killifish.

Research questions:

1. Do California killifish form daily otolith rings?
2. Do California killifish otolith increment widths correlate with changing food amount?
3. How do indirect measurements of growth (daily otolith increments, RNA:DNA index) compare to a direct measure (Fulton's *K*)?

Overall, comparing both indirect and direct measures of relative growth will allow us to assess reliability and sensitivity of these measurement types to indicate past food intake. Evaluating these relationships in a controlled setting provides a clearer understanding of how variable food rations can affect these growth indices, which would be more difficult to evaluate in the wild. Lastly, learning how to better monitor fish health could be then used as a potential tool to assess how well wetlands are supporting fish populations in the wild.

CHAPTER 2: The effect of different feeding regimes on recent nutritional and growth measurements in juvenile California killifish (*Fundulus parvipinnis)*

2.1 Introduction

Reliable and sensitive measures of nutritional condition for fishes are an important tool to evaluate the relative food availability in the environment. One way to estimate food availability is through the interpretation of fish growth history using otoliths. Otoliths are calcium carbonate structures in the inner ear that are used for balance and hearing. Otoliths grow over time and often deposit daily and annual growth rings that can be used to back calculate growth rates in teleost fish (Campana *et al.*, 1997; Radtke and Dean, 1982; Radtke, 1987; Morales-Nin 2000). Morphological indices, such as Fulton's condition factor (known as Fulton's *K*), presents an alternative, non-lethal condition index that uses mass and length to estimate changes in nutritional condition (Suthers, 1998). In addition, the ratio of ribonucleic acid to deoxyribonucleic acid (RNA:DNA index) has long been used as a biochemical indicator for physiological and recent nutritional status of aquatic organisms (Holm-Hansen *et al.*,1968). This method has been used in various aquatic organisms, including phytoplankton (Berdalet and Dortch, 1991) and juvenile/adult fish (Smith and Buckley, 2003), to show changes in nutritional condition and metabolic activity caused by starvation or other forms of stress (e.g., hypoxia and thermal changes; Bulow, 1970).

Comparing these proxies (otolith, somatic, biochemical) for fish growth is challenging because there are many interacting physiological mechanisms in fish energy metabolism and muscle growth. For example, a deficiency in the required ten essential amino acids in fishes' diet could lead to amino acid catabolism resulting in a loss of feeding efficiency and sub-optimal protein synthesis during growth (Dutta, 1994). Furthermore, a variety of intrinsic (e.g., fish size, developmental stage) and extrinsic (e.g., temperature, water chemistry; Ferron and Leggett, 1994; Suthers, 1998) factors can affect otolith morphogenesis and somatic growth patterns (Mahé *et al.*, 2019).

More specifically, estimating growth in juvenile fish is advantageous because immature fishes redirect food uptake more towards somatic growth and thus provide more apparent responses to food levels (Ehrlich, 1974). Once fish reach sexual maturity, the growth pattern often changes to divert more energy utilization toward reproductive organs, such as gonadal tissue, and fat accumulation (Dutta, 1994). In nature, researchers may examine fish growth rates by repeatedly sampling a population of marked fish with known sizes, however these estimates from recaptured individuals may be biased due to size-selectivity mortality and the logistical challenges of recapturing an adequate number of marked fish (Fisher and Pearcy, 1988, 2005). Since in the wild there are various factors that need to be considered when examining fish growth, creating a controlled laboratory study to examine the effects of food in juvenile fish is a critical first step to provide a clearer understanding of how to infer past food conditions from wild-caught individuals.

Evaluating growth conditions in organisms living in highly disturbed habitats can be a valuable tool to assess habitat quality in terms of food accessibility and functionality. Wetland loss and degradation has been substantial worldwide, thus monitoring prey availability through fish health (i.e. evaluating fish assemblages and diversity) will provide reference as to how well these fragmented, complex systems are operating (Zedler, 1996). This is especially important to assess in the remaining Southern California salt marshes, which are coastal wetlands, that support various endangered and sensitive species (Zedler, 1996). Furthermore, these tidal marshes are characterized by hydrologic conditions in which periodic flooding in the intertidal regions aids animals in accessing a greater diversity of food at the marsh surface (Zedler, 1983). Thus, maintaining strong links between adjacent ecosystems (uplands, riparian corridors, and nearshore waters) and fewer barriers for animal movement will support biodiversity within salt marshes (Zedler, 1996). Finding more relevant information to strengthen habitat quality assessments are essential to advance ecosystem-based restoration decisions. More specifically, linking the impact of past prey availability to biological information such as fish health could be a powerful tool to gain a different perspective on habitat quality and functionality of these coastal ecosystems.

The California killifish, *Fundulus parvipinnis,* could be an excellent bioindicator species throughout the Pacific coast of North America due to its naturally high abundance and ecologically important role in estuarine food webs (Kwak and Zedler, 1997). California killifish are non-migratory species that

spawn in the nearshore habitats during spring high tides at night from April to June (Bernardi and Talley, 2000) and largely utilizes vegetated marsh surfaces for feeding, therefore this species is heavily reliant on the productivity of these flooded shallow marsh channels (West and Zedler, 2000). Furthermore, this species is an important food source for higher trophic level species making them a vector for transferring energy and nutrients off the marsh surface to deeper habitats (Kneib, 1997; Talley, 2000; McMahon *et al.,* 2005). California killifish growth is heavily dependent on productivity from the salt marsh habitat, with individuals that undergo tidal migrations consuming six times as much food relative to those who were restricted to the subtidal (Rickets *et al.*, 1985; West and Zedler, 2000). A bioenergetics model predicted that California killifish would grow 20-44% faster with 2-3 hours of vegetated marsh access than individuals without access (Madon *et al.*, 2001). This suggests that fish growth can represent marsh function to a certain degree, as adequate flooding of marsh surface can be related to fish growth. Assessing nutritional indicators of fishes, especially across the highly disturbed Southern California wetlands, will provide more biological evidence of the environmental quality of coastal habitats.

This controlled laboratory study allowed for a precise evaluation in how two different food rations (high vs low feed) affected three measures of fish health: otolith-based growth proxies, somatic growth (Fulton's condition factor *K*), and biochemical indicator (RNA:DNA index) in juvenile California killifish over the span of 28 days. Understanding how food rations affect indirect and direct growth estimates in juvenile California killifish will provide answers to if

these indicators can portray past food availability, and how these indices compare in response to different food levels.

2.2 Materials and Methods

2.2.1 Fish Collection

Juvenile killifish (2.3-2.9 cm, standard length) were collected with a 3 mm mesh beach seine from the Kendall-Frost Reserve located in Mission Bay, California (32.79117°N, 117.23013°W) during low tide on 25th June 2020, 28th June 2020, and 10th July 2020 (Figure 2.1). On the last day of field sampling approximately a dozen of the fish were frozen on-site for RNA:DNA analyses as reference for this natural population. Fish were transported back to the aquarium facilities at the University of San Diego in containers with portable aerators.

2.2.2 Alizarin Red Stain Immersion

On 21st July 2020 about 170 fish were immersed in 400 mg/L alizarin red stain (ARS) and seawater for 24h to mark their otoliths to validate daily increment formation. The following day the fish were distributed into three replicates for high feed treatment and low feed treatment treatments (Figure 2.2). The following summer we performed a second immersion attempt with juvenile killifish that were fed ad libitum in Mission Bay water for the sole purpose of marking their otoliths (see appendix A.1).

Experiment: Different feeding regimes to test measures of growth

A total of 111 marked fish (ranging from 2.3-2.9 cm, standard length) that survived the immersion period were allocated into six tanks. For high feed treatment there were 10 fish in each of the 3 tanks (density of 0.104 g of fish/l of seawater) and 27 fish in each of the 3 tanks for low feed treatment (density of 0.140 g of fish/l of seawater). Tanks were attached to a larger circulation system that maintained water temperature at 20±1°C, salinity at 36±1 ppt, and the room used a light/dark cycle at 12h/12h.

Two designated feeding treatments described below:

1) *High feed:* fish fed a ratio of 0.1g/fish twice daily throughout experiment
2) *Low feed:* fish fed a ratio of 0.1g/fish once daily throughout experiment

Fish were fed frozen, enriched brine shrimp nauplii with a feeding ratio of approximately 0.1 g/fish/day and 0.2 g/fish/day, which is similar to estimates of their natural daily food consumption (Pérez-España *et. al.*, 1998). Fish were evaluated daily by visual inspection of physical health for the duration of the feeding regime study, with any dead or distressed fish immediately removed from the tanks. Feeding rations were adjusted to keep the daily feeding ratio constant depending on the number of fish. Originally the low feed individuals were designated to zero food at the start of the experiment, but due to high mortality rates (33/80 total fish) these fish were shifted to constant daily low feed rations by day 4 (Figure 2.11). A random subset of at least 20 (range was 20-25) individuals

from each treatment was taken at the beginning of the feeding regime study and on a weekly basis to collect standard length (nearest 0.1 mm) and mass (nearest 0.01 g). Measured individuals were returned to their respective tanks. At the end of the 28-day feeding regime experiment, the remaining 43 fish (n = 21 high feed, n = 22 low feed) were euthanized by elevating carbon dioxide levels and stored in a freezer (-80°C) for indirect measurements (otolith increment widths, RNA:DNA index). Sex was not determined due to the lack of distinguishable gonads and sexual dimorphic characteristics in immature killifish (Fritz, 1976).

2.2.3 Otolith Preparation

All fish that survived the feeding experiment (n = 43 fish) were sampled for otolith extraction and future otolith aging. Right sagittal otoliths were extracted from the cranial cavity and mounted proximal face down with cyanoacrylate glue onto a microscope slide. Otoliths were polished with a variety of abrasive 3M Wetordry® micron graded polishing paper (30 micron to 1 micron) until the primordium was clear.

2.2.4 Otolith Age Validation

Sanded otolith sections were pictured at 40x under normal transmitted light and viewed under fluorescent light using an Olympus BX-51 equipped with TRITC filter cube (excitation filter: 510-550 nm, emission/barrier filter: 590 nm) to decipher ARS marks from the survivors. ARS marks were not detected, however marks were detected under the fluorescence microscope from the second

immersion attempt from the two individuals that survived (see appendix A.1). Otolith rings were counted twice blindly from the initial ARS mark to the edge of the otolith. Comparing the mean count of increments outside the ARS mark and the number of days post-marking showed if killifish grow daily increments.

2.2.5 Total Otolith Increment Count

To determine if width of otolith increment was affected by food consumption, all discernable otolith radius and increments ($\pm$ 0.001 μm) were measured from the surviving fish in each feeding treatment using plugin ObjectJ with modification to the open-source image program ImageJ (Schneider *et al.*, 2012; Vischer and Nastase, 2021). Otolith radius was measured from the nucleus (center) of the otolith to the farthest edge, and then each increment along the marked otolith radius was measured to calculate otolith increment widths. If the total increment counts differed by more than 3 increments then a third count was performed. If the difference was less than 3 increments then one of the readings was randomly chosen for data analysis. If the difference was greater than 3 increments for all three readings then the otolith was discarded (n = 0). Lastly, indirect measurements of recent otolith growth were calculated by finding the mean width of the last 3 completely formed increments as another method to compare between feeding treatments (Somarakis and Nikolioudakis, 2007).

2.2.6 Weekly Somatic Growth Rates

Total length (± 0.1 mm), standard length (± 0.1 mm), weight (± 0.001 g) and Fulton's Condition Factor (K = (weight/SL^3x 100)) was measured on a weekly basis on a random subset of 20-25 fish per treatment during the feeding regime experiment. Weight and length measurements were also taken on all the fish that died during the experiment and the individuals who survived the entire experiment.

2.2.7 RNA:DNA Index Preparation and Analysis

RNA:DNA index is a reliable biochemical indicator for physiological and recent nutritional status of aquatic organisms (Holm-Hansen *et al.*, 1968). RNA:DNA Index values were calculated on all the survivors using the SYBR Green II fluorometric technique to detect the nucleic acid concentrations by spectrofluorescence from the frozen dorsal muscles of killifish. All sample preparation and measurements of RNA and DNA were performed at University of San Diego using a modification of methods outlined by Berdelat *et al.*, 2005b. Approximately 0.006g of white muscle was extracted from frozen individuals and then homogenized with 5 mM Tris (pH 8.0). 1% STEB (1% Sarcosyl, 1mM EDTA, 5 mM Tris, pH 8.0) was added to the natural samples and vortexed for 30 minutes to disrupt cells. After homogenates were centrifuged for 2 min at 10,000 rpm, aliquots from supernatants were used to estimate total DNA and RNA concentrations. The emitted light from samples was recorded with a microtiter fluorescence reader (Thermo Labsystems, Fluoroskan Ascent FL) and the amount

of nucleic acids expressed in units of μg (mg muscle tissue)$^{-1}$ was compared to fluorescence values of RNA (R6625) and DNA (D4764) standard curves of known concentrations (Foley *et al.*, 2016).

Some studies have advocated caution in the accuracy of this biochemical index due to the high risk for potential RNA degradation in laboratory processing and the potential lack of intercompatibility between RNA:DNA index values among laboratories employing different spectrofluorometric techniques (Grémare and Vétion, 1994). The difference in values from specific techniques can be resolved if the ratio of the slopes of the DNA and RNA standard curves are provided (Caldarone *et al.*, 2006) and furthermore we followed a rigorous protocol to detect as low as 1 ng for DNA (Berdalet *et al.*, 2005a, 2005b).

2.2.8 Statistical Analyses

All data analyses were performed in RStudio (R Core Team 2018). Two sample t-tests were used to examine the effects of variable feeding regimes on multiple dependent variables (weekly Fulton's *K*, otolith width increments, and RNA:DNA index) (Johnson *et al.*, 2002). A 2-tailed binomial test was performed on the increments before and during the feeding experiment to determine if the high feed increments differed from low feed increments. All graphs were produced using Microsoft Excel (v. 16.47.1, Microsoft Corporation 2021), with the exception of boxplots, which were produced using RStudio (R Core Team 2018).

2.3 Results

2.3.1 Age Validation

Using 400 mg/l ARS for a 24 h immersion period yielded inconsistent results, from no mark to prominent marks with varying mortality rates (65-.05% survivorship) during the immersion trials. The first immersion experiment failed to produce an ARS mark in the fish otoliths, but a second immersion attempt presented clear ARS marks under fluorescent light in only a few individuals (2 out of the 40 who survived) (see appendix A.1). Daily deposition of otolith increments was validated, with the mean count of the rings after the ARS mark matching the number of days (7) post-marking, (Figure 2.3-2.4).

Indirect & Direct Growth Indices

2.3.2 Recent Otolith Growth

Recent otolith growth had less of a response to the two feeding conditions, in comparison to the other two indices, based on the two sample t-test with the mean recent otolith growth (average of last 3 increments) being 5.67$\pm$.52 µm for high feed and 5.35$\pm$.67 µm for low feed; $t_{39.45}$=1.76, p=.086. Low feed individuals showed higher variance of 0.56 μm^2 in recent otolith growth measurement compared to high feed individuals with a variance of .39 μm^2 (Figure 2.5). Furthermore, width of recent otolith growth was not correlated with Fulton's *K* ($F_{1,40}$ =1.06, p-value =.31), with R^2 of 0.03 or RNA:DNA index ($F_{1,40}$ =1.61, p-value =.21), with R^2 of 0.04.

2.3.3 RNA:DNA Index

The RNA:DNA index values for high fed fish that survived the entire experiment (mean=7.26±1.93) were significantly higher than the low fed fish (mean=3.05±.94); $t_{3.66}$=7.16, p=.003 (Figure 2.10). Slopes of the standard curves (m_{DNA}/m_{RNA}) were calculated as a means to facilitate inter comparability with other RNA:DNA index values from different laboratories that may utilize slightly different spectrofluorimetric protocol. This method assumes that standard curves are linear and the y-intercepts were near zero after subtracting the blank reagents (Caldarone *et al.,* 2006). Slope of the DNA standard curve (m_{DNA}) was 219.1 and the slope of the RNA standard curve (m_{RNA}) was 32.83 to produce a ratio of standard curves (m_{DNA}/m_{RNA}) of 6.7. There was a strong positive correlation between calculated values of Fulton's *K* and RNA:DNA index (correlation coefficient =.61; $F_{1,41}$ =24.42, p<.001), with R^2 of 0.37.

2.3.4 Otolith Increments Over Time

To test if food consumption influences daily otolith increment lengths, we first performed a two-tailed binomial test to determine if fish in the low and high feed treatments held similar otolith growth rates prior to the start of the feeding experiment. We conclude that there was no evidence that the otolith growth rates were similar with a p-value < .05. In fact, despite our efforts to ensure random assignment of treatments, on average the individuals experiencing high feed treatment had slower otolith growth (smaller increment sizes) before the experiment started. Running the two-tailed binomial test during the feeding

experiment revealed a p-value > .05, meaning that there was no difference in otolith growth rates for each of the treatment types. Over the duration of the feeding experiment, there was a shift from high feed increments consistently being measured smaller (time zero through 11th day of the experiment) to gradually increasing in size and ultimately surpassing low feed increments (12th day to the final day of the experiment) (Figure 2.6). This implies that there was over a week delay between changes in feeding regime to be reflected in otoliths and the large standard error bars for each mean otolith increment length reveals that these mean values were less precise.

2.3.5 Fulton's Condition Factor (K)

At the start of the feeding experiment, Fulton's *K* value was ~1.25g/cm^3 regardless of treatment. There was no difference between the Fulton's *K* for the high feed and low feed during week 1 and 2 based on the two sample t-tests. On the other hand, during weeks 2 ($p < .05$) and 4 ($p < .001$) there was a significant difference between the *K* values for each treatment (Table 2.1). Simple linear regression of the Fulton's Condition Factor examined in low feed treatment showed a significantly lowered condition factor over time ($R^2 = 0.40$, $F_{1,11}=7.25$, $p<.05$) (Figure 2.7). Individuals who died during the experiment measured lower for this condition factor than the living fish, high feed fish had an average Fulton's *K* of 1.04g/cm^3 and low feed fish had an average Fulton's *K* of .79g/cm^3.

2.3.6 Normalized Fulton's *K* and Otolith Increments

To better compare average Fulton's *K* and daily otolith increments on a more standardized scale, we normalized both measurements from day 0 (start of the experiment) as reference to examine how each type of index changed from that time point (Figure 2.8-2.9). Normalizing data will better highlight the changes from the beginning values for each group since the high feed individuals displayed slower otolith growth rates originally.

By the beginning of week 2, Fulton's *K* value for high feed fish was approximately 1 standard deviation above the mean Fulton's *K* value at day 0, while low feed fish had relatively the same Fulton's *K*. At week 3, Fulton's *K* continued to be greater for high feed fish. During week 4, high feed individuals had Fulton's *K* values that were nearly 1 standard deviation above the mean initial value again in comparison to the food-deprived individuals with approximately .5 standard deviation below the mean initial value.

The normalized daily otolith increments showed both a more variable and stronger response to the feeding regimes. Most of the time, high feed individuals showed faster average otolith growth represented by increasing widths of otolith increments over time, with the highest being nearly 10 standard deviations above the mean initial increment at day 21. Similarly, the low feed fish had continuously smaller increment values from day 7 to day 28 with the lowest being nearly 12 standard deviations below the mean initial value at the very end of the experiment.

2.4 Discussion

Our study revealed that different levels of food intake by killifish was reflected in three measures of nutritional status (otolith increment growth, Fulton's *K*, and RNA:DNA index) to different extents, and provides confirmation that this species produces daily otolith increments. Daily otolith increments widths did not show a difference between treatment types, possibly due to the high variability in the microstructure readings and time-lag effect between feeding events and response of increments. However, the normalized increment widths plotted over time did show more of the expected pattern with a gradual increase for high feed and larger decrease in increment width for low feed. There was a clearer pattern in the response of Fulton's *K* to food rations, with weeks 2-4 being significantly different from one another. One potential reason for why Fulton's K in high feed individuals inconsistently grew larger could be due to individual variation in feeding, which is hard to monitor with living fish. Lastly, RNA:DNA index showed a large difference in respect to past food uptake, however evaluating on a weekly basis would have given us more clarity on how sensitive this biochemical indicator is. Overall, Fulton's K and RNA:DNA index were more correlated to each other as measures of relative growth for killifish. Otolith growth also showed a response to food uptake, however caution should be exercised when using this indirect measurement of growth due to the highly varied response to past food levels.

2.4.1 Marking Quality and Application

Validating daily otolith increment formation is a critical first step before interpretation of otolith growth patterns can be made, and to examine life history traits for effective fishery management (Campana and Neilson, 1985; Jia and Chen, 2009). The 65% survivorship from the first immersion trial and the experimental fish eating almost immediately following immersion allows us to assume that chemical marking did not induce long-term damage or severe enough stress to result in poor fish growth performance. Only two fish survived and were successfully marked by the second marking attempt, however otolith marks were clearly visible under fluorescence microscope, and our findings support that Alizarin Red Stain can lead to otolith mark retention in fish at early developmental stages at a lower cost than other fluorochrome dyes (Beckman and Schulz, 1996; Eckmann *et al.,* 1998; Eckmann, 2003; Baer and Rösch, 2008).

One concern was the potential for the fish to experience high stress during the immersion period since high concentrations of certain fluorochromes can become toxic to fish. This could explain the high mortality during the original immersion experiment before the feeding experiment (65% survivorship) and the second marking trial experiment (.05% survivorship). Excessive mortalities, low marking success, and apparent stress during the immersion period were seen at concentrations of 400 mg/L ARS for larval immersions (Beckman and Schulz, 1996). Variable mortality rates (<10 to 100%) were also observed when mass marking *Coregonus albula* (L.) embryos at concentrations of 150 mg/l ARS or higher (Eckmann *et al.*, 1998). To combat these high mortality rates, a chemical

stabilizer, such as NaOH, is often used to maintain the pH of the water to obtain the best marking effect (Baer and Rösch, 2008). In the current study, water quality was checked prior to adding in ARS and strong aeration was used to raise the pH, but pH was not continuously monitored once fish were immersed. To examine if pH does decline, we completed a trial test monitoring the pH levels with only 400 mg/l ARS, and this showed no drastic decline in pH after 24 hrs.

Another potential improvement to produce better marking success would be to test at a younger development age since for some species the best life stage for mass-marking with ARS is at the embryonic or larvae stage (Nagiec et al., 1995; Beckman and Schulz, 1996; Eckmann et al., 1998). Furthermore, we recommend adding more air pumps to the ARS solution to enhance the circulation in the system as well as using larger containers to decrease the potential stress of overcrowding the fish during the immersion period. Other potential causes for high mortality rates may be due to overcrowding, lack of oxygen, excessive stress or a combination of these events. There is always a danger to using any chemical for immersion marking; thus it is important to assess which developmental stage and which fluorochrome dye is optimal to safely mark the calcium carbonate structure in growing fish (Eckmann, 2003).

2.4.2. Otolith Recent Growth Findings and Context

Past studies have shown a strong relationship between somatic growth rates and otolith growth rates (Radtke, 1989; Iglesias *et al.,* 1997). For example, a study on *Engraulis anchoita* larvae showed that the otolith growth rings

correlated more strongly with fish size and somatic growth than with the nutritional condition as measured by the RNA:DNA index (Do Souto *et al.*, 2019). In contrast, the otolith growth for California killifish did not show strong correlations with either the somatic growth or RNA:DNA index. Low feed individuals showed higher variance in recent otolith growth, which may suggest some individuals were able to out-compete others when feeding period occurred. Thus over the duration of the feeding experiment, the individuals who were better competitors would continue to be healthier and have better otolith growth than poorer competitors.

Otolith deposition is influenced by various exogenous (e.g., temperature, feeding conditions) factors and intrinsic (e.g., developmental stage, fish size) factors, thus with this controlled study we were able to only focus on how food levels would alter otolith growth (Campana and Neilson, 1985). Otolith deposition rates varied dramatically among groups of herring larvae (*Clupea harengus*) due to differences in individual growth rate, which is controlled by level of activity, and herring larvae were observed to grow faster in larger tanks than smaller ones (Geffen, 1982). Furthermore, overcrowding can lead to decreased growth rates and constant stress can lead to adverse state of welfare in fish (Stevens *et al.*, 2017). Although the densities for the low feed treatment were higher (27 fish/tank) in comparison to high feed (10 fish/tank), the low feed treatment fish were placed in larger tanks for a nominal difference of .036 g of fish per liter of seawater. Therefore, if small tank size inhibited swimming activity and growth in killifish then this factor would most likely have equally impacted

both treatments as the densities were relatively similar to each other. However, since smaller tank sizes can confound potential growth in fish, larger enclosures should be considered for future studies to promote better conditions for maximum growth in fish. Water quality was also relatively the same across all experimental tanks due to the flow-through system that maintained water temperature and salinity throughout the experiment. One potential reason for why recent otolith growth showed less of a response to food amount may have been the inability to track individual feeding levels, as stated before, some of the fish in the food-deprived treatment may have been able to out-compete others to access more food. Therefore different feeding rates among food deprived individuals would most likely present different growth rates and reconfirm why there was a larger range of variance for recent otolith growth. These environmental and physiological factors are difficult to assess in a non-laboratory setting, and therefore otolith microstructure analysis should be used with caution in those situations.

The effect of food on otolith growth was more apparent in the standardized dataset, in which generally the average otolith increment size for high feed stayed around 0-5 standard deviations above the initial mean increment size and the low feed had a more continuous decline in otolith increment size over time to 11 standard deviations below the initial mean increment size. However, a lagged response of otolith increments to changes in nutritional status is not uncommon, as incremental growth is dependent on energy reserves, fish size, and potentially temperature (Hüssi and Mosegaard, 2004). Because of the slow,

lagged changes in daily otolith increment widths, we conclude that otolith growth may not be as sensitive to food uptake as the other indices.

2.4.3 Fulton's Condition Factor (*K*) Findings and Context

By week two of the experiment, there was a significant difference in Fulton's *K* between feeding regimes (t-test, $p<.05$). This result suggests that length and mass measurement respond to food availability in at least a two-week time period. The Fulton's *K* for the low feed treatment declined much more than that of the high feed treatment throughout the experiment, which is expected for fish being deprived of food. There was a significant difference in mean of Fulton's *K* in weeks 2-4 of the experiment, which shows that this value can fluctuate on a rather quick timescale and be a reliable indicator for relative food conditions. A food deprivation study on European sprat juveniles (*Sprattus sprattus*) showed that Fulton's *K* can change rapidly, with a significant decrease in Fulton's *K* after 4 days (Peck *et al.*, 2015). However, when normalized this approximation explained the least amount of variability in sprat growth rate, while otolith growth and RNA:DNA index had more rapid responses during food deprivation (Peck *et al.*, 2015). In this current study, since all the fish were fed the same type of prey during the experiment, we did not have to evaluate if there was bias between different prey types affecting Fulton's *K*. On the other hand, in a natural setting with access to upper marsh surface, killifish populations can consume a mixture of prey including bivalves, insects, and mainly amphipods (Robinson-Filipp, 2021). Due to this varied diet and bias, the type of prey may

need to be more considered when sampling this index from natural populations since these individuals may have higher Fulton's K values associated with certain prey types. Overall, this morphometric index proved to be an easy, non-lethal way to measure index of condition, with a 2-week lag for killifish.

2.4.4 RNA:DNA Index Findings and Context

This work confirms that nucleic acid quantification can reflect past food uptake since the RNA:DNA index values were significantly different from each other based on treatment type. Additional studies have confirmed that this biochemical indicator can depict poor feeding conditions rapidly and is closely related to recent growth rates for various organisms (Buckley, 1984; Bergeron, 1997). During the starvation trials, lowered RNA:DNA ratios were due to the reduction of protein synthesis associated with the drop in rRNA quantity in the muscles and a diminished efficiency of amino acid incorporation from the poor diet (Ferron and Leggett, 1994). RNA:DNA ratios were even seen to be correlated with diel periodicity in *Sciaenops ocellatus* larvae, in which significant declines in RNA:DNA ratios were observed during nocturnal periods (0000, 0400 hours) and peaked around 1200 hours regardless of temperature differences (Rooker and Holt, 1996). The effect of food deprivation on RNA:DNA ratios of killifish is in agreement with the various findings that this biochemical indicator will drastically decrease due to a reduction in food availability and can be a useful indicator in recent growth rates (Buckley, 1980; Lowery and Somero, 1990; Richard *et al.*, 1991). However, to better understand how reliable RNA:DNA index may

represent nutritional status for killifish, future studies should evaluate how this biochemical indicator changes in the time span of hours to a few days. While we could not determine this in this study, we would expect a significant disparity between the two values most likely within less than a week's time based on past studies.

It is essential to calculate the ratio of the slopes of the standard curves (m_{DNA}/m_{RNA}) as a means to facilitate comparison of RNA:DNA index values from other laboratories that use different spectrofluorometric protocols. Inter protocol differences (i.e., specific fluorophore, protein dissociation chemical being used, and enzyme incubation period) may lead to drastic alterations to the calculated RNA:DNA index values. Since the standards and reagents were not exactly the same as Caldarone *et al.*, these minor differences may explain why this study detected a different m_{DNA}/m_{RNA} of 4.3 in comparison to our 6.7 value (Caldarone *et al.*, 2006).

Individuals sampled directly from the field had RNA:DNA index values in between the high feed and low feed treatment individuals (Figure 2.10). These fish, representing the natural population, were sampled when the previous nighttime tides were greater than 1 meter in height, and thus we assume that the channels were flooded and the vegetated marsh surface was accessible. We assume that these individuals had less accessibility to capture food since the last high tide, thus the average RNA:DNA index values represents the natural population's satiation levels after less than 12 hours from the feeding event and the previous days of feeding. The lower range in RNA:DNA index values for

these wild-caught individuals suggests that these populations have greater capacity to consume more food than they did at the time of sampling (Figure 2.10). We would most likely see a higher RNA:DNA index if sampling times took place during high tide right after or when the vegetated marsh surface was accessible.

2.4.5 Limitations to a Controlled Study & Recommendations

Laboratory studies allow for more control of variables, allocation of treatment types, and better replication, yet there are a number of disadvantages that arise with a laboratory setting as well. One major disadvantage with an artificial environment is that this type of unrealistic setting may lead to a lack of ecological realism. However, controlling the densities of fish per tank and specified food rations are important components in the context of this experiment to detect if food treatment does cause an impact on fish growth. Another disadvantage is that stress from an artificial environment can affect fish growth. Regardless of treatment type, the fish that died during the feeding experiment held lower average Fulton's *K* compared to the start of the experiment, which may suggest that the environment was inducing stress and in turn reduced body condition. In addition, the dramatic increase in mortality rate for the temporary starvation period for the low feed treatment fish indicated that juvenile California killifish are deeply reliant on food resources to survive at this stage of development. A solution to gain more applicability from experimental results is to create a mesocosm experiment to better mimic natural field conditions or to

recapture tagged fish living in a natural marsh at a later date. However mesocosm experiments can be expensive and re-capturing efforts could disturb endangered bird species and other organisms in the Kendall-Frost Reserve (Zedler *et al.*, 1992).

2.4.6 Implications

The two main factors that govern habitat quality are water temperature and food availability (Gibson, 1994). Our results show that these three nutritional indices (recent otolith growth, Fulton's *K*, and RNA:DNA index) can reflect relative food conditions for California killifish across a number of timescales, and therefore may indicate the health of this estuarine fish species. This in turn can provide a better indication of prey availability or feeding success in their surrounding habitats. Monitoring fish health across different spatial and temporal scales is important, since organisms can act as indicators of the environmental status for that region. For example, the health of flatfish was used as a bioindicator to identify contaminated nursery areas because the organisms showed a reduced biological performance in terms of low abundance and somatic growth (Gilliers, *et al.*, 2006). Another study performed by Eastwood and Couture (2002) showed pollutants impairing fish health, in which yellow perch (*Perca flavescens*) sampled in contaminated metal environments displayed higher liver metal concentrations, lower relative condition factors, and slower fish growth rates (smaller for a similar age). Since food availability affects the nutritional conditions of juvenile fishes rather rapidly, it would be important to also

investigate how temperature and polluted waters play a role in determining these growth rates as well.

We recommend sampling across various wetlands where California killifish reside in to provide more insight in how different environmental factors directly and indirectly affect fish health status. Gaining a deeper understanding of how to monitor fish health may paint a clearer picture of the prey availability as well as insight as to how well these local ecosystems are supporting fish populations. Consequently, this will inform better prioritization decisions for conservation and management efforts of these heavily degraded areas.

2.5 Conclusion

This study provides a better understanding of (1) daily validation of otolith increments for the California killifish, (2) the effect of amount of food on nutritional indices for this species, and (3) how these nutritional indices compare with each other at different food levels. The shift in daily otolith increments in response to food was best shown in the normalized average otolith increment values; however there was high variance for each increment regardless of treatment. There was also weak evidence to show that recent otolith growth (avg. of last 3 increments) can convey food amount in this species. There was a continuous decrease in body condition as shown by the Fulton's *K* when killifish experienced low levels of food and by day 14 there was a significant difference in Fulton's K for each treatment type. Unlike otolith increments this morphometric index always measured greater for the high feed individuals than the low feed

treatment fish. Lastly, RNA:DNA index showed a significant difference with the high feed fish having higher RNA:DNA index values at the only time point of sampling which was day 28. Individuals sampled directly from the Kendall-Frost Reserve held a RNA:DNA index closer to the RNA:DNA index value representative of the low feed treatment type, which indicates that this certain population has the potential to consume more than they did at the time of capture.

2.5.1 Recommendation to Test Sensitivity of RNA:DNA index

RNA:DNA index showed a significant difference between the two treatments and we would expect this to be true for shorter time scales as well. Without weekly sampling of RNA:DNA index it is impossible to confirm from this study how rapidly this index changes based on condition in killifish. However, based on numerous nucleic acid quantification studies with larval/juvenile fish, this index appears to be a more rapid way of assessing health (i.e., feeding history) than Fulton's *K* (De Raedemaecker *et al.*, 2012). We suggest further investigation into nucleic acid quantification techniques at a shorter time-scale (in days) to better evaluate this relationship for this species.

2.5.2 Recommendation to Evaluate Nutrition Indices on Broader Scale

Lastly, we recommend future sampling of killifish populations across multiple sites to evaluate spatial and temporal variation in these condition indices. These nutritional indices may enable better characterization of the sites in terms of prey availability and optimal condition for fish growth. Since water

temperature is one of the most prominent hydrological variables to affect biological performance (i.e., food rate and swimming activity) and fish growth, sampling environmental factors across multiple estuarine habitats will provide better inference to a temperature optima for this species (Fonds *et al.,* 1992; Lankford and Targett, 1994; Mallekh *et al.,* 1998). A previous work has shown temporally stable spatial trends in Fulton's K, but variable RNA:DNA index patterns among juvenile plaice (*Pleuronectes platessa*) and dab *(Limanda limanda*) potentially due to short-term variations in food availability or habitat characteristics (De Raedemaecker *et al.,* 2012). Thus, nucleic-based condition indices appear to be a better reflection of short-term fluctuations in environmental conditions than Fulton's *K*. Future experiments should assess different temperature conditions and prey type to better standardize this biochemical index for killifish.

Our findings from this current study, support that Fulton's *K* or RNA:DNA index appear to be more reliable indices of growth as reference for past prey availability. Surveying these nutritional indices against more environmental conditions across coastal estuaries will deepen our understanding of prey volumes in one of the most anthropogenically degraded ecosystems. Furthermore, finding more indicator species to provide insight into changes and disturbances to the local environment is one approach to support better conservation for estuarine fish.

TABLES

Table 2.1 T-values and p-values from two sample t-test of weekly Fulton's condition factor, K (g/cm^3) throughout the 28-day experiment.

	Treatment type									
	High feed			Low feed			95% CI for Mean			
Fulton's condition factor (*K*)	*M*	*SD*	*n*	*M*	*SD*	*n*	Difference	*t-value*	*df*	*P* Value (2-tail)
K-values: week 1	1.43	0.27	25	1.37	0.27	24	-0.23, 0.35	0.55 (ns)	4	.62
K-values: week 2	1.47	0.17	23	1.26	0.16	25	0.07, 0.36	4.09*	4	.02
K-values: week 3	1.34	0.12	20	1.21	0.2	24	8.81E-04, 0.27	2.79	4	.05
K-values: week 4	1.39	0.09	20	1.14	0.09	22	0.23,0.28	27.50***	4	<.001

*p < .05; **p<.01; *** p < .001; ns, not significiant (p > .05)

FIGURES

Figure 2.1 Map of the fish collection site in Southern California, USA, where juvenile California killifish (*Fundulus parvipinnis*) were collected for the feeding regime experiment. California killifish were collected from this site using beach seine nets during low tide on the 25th June 2020, 28th June 2020, and 10th July 2020.

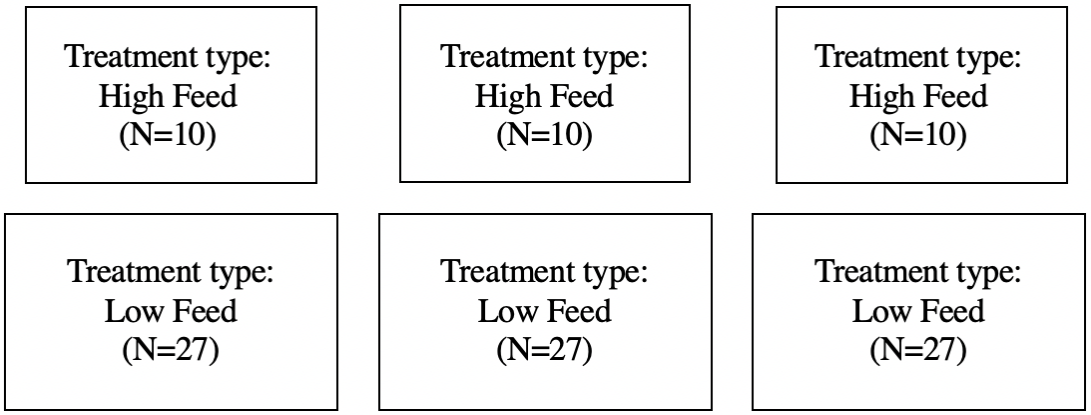
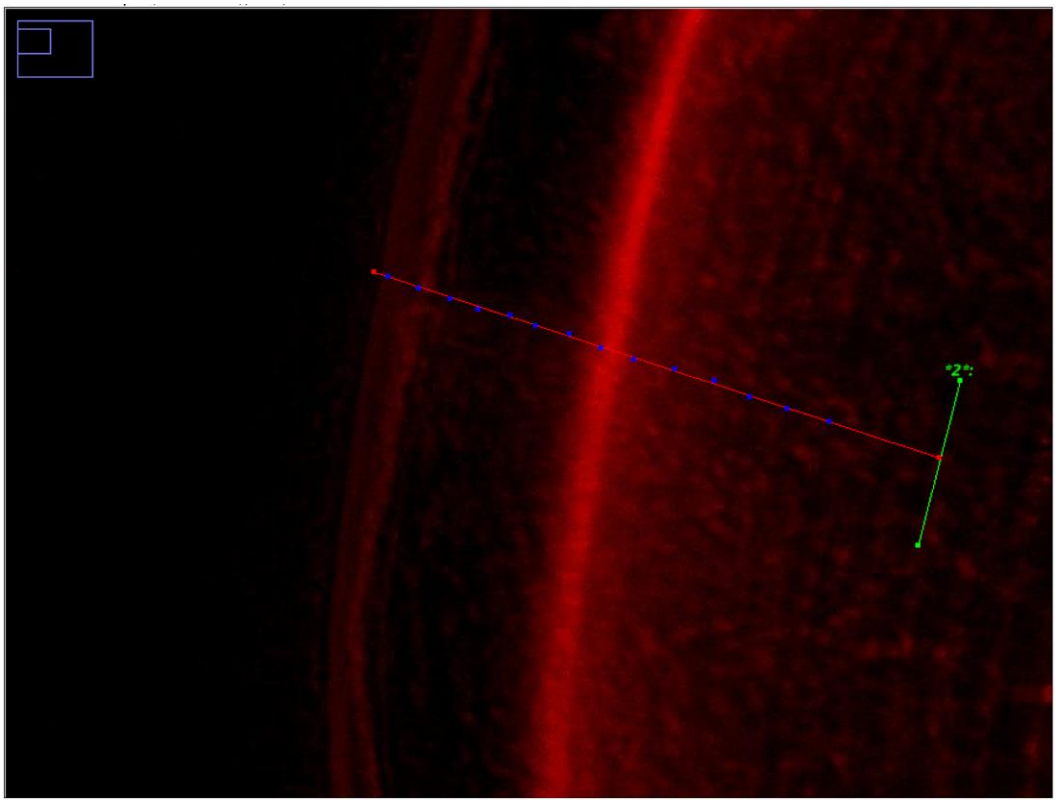


Figure 2.2 Assignment of number of juvenile California killifish to each feeding treatment (high vs low feed). High feed treatment tanks had a density of .104 g of fish per liter of seawater and low feed treatment tanks had a density of .140 g of fish per liter of seawater.

Figure 2.3-2.4 Alizarin red stain (ARS) mark shown in California killifish sagittal otolith at 20x.



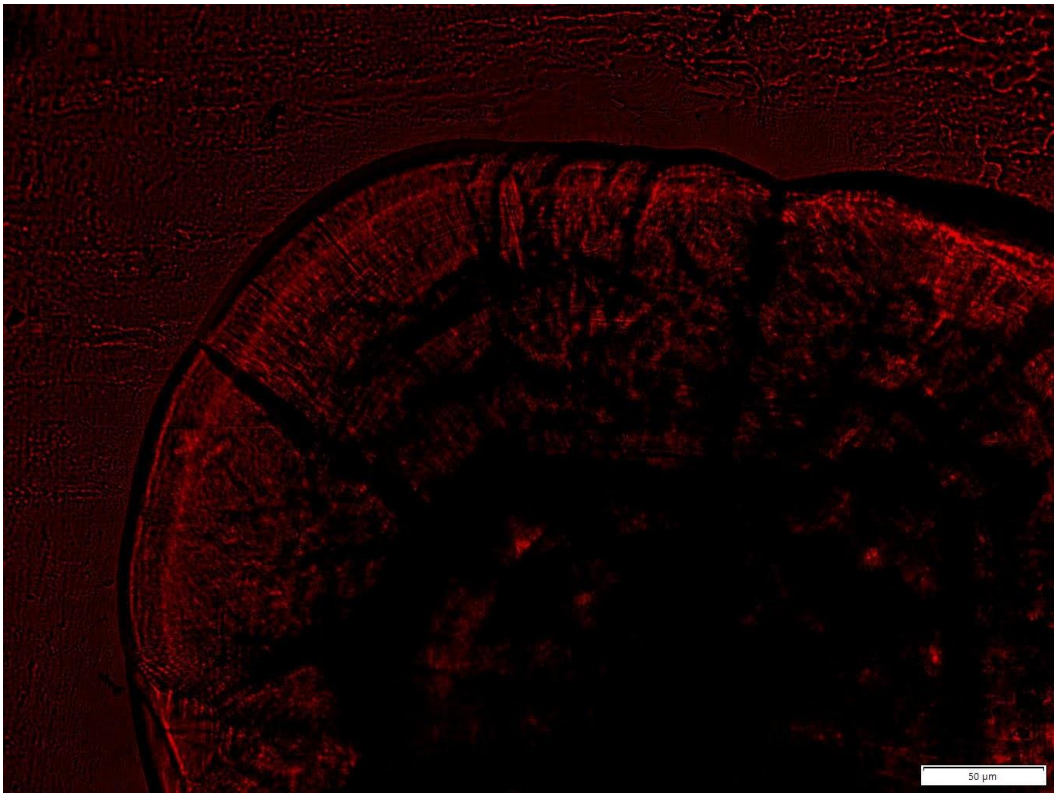


Figure 2.5 Boxplot of the recent otolith growth (average of last 3 increments) in 43 otoliths of juvenile California killifish experiencing high feed and low feed treatments.

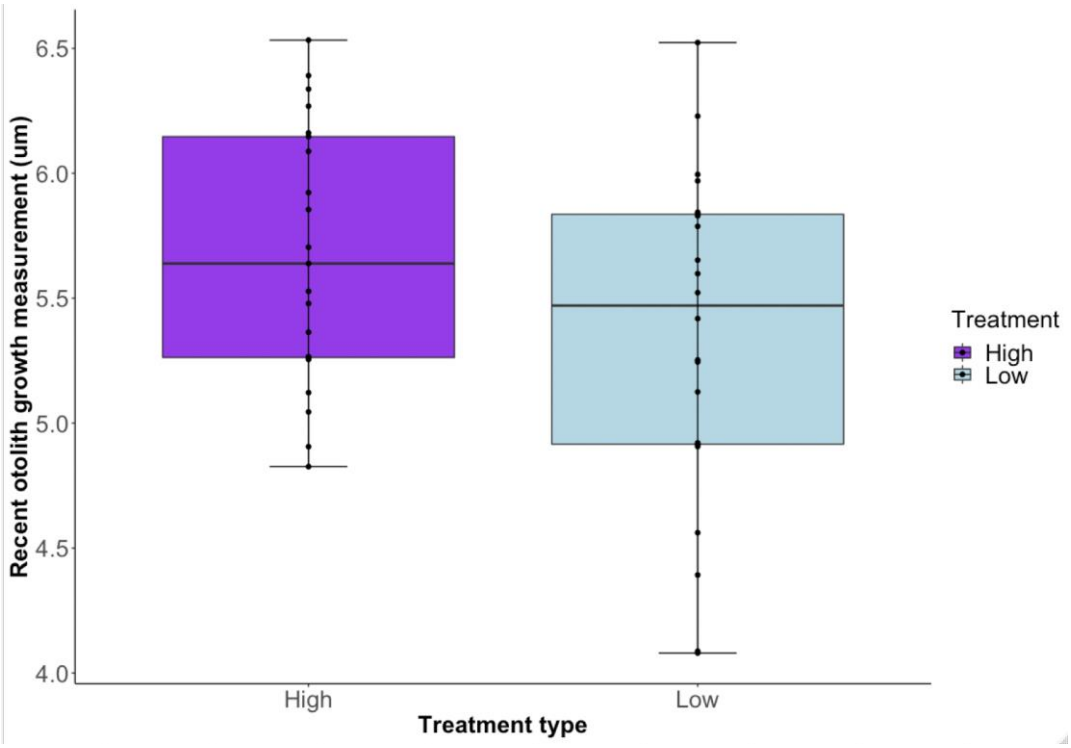


Figure 2.6 Scatter plot of the average otolith increment size in 43 otoliths of juvenile California killifish experiencing the high feed and low feed treatments. Grey dashed line represents the start of the 28-day experiment and the error bars display $\pm$ 1 standard error.

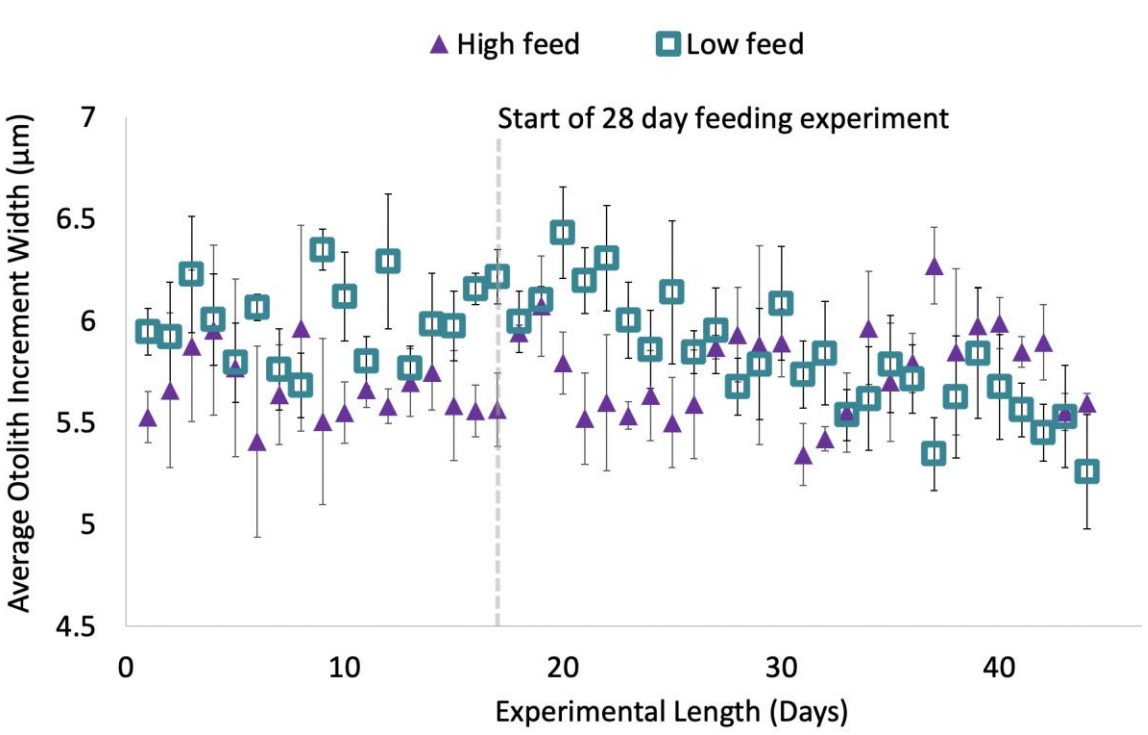

High feed
Low feed
Start of 28 day feeding experiment
Average Otolith Increment Width (µm)
7
6.5
6
5.5
5
4.5
0
10
20
30
40
Experimental Length (Days)

Figure 2.7 Scatter plot of the weekly Fulton's condition factor, K (g/cm^3) calculated from the weekly random sampling of length and weight measurements from juvenile California killifish throughout the 28-day experiment. Error bars display $\pm$ 1 standard error.

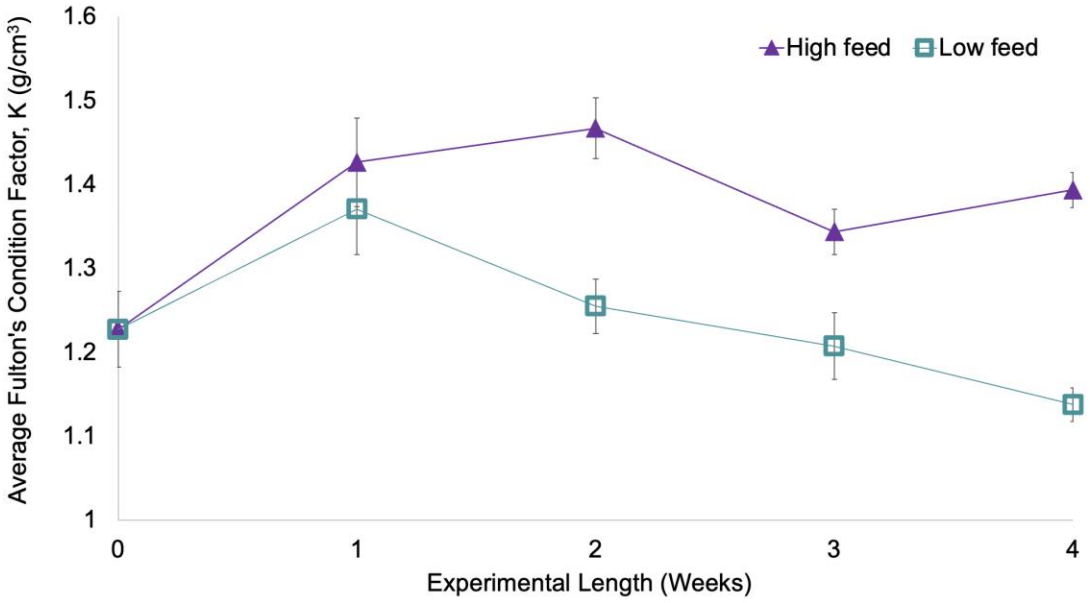


Figure 2.8 Normalized average otolith increments (µm) in respect to the average otolith increment value at day 0 for each treatment. Error bars display ± 1 standard error.

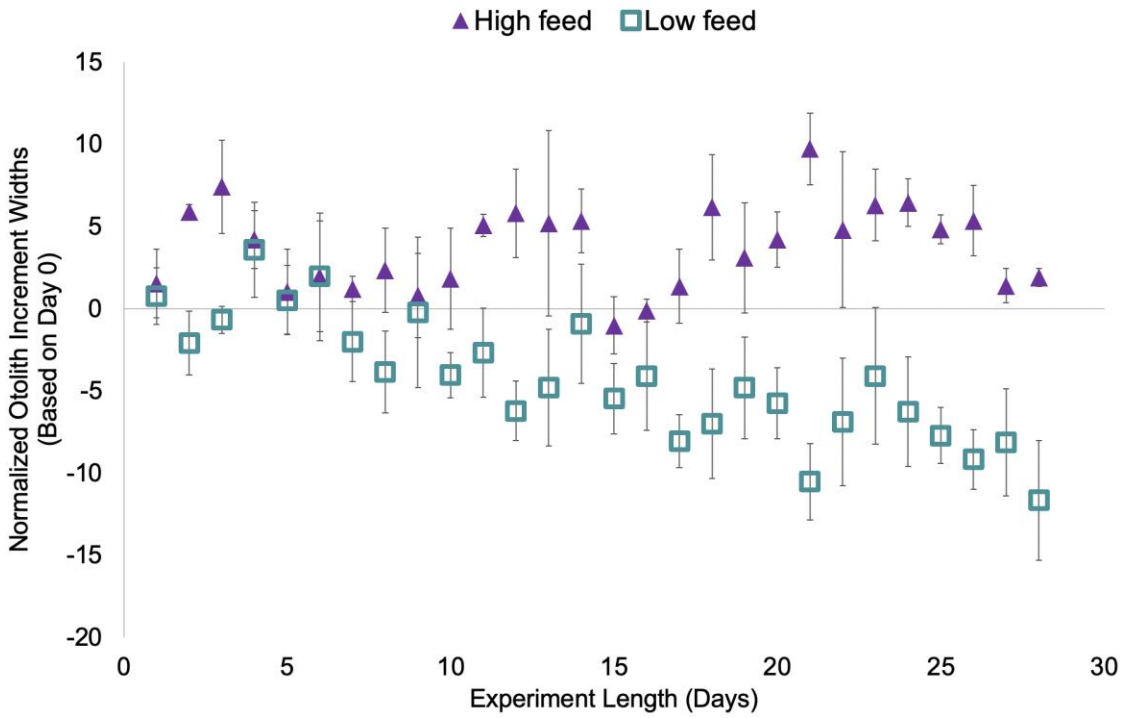


Figure 2.9 Normalized average Fulton's Condition Factor, K (g/cm^3) with respect to the average value at day 0. Error bars display ± 1 standard error.

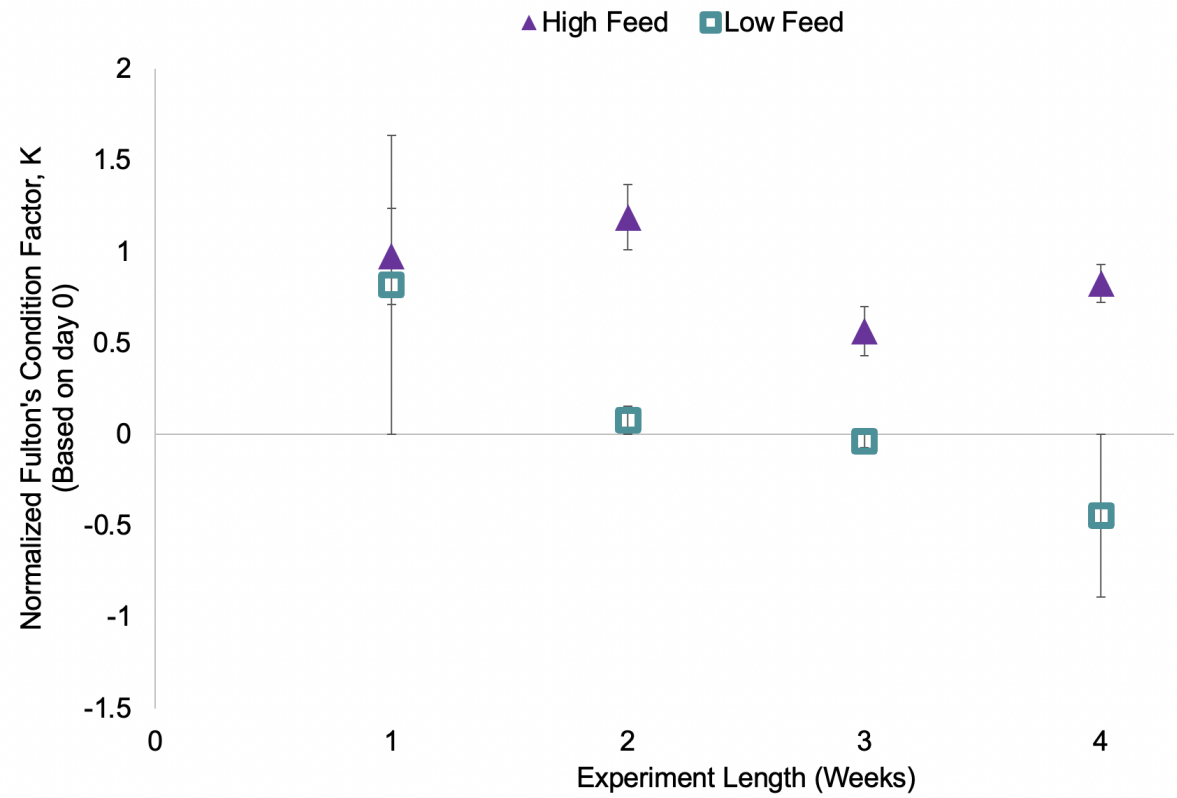


Figure 2.10 Boxplot of the range of RNA:DNA index values for each treatment type (high feed vs low feed) as well as juvenile fish that were captured directly from the Kendall-Frost Reserve.

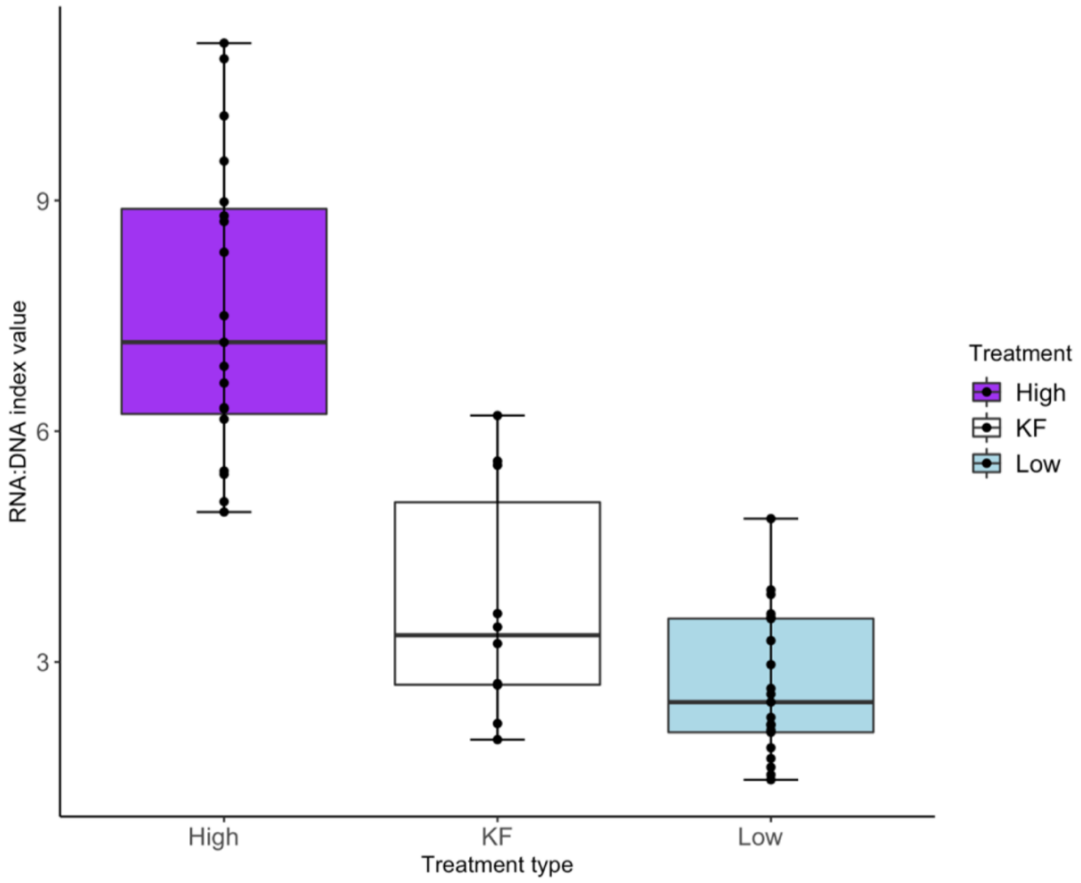
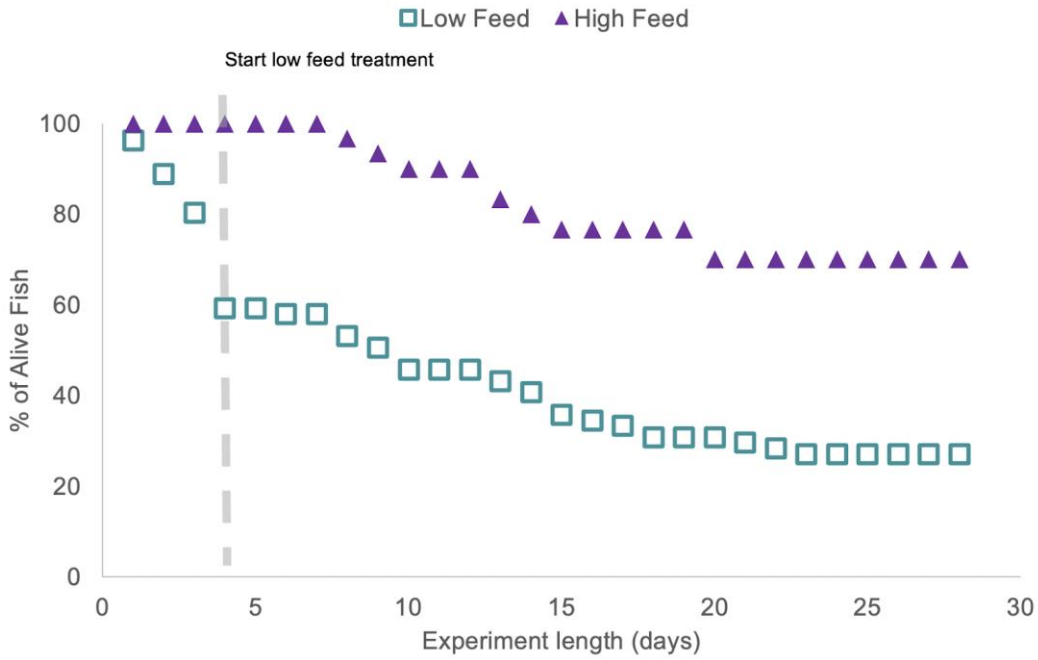


Figure 2.11 Scatter plot of the daily mortality count of juvenile California killifish for both treatments throughout the 28-day experiment length represented as percent of fish that were alive. Low Feed treatment was originally zero food, until low feeding treatment was established (half the feeding rate of the high feed treatment) by day 4 due to an apparent increase in mortality rate.



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APPENDIX

Appendix A.1 - Immersion experiments for marking otoliths

In summer 2020, juvenile killifish caught from Kendall-Frost Reserve were placed in 400 mg/L ARS for 24 hours with approximately 65% survivorship and the survivors were used for the 28-day feeding experiment. There was no ARS mark detected under normal and fluorescence light at the end of the feeding experiment. A second immersion experiment attempt was done in summer 2021 with the juvenile California killifish caught at the opening of Tecolote Creek in Mission Bay, California (32°46'15.2"N 117°12'29.9"W). Fish sampling took place during low tide on the 6th June 2021 and 40 individuals were placed in 400 mg/L ARS for 24 h and 30 individuals in 200 mg/L ARS for 12 h. Higher concentration had a low survival rate (.05%) and ARS mark was distinct only under the fluorescent light. We found better marking success when the ARS was completely dissolved in water by first mixing the chemical in 1 liter containers with magnetic stirrers. All the individuals survived the 200 mg/L, but there was no ARS mark formed. A more in-depth analysis of their microincrements was beyond the scope of this project, but is required in order to make better inferences about major life history traits for killifish.

Appendix A.2- Metacercaria Infection Rate

This fish species is a second intermediate host to a brain-encysting trematode called *Euhaplorchis californiensis*. Mean abundance generally increases with host size and most fish are infected with 100 to 1,000s of *Euhaplorchis californiensis* (Shaw *et al.*, 2010).

Length of fish (cm)	Metacercaria Infection Rate (number of metacercaria/fish)
SL < 2.4 cm	640 metacercaria/fish
SL 2.4-2.9 cm	819 metacercaria/fish
SL > 2.9 cm	701 metacercaria/fish
Total average	763 metacercaria/fish