PERFORMANCE OF DIFFERENT DIET TYPES ON LARVAL REARING OF THE THREATENED DEVILS RIVER MINNOW (*DIONDA DIABOLI*)

A Thesis

by

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ABSTRACT

Dionda diaboli is a threatened species of algivorous minnow endemic to spring-fed creeks and rivers of the Rio Grande drainage in south central Texas and adjacent regions of Mexico. Populations of *D. diaboli* are decreasing due to drought, habitat degradation (including the introduction of invasive piscivorous fishes and over- pumping of water). Therefore, this species is the focus of a federal captive rearing and maintenance program. Development of controlled larval rearing protocols is crucial for successful captive rearing and ultimately wild stock enhancement and species survival. This study explores the utility of four different diets for use in captive rearing of *D. diaboli*. Sixteen-day-old post-hatchings were stocked for 130 days in 20, 7.8-L flow-through tanks and fed four different diets, including two live feed diets (*Artemia* nauplii or mixed zooplankton) and two prepared feed diets (protein flakes or algal gel). During 16-46 days post hatch (dph), specific growth rate (SGR) for length (SGR_L) and weight (SGR_W) of individuals was highest for fish fed a diet of *Artemia* nauplii (2.71 mm/d SGR_L, 2.84 mg/d SGR_W) and algal gel (2.21 mm/d SGR_L, 2.41 mg/d SGR_W). The SGR_L of zooplankton-fed fish during this time was 2.04 mm/d followed by protein flakes at 1.96 mm/d. SGR_W for individuals fed protein flakes was 2.19 mg/d and was followed by individuals fed zooplankton at 1.96 mg/d. For successive time periods, there was a gradual shifting in highest to lowest grow rates per diet for both length and weight, with protein-flake-fed fish achieving the highest SGR for each successive sampling period beginning with the 46-76 dph time period for length (0.56 mm/d), and the 76-106 dph time period for weight (0.75 mg/d). Zooplankton fed fish achieved the poorest SGR for

both length and weight for all time periods after 46 dph, and never developed external or internal morphology beyond that equivalent to 64 dph in normal development. Overall survival was highest for fish fed a diet of algal gel (100%) followed by fish fed *Artemia* nauplii and protein flake (99.7%) and lowest for fish fed zooplankton (77%). The number and length of intestinal coils was considered "normal" for 136 dph juveniles fed on a diet of *Artemia* nauplii, algal gel and protein flakes, while number and length of intestinal coils for 136 dph juveniles fed on a diet of zooplankton were comparable to those of "typical" 64 dph larvae. Juveniles at 136 dph fed algal gel and protein flakes exhibited higher quantities of visceral fat than those juveniles fed on a diet of either *Artemia* nauplii or zooplankton. Though overall SGR for both length and weight was greatest with the algal gel diet (1.05 mm/d SGR_L, 1.65 mg/d SGR_W), observed growth trends throughout the study suggest that nutritional requirements may change continually throughout the development of *D. diaboli*, and an optimal diet should satisfy the physiological and metabolic demands of different ontogenetic stages to ensure optimum growth.

DEDICATION

I dedicate my thesis work to my mom, the late Gloria C Caccavale. She gave me unending support, and love, and constantly encouraged and nurtured my love of science and nature.

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INTRODUCTION

Dionda diaboli Hubbs and Brown is a threatened species of freshwater fish (USFWS 1999) endemic to a small portion of the Rio Grande drainage basin in Texas and Mexico. Known commonly as the Devils River minnow, *D. diaboli* is an inhabitant of clear spring - fed waters and is present in San Felipe and Pinto creeks and the Devils River of south central Texas, but is either rare or extirpated in Sycamore Creek in Texas and in the Río San Carlos and Río Sabinas in Mexico (Garrett et al. 1992, U.S. Fish and Wildlife Service 2005). Prior to 2001, only two populations of Devils River minnows were known; one in the Devils River and the other in San Felipe Springs. Garrett et al. (2004) reported the discovery of an additional population in Pinto Creek, Kinney County, Texas. At the current time, this population is considered to be the most threatened as a result of reduced spring flows resulting from excessive pumping from the associated aquifer (Garrett et al. 2004). Threats to the survival of the species include loss of habitat due to primarily reduced flow of springs, competition with introduced species, and degradation of water quality (Garrett et al. 1992). Hanna (2011) found the decline in the population in Pinto Creek has reached nearly three orders of magnitude and this population has lower genetic variation than the population in the Devils River. Individuals of *D. diaboli* were not found in Pinto Creek in the Fall of 2013, and only a small number of adults were observed during a survey conducted in Spring 2014 (personal observation), of which three were brought back to SMARC (San Marcos

Aquatic Resources Center, USFWS). Recent surveys in Pinto Creek by USFWS staff resulted in few (2014) or no individuals (2015) of *D. diaboli* (personal observation).

Very little is known about the biology of the Devils River minnow. Harrell (1978) described *D. diaboli* as a channel species that shifts to riffles after flooding, preferring fast-flowing spring - fed water over gravel. Cohen (2008) described *Dionda* as herbivorous, consuming detritus, algae, diatoms and plant material. McMillan (2011) reported the gut contents of adult *D. diaboli* from Pinto Creek to consist primarily of detritus (53%), blue-green algae (21%) and filamentous algae (7%), with some individuals also containing low numbers of aquatic invertebrates within their stomachs.

Most herbivorous fishes begin their lives as carnivores (Horn 1989). As is typical in members of Cyprinidae, larvae of *D. diaboli* are zooplanktivorous post hatch and feed directly from the water column (personal observation). Herbivory (or algivory) in later life stages is the result of an ontogenetic dietary shift (German et al. 2014). Ontogenetic trophic shifts are changes in diet that occur during the life of an individual and are common amongst vertebrates that are herbivores as adults (White 1985). Such shifts present an opportunity to investigate the developmental and physiological requirements of herbivory, as they represent the point at which herbivory becomes a feasible trophic specialization (Day et al. 2011). Juvenile fish have an apparent inability to thrive on an herbivorous diet, and this may be due to a need to meet an elevated protein demand (Day et al. 2011) or the alimentary systems of juveniles may lack the ability to adequately

process plant matter, as trophic shifts tend to coincide with gut lengthening (German and Horn 2006).

The 1998 Devils River minnow Conservation Agreement (USFWS 1999) recommended maintenance of captive populations of the Devils River minnow for reintroduction. In 2000, the San Marcos National Fish Hatchery and Technology Center (now the San Marcos Aquatic Resources Center, SMARC), U.S. Fish and Wildlife Service began captive propagation attempts in indoor systems. Since 2000, numerous techniques have been developed to improve survival and growth of hatchery-produced fish (Gibson et al. 2004; Gibson and Fries 2005; Fries and Gibson 2010).

Despite its threatened status, very few studies have investigated captive reproduction of Devils River minnows. Those studies conducted to date have focused on abiotic factors associated with reproduction, including temperature tolerance (Fries and Gibson 2010), substrate preference (Gibson et al 2004) and water flow (Gibson and Fries 2005). Surprisingly, there has been no attempt to investigate the effects of nutrition on captive reproduction in this threatened species.

At present, after hatching, captive propagated Devils River minnow larvae are fed a diet of *Artemia* nauplii (newly hatched live brine shrimp) from week 1 after hatching (post egg sac adsorption) until about 2 months where a 60/40 mix of Pentair's 45% protein *Spirulina* flake and 41% protein krill/plankton flake is slowly introduced into their diet, becoming 100% flake after about 4 months. At the present time, the success rate of hatching fry to adulthood is approximately 1.5% (personal observation

and husbandry records). After 25 to 60 days post hatch (dph), the author noticed large percentages of larvae became very thin, stopped eating and eventually died. Past records and speaking to the previous minnow culturist revealed this was indeed a consistent pattern. External examination of live individuals, as well as histological examination of deceased individuals revealed no known parasite or virus that might be causing this situation (personal communication with Dr. Teresa Lewis Supervisory Fish Biologist, Southwestern Fish Health Unit Leader, Southwestern Native Aquatic Resources and Recovery Center, Dexter, NM). Based on the limited available information regarding the diet of adult *D. diaboli*, it is possible that an essential nutritional component is lacking from the diet of captive-raised individuals that needs to be introduced during the larval phase. In many species of fishes, poor growth rate and poor survival of early developmental stages occur as a result of inappropriate rearing conditions (Shields 2001). Larval rearing is critical, and the development of rearing technology is essential for the conservation of threatened and endangered species of fishes.

Success of larval rearing depends mainly on the availability of suitable diets that are readily consumed, efficiently digested and that provide the required nutrients to support higher growth and health (Sarkar et al. 2006.). Growth or net nutrient deposition is the most accurate and important tool in studying fish feed efficiency and nutrient requirements (Belal 2005). Due to the low number and possible extirpation of *D. diaboli* from Pinto Creek, is it imperative to develop successful propagation practices to increase the survival rate of captive propagated individuals. The present study aims to evaluate

growth performances and survival rates of *D. diaboli* post-hatchlings fed on four different diets comprising live and dry diet under experimental flow-through hatchery systems. There are no studies which have documented the ontogenetic changes (resulting in change in foraging strategies) in the trophic apparatus and gut of *D. diaboli* to assess at which point in development, morphological traits associated with the adult diet first appear. Ontogenetic shifts have been documented in other herbivorous fishes and have been attributed to differences in habitat use or nutritional requirements (Watson et al. 2009); however the occurrence of ontogenetic shifts has never been suggested for any *Dionda*.

Because the Pinto population of *D. diaboli* is nearly extirpated, and culture methods are focused solely on this population, it is imperative that invasive techniques such as analyzing gut contents or mass examination of internal morphology be limited to more stable populations of *D. diaboli*, such as those found in the Devils River. McMillan (2011) did find differences in gut content between different *Dionda* populations, with the Pinto adults having a greater proportion of detritus and diatoms within the intestines.

It is the hope this study will shed light on an optimal diet that reflects developmental changes in behavior of captive-reared *D. diaboli* from the Pinto population, and whether the timing of ontogenetic shift can be traced to forced diet choices of growing larvae, translated into growth changes as larvae mature into juveniles. A greater understanding of larval diet requirements, and associated

developmental changes that occur in captive-reared fish might provide insight into habitat and foraging requirements for threatened *D. diaboli* in the wild.

MATERIALS AND METHODS

Pilot Study

Beginning in March 2014 through June 2014, batches of captive-reared larvae of *D. diaboli* were exposed to four diets from 16 days post-hatch (dph) until approximately 90 dph. Larvae were kept in 18.6-L aquaria in various densities of 50 to 150 individuals. This prior exposure was to ensure all diets would be accepted readily by the different ages of larvae, and that no significant mortalities would result from feeding a certain diet. It was determined that larvae did not thrive on any diet except *Artemia* nauplii from day 8 (egg yolk absorption) to approximately day 16 dph. *Artemia* nauplii, ground protein flake and algal gel were accepted by larvae after 16 dph. It was found that zooplankton needed to be filtered to 150 *um*. All breeding culture activities of adult wild stock *D. diaboli* from Pinto Creek were suspended from July 2014 through September 2014 to ensure a large batch of fry for the main experiment, which began in October 2014. Measurements of total length (TL) and weight (WT) of a 20% subset (32 individuals) of larvae were taken at 16 dph for later growth comparisons.

Feeding Experiment

Beginning in October 2014, *D. diaboli* offspring were obtained from captive adults via induced spawning techniques developed by Gibson and Fries (2005). The post-hatchings were obtained from one spawning event that produced approximately nine hundred eggs which were divided between two 18.62-L aquaria. Eight days post hatch larvae (yolk sac was absorbed) were fed newly-hatched *Artemia* nauplii until day 14, before the commencement of the experiment. Fry were starved for 2 days prior to beginning feed trials at 16 dph, in order to simulate a feed transition for those larvae that were continued to be fed *Artemia* nauplii for the duration of the study. Larval-rearing experiments were conducted across twenty 7.8-L tanks maintained on a common flow-through system utilizing well water from the Edward's Aquifer. Larvae were stocked at a density of eight individuals per 7.8-L and maintained at a temperature of 22 ±1 ˚C and subjected to a 12:12 hour light: dark cycle for the duration of the experiment. Water quality parameters (dissolved oxygen, percent saturation, pH, and conductivity) were measured once per week. Tanks were randomly assigned one of four different diet types (5 replicates per diet) and fishes were fed ad libitum twice per day. Each morning before first feeding, each tank was siphoned of leftover food from the day prior. The behavior of fish in each tank was noted 10 minutes before, during and 10 minutes after feedings. Twelve additional 7.8-L tanks were set up with three replicates of each food treatment, as a backup in the event of mortality in the twenty main treatment tanks in order to maintain densities.

(The remaining approximately 700 members of this cohort were reared in duplicate abiotic conditions, in 1.82 m x 0.6 m fiberglass tanks, with densities of 200 individuals and fed a diet of protein flakes and algal gel after 30 dph).

The four diet types consisted of the following: (1) *Artemia* nauplii (Great Salt Lake *Artemia* cysts) freshly hatched every 2 days; (2) zooplankton, (predominantly *Daphnia* sp.) produced in 0.04-ha ponds on hatchery grounds and then filtered through 150-*um* screens before feeding to larvae; (3) ground protein flakes comprised 60/40 mix of Pentair's 45% protein *Spirulina* flake and 41% protein krill/plankton flake, and mixed with water before feeding; and (4) an algal gel comprising a pureed protein/vegetable mix of kale (30%), spinach (30%) , mysis shrimp (15%), *Nannochloropsis* (25%), water and unflavored gelatin (for binding). A proximate analysis of each diet type (Table 1) was performed by the Fish Nutrition Laboratory (Department of Wildlife and Fisheries Sciences, Texas A&M University) and crude fiber composition was performed for the algal gel and protein flake diets (Table 2) by Eurofins Scientific Inc. (Des Moines, IA).

Table 1. – Proximate composition analysis of flake, algal gel, *Artemia* and zooplankton diets

Sample Id	Dry Matter	Moisture	Ash (Dry)	Ash (Wet Basis)	Protein (Dry-Matter Basis)	Protein (Wet Basis)	Lipid (Dry-Matter Basis)	Lipid (Wet Basis)
Flakes	95.35	4.64	12.59	12.01	45.03	42.93	11.49	10.96
Gel	7.99	92.00	6.93	0.55	89.42	7.15	8.52	0.68
Artemia	4.95	95.04	14.19	0.70	53.36	2.64	21.74	1.07
Zooplankton	6.00	93.99	26.68	1.60	56.83	3.41	23.74	1.42

Table 2. – Crude fiber analysis of algal gel and flake diets

Sample ID	Fiber, Crude	Fiber, Acid detergent
Gel	<0.2%	0.40%
Flakes	1.30%	2.90%

At the termination of each 30-day feeding period (46 dph, 76 dph, 106 dph and 136 dph), all individuals were removed from each treatment tank and immersed in 1 L of well-water containing 0.75 mg of analgesic MS-222(tricaine methanesulfonate, Western Chemical).The length of each individual was then measured using a pair of digital calipers to the nearest 0.01 mm. Weight was measured collectively at the end of the first 30 days (46 dph) by transferring all individuals from a single tank to a fine mesh net (dried briefly on paper towels to reduce the amount of water) that was then placed on a Scout Pro SPE402 electronic scale (Ohaus Corporation, NJ). Individuals were allowed to fully recover in a container of fresh well water before being transferred back to the original treatment tank. Weight measurements taken on 76, 106 and 136 dph, were obtained individually, as individuals were large enough to survive this extra handling.

One way repeated measures ANOVA were performed using SPSS 20.0 to examine differences in length and weight over the course of the 4-month study. Dietary treatments were considered the fixed effect and blocks considered the random effects. Prior to the ANOVA, Chi-Square Goodness of Fit test was used to evaluate normality. Normality was violated for both data sets and therefore the data was transformed ($-\log_{10}(x + 1)$ for resulting ANOVA analysis. Differences were considered significant at a P value < 0.05. Specific Growth Rate for Length or Weight (SGR_L or SGR_W) was calculated as follows:

$$SGR_{(L or W)}\ (\%\text{gain (L } or \text{ W)/day}) = \left[\frac{(\text{Log}_n\text{Final fish (L } or \text{ W). } - \text{Log}_n \text{ Initial fish (L } or \text{ W.})}{\text{Time Interval}}\right] 100$$

In addition, permission was obtained to perform necropsies on 38% of the fish in each treatment tank (for a total sample size of 60 juveniles) in order to compare external morphological characteristic as well as the shape and length of intestines (straightened and measured from esophagus to rectum) among individuals from the four different diet treatments. Gut length (GL) was measured for 136-dph juveniles at the end of the experiment (n = 60) and the arrangement of the coils or loops were examined qualitatively and the number of intestinal coils or loops quantified (a coil was defined as the intestine forming a complete oval shape which crossed over itself; a loop did not cross and appeared as an open "U" shape). General morphological examination and gut

length measurements were taken using an Olympus SZ-11 scope Nikon DS-5M digital camera with an ocular micrometer. Intestinal coils or loops were counted prior to removal of the viscera from the body cavity. The viscera were then removed and the intestine uncoiled, without stretching, and the total GL (± 0.5 mm) was measured from the beginning of the esophagus to the rectum. In addition, the presence/absence of fat deposits within the body cavity was also examined and quantified using a Mettler Toledo AB204 analytical precision balance.

RESULTS

General Observations

All individuals involved in the study from 16 dph to 136 dph, actively fed on all offered diets. Individuals typically remained in the upper portion of the water column, though those individuals fed protein flakes and algal gel remained low in the water column while feeding. Those fish fed *Artemia* nauplii generally appeared to be less active when compared with those fed zooplankton. Zooplankton-fed fish actively chased zooplankton in the upper water column. All individuals generally exhibited "protruding" full stomachs approximately 10 minutes after initial feeding. After approximately 68 dph larger individuals fed on the diet of *Artemia* nauplii started to exhibit "chasing" and territoriality behaviors with cohorts when *Artemia* nauplii were first introduced into the tank. Little to no chasing was observed in fish fed protein flakes or algal gel.

Specific Growth Rate

The Specific Growth Rate (SGR) was calculated to determine the growth performance (TL and WT) during the experimental period. Due to differences in SGR with regard to lengths and weights per diet treatment, these were first analyzed as separate dependent variables per time period (16-46 dph, 46-76 dph, 76-106 dph and 106-136 dph).

Weight (WT) Measurements

Standard weight of 16 dph mesolarave (20%, 32 individuals) averaged 7 mg ± 0.2. Mauchly's Test of Sphericity for the weight variable was violated for the within subject effect of weight vs time and therefore, corrected Greenhouse-Geisser values were used.

Tests of between-subject effects for WT illustrate how treatment had a significant effect across the entire duration of the study ($P<0.001$) (Table 3). A value of $P < 0.001$ for pairwise WT comparisons as a result of Bonferoni post hoc test show the mean difference between treatment levels (diet vs time) was statistically significant ($P<0.005$) for 80% of comparisons (Table 4).

Table 3. Tests of between subject effects (WT) from one way repeated measures ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	259.66	1	259.66	17898.83	0
Treatment	3.29	3	1.09	75.67	0
Error	0.23	16	0.01		

Table 4. Pairwise comparisons for weight (WT) of Devils River minnows fed four different diets

Treatment I	Treatment J	Mean Difference (I - J)	Std. Error	Sig. [b]	95% Confidence Interval for difference [b]	
					Lower Bound	Upper Bound
Artemia nauplii	2	***0.139***	0.038	0.002	0.058	0.219
	3	0.051	0.038	0.201	-0.03	0.132
	4	***0.518***	0.038	.000	0.437	0.598
Protein Flakes	1	***-0.139***	0.038	0.002	-0.219	-0.058
	3	***-0.088***	0.038	0.035	-0.169	-0.007
	4	***0.379***	0.038	.000	0.298	0.46
Algal gel	1	-0.051	0.038	0.201	-0.132	0.03
	2	***0.088***	0.038	0.035	0.007	0.169
	4	***0.467***	0.038	.000	0.386	0.547
Zooplankton	1	***-0.518***	0.038	.000	-0.598	-0.437
	2	***-0.379***	0.038	.000	-0.46	-0.298
	3	***-0.467***	0.038	.000	-0.547	-0.386

Italics. The mean difference is significant at the 0.05 level

[b] Adjustment for multiple comparisons: Least Significant Difference

Table 5 and Figure 1 display SGR_W per diet treatment for each 30-day time period expressed as milligrams per day (mg/d). The 16-46 dph period growth rates for weight gain were the highest across all diets, with individuals fed a diet of *Artemia* nauplii achieving the most weight gain during this time period (2.84 mg/d) and fish fed the zooplankton diet the least (1.96 mg/d). The SGR_W for zooplankton-fed fish dropped dramatically beginning 46 dph and continued to exhibit the lowest amount of weight gain for the entire 136-dph time frame (0.31 mg/d, 0.26 mg/d, 0.14 mg/d). SGR_W of individuals fed the diets of *Artemia* nauplii, protein flake or algal gel decreased during 46-76 dph, with *Artemia*-nauplii-fed fish conversely exhibiting the second lowest SGR_W

(0.4 mg/d) during the 76-106 dph time period, while the individuals fed the protein flake or algal gel diets exhibited the same SGR_W during this time (0.75 mg/d). Fish fed the diet of algal gel exhibited the highest SGR_W for the entire study period (1.65 mg/d), compared to fish fed protein flake (1.60 mg/d), *Artemia* nauplii (1.59 mg/d) or zooplankton(1.13 mg/d).

Table 5. Growth Rate (milligrams per day (mg/d)) for weight (SGR_W) of Devils River minnows fed four different diets from 16 dph through 136 dph

Treatment	Time Period(dph)				
	16-46	46-76	76-106	106-136	Overall Growth Rate
Artemia	2.84	1.39	0.4	0.19	1.59
Flakes	2.19	1.25	0.75	0.62	1.6
Gel	2.41	1.4	0.75	0.45	1.65
Zooplankton	1.96	0.31	0.26	0.14	1.13

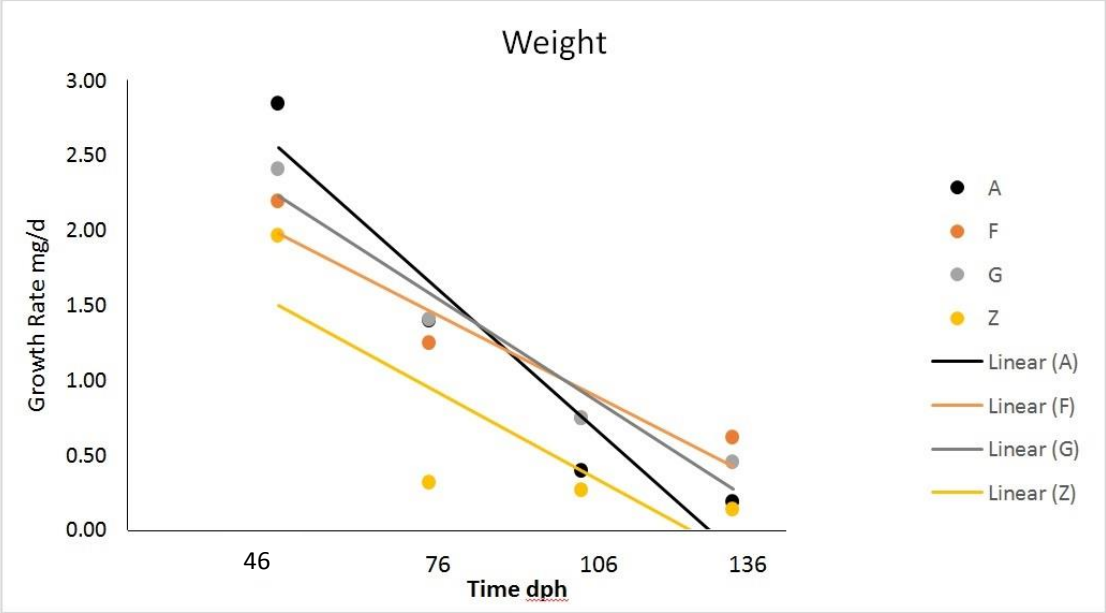


Figure 1. SGR_W (mg/d) for four diet treatments; *Artemia* nauplii (A), protein flake (F), algal gel (G), and zooplankton (Z) from 46 dph through 136 dph

Total Length (TL) Measurements

Total length of 16 dph mesolarvae (20%) averaged 5.5 mm ± 0.3. Mauchley's test of Sphericity for TL (Table 7) demonstrated a significant (P>0. 005) difference and thus sphericity was not violated.

Table 6. Sphericity Assumed values (TL) for tests of within-subjects effects

Source	Type III Sum of Squares	df	Mean Square	F
Time	0.667	3	.222	1183.857
Time*Treatment	0.063	9	.007	37.201
Error(Time)	0.009	48	.000	

Tests of between-subject effects for length illustrate how treatment had a significant effect across the entire duration of the study (Table 7). A value of $P < 0.002$ for pairwise length comparisons as a result of Bonferoni post hoc test showed the mean difference between treatment levels were statistically significant ($P<0.005$) for 80% of comparisons (Table 8).

Table 7. Tests of between subject effects (TL) from one way repeated measures ANOVA

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Intercept	137.131	1	137.131	134360.599	.000
Treatment	.251	3	.084	81.885	.000
Error	.016	16	.001		

Table 8. Pairwise comparisons for length (TL) of Devils River minnows fed four different diets

Treatment I	Treatment J	Mean Difference (I - J)	Std. Error	Sig. [b]	95% Confidence Interval for difference [b]	
					Lower Bound	Upper Bound
Artemia nauplii	2	***0.055***	0.010	0.000	0.034	0.077
	3	***0.038***	0.010	0.002	0.016	0.059
	4	***0.152***	0.010	.000	0.130	0.173
Protein Flakes	1	***-0.055***	0.010	.000	-0.077	-0.034
	3	-0.017	0.010	0.103	-0.039	0.004
	4	***0.097***	0.010	.000	0.075	0.118
Algal gel	1	***-0.038***	0.010	0.002	-0.059	-0.016
	2	0.017	0.010	0.103	-0.004	0.039
	4	***0.114***	0.010	.000	0.093	0.136
Zooplankton	1	***-0.152***	0.010	.000	-0.173	-0.130
	2	***-0.097***	0.010	.000	-0.118	-0.075
	3	***-0.114***	0.010	.000	-0.136	-0.093

***Italics*. The mean difference is significant at the .05 level**

[b] Adjustment for multiple comparisons: Least Significant Difference

Table 9 and Figure 2 demonstrate the SGR_L per diet treatment for each 30-day time period. For the 16-46 dph period, SGR_L was the highest across all diet treatments; *Artemia* nauplii (2.71 mm/d), algal gel (2.21 mm/d), zooplankton (2.04 mm/d) and protein flake (1.96 mm/d). The *Artemia*-nauplii-fed fish had the highest SGR_L (2.71 mm/d) during this time period and protein flake-fed fish the lowest SGR_L (1.96 mm/d). The SGR_L for zooplankton fed fish decreased dramatically beginning at 46 dph and continued to exhibit the lowest SGR_L (0.25 mm/d, 0.11 mm/d, 0.10 mm/d) for the entire 136-dph time period. The SGR_L for fish fed *Artemia* nauplii, protein flake or algal gel decreased during the 46-76 dph, 76-106 dph and 106–136 dph time periods. Protein-flake-fed fish displayed the highest SGR_L at 46-76 dph (0.56 mm/d), 76-106 dph (0.34 mm/d) and 106-136 dph (0.21 mm/d) time periods. SGR_L for algal-gel-fed fish was second to protein-flake-fed fish during these time periods (0.52 mm/d, 0.32 mm/d, 0.18 mm/d, respectively), while SGR_L for *Artemia*-nauplii-fed fish was third (0.53 mm/d, 0.17 mm/d, 0.10 mm/d) and SGR_L for zooplankton-fed fish was fourth (0.25 mm/d, 0.11mm/d, 0.10 mm/d). The *Artemia*-nauplii and algal-gel-fed fish exhibited the highest overall SGR_L for the entire study period (1.05 mm/d).

Table 9. Growth Rate (millimeters per day (mm/d)) for length (SGR_L) of Devils River minnows fed four different diets from 16 dph through 136 dph

Treatment	Time Period (dph)				
	16-46	46-76	76-106	106-136	Overall Growth Rate
Artemia	2.71	0.53	0.17	0.1	1.05
Flakes	1.96	0.56	0.34	0.21	1.04
Gel	2.21	0.52	0.32	0.18	1.05
Zooplankton	2.04	0.25	0.11	0.1	0.9

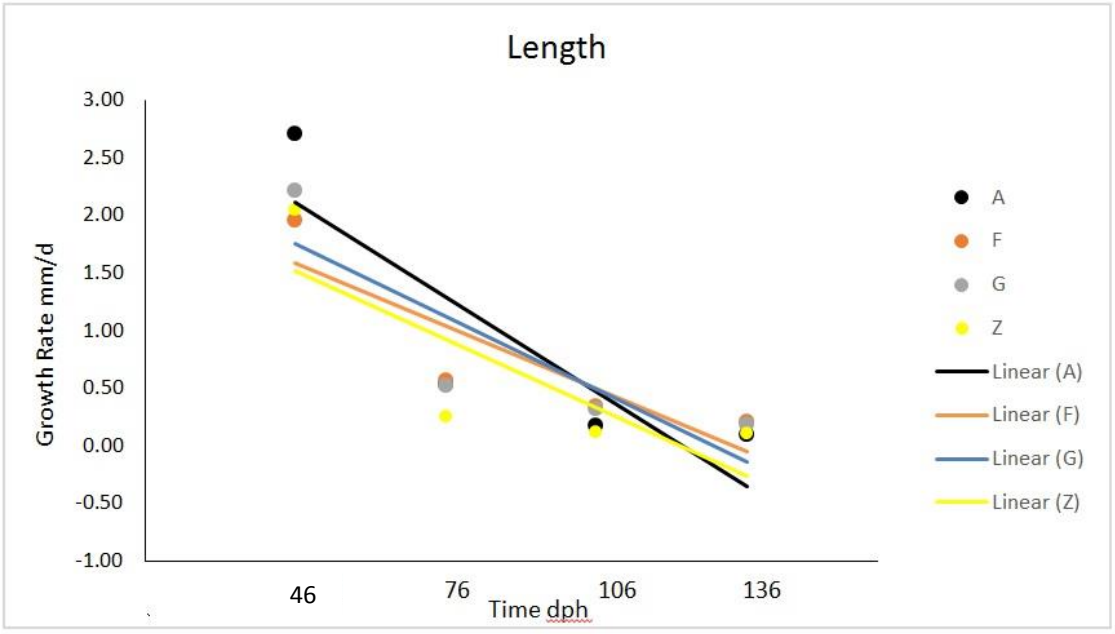


Figure 2. SGR_L (mm/d) for four diet treatments; *Artemia* nauplii (A), protein flake (F), algal gel (G) and zooplankton from (Z) 46 dph through 136 dph

Mortality

The mean survival of *D. diaboli* post-hatchings fed the four different diets during the experimental period varied from 100% to 77.5%. All individuals fed the algal gel diet survived to the end of the 136-day study. Those individuals reared on the diet of *Artemia* nauplii or protein flakes exhibited similar survival (97.5%) and those fish fed on a diet of zooplankton exhibited the lowest survival (77.5%).

Morphology

External morphology differences were exhibited by 136-dph juveniles fed zooplankton in comparison to juveniles fed the diets of *Artemia* nauplii, protein flakes or algal gel. Juveniles fed *Artemia* nauplii, protein flakes or algal gel exhibited similar size ranges and morphometric and melanophore characteristics to those of typical juveniles of *D. diaboli* (based on 128-dph juveniles described by Hulbert et al. 2007) (Figure 3). However, juveniles fed the diet of zooplankton exhibited size range and morphometric characteristics similar to 64-dph juveniles and melanophore characteristics similar to 32-dph metalarvae (Hulbert et al 2007). All 136-dph juveniles, regardless of diet type, exhibited a rounded or wedge-shaped dark caudal spot at the base of the caudal fin. However, only those juveniles fed the diets of *Artemia* nauplii, protein flakes or algal gel, also exhibited prominent melanophores along the posterior edge of dorsal and lateral body scales and were prominent on the dorsum and extending to just below the

midlateral stripe, which was dark and densely populated with melanophores. Such characteristics were not observed in 136-dph zooplankton-fed juveniles (Figure 4).

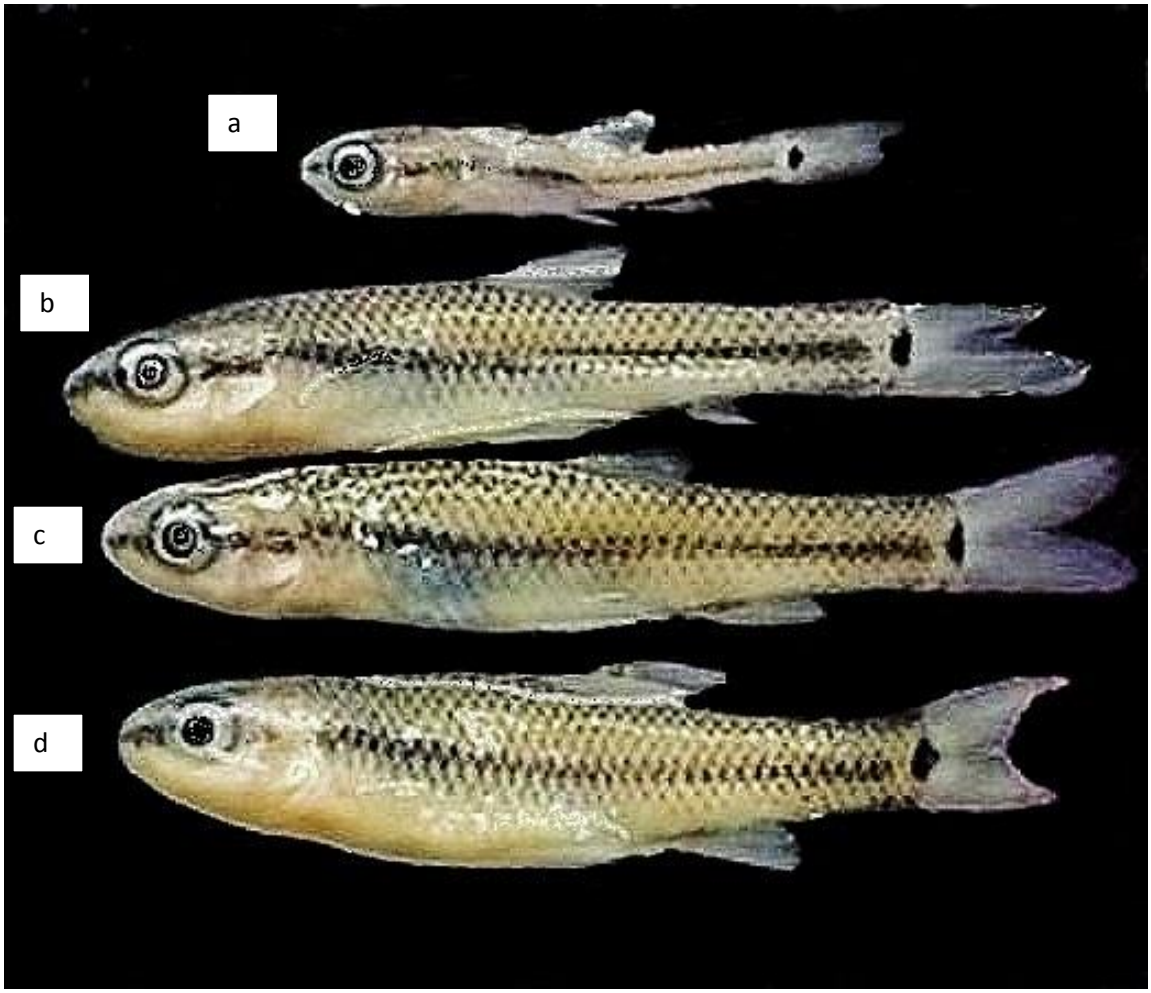


Figure 3. 136-dph juveniles fed zooplankton (a), protein flakes (b), *Artemia* nauplii (c) and algal gel (d)

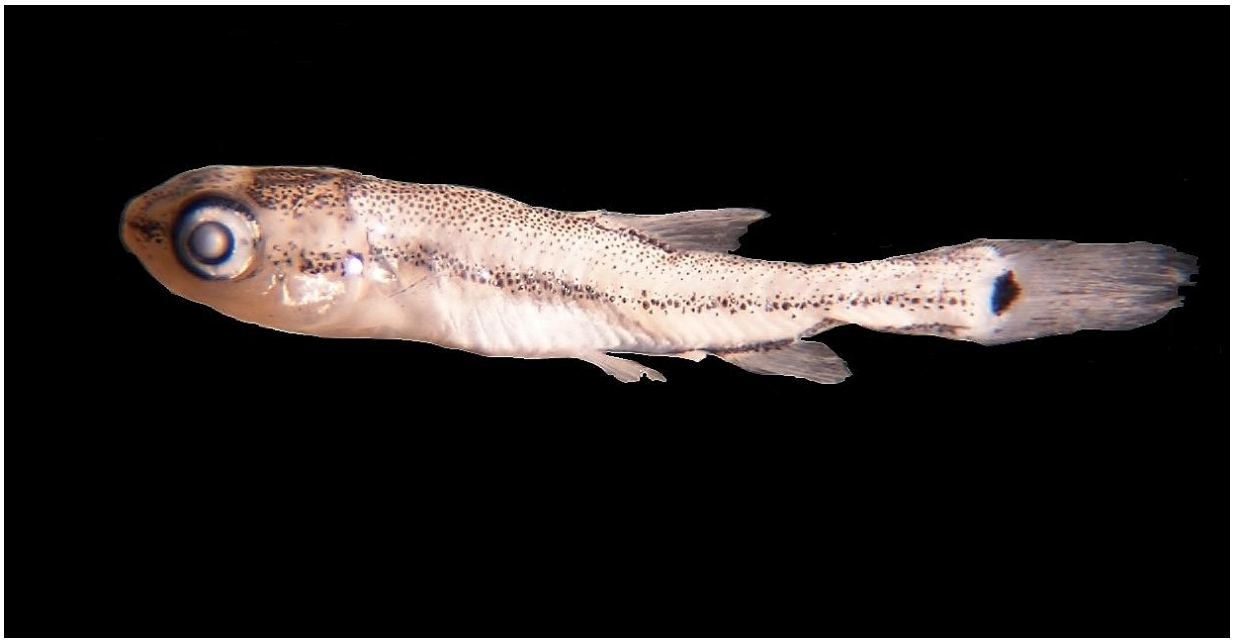


Figure 4. Melanophore characteristics in 136-dph zooplankton-fed juvenile resembling 64-dph larvae in pigmentation

Internal morphology characteristics, specifically intestinal length and intestinal coiling (or loops) of 136-dph juveniles fed the diet of zooplankton also differed in comparison to juveniles fed diets of *Artemia* nauplii, protein flakes or algal gel. On average, fish fed on zooplankton had much shorter intestines (8.2 mm ± 1.2) than those fed *Artemia* nauplii (23.5 mm ± 1.9), protein flakes (32.4mm ± 2.8) or algal gel (24.1 mm ± 2.4) (Table 10). Also, 136-dph juveniles fed diets of algal gel or protein flake exhibited three to four intestinal coils (Table 10;Fig 5a, 6a); whereas, those fed *Artemia* nauplii exhibited two to three coils (Table 10; Fig 5b, 6b). The 136-dph fish fed the diet

of zooplankton developed an uncoiled intestine with only a single loop (Table 10; Fig 5c and 6c).

Table 10. Internal morphological intestinal characteristics (mean ± SD) of 136-dph juveniles fed four different diets

Diet	N	Coils	Intestinal Length (mm)		
			Mean	Range	SD
Artemia nauplii	15	2.5-3.5	23.51	20-26	1.9
algal gel	15	3.5-4	24.09	21-28	2.4
protein flakes	15	3.5-4	32.36	27-36	2.8
zooplankton	15	0	8.2	6-10	1.2

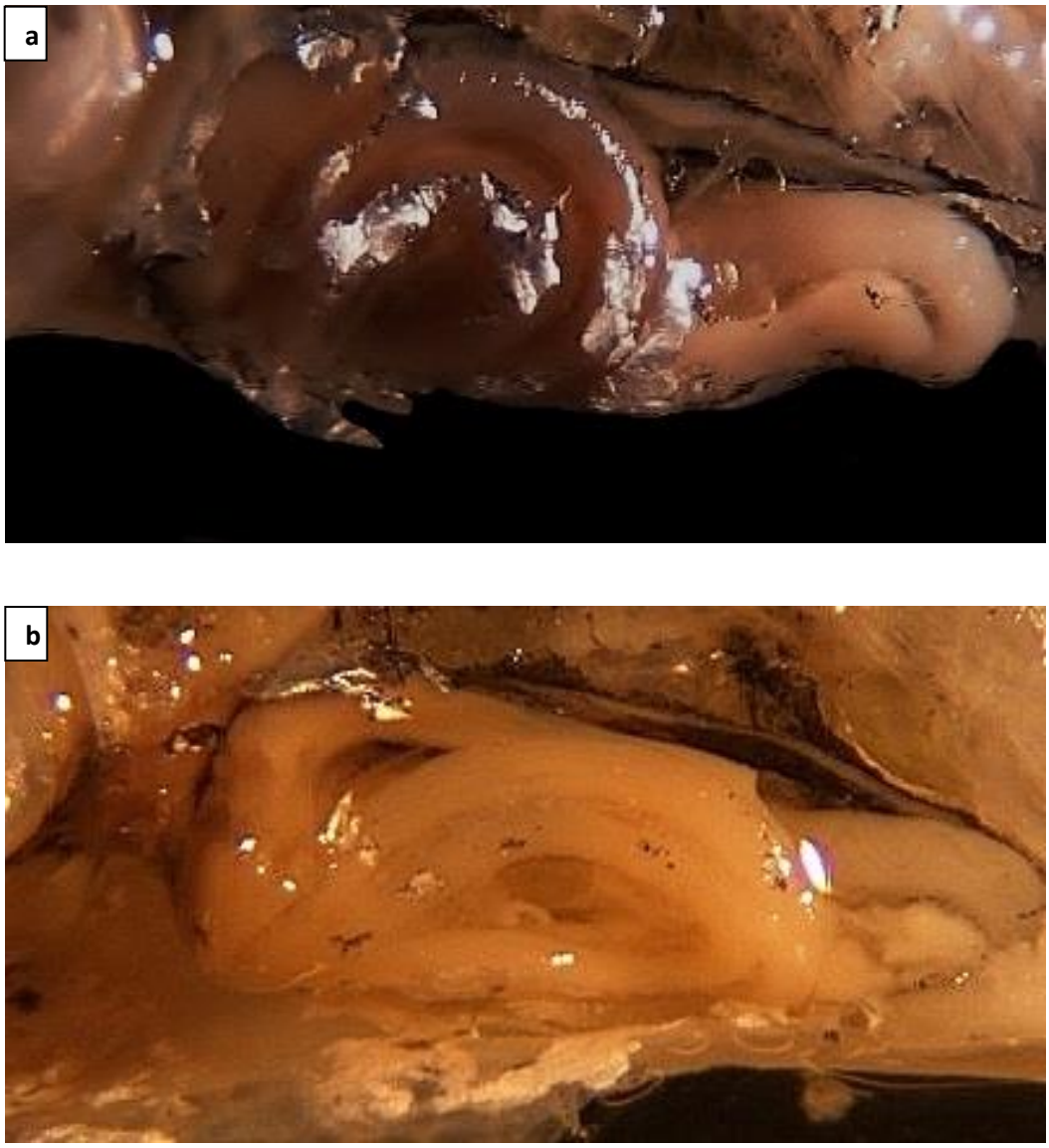


Figure 5. Intestinal coiling of 136-dph juveniles fed protein flakes and algal gel (a), *Artemia* nauplii (b) and zooplankton (c)

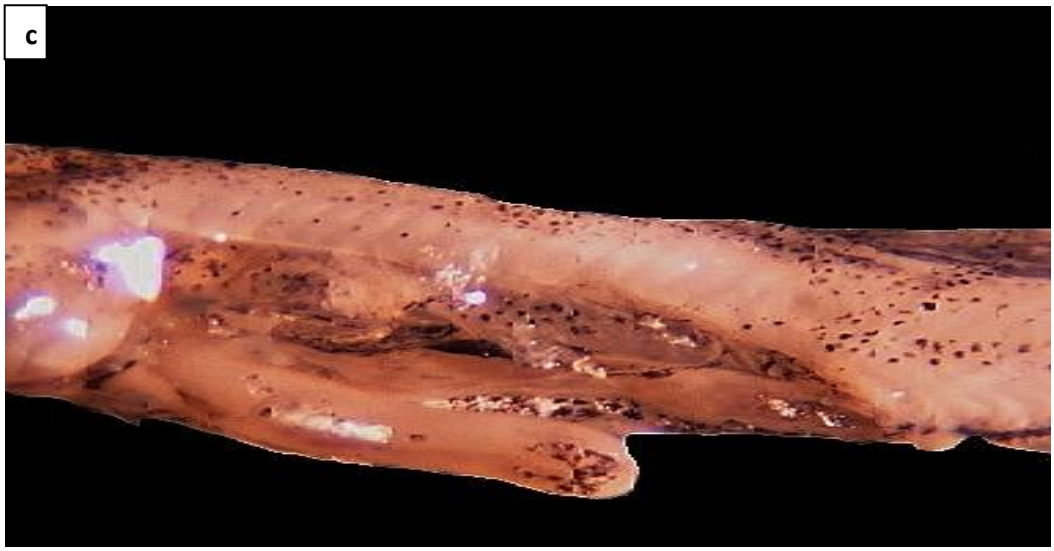


Figure 5. Continued

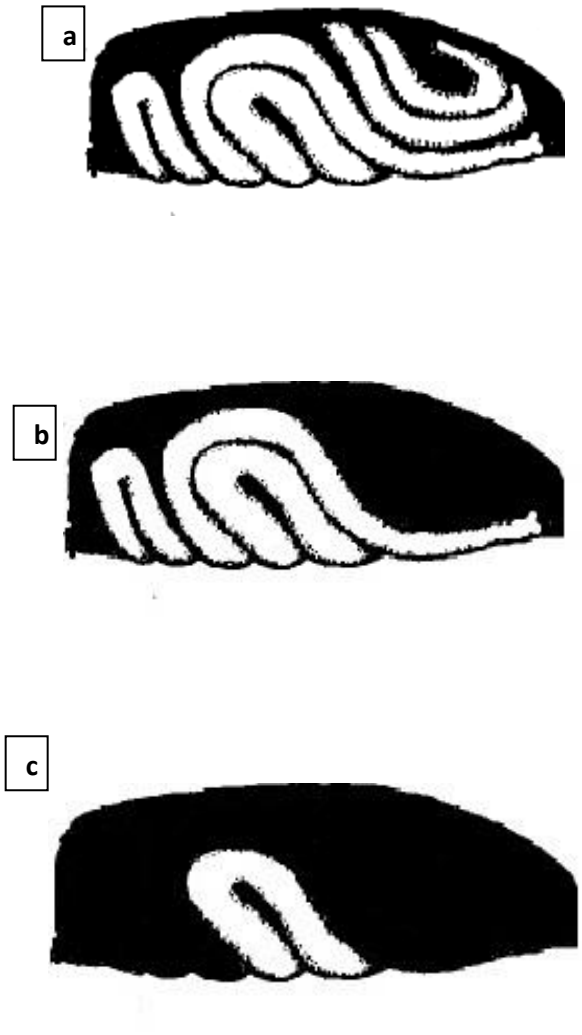


Figure 6. Drawing of coiling in fish fed protein flakes and algal gel (a), *Artemia* nauplii (b) and loop in zooplankton-fed fish (c) at 136 dph

The presence/absence of fat deposits in the body cavity of 136-dph juveniles was also examined (Table 11). The 136-dph juveniles fed the diets of *Artemia* nauplii or zooplankton exhibited little (1.6 mg ± 0.49) or no fatty deposits, respectively, within the body cavity (Fig. 7a and 7b). In comparison, 136-dph juveniles fed a diet of protein flakes or algal gel exhibited greater quantities of fatty deposits (7.94 mg ± 0.81 and 11.04 mg ± 1.48, respectively) throughout the body cavity (Fig 7c and 7d).

Table 11. Quantitiative comparison of visceral fat observed in 136-dph juveniles fed four different diets

Diet	N	Visceral fat (mg)		
		Mean	Range	SD
Artemia nauplii	15	1.6	0.7-2.4	0.49
algal gel	15	11.04	8.0-13.2	1.48
protein flakes	15	7.94	6.3-9.7	0.81
zooplankton	15	0	0	0

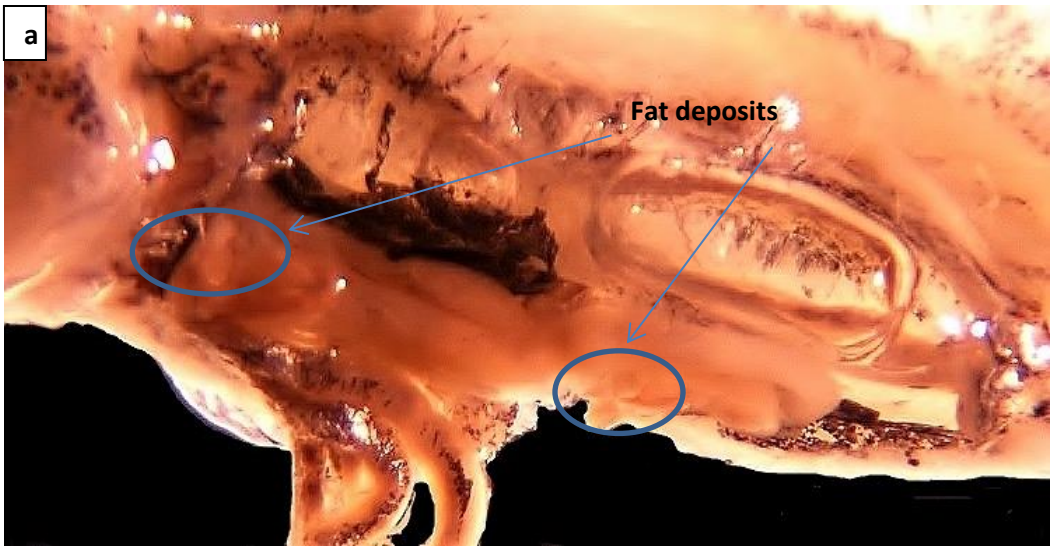
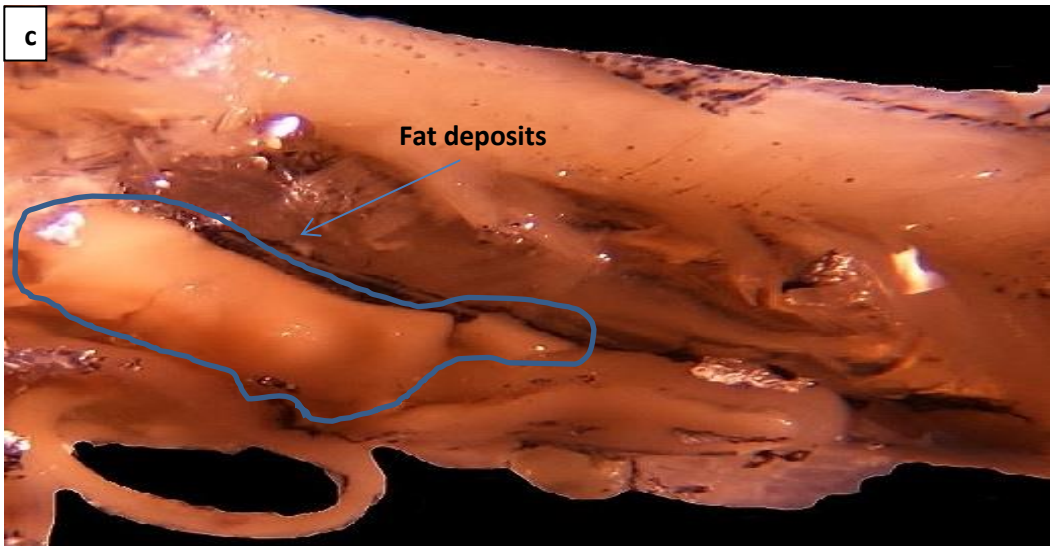


Figure 7. Fatty deposits in 136-dph juveniles fed *Artemia n*auplii (a), zooplankton (b), protein flakes (c) or algal gel (d).



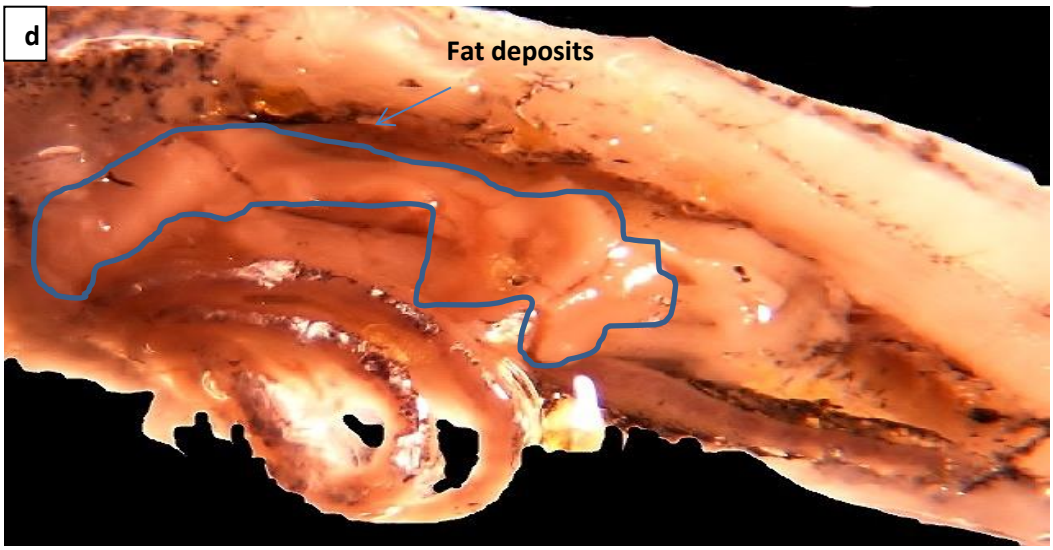


Figure 7. Continued

DISCUSSION

This is the first study on the rearing of *D. diaboli* from fry through juvenile stages, utilizing different diets in an attempt to achieve maximum growth rates. The success of captive propagation efforts is essential to the recovery of this species, and providing essential dietary requirements at the appropriate life history stages is imperative to the successful development from fry to juvenile.

Growth

SGR_W was initially highest for 46-dph larvae fed *Artemia* nauplii (2.84 mg/d), compared to those fed algal gel (2.41 mg/d), protein flakes (2.19 mg/d) or zooplankton (1.96 mg/d). Overall, the gel diet produced the highest SGR_L (1.05 mm/d) and SGR_W (1.65 mg/d) by 136 dph. During the first few days of exogenous feeding, larval fishes are frequently reported to exhibit high growth rates, in some cases up to 60% of body mass per day (Terjesen et al. 1997) and multiply their body mass to adult size by a factor of 10^5 - 10^7 (Finn et al. 2002) . The SGR_W values for fish fed *Artemia* nauplii are in line with growth rates for larvae of other species of fish during this time period. It was expected *a priori* that 46-dph larvae fed zooplankton would perform equally as well as those fed *Artemia* nauplii. However, 46-dph larvae fed zooplankton exhibited the lowest weight gain during this time period. Protein content (wet basis) for the zooplankton diet (3.4%) was actually higher than the *Artemia* nauplii (2.6%), so one would expect that higher protein concentration in a prey item would correlate with better growth at this

stage of development. Ostaszewska et al. (2008) found that growth increased with increased protein consumption in the cyprinid *Vimba vimba*, but it was the type and quality of protein that mattered most. Cowey (1994) suggested it was the type of amino acids present within the protein source that was of most importance for growth in fishes, especially during the initial growth stage. Fishes experience an exponential increase in growth that diminishes as an individual approaches sexual maturity (Diana 2004). It is interesting that during the 46-76 dph, 76-106 dph and 106-136 dph time periods, there was a subtle shift in the growth performance of fish fed the *Artemia* nauplii, algal gel and protein flake diets. As can be observed from table 5, during those successive three time periods the SGR_W from highest to lowest were algal gel (1.4 mg/d), *Artemia* nauplii (1.39 mg/d), then protein flake (1.25 mg/d) for the 47-76 dph period, algal gel and protein flake (0.75 mg/d), then *Artemia* nauplii (0.4 mg/d) for the 76-106 dph time period, and then protein flake (0.62 mg/d), algal gel (0.45 mg/d) and *Artemia* nauplii (0.19 mg/d), during the 106-136-dph period. It is possible that this change in SGR_W among fish fed these three diets may be reflective of the ontogenetic changes (morphological, physiological and functional) that may occur as larvae shift from carnivorous to omnivorous, to herbivorous feeding habits. The change in diet from carnivory to herbivory has been explained by several authors as being related to nutritional, energetic and ecological considerations (Hofer 1982; German et al. 2014; Infante et al. 2008; Hjelm et al. 2000; Drewe et al. 2004). Numerous species of fishes that consume microalgae increase the algal (fiber) proportion of their diet as they increase in size (Horn 1989).

One would expect the rate of weight gain to slow as the larvae pass into juvenile stages. However, it is the progression and sequence of this SGR_W when compared to diet that may be insightful: from a live, low-fiber diet (*Artemia* nauplii), to one of higher fiber (algal gel) to the protein flake diet (highest fiber and lipid content) showing the highest SGR_W at the juvenile stage (106-136 dph), when compared with the other diets during this period. The SGR_L followed a similar pattern, in that the fish fed the *Artemia* nauplii diet exhibited the highest SGR_L during the 16-46 dph time period (2.71 mm/d) with fish fed the algal gel diet being second (2.21 mm/d) followed by the zooplankton fed fish (2.04 mm/d). As illustrated in table 9, during the successive time periods after 16-46 dph, the protein-flake-fed fish led consistently, followed by the algal-gel-diet fed fish. Again this represents a gradual increase in the utilization of fed diets exhibiting the highest crude fiber and lipid content. Crude fiber content is an important source of dietary fiber (Toko et al. 2008). Dietary studies on the Rio Grande silvery minnow (*Hybognathus amarus*) have demonstrated that the timing of the introduction of manufactured feed (after initial feeding with *Artemia* nauplii) is more important for growth and survival than feed type (Caldwell et al. 2005). Brett and Groves (1979) proposed that both omnivorous and herbivorous species of catfish and carp appear to be able to use dietary carbohydrates as an effective energy source, and that protein content was less important in these species. A general diet relationship between natural feeding habits and dietary protein requirements has been noted where herbivorous and omnivorous species of fishes are able to use lower concentrations of dietary proteins, while more carnivorous species may require higher concentrations of proteins (Royes

and Murie 2006). Gatlin (2001) suggested that herbivorous and omnivorous species of fish have an optimum crude protein requirement that ranges between 25 - 35% of the diet; whereas, more carnivorous species of fishes may require higher levels of crude protein between 40 - 50%.

Very little is known about the feeding habits of *D. diaboli* in the wild. McMillan (2011) investigated the gut contents of adult individuals of *D. diaboli* from two different localities (Devils River and Pinto Creek) which revealed a number of unexpected differences between the localities. Individuals examined from Pinto Creek had narrower diet breadths and consumed a larger proportion of amorphous detritus, than individuals from the Devils River.

It is unclear why fish fed the zooplankton diet exhibited the poorest growth (both SGR_L and SGR_W) and highest mortality throughout the entire length of the study. Hokanson and Lien (1986) found that fish fed zooplankton diets yielded significantly poorer growth than other diet treatments, and were inferior in diet quality to *Artemia*. They suggested that both the taxa and size composition of zooplankton are important attributes influencing both growth and survival of larvae. Dabrowski (1984) suggested that nutritional composition of zooplankton were dependent on origin, season and species. Similar diet studies on the Rio Grande silvery minnows (*H. amarus*) concluded that mortality was very high when larvae were fed live rotifers (Caldwell et al. 2005). The same authors concluded that *Artemia* nauplii were essential for the successful rearing of Rio Grande silvery minnows from time of yolk resorption through the end of

the larval stage (approx. 15 dph) (Caldwell et al. 2005). Observations of *D. diaboli* made during actual feeding times illustrated those individuals fed zooplankton exhibited high energy expenditure when "chasing" zooplankton and had the most swimming activity when compared to those post-hatchlings fed *Artemia* nauplii, algal gel and protein flake diets. Zooplankton were fed at the same density as *Artemia* nauplii (approximately 110 individuals/0.05 ml), but perhaps were of poorer nutritional quality. Also, the bright color of *Artemia* nauplii and their continuous movement has been suggested to render them more perceptible to larval fishes (Leger et al. 1987). Though the zooplankton ponds from which zooplankton were obtained for this study were fertilized with the same quantity and quality of materials that are used by other aquaculturists (personal communication A.E. Woods Fish Hatchery, Texas Parks and Wildlife, San Marcos, TX), it is possible that essential nutritional elements were lacking in the 150 *um* sized zooplankton fed to *D. diaboli* in this study, perhaps the result of poor nutrient absorption by adult zooplankton (Lukas et al. 2013). At the end of the study, it was determined, through molecular assays, that the zooplankton population produced in the ponds at SMARC, were infected with microsporidia. Though initial analysis currently suggests that fish fed zooplankton have not become infected with microsporidia, there is evidence to support the premise that bacterial infection of zooplankton, such as *Daphnia*, affects its body stoichiometry, altering levels of carbon, nitrogen and phosphorus (Frost et al. 2008). These chemical changes are dependent on the food source of *Daphnia* and the extent and progression of the bacterial infection (Frost et al. 2008). It is possible, though difficult to confirm, that the presence of microsporidia was another factor responsible for

the poor growth exhibited by individuals of *D. diaboli* fed the diet of zooplankton in this study.

Morphology

The external appearance of 136-dph juveniles of *D. diaboli* reared in this study on diets of *Artemia* nauplii, algal gel, or protein flakes were comparable to 128-dph juveniles described by Hulbert et al. (2007). The external appearance of 136-dph juveniles raised on a diet of zooplankton during this study differed markedly from "typical" juveniles. Externally, these juveniles exhibited pigmentation patterns and lengths comparable to those of much earlier stages in Hulbert et al. (2007), 32 and 64 dph, respectively (figure 3 and 4). This "stunted" external appearance might be related to nutritional deficiencies and/or extra energy expenditure associated with the capture of live zooplankton. Though larvae of *D. diaboli* have not been observed free swimming in the wild, observations on larvae (≤30mmTL) in self-perpetuating populations maintained in 12.2m concrete outdoor raceways suggest that they feed and seek refuge within dense patches of aquatic vegetation (pers. obs.). Contrary to older juvenile and adult individuals of *D. diaboli*, larvae were rarely seen emerging from planted areas when offered supplemental feed (pers. obs.). When yearly counts are performed on these outdoor populations, individuals greater than 30 mm TL are often caught by seine while juveniles less than 30 mm TL must be physically separated from within the plant material (pers. obs.).

Interestingly, there were also marked differences in the visceral anatomy of 136-dph juveniles fed the diet of zooplankton, compared to those fed the other diets. These differences involved the length of the gut and the number of intestinal coils or loops, which were shorter and less coiled in juveniles fed the diet of zooplankton (Figures 5c, 6c) compared to juveniles fed the diet of *Artemia* nauplii, algal gel or protein flake (Fig 5a, 5b, 6a, 6b). Such differences in visceral anatomy could be the results of multiple different factors. During development, the digestive tract is considered to play an important role in the maintenance of body homeostasis in fishes (Ostaszewska et al. 2008). An inappropriate diet composition may induce disturbances in digestive tract development and function, which may result in reduced growth and development (Ostaszewska et al. 2008). The structure and relative length of the intestinal tract in teleost fishes is not only influenced by diet but also by the form and size of the body and by phylogeny (Goldschmid et al. 1984). Short guts are generally indicative of carnivory in fishes, while longer guts are generally indicative of diets with high proportions of indigestible material, such as in omnivory and herbivory, where longer guts are considered important for extracting nutrients from nutrient-poor foodstuffs that require greater digestive processing times (Hofer 1982; Horn 1989). In cyprinids, intestinal loops and coils are proportional to the relative length and space available within the body cavity; the longer the gut, the more complex the coiling pattern (Junger et al. 1989). Intestines of omnivorous cyprinids are generally straight throughout most of the larval period and eventually develop an S-shaped loop by, or during, the juvenile period (Junger et al. 1989). Members of the genus *Dionda* are classified as herbivores based on

the lack of a defined stomach and the presence of a long coiled intestine, however, little quantification of their diets has been published to verify this (McMillan 2011). In *D. diaboli*, the initially straight intestine first develops a single loop at approximately 32 dph before transferring into a multi-looped, convoluted intestine by 128 dph (Hulbert et al. 2007). When comparing the intestines of the 136-dph juveniles (~18 mm TL) fed a zooplankton diet in this study to that of the stages in the development of a typical *D. diaboli* available from Hulbert et al. (2007), it is clear that the intestinal tract of the former never progressed passed the single loop stage of a typical 32-dph larvae (~12 mm TL), regardless of the differences in size.

In comparison to those individuals fed zooplankton, it is interesting that *Artemia*-nauplii-fed fish developed the degree of intestinal coiling (and TL) seen in cohorts on an herbivorous diet. Observations conducted before the first and second daily feeds, showed that individual *D. diaboli* fed a diet of *Artemia* nauplii continued to feed on dead *Artemia* on the bottom of the tanks between feedings. As *Artemia* deteriorate, nitrogen concentrations increase (Frost et al. 2008). Nitrogen enrichment has been documented in organisms that are nutrient limited and must use fat reserves to persist (Gannes et al. 1997). Perhaps the decaying *Artemia* nauplii partly provided the nutritional requirements similar to those found in herbivorous diets, and hence the developments of intestinal coils were comparable to those of *D. diaboli* individuals fed a diet of algal gel or protein flakes.

Lastly, there were significant differences in the quantities of visceral fat observed within the body cavities of the 136-dph juveniles fed *Artemia* nauplii, algal gel, protein flake or zooplankton. Fish fed the diet of zooplankton accumulated little to no detectable fat bodies (which were not quantified). Fish fed a diet of A*rtemia* nauplii exhibited very low visceral fat deposits (1.6 mg ± 0.49). In comparison, there were much greater deposits of fat in the viscera of 136-dph juvenile fish fed the diets of protein flake or algal gel (Table 11). Visceral fat deposits have not been observed in wild caught adult *D. diaboli* (personal communication Randy Gibson SMARC and McLean Worsham TSU), and the status of visceral fat deposits in larvae or juveniles born and raised in the wild is unknown. Caldwell et al. (2005) determined visceral fat quantities to be highest in Rio Grande silvery minnow juveniles fed Aquatic Ecosystems (Pentair) protein flakes, when compared with other feeds. When 2% of fish from this study and their cohorts (which were released into outdoor concrete raceways at 180 dph) were examined at 270 dph, none exhibited any evidence of visceral fat. Perhaps the accumulation of fat correlates to being reared in small systems on manufactured feeds, and once juveniles are moved to a larger more "natural" environment, these stores are then depleted.

CONCLUSIONS

It is evident from this study that live feed is essential for best growth during the first 30 days of rearing *D. diaboli* larvae. Although this study demonstrates that *D. diaboli* fed *Artemia* nauplii exhibited the highest growth rate for the 46 dph time period, it was the individuals fed the plant-based diets (algal gel or protein flakes) that attained the highest growth rates for the 76 dph, 106 dph and 136 dph time periods, possibly suggesting that an optimal diet for captive *D. diaboli* is one which transitions from predominantly animal protein to plant based, as the larvae develop toward the juvenile stage. Captive propagation techniques need to incorporate and reflect the changing foraging strategies and diet requirements that occur in wild populations if mass production and reintroduction of this species is to be successful and long lasting. Dietary requirements and trophic ecology of *Dionda diaboli* are important, not only to the conservation of the species, but to provide insight into the ecology of other members of the genus, as well as other spring-associated species of minnows that are subject to the same threats as *D. diaboli.*

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