Transitioning from algae to clay as turbidity agents: Timing, duration, and transition rates for larval sablefish (*Anoplopoma fimbria*)

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Abstract

Clay (claywater) can substitute for algae (greenwater) as a turbidity agent after the first week of feeding for larval sablefish (*Anoplopoma fimbria*), reducing dependence on expensive algae. However, more information is needed to optimize the timing and rate of transition from greenwater to claywater, and to determine whether claywater can be used until the end of the larval period. This study compared four turbidity schedules through the entire 35-day larval period: greenwater transitioned to claywater on day 8, greenwater transitioned to claywater on day 18, greenwater throughout, and claywater throughout. Both gradual and sudden transitions were explored on days 8 and 18. Transitioned larvae, compared to non-transitioned larvae, had lower feeding rates the day after sudden day-8 transitions, but not the day after sudden day-18 transitions, and had lower body weights seven days after sudden day-8 and day-18 transitions. Gradual transitions (over four days instead of one) did not alleviate these negative effects on feeding rates and weight. However, by the end of the larval period, larvae in both transitioned treatments had higher body weight and biomass than larvae from non-transitioned treatments. This suggests that the short-term negative effects on feeding and body weight in transitioned larvae were followed by greater growth benefits. Transitioning at day 8 was the most cost effective, and transitioning at day 18 maximized biomass. Both treatments that transitioned from greenwater to claywater were better than the traditional greenwater-throughout treatment in algal cost savings, biomass, and wet weight per larva. This study provides detailed methods for the use of claywater to further reduce monetary cost and improve production for sablefish aquaculture.

Keywords: Clay, Algae, Greenwater, Larvae, Turbidity, Feeding, *Anoplopoma fimbria*

1. Introduction

Algae plays an important role in marine fish larviculture. In clear, non-turbid water, larvae of many species swim against tank side walls (“wall-nosing”), feed poorly, and die (Boehlert and Morgan, 1985; Cobcroft et al., 2012). Turbidity, usually provided by algae, can improve visual contrast in tanks, helping larvae orient themselves and see prey (Naas et al., 1992; Utne-Palm, 2002; 2004). The mix of algae and seawater is known as “greenwater,” and may also have other functions for young larvae, including stimulation of gut enzymes (Cahu et al., 1998), influences on the microbiome (Attramadal et al., 2012; Pierce et al., 2019), nutrition (Reitan et al., 1998; van der Meeren et al., 2007), and feeding stimulation (Lee et al., 2016).

In aquaculture, mortality and daily operational costs during the larval stage are higher than any other life history stage. Over 90% of lifetime mortality occurs during the larval stage (Hjort, 1914). For species requiring turbidity, greenwater improves performance, but can promote opportunistic and pathogenic bacteria and subsequently compromise fish health (Attramadal et al., 2012; Pierce et al., 2019; Stuart et al., 2015). Further, greenwater is labor-intensive to culture and expensive to purchase.

Clay is an inexpensive and inorganic alternative to algae for creating turbidity, but effects of claywater on larval growth and survival have differed among studies. For instance, larval growth or survival in claywater has been inferior (Daugherty, 2013), superior (Attramadal et al., 2012; Stuart et al., 2015), or equivalent to that in greenwater (Daugherty, 2013). Relative performance of greenwater versus claywater appears to vary among fish species and methodologies

(Daugherty, 2013). Most studies on claywater have taken an “all or none” approach, using either greenwater or claywater for the entire larval period.

Temporal aspects may also determine whether clay or algae is the superior turbidity agent. In a study on larval sablefish (*Anoplopoma fimbria*), claywater led to poor survival if used for the first week of larval rearing, but higher growth (and equal survival) if greenwater was transitioned to claywater at the beginning of the second week of larval rearing (Lee et al., 2017a). Such temporal aspects should be considered before claywater is ruled out for a species. For species that have been shown to benefit from claywater, understanding temporal aspects and testing more complex alternative schedules of switching between greenwater and claywater may further improve larviculture methods.

Sablefish require turbidity for the first 30-35 days of larval rearing (Lee et al., 2017a), but no previous study has compared their performance in greenwater versus claywater beyond the first two weeks. In this study, we tested whether claywater could continue to be successfully utilized through to the end of the 35-day larval period. We also compared two schedules (day 8 and day 18) and rates (sudden and gradual) for transitioning from greenwater to claywater.

2. Methods

2.1. Spawning and production

Sablefish broodstock collection, incubation, and early rearing are described in Cook et al. (2015). Briefly, broodstock were collected by longline from the Washington Coast. Eggs were artificially fertilized and held in incubators for 12 days, then transferred to silos. The rooms were kept dark, and seawater was 5 ˚C to simulate the deep and cold waters that developing eggs normally encounter in nature. Upon yolk depletion (approximately 46 days after fertilization), larvae were transferred from silos to the rearing tanks.

2.2. Tanks

Rearing tanks were 91 cm tall with a diameter of 102 cm at the top of the tank and 99 cm at the bottom. Tanks were filled with seawater to a height of 78 cm (Experiment 1, 615 L volume, stocked at 7.3 larvae per L) or 53 cm (Experiment 2, 412 L, stocked at 7.8 larvae per L). Water height was maintained by an external standpipe, while a 10 cm (internal diameter) mesh-lined internal center standpipe prevented larvae from exiting the tank. In experiment 1, new water initially flowed into each tank at a rate of 1 liter per minute (LPM), increased to 2 LPM on day 16, and increased to 2.4 LPM on day 21. In experiment 2, flow started at 0.7 LPM and was increased to 0.9 LPM on day 20. For both experiments, light intensity at stocking was 20-30 lux at the water surface and was increased to 80-90 lux after 10 days.

Concentrated solutions of greenwater or claywater were pumped from source tanks into rearing tanks approximately three times per hour to maintain desired turbidity (approximately 11 Nephelometric Turbidity Units, NTU). The algae was *Nannochloropsis oculata* Instant Algae (Reed Mariculture, Campbell, CA, USA). Clay was Kentucky Ball Clay OM4 (Kentucky-Tennessee Clay Company, Roswell, GA, USA). Water temperature was 8.4 to 10.5˚ C at stocking, and gradually increased to 14˚ C by day four. Periodic tank maintenance included siphoning tank bottoms, wiping tank surfaces after partial tank draining, and rinsing center standpipes. Larvae were fed three times per day, starting with live rotifers at stocking, then live *Artemia franciscana* (INVE Aquaculture, Salt Lake City, Utah, USA) starting on day 16, and dry prepared feeds (Otohime fish diet, Marubeni Nisshin Feed Co., Tokyo, Japan) starting on day 29. Experiments ended when larvae completed weaning onto dry prepared feed on day 35. General rearing and greenwater/claywater methods can be found in Cook et al. (2015) and Lee et al. (2017a).

2.3. Experiment 1—Greenwater, claywater, and transition date

The experiment was conducted over 35 days in 16 rearing tanks, divided among four treatments (n=4 per treatment, Table 1). Treatment G8C received greenwater for the first seven days, switched to claywater on day 8, and remained on claywater until the end of the 35-day larval period. Treatment G18C received greenwater for the first 17 days, switched to claywater on day 18, and remained on claywater until the end of the 35-day larval period. Treatment G35 received greenwater throughout, and treatment C35 received claywater throughout.

Feeding was characterized by quantifying rotifers in larval guts one day after each transition (on days 9 and 19). On both days, 30 minutes after rotifers were added to each tank, 10 larvae were removed from each tank and examined under a microscope by a treatment-blind observer. On day 9, larval bodies are transparent and individual rotifers are easily identified, so the number of rotifers in the larval guts were counted. In each tank for day 9, the number of rotifers counted in 10 larvae were summed and divided by 10 to calculate the average number of rotifers per gut. By day 19, the large quantities of rotifers packed into the larval guts made counting difficult, so percent gut fullness was visually estimated.

Body weights were quantified seven days after each transition (dry weights on days 15 and 25), and at the end of the experiment (wet weights on day 35). For dry weights, ten larvae were removed from each tank, dried overnight in an oven, and group weighed. Weights were divided by ten to calculate dry weight per larva. For wet weights, all surviving larvae in each tank were group-weighed while wet ("biomass"), then counted. For each tank, biomass was divided by the number of survivors to calculate average wet weight per larva.

2.4. Experiment 2—Rate of transition

The second experiment repeated G8C and G18C treatments and thus added replication for these two treatments, but half of the tanks within each treatment were transitioned either suddenly or gradually, creating four treatments (G8C-sudden, G8C-gradual, G18C -sudden, G18C-gradual; n=4 per treatment). The claywater-only (C35) and greenwater-only (G35) treatments were not repeated in experiment 2.

G8C-sudden and G18C-sudden were switched from greenwater to claywater suddenly (greenwater in the peristaltic pump source tank was emptied, then refilled with claywater), as was done in experiment 1, whereas G8C-gradual and G18C-gradual were switched gradually over four days, beginning at day 8 for G8C-gradual, and beginning at day 18 for G18C-gradual. On the first day of the gradual transition, concentrated greenwater in the pump source tank was replaced with a 75% greenwater concentrate / 25% claywater concentrate mix. This 75/25 mix was changed to 50/50 on the second day and 25/75 on the third day, reaching full claywater concentrate (0/100) on the fourth day.

The sampling procedures used in experiment 1 for feeding and dry weights were repeated, with the exception that feeding data were not collected after the day-18 transition. At the end of the experiment, biomass, survival, and wet weight per larva were quantified following methods from experiment 1.

2.5. Statistics

2.5.1. Comparisons

Feeding and body weight comparisons were made one day (feeding) or seven days (body weight) after the transitions on days 8 (G8C) and 18 (G18C). The recently-transitioned tanks were compared to non-transitioned tanks. After the day-8 transition in experiment 1, the G8C (n=4) transitioned tanks were compared to the non-transitioned tanks (n=8, four from G35 and four from G18C). G18C was included as a non-transitioned tank because it had not yet transitioned by the sampling day. After the day-8 transition in experiment 2, G8C (n=8) was compared to G18C (n=8), which again had not yet transitioned by the sampling day. After the day-18 transition in experiment 1, G18C (n=4) was compared to G35 (n=4). No comparison was made for the day-18 transition in experiment 2, since all tanks had begun transitioning by day 18. In experiment 2, four out of the eight transitioned tanks were transitioned gradually, but all transitioned tanks were included in analyses regardless of transition speed because transition speed (gradual versus sudden) did not affect feeding, body weight, or survival (see below, $p > 0.05$, Table 2). Tank was the unit of replication for all data in all experiments.

2.5.2. Next-day feeding effects after transitions

We tested whether transitions affected feeding the next day. For day-8 transitions, a general linear model examined the effects of treatment (transitioned versus non-transitioned), experiment (1 versus 2), and the interaction between treatment and experiment on feeding on day 9. For day-18 transitions, a t-test compared feeding on day 19 between transitioned and non-transitioned treatments.

2.5.3. One week growth effects after transitions

To determine whether transitions had short-term effects on body weight, a general linear model examined effects of experiment (1 versus 2), transition day (8 or 18), treatment (transitioned versus non-transitioned), the interaction between transition day and treatment, and the interaction between treatment and experiment on body weight seven days after each transition.

2.5.4. Effect of transition speed

For experiment 2, t-tests compared suddenly-transitioned and gradually-transitioned tanks for feeding (one day after transition) and dry weight (seven days after transition). To evaluate long-term effects on body weight and survival (day 35), t-tests compared suddenly-transitioned and gradually-transitioned tanks for biomass, survival, and wet weight per larva.

2.5.5. Treatment effects at the end of the larval period

For experiment 1, ANOVA followed by Tukey-Kramer HSD tested for differences among treatments. For experiment 2, a two-way ANOVA tested for differences among treatments in biomass, number of survivors, and wet weight per larva on day 35.

3. Results

3.1. Experiments 1 and 2—Next-day feeding effects after transitions

Transitioning on day 8 significantly reduced feeding on day 9 (Figure 1, $p < 0.05$), but there was no significant effect of experiment (1 and 2) and no significant interaction between treatment and experiment. Transitioning on day 18 did not significantly affect feeding on day 19 (Figure 1, $p > 0.05$).

3.2. Experiments 1 and 2—One-week growth effects after transitions

For day-8 and day-18 transitions, body weight one week after the transition was significantly lower than non-transitioned larvae (Figure 2, $p < 0.01$). There were also significant effects of experiment (Figure 2, $p < 0.01$) and transition day (Figure 2, $p < 0.01$). The transition day effect reflects the higher body weight of older larvae (weighed seven days after the day-18 transition versus seven days after the day-8 transition). There were no significant interactions between transition day and treatment, or between treatment and experiment ($p > 0.05$).

3.3. Experiment 1—Treatment effects at the end of the larval period

At the end of experiment 1 (day 35), there were significant treatment effects on biomass [Figure 3, $F(3, 12) = 69.87$, $p < 0.0001$], number of survivors [Figure 3, $F(3, 12) = 61.38$, $p < 0.0001$], and wet weight per larva [Figure 3, $F(3, 12) = 19.26$, $p < 0.0001$]. C35 (all claywater) had significantly lower biomass and number of survivors than all other treatments (Figure 3, $p < 0.05$) and significantly lower wet weight per larva than G8C and G18C (Figure 3, $p < 0.05$). G8C and G18C had significantly higher biomass and wet weight per larva than G35 and C35 (Figure 3, $p < 0.05$), and higher survival than C35 (Figure 3, $p < 0.05$).

3.4. Experiment 2—Effect of transition speed

Compared to sudden transitions, gradual transitions at day 8 and day 18 did not significantly affect feeding on the day after the transition, body weight seven days after the transition, survival, biomass, or wet weight per larva (Table 2, $p > 0.05$).

3.5. Experiment 2—Treatment effects at the end of the larval period

At the end of the experiment (day 35), larvae that transitioned on day 18 had significantly higher biomass than larvae that transitioned on day 8 [Figure 4, $F(1, 12) = 7.58$, $p < 0.05$], but there were no significant effects of transition day on number of survivors [Figure 4, $F(1, 12) = 2.45$, $p > 0.05$] or wet weight per larva [Figure 4, $F(1, 12) = 3.90$, $p > 0.05$]. There were no significant effects of transition speed [biomass: $F(1, 12) = 0.07$, $p > 0.05$; survivors: $F(1, 12) = 0.01$, $p > 0.05$, wet weight per larva: $F (1, 12) = 0.15$, $p > 0.05$] or the interaction between transition day and transition speed [biomass: $F(1, 12) = 3.40$, $p > 0.05$; survivors: $F(1, 12) = 2.95$, $p > 0.05$, wet weight per larva: $F (1, 12) = 0.03$, $p > 0.05$].

4. Discussion

This study covered the entire larval rearing period for sablefish, extending findings from our 2017 study which focused on the first 14 days of the larval period (Lee et al., 2017a). Consistent with the 2017 study, claywater was a poor substitute for greenwater if used during the first seven days of larval rearing. The present study showed that claywater can be used from day 8 to the end of the larval period (day 35). Compared to the traditional all-greenwater method, biomass was higher if claywater was used from day 8 to day 35, and even higher if the transition was delayed to day 18. Compared to non-transitioned larvae, the transition led to reduced feeding one day after the day-8 transition, and lower body weight seven days after the day-8 and day-18 transitions. Despite the negative short-term effects of transitioning on feeding and body weight, subsequent growth led to a reversal in body weight differences between transitioned and non-transitioned larvae, with transitioned larvae having higher body weights by the end of the larval period (day 35). Transition speed (sudden versus gradual) did not affect feeding, body weight, or survival.

4.1. Short-term effects on feeding and body weight

Short-term negative effects of transitioning on feeding and body weight, followed by accelerated growth relative to greenwater, suggest that there are both negative and positive effects of claywater that vary temporally. Previous studies have documented positive and negative effects of claywater. Claywater can be superior to greenwater in part because claywater is inorganic, does not promote as much opportunistic bacterial growth, and can aggregate and sink organic matter to tank bottoms (Attramadal et al., 2012; Stuart et al., 2015). Clay has been shown to damage gills of larval fish, and increase deformity rates, but only at concentrations much higher than those used in aquaculture applications (Hess et al., 2015; Zhang et al., 2019).

Claywater may have more immediate benefits in tanks with poorer water quality. Compared to non-transitioned larvae, the 2017 study found higher larval body weights seven days after the greenwater-claywater transition, while the present study found the opposite—lower body weights in transitioned larvae, though that difference was reversed by the end of the larval period. Tank size, cleaning regimens, and thus water quality may have differed between the 2017 and the present study. The 2017 study used small (37L) tanks that were not cleaned during the course of the 14-day experiment, whereas the present study utilized 500L tanks with scheduled cleanings, over 35 days. Further, tank surfaces (tank walls, bottom, and standpipe) tend to become fouled with biofilms (Karunasagar et al., 1996), and are more likely to come into contact with larvae in smaller 37 L tanks, since smaller tanks have higher ratios of tank surface areas to water volume. The benefits of claywater may have been stronger and more immediate for the 2017 study if levels of organic matter or pathogens increased earlier and faster than in the present study. Organic and microbial loads and communities can vary through time in larval sablefish rearing tanks (Pierce et al., 2019), and could play a role in generating temporal changes in body weight effects such as those observed in our studies. Tanks with higher organic or bacterial loads might benefit from earlier transitions (e.g. on day 8), and cleaner tanks might benefit from later transitions (day 18). In marine aquaculture, organic loads and tank hygiene can vary both predictably (with stocking density, feed type, feed density, water temperature) and unpredictably (with feed quality, quality of incoming water, stochastic microbial growth). Despite differences in the timing of growth patterns between the 2017 and the present studies, the implications for aquaculture from both studies remain unchanged; that is, transitioning from greenwater to claywater is superior to remaining on greenwater through the entire larval stage.

In another study, bacterial cell counts increased in larval sablefish tanks after a transition to claywater, but the bacteria potentially may have been bound to the cells and biologically unavailable to the larvae (Pierce et al., 2019), and more recent work suggests that the increase is temporary (Pierce et al, unpublished data). Microbial communities in water also differ from communities on tank surfaces and larval skin (Pierce et al., 2019). Claywater has also been associated with reductions in vibrios (Attramadal et al., 2012; Pierce et al., 2019; Stuart et al., 2015).

4.2. Transitions

The short-term negative effect of transitioning on body weight in this study may be related to the reduced feeding observed shortly after the transition (day 9 feeding, Figure 1). Reduced feeding after the transition might result from general but temporary stress associated with the transition, or might indicate that claywater is a poorer visual medium than greenwater for young larvae. While larval marine fish use vision to target prey in the near field, their vision is generally poor and sensitive to environmental conditions (Utne-Palm, 2002). Further, the visual system of larval sablefish changes during early larval rearing (Britt, personal communication). Older and larger larval fish are also generally better at capturing prey for multiple reasons (China and Holzman, 2014; Knutsen and Tilseth, 1985; Miller et al., 1993), and therefore may be less sensitive to disruptions from transitions. The visual systems of older larvae may also be generally better at identifying prey in the claywater medium. These factors might explain why larvae that transitioned on day 18 did not show significant reductions in feeding (Figure 1) and had higher final biomass than larvae that transitioned on day 8. Interestingly however, those larvae that transitioned on day 18 still showed negative short-term effects on body weight (Figure 2). More work is needed to elucidate the mechanisms and temporal changes to the mechanisms behind short-term transition costs.

Gradual transitions did not improve feeding or body weight, but other methodological changes might help. While biomass and wet weight per larva were increased in transitioned tanks, those performance measures might be increased even further if the short-term effects on feeding and body weight are eliminated or minimized. Cultured marine fish larvae are commonly susceptible to starvation (Yin and Blaxter, 1987). If reduced feeding during transitions causes the negative short-term effects on body weight, then higher rotifer densities in the few days following transitions might ameliorate both. Feeding rates are largely influenced by encounter rate, which can be increased with higher feed densities (Mackenzie et al., 1994; Puvanendran and Brown, 1999). Rotifer density positively affects body weight and survival in young sablefish larvae (Lee et al., 2017b).

4.3. Treatment effects at the end of the larval period

Both transition timing options (G8C and G18C) resulted in higher biomass by the end of the larval rearing period, relative to continued rearing in greenwater, but the choice to transition on day 8 versus day 18 will depend on the trade-off between reduced greenwater costs and increased larval production and vary with production goals and conditions unique to each hatchery or hatchery production run. For a 5000 L flow-through aquaculture rearing tank that receives 5000 L of new water once every four hours, greenwater for the entire 35-day larval rearing period would cost $1,566 per tank (at $71 per L of algal concentrate). If greenwater is transitioned at day 18, the cost for turbidity is almost halved to $788 per tank, and for a tank transitioned on day 8, the cost is $322 per tank (at $71 per L of algal concentrate and $0.85 per kg of clay). Transitioning at day 8 maximizes cost savings associated with switching from expensive greenwater to inexpensive claywater, while transitioning at day 18 maximizes biomass. The preferred timing for starting claywater will depend on factors such as labor and supply costs, tank space availability, algae costs (purchase or culture costs), and organic loads. For example, day-18 transitions may be preferred during hatchery production runs that stretch to reach a high target production number, whereas day-8 transitions may be preferred when the cost per fingerling is more important, when algae prices rise, or when live feeds are dirty.

5. Conclusions

For marine fish aquaculture, the larval stage is the most expensive and highest-mortality stage on a per-day basis, thus improvements to these factors will have positive impacts on the aquaculture industry and seafood availability. Currently, greenwater appears to be necessary during the first week of larval sablefish rearing, but transitioning to claywater at day 8 or day 18 leads to higher biomass by the end of the larval stage, providing two methods that can reduce larval rearing costs while increasing larval production. Areas for future research could include the identification of novel methods for minimizing greenwater use during the first week, or for reducing costs associated with transitioning from greenwater to claywater. Future studies could also investigate other potential effects of clay and algae on important variables such as behavior and deformities.

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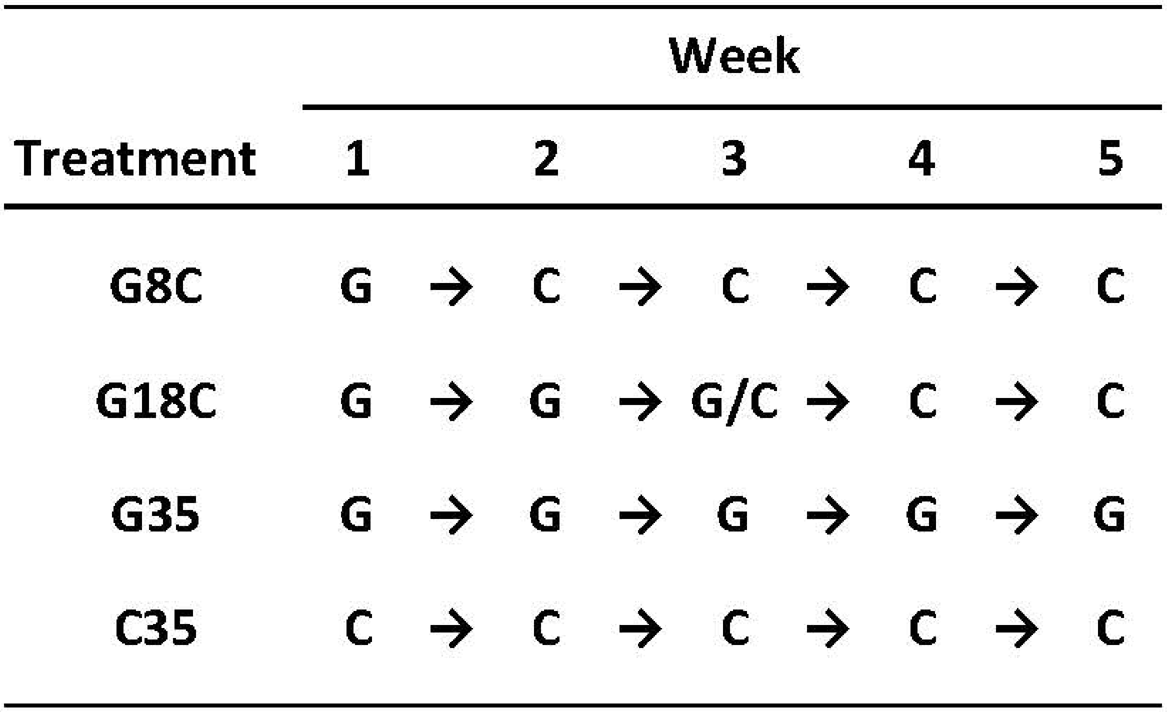
Table 1. Treatments in experiment 1 differed in their use of turbidity agents during the 35-day experiment. G = Greenwater; C = Claywater. Treatment G8C used greenwater before transitioning to claywater on day 8 (n=4). Treatment G18C used greenwater before transitioning to claywater on day 18 (n=4). Treatments G35 and C35 used greenwater and claywater, respectively, throughout the experiment (n=4 per treatment). Experiment 2 repeated treatments G8C and G18C (n=8 per treatment), but half of the tanks in each treatment were transitioned to claywater gradually over four days, instead of one day.



	Week								
Treatment	**1**		**2**		**3**		**4**		**5**
G8C	G	→	C	→	C	→	C	→	C
G18C	G	→	G	→	G/C	→	C	→	C
G35	G	→	G	→	G	→	G	→	G
C35	C	→	C	→	C	→	C	→	C

Table 2. Mean (± S.E.M.) feeding, dry weight, and end-of-experiment survival, biomass, and wet weight per larva, for sudden and gradual transitions at days 8 and 18. Sudden and gradual treatments did not lead to significant differences in any measure ($p < 0.05$).

	1 day after transition	**7 days after transition**	**End of experiment**		
Clay transition timing	**Rotifers in gut (#)**	**Dry weight (mg)**	**Survival (%)**	**Biomass (g)**	**Wet weight per larva (mg)**
Sudden (G8C)	**14.23 ± 4.84**	**0.89 ± 0.10**	**20.74 ± 1.05**	**71.00 ± 2.97**	**106.88 ± 0.89**
Gradual (G8C)	**11.73 ± 0.90**	**0.89 ± 0.11**	**22.92 ± 1.90**	**79.75 ± 6.34**	**108.56 ± 4.32**
Sudden (G18C)	**not collected**	**3.83 ± 0.52**	**24.70 ± 0.79**	**90.00 ± 1.78**	**113.41 ± 2.60**
Gradual (G18C)	**not collected**	**4.00 ± 0.82**	**22.60 ± 0.97**	**83.50 ± 4.01**	**114.11 ± 3.34**

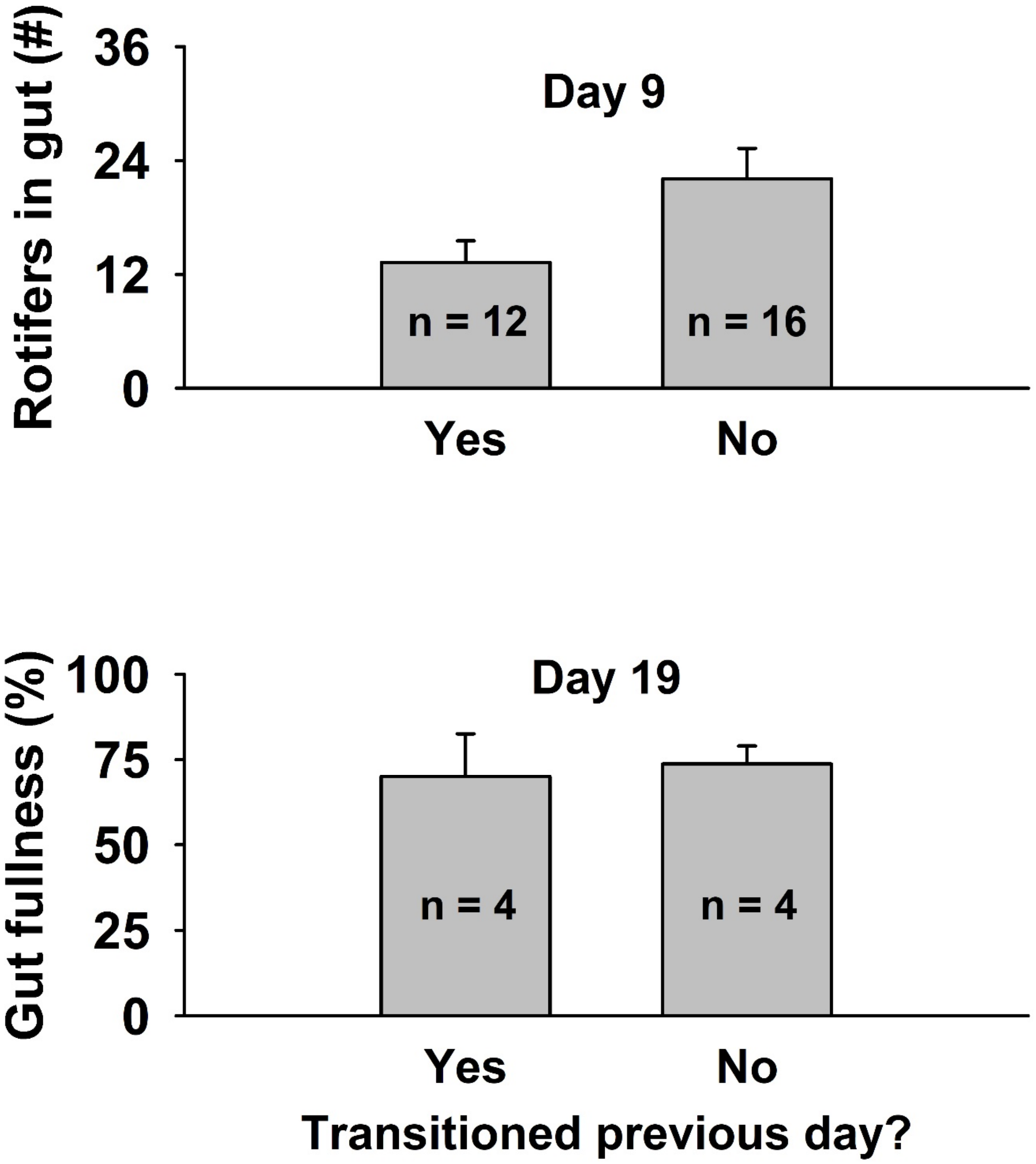


Figure 1. Feeding one day after the start of transitions to claywater, compared to larvae that remained on greenwater. There was a significant difference on day 9 ($p < 0.05$), but not day 19.

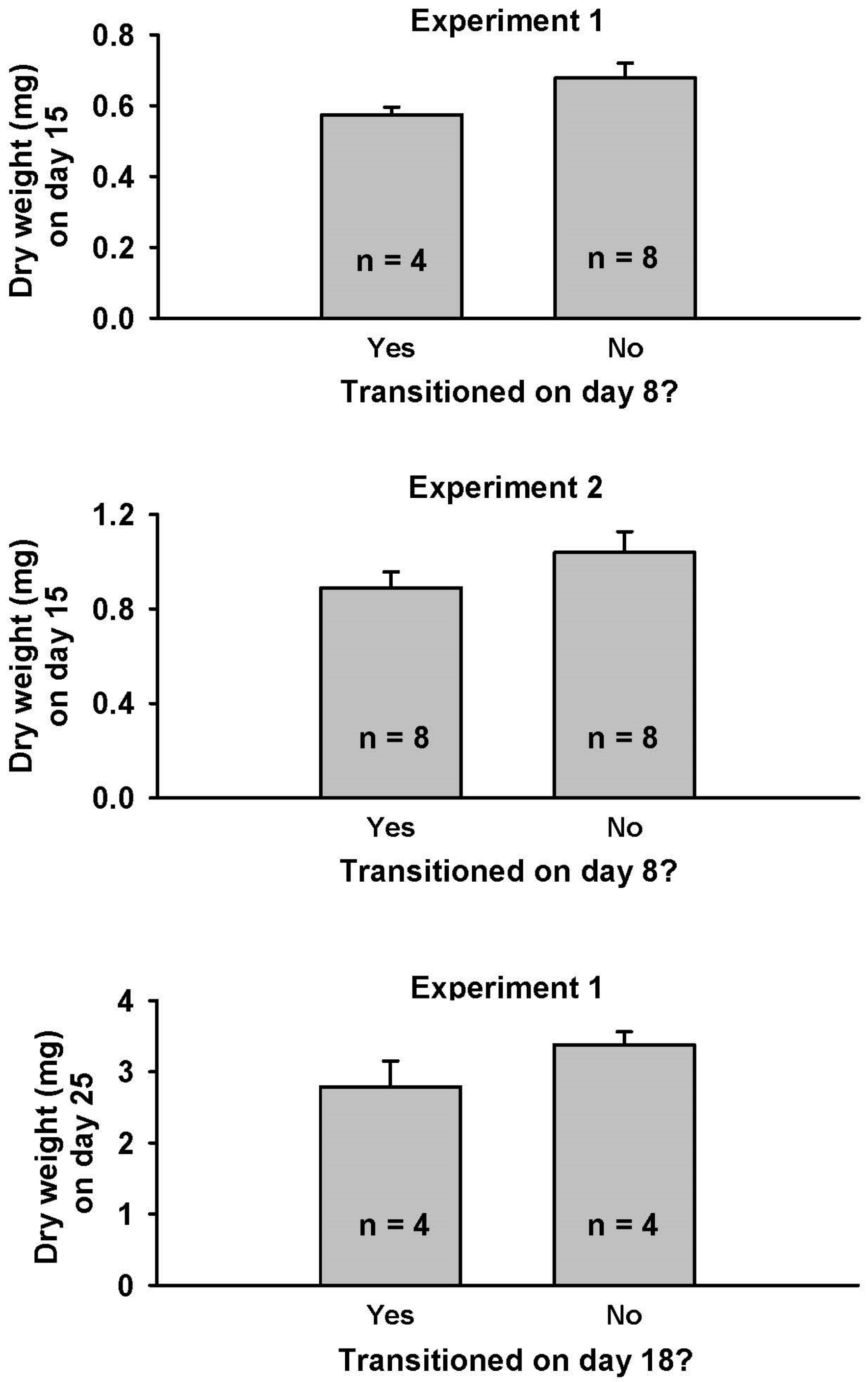


Figure 2. Dry weight per larva seven days after tanks were transitioned to claywater or kept on greenwater, for transitions at days 8 and 18. Dry weight was significantly lower for transitioned larvae ($p < 0.01$).

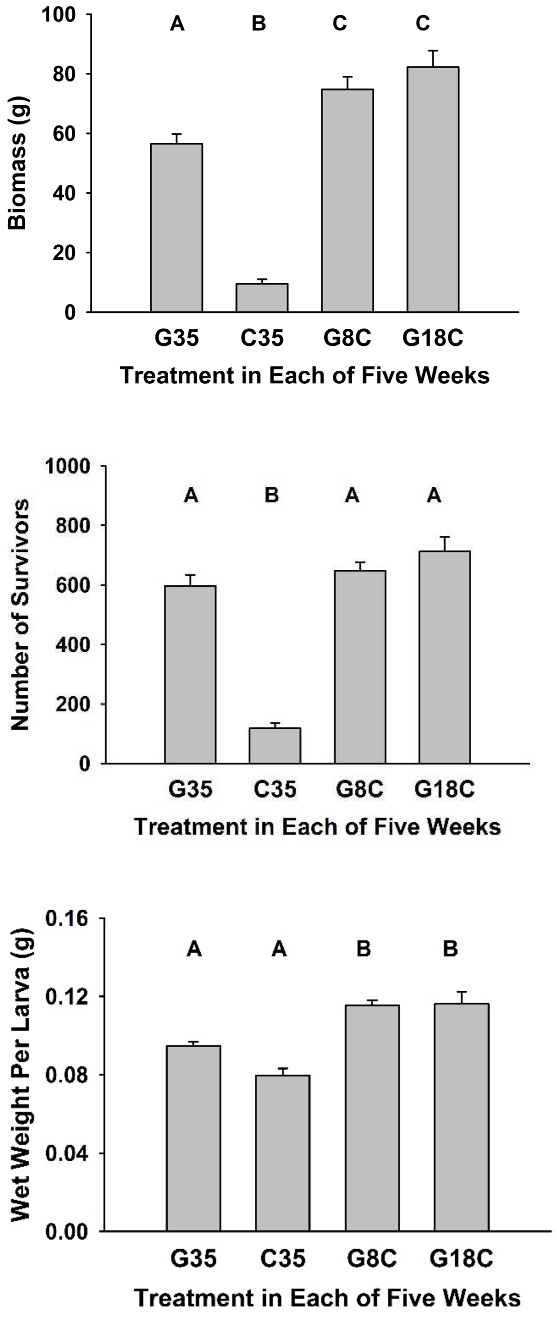


Figure 3. Experiment 1, biomass, number of survivors, and weight per larva, for tanks with greenwater throughout (G35, n=4), tanks with claywater throughout (C35, n=4), and tanks transitioned from greenwater to claywater on day 8 (G8C, n=4) or day 18 (G18C, n=4). Different letters reflect significant differences ($p < 0.05$).

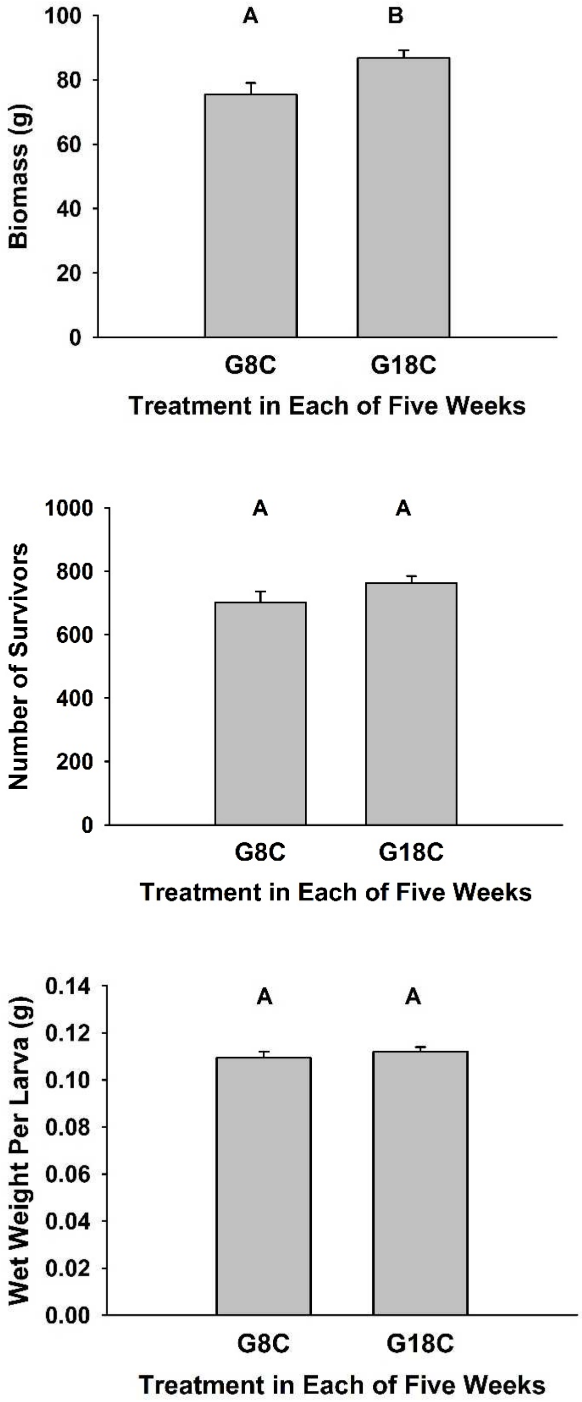


Figure 4. Experiment 2, biomass, number of survivors, and weight per larva, for tanks transitioned from greenwater to claywater on day 8 (G8C, n=8) or on day 18 (G18C, n=8). Different letters reflect significant differences ($p < 0.05$).