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Article

Feeding Behavior of Larval Zebrafish: Effects of Two Brine Shrimp Strains

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Abstract

Larval zebrafish husbandry regimens often use live rotifers or paramecia during the first 2-4 weeks of feeding, then transition to using live brine shrimp (*Artemia*). This practice is based on the assumption that the small larval gape better accommodates smaller prey, as opposed to larger *Artemia*. Once Artemia are introduced into the diet, protocols often use San Francisco Bay (SFB) *Artemia franciscana* because this strain is reported to be smaller than the Great Salt Lake (GSL) strain. We tested feeding preferences of larval zebrafish for either strain from 5-15 days post fertilization (dpf). Daily, intestinal contents were imaged through the body wall of live larvae following feeding. We found that larval zebrafish captured the SFB strain more successfully than the GSL strain until 13 dpf, yet larvae fed either strain exhibited similar progression to metamorphosis. We tested for length and width differences between *Artemia* strains and found no size differences for newly-hatched nauplii. However, after 48 hours of culturing, GSL metanauplii were significantly longer than SFB metanauplii. In sum, newly-hatched *Artemia* of either strain can be included in the larval zebrafish diet, though it is important to confirm that larvae sufficiently capture the live prey.

Keywords: *Danio rerio*; *Artemia*; zebrafish husbandry

1. Introduction

The zebrafish (*Danio rerio*) is a small, omnivorous, freshwater teleost that tolerates wide ranges in temperature, salinity, and pH [1–3]. Zebrafish are a popular model organism and are easy to maintain in the laboratory, as shown by the many different successful husbandry regimens, reviewed in Wilson [4] and Licitra et al. [5]. Husbandry regimens vary in their details, but a general practice for maintaining adult zebrafish is to supply a formulated feed at least once per day along with a daily supplement of live *Artemia* (brine shrimp). Typically, *Artemia* are cultured from cysts and collected after a day or two of culture. Although zebrafish do not eat *Artemia* in the wild, *Artemia* are a popular dietary supplement because they are highly nutritious and simple to culture [6].

During the larval stage, zebrafish are quite vulnerable and raising them successfully requires more effort than maintaining juveniles or adults. Zebrafish are typically considered to enter the larval stage when the embryo hatches from its chorion and becomes free-swimming, at 3 days post fertilization (3 dpf). At this stage, larvae are 3.5 mm in total length [7]. The time to reach the metamorphic stage is variable, but most larvae will have begun metamorphosis between 15 – 21 dpf [8]. To support maturation, husbandry recommendations for larvae typically call for at least two daily feedings using a formulated commercial feed, as well as a supplement of either live *Paramecium* or *Brachionus* (rotifers) during the first weeks of life [6,9,10]. The rationale for this feeding regimen is that paramecia (150-250 μm) and rotifers (~150-250 μm) are small and thus easily captured and ingested by larval zebrafish [10]. By contrast, *Artemia* nauplii are approximately two to four times larger in length than paramecia or rotifers [10,11], and it is generally thought that the relatively large size of *Artemia* nauplii makes them unsuitable for newly-feeding larval zebrafish. Therefore, zebrafish

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laboratories often use rotifers or paramecia as the first live food, then transition to *Artemia* when larvae are around two to four weeks old [4,12–15].

Despite these recommendations for waiting to introduce *Artemia* into the feeding regime, our lab has routinely achieved good results raising larval zebrafish without using paramecia or rotifers as a live food source. Instead, we supplement the larval zebrafish diet with live *Artemia franciscana* nauplii, with the first feeding beginning at 5 dpf [8]. Our success with this approach suggests that larval zebrafish are adept at capturing *Artemia* from shortly after hatching. However, the feeding behavior of larval zebrafish capturing and ingesting live brine shrimp has not been previously investigated.

Here, we test the ability of larval zebrafish to capture newly-hatched, live *Artemia* nauplii. Our results show that larval zebrafish initially have a low success rate but rapidly become more adept at capturing *Artemia* as they grow. We raise cohorts of zebrafish on two different *A. franciscana* strains, San Francisco Bay (SFB) and Great Salt Lake (GSL), and find that feeding with either strain of *Artemia* leads to similar maturation rates for larval zebrafish. However, zebrafish larvae are more successful at capturing the SFB strain, with at least 50% of larvae eating a shrimp by 9 dpf, as measured by the presence of *Artemia* in the intestine. By contrast, we find that larvae fed the GSL strain reach 13 dpf before eating shrimp with a similar success rate. To better understand these unexpected differences in feeding behavior, we investigate whether there are size differences between the shrimp strains. Morphometric analyses show no size difference in length or width between the strains for newly-hatched nauplii collected 24 hours after culturing. However, significant differences in length are apparent after 48 hours of culture. We conclude that, when choosing which of these strains to include in a larval husbandry regimen, size of *Artemia* is not a significant factor if newly-hatched nauplii are used. Thus, either strain of newly-hatched *A. franciscana* can support zebrafish maturation.

2. Materials and Methods

2.1. Zebrafish Husbandry

Wild-type zebrafish of the AB line and from a local pet store were maintained following standard methods [6]. Adults were maintained on a commercial recirculating system with a daily light cycle of 14 hours light, 10 hours dark. Fertilized eggs were collected from overnight mating crosses and bleached [6]. After bleaching, embryos were transferred to fish bowls at a density of 50 embryos per bowl. The bowls contained 0.5x E3 medium (2.5 mM NaCl, 0.085 mM KCl, 16.5 mM $CaCl_2$, 16.5 mM $MgSO_4$) supplemented with 0.01% methylene blue. The bowls were incubated at 28-29°C until 5 dpf, when the larvae were transferred to a custom tabletop nursery as previously described [8]. Briefly, at 5 dpf, larvae were transferred to 0.8 L tanks (Aquaneering) at a starting density of 50 larvae per tank in approximately 250 mL of 0.5x E3 medium. Tanks were maintained at 28-29°C in the custom tabletop nursery on a natural light cycle. Larval zebrafish were fed daily with a formulated food mix at 9 am and 3 pm and live, newly-hatched *Artemia* at noon. For 5-10 dpf larvae, the food mix consisted of equal parts Golden Pearl (GP) reef and larval fish diet 5-50 micron size and GP 50-100 micron size (Brine Shrimp Direct). For 11-15 dpf larvae, the food mix consisted of equal parts GP 50-100 and GP 100-200. Food mix (1 g/L) was suspended in 0.5x E3 medium for delivery to the tanks. Larvae were fed 1 mL per 10 fish.

2.2. Institutional Review Board Statement

All vertebrate animal procedures were approved by the Institutional Animal Care and Use Committee of Appalachian State University.

2.3. Brine Shrimp Husbandry

Encapsulated cysts of *Artemia franciscana* San Francisco Bay strain and Great Salt Lake strain were purchased from Brine Shrimp Direct and stored at 4°C with desiccant. Cysts were cultured in

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approximately 725 mL of 30% sea water (Instant Ocean) in a tabletop culture dish (Hobby, Dohse Aquaristik) at room temperature or in a water bath at 26-27°C. To make 30% sea water, Instant Ocean sea salts were dissolved in RO/DI water and aerated for at least 1 hour prior to use. For feeding zebrafish and for morphometric analyses, free-swimming *Artemia* were collected in a sieve by directing light over the sieve to attract the shrimp. Shrimp were rinsed thoroughly in 0.5x E3 medium and immediately fed to larval zebrafish.

2.4. Feeding Tests

Larval zebrafish feeding tests were performed using larvae from 5-15 dpf. Before testing, one tank was randomly selected and handled following the normal husbandry regimen with one modification: at the last feeding of formulated food, GP 5-50 (pale color) was substituted for the usual food mix (orange color) to enable unambiguous distinction between ingested brine shrimp (orange) and any remnants of the previous meal. The meal of pale formulated food was typically administered in the morning, 3 hours before the brine shrimp meal at noon. In some cases, the pale formulated food was instead administered in the evening, and the brine shrimp meal was delivered at 9 am the next morning. For the shrimp feeding tests, larvae in the selected tank were fed live, newly-hatched (instar I) brine shrimp nauplii and allowed to feed for 15-30 minutes before being collected for imaging. Each day, larvae were sampled from a new tank that had not been previously sampled so that each fish was sampled once, except for one tank that was measured on two subsequent days. Each sampled fish was assessed for whether or not a brine shrimp had been ingested. Multiple mating pairs were used for each experiment to generate multiple clutches. The SFB feeding test was performed two independent times and the GSL feeding test was performed once. On each imaging day, either a subset of the larvae from one tank was randomly selected for imaging or all larvae in the tank were imaged.

2.5. Larval Zebrafish Imaging

Larvae were anesthetized using several drops of MS-222 (4 mg/mL, pH 7) or by gradual addition to the tank water of ice chips made from 0.5x E3 medium, as described previously [16]. Immediately after anesthetizing, a wide bore fire-polished Pasteur pipet was used to transfer larvae to a mold (#TU-1, Adaptive Science Tools) for imaging. The mold was made with 3% agarose in 0.5x E3 medium. Larvae were positioned laterally on the mold using a loop of fishing line. The intestinal contents were imaged through the transparent body wall using an Olympus SZX12 stereomicroscope. Larvae were immediately transferred to a recovery tank containing pre-warmed 0.5x E3 medium. Images were analyzed for presence or absence of *Artemia* in the intestinal bulb.

To video record fish during feeding, a custom fish tank was constructed using glass slides and cover glass: 75 x 50 x 1 mm glass slides (Ted Pella Inc.), 25 x 75 x 1 mm microslides (VWR), and 36 x 60 mm number 1.5 cover glass (Ted Pella Inc.). The glass was cut using glass pliers (Ted Pella Inc.) to make a chamber that was 45 mm length x 35 mm height x 25 mm depth. The edges were glued together using aquarium sealant (Aqueon). The finished tank held 30 mL 0.5x E3 embryo medium. Several larval zebrafish at 9 dpf were transferred to the tank shortly before feeding brine shrimp and recording.

2.6. Artemia Morphometrics

Free-swimming *Artemia* were collected either 24 or 48 hours after the start of the culture and were immobilized using club soda added to the culture medium as a narcotizing agent [17]. *Artemia* were randomly selected with respect to sex. To sort metanauplii from nauplii in 48 hour cultures, we identified nauplii as shrimp with unsegmented bodies in the post-mandibular region, while metanauplii were recognized by segmented bodies, as described in Schrehardt [18]. Immobilized *Artemia* were transferred with culture medium to a 0.1 mm stage micrometer (Peak Glass scale 50) and imaged using an Olympus SZX12 stereomicroscope with a 0.5x plan fluorite objective, 70 mm

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working distance. *Artemia* were positioned on the micrometer and imaged using transmitted light and an external light source. For consistency, each shrimp was oriented flatly on the dorsal side with the median naupliar eye (central eye spot) visible. Digital images were analyzed using ImageJ [19]. For each image, the software was recalibrated using the stage micrometer before taking a measurement. Body length was determined by measuring the maximum length down the midline of the body from anterior to posterior. Width was determined at the widest point, excluding the appendages. For length and width, measurements were performed two independent times per strain and developmental stage by two different people.

2.7. Statistical Analysis

All statistics were performed using GraphPad Prism 7. For comparisons of shrimp length by strain and age, two-way ANOVA was used, followed by Sidak's multiple comparisons test. For comparisons of shrimp width by strain and age, two-way ANOVA was used, followed by Tukey's multiple comparisons test.

3. Results

3.1. Larval Zebrafish Feeding Tests

In previous studies, we found that zebrafish larvae can be raised successfully using newly-hatched *Artemia* as a supplemental live food beginning with the onset of exogenous feeding at 5 dpf [8]. However, this practice may not be typical, as only a few reports use a similar husbandry regimen. For young larval zebrafish, the gape is generally thought to be too small to ingest *Artemia* [10,15,20]. Nevertheless, casual observation shows that shortly after delivering brine shrimp to a tank, at least a few larvae appear to have orange intestines, indicating they have ingested shrimp. Video recording of 9 dpf larvae suggests that suction feeding is used (Figure 1 and Supplemental Video S1), and thus gape may not limit their ability to capture relatively large *Artemia*. Because the success rate for larval zebrafish capturing *Artemia* has not been formally investigated, we determined the capture success rate for larvae from 5-15 dpf. Because larval zebrafish have transparent body walls, our strategy was to image the intestinal contents through the body wall of live larvae to determine the presence or absence of shrimp (Figure 2, panels). Newly-hatched live *A. franciscana* SFB strain nauplii were delivered daily to tanks of wild-type zebrafish larvae. After 15-30 minutes of feeding, larvae were collected, anesthetized, and imaged. We found that larvae initially had limited success in capturing shrimp at 5 dpf and gradually became more adept with age (Figure 3 graph).

Next, we asked whether larvae would show a difference in ability to capture a different strain of *A. franciscana.* We raised zebrafish larvae from 5-15 dpf using the same feeding strategy as above, but using the GSL strain instead of SFB. As shown in Figure 2, larvae younger than 13 dpf captured the GSL strain less successfully than age-matched larvae fed the SFB strain. Given this delay in capturing shrimp, we asked whether larval progression to metamorphosis would be delayed. To assess this, we used swim bladder morphology to determine the percentage of larvae that were metamorphic at 15 dpf after being maintained on the GSL-supplemented diet versus larvae maintained on the SFB-supplemented diet. The swim bladder is an easily identifiable characteristic, as the larval swim bladder consists of a single, posterior lobe while larvae that have begun metamorphosis develop a second, anterior lobe [21]. Based on swim bladder development at 15 dpf, 25% of GSL-supplemented larvae were metamorphic (of 44 screened), while 21% of SFB-supplemented larvae were metamorphic (of 58 screened). Therefore, we concluded that the lack of early feeding on *Artemia*, particularly when feeding with the GSL strain, did not delay progression to metamorphosis.

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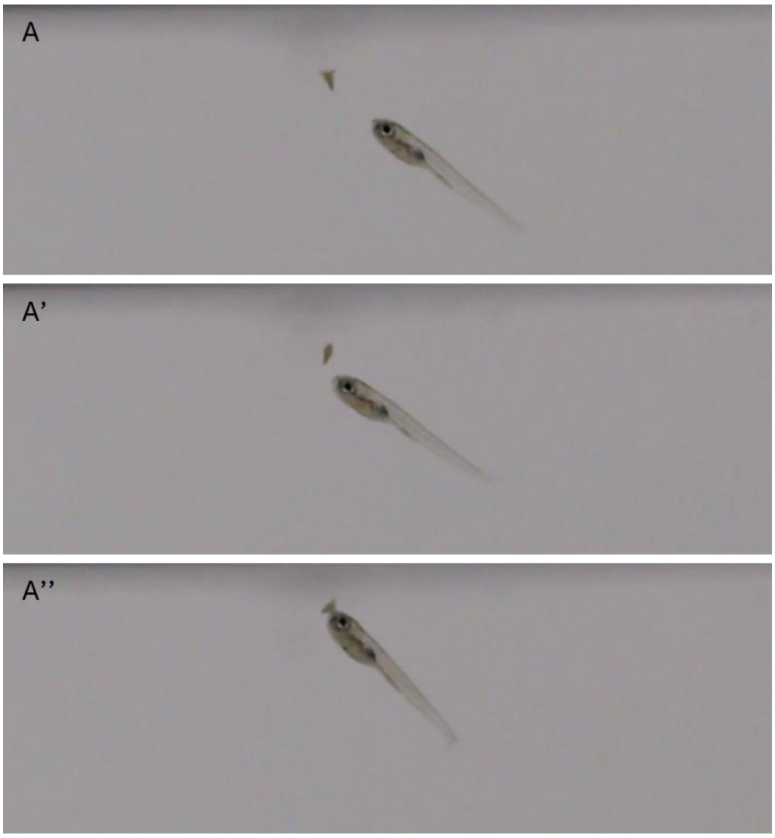

Figure 1. Larval zebrafish, 9 dpf, approaching a brine shrimp. Still frames of a single larva from one video. Representative series from two videos.

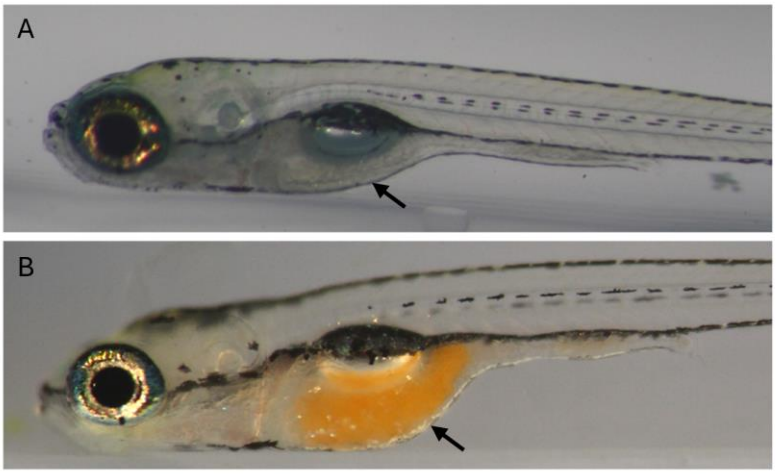

Figure 2. Zebrafish intestinal contents imaged through the body wall, live 9 dpf larvae. A) Empty intestine. B) Intestine with brine shrimp. Both images taken within 30 minutes after delivering shrimp to the tank. Lateral view, anterior to the left. Arrow: Intestine.

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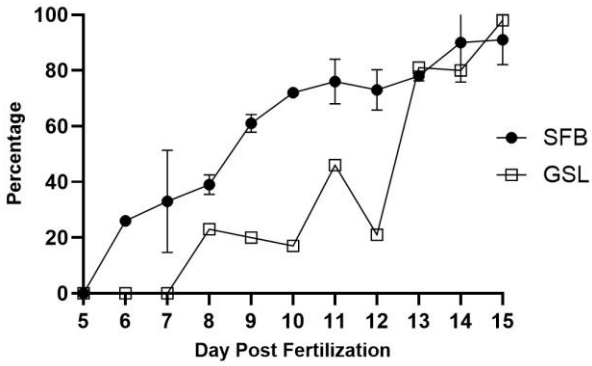

Figure 3. Capture success rate for zebrafish larvae fed *A. franciscana*. SFB: San Francisco Bay strain, GSL: Great Salt Lake strain. Sample sizes are in Appendix A, Table A1.

3.2. Artemia Morphometrics

We next sought to determine whether size differences between the two shrimp strains contributed to the observed differences in larval zebrafish feeding behavior. To develop a strategy for measuring the brine shrimp, we first considered that nauplii have a relatively large labrum that projects from the ventral surface, which might tilt the nauplius enough to produce a foreshortened image when positioned on the ventral side (dorsal view). Additionally, the lateral appendages (antennae) curve ventrally at rest when nauplii are narcotized and could also contribute to tilting and foreshortening. Therefore, we tested whether the orientation mattered for length measurements. To do so, free-swimming nauplii of the SFB strain were collected after 24 hours of culture. We imaged 19 newly-hatched SFB nauplii in both orientations (dorsal and ventral views) and measured the maximum length (Figure 4). We found that ventrally-positioned nauplii (in dorsal view) showed a shorter average length and a larger standard deviation (437.5 ± 38.13 µm) compared to orienting the same nauplii dorsally (in ventral view), which resulted in an average length of 460.9 ± 29.02 µm. The difference in length was statistically significant at $P < 0.0001$ (paired t-test, one-tailed). Therefore, we concluded that orienting the nauplius on its relatively flat, dorsal side improves the accuracy and precision of length measurements.

For subsequent *Artemia* morphometric analysis, length measurements of both strains were made with each nauplius oriented on its dorsal side (Figure 5). Because young zebrafish larvae showed relatively poor capture success when fed the GSL strain of *Artemia*, we asked whether the GSL strain shrimp are significantly larger than the SFB strain and thus more difficult to capture. We first measured shrimp size at 24 hours after the onset of culturing, as our larval zebrafish feeding regimen utilizes 24 hour cultured brine shrimp. A comparison of the SFB and GSL strains by length at 24 hours was not significantly different ($P = 0.9594$, Table 1). We further tested for a size difference by measuring maximum width of the same individual nauplii (Table 1). This test also failed to distinguish between the strains ($P > 0.9999$). We concluded that there was no significant size difference between newly-hatched (24 hour cultured) SFB and GSL strains of brine shrimp.

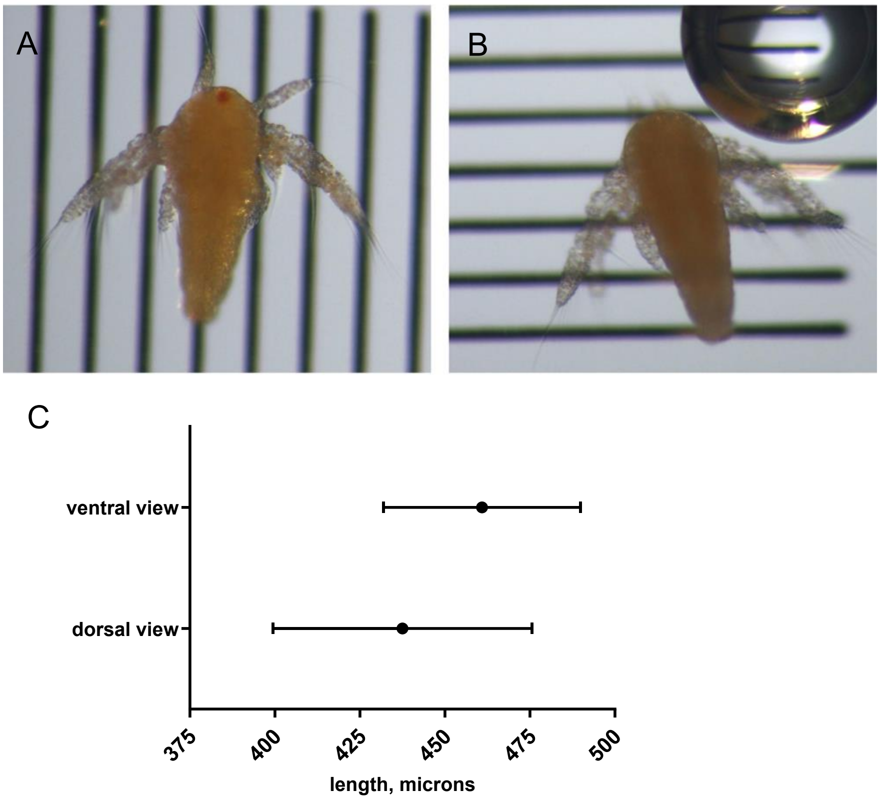

Figure 4. *A. franciscana* SFB strain, length at 24 hours after culturing. A) Ventral view, live. The dark red median eye spot is visible. B) Dorsal view of the same shrimp. The shrimp appears shorter. C) Comparison of measurements made in ventral versus dorsal view. Ventral view, mean and standard deviation, 460.9 ± 29.02 µm. Dorsal view, mean and standard deviation, 437.5 ± 38.13 µm.

Table 1. Morphometrics of *A. franciscana* strains. SFB: San Francisco Bay, GSL: Great Salt Lake.

	24 hours		48 hours	
	SFB	GSL	SFB	GSL
Length, mm	467 ± 28	470 ± 28	686 ± 64	746 ± 96
n =	36	51	54	49
Width, mm	164 ± 12	164 ± 10	164 ± 17	163 ± 15
n =	30	30	30	30

Because these size comparisons contradicted the literature [11,22,23] showing that the GSL strain is larger than the SFB strain, we tested whether a difference would become apparent after an additional day of culture. Because *Artemia* hatch continuously, by 48 hours of culture the population is a mix of metanauplii and newly-hatched nauplii. Therefore, we sorted the metanauplii from obviously younger nauplii (Figure 5) based on morphological criteria [18]. We found that, when measuring metanauplii after 48 hours of culture, the strains were significantly different in length (Table 1, $P < 0.0001$). However, we detected no significant difference in width between strains at 48 hours ($P > 0.9649$) or when comparing the newly-hatched nauplii with the metanauplii ($P > 0.9823$). This suggested that, between 24 and 48 hours of culturing, shrimp of both strains primarily grew in length.

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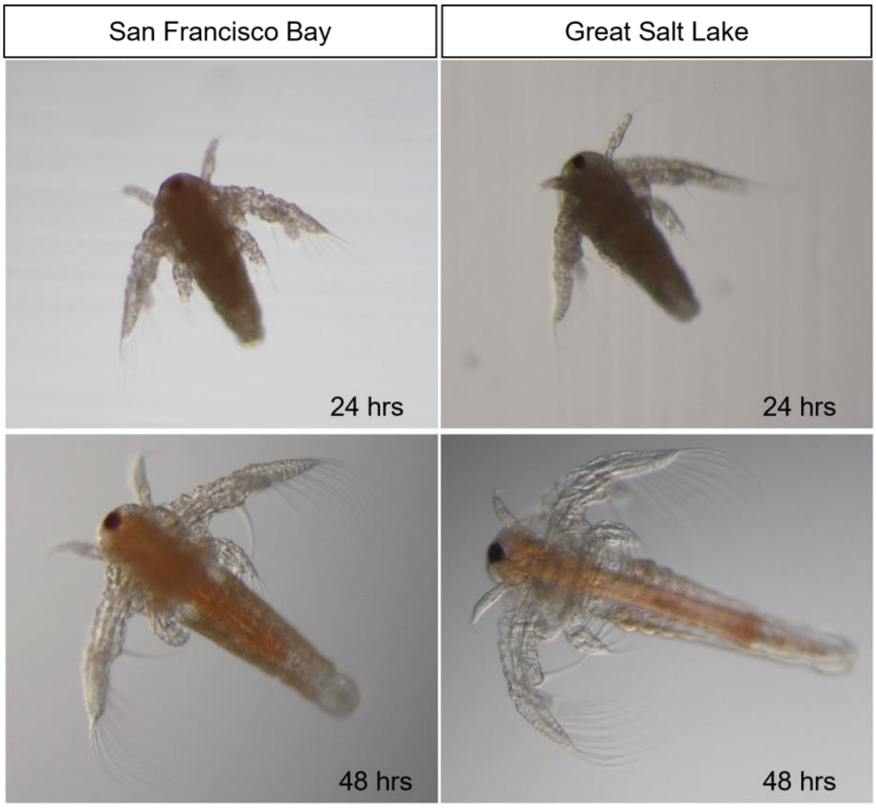

Figure 5. Shrimp strain size comparison at two time points. Live, ventral views.

4. Discussion

The larval zebrafish feeding tests confirmed our casual observations that larvae can capture live *A. franciscana* beginning shortly after the onset of external feeding. Indeed, some researchers, including ourselves, have reported raising larval zebrafish successfully with *Artemia* as the only live food included in the diet [4,8,24,25]. Here, we find that the small larval gape does not prevent capturing relatively large prey, similar to other teleosts. Bremigan and Stein [26] showed that at least some larval teleosts eat zooplankton that are larger than larval gape. Our video recordings suggest that larval zebrafish use suction feeding to ingest the nauplius whole, without biting or otherwise crushing it. This may be analogous to how other teleosts with small gapes ingest prey. For example, seahorses and pipefish, despite having narrow jaws and small buccal cavities, are able to capture relatively large prey, including prey larger than their gape [27,28].

Our tests also demonstrated that larval zebrafish become more adept at capturing prey over time, similar to other larval species [29]. For larval zebrafish, it is not clear whether increased prey capture over time is the result of physical maturation or learning. Studies by Cox and Pankhurst [30] showed that greenback flounder larvae learn to capture prey, such that earlier exposure to live prey improves later capture ability. With a potential learning curve in mind, we routinely introduce brine shrimp to larval zebrafish starting at 5 dpf despite few larvae capturing shrimp in the first few days of external feeding.

To attempt to identify why young larval zebrafish were less successful at capturing the GSL strain compared with the SFB strain of *Artemia*, we measured individual shrimp of both strains and found no differences in length or width after 24 hours of culture. This was unexpected, as zebrafish husbandry recommendations generally call for using the SFB strain of *A. franciscana*, based in part on early studies reporting that the SFB nauplii are smaller than the GSL nauplii [11,22,23,31,32]. However, we found that the SFB nauplii had a larger average length than previous reports indicated. Discrepancies between studies may result from employing different methods. In the earlier literature, the methods for measuring length were often not reported in detail, making it difficult to compare across studies. For example, some studies report using a microscope with an ocular micrometer (increments not specified), with no further details. We found no studies that reported how the nauplii were oriented, and here we have shown that measuring in dorsal view underestimates the length. Further, previous studies did not always report whether nauplii were live or fixed for the analysis. Fixation may introduce artifacts, including when using Lugol's solution, a common fixative for zooplankton. For example, Vanhaecke and Sorgeloos [11] reported fixing nauplii in 5% Lugol's solution before measuring length. Lugol's solution is known to swell or shrink tissues, depending on the length of fixation [33–35].

Despite potential artifacts and differences in methodologies for measuring size, previous studies spanning multiple years and sampling different populations consistently demonstrate that the SFB strain is smaller than the GSL strain beginning with instar I nauplii. Evaluations of multiple strains from different geographical locations suggest that naupliar size and nutritional value vary not just between strains, but within populations of the same strain and from year to year, likely from changing environmental influences [36–38]. Thus, it is difficult to predict the precise biometric characteristics of a specific sample. In addition, simply measuring length and width may not be a good predictor of overall size. We did not measure volume, which would account for the appendages that project from the body including the antennae, mandibles, and ventral labrum. The sizes of these structures may vary by *Artemia* strain, and therefore volume measurements may show that the SFB strain used in the current study is indeed smaller than the GSL strain. We conclude that, while our measurements of the SFB strain of *Artemia* were larger than expected, the size of the strain did not hinder larval feeding, and the SFB strain was more often captured by larvae compared with the GSL strain.

Finally, we include the SFB strain in our larval feeding regimen not only because the larval zebrafish capture these more readily at younger ages, but because several studies have shown that the SFB strain is more nutritious than the GSL strain, reviewed in Leger et al. [39]. The SFB strain is typically recommended by commercial suppliers and by husbandry resources because it is higher in highly-unsaturated fatty acids (HUFA) than the GSL strain, and therefore likely better supports the rapid growth of larval fish. A drawback is the higher cost of the SFB strain. To mitigate cost, we raise larvae using the SFB strain until 15-21 dpf when the larvae have gone through metamorphosis. At that point, they are considered juveniles, are transferred from static tabletop nurseries to a recirculating system, and the diet is adjusted to include the GSL strain instead of the SFB. In the current study, while we did not find differences in time to metamorphosis for larvae fed on either strain, future studies should track additional metrics, for example, whether choice of *Artemia* strain leads to differences in fecundity.

Supplementary Materials: The following supporting information can be downloaded at website of this paper posted on Preprints.org, Video S1: Larval zebrafish approaching brine shrimp.

Author Contributions: Conceptualization, M.K.; methodology, M.K., S.S., T.D.; validation, M.K., S.S. and T.D.; formal analysis, M.K.; investigation, M.K., S.S., T.D.; resources, M.K.; writing—original draft preparation, M.K.; writing—review and editing, S.S., T.D.; project administration, M.K. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The vertebrate animal study protocols were approved by the Institutional Review Board (or Ethics Committee) of Appalachian State University, protocol number 2022-003 approved 08/19/2022, and protocol number 2023-0057 approved 03/16/2023.

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Appendix A.1 Sample Sizes

Table A1. Sample sizes for Figure 3, capture success study.

Day post fertilization	SFB	GSL
5	50	50
6	50	50
7	85	50
8	87	43
9	149	40
10	88	36
11	93	48
12	72	34
13	70	47
14	25	49
15	70	45

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