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Influence of Temperature on Yolk Resorption by Centropomus undecimalis Larvae

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Influence of Temperature on Yolk Resorption

by *Centropomus undecimalis* Larvae

by

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DEDICATION

I dedicate this thesis to God, who blesses me every day. To my parents, Laureano II and Acela María for their effort and love. To my husband Juan and my son Jeronimo who has come to fill my life, and the Wayuu community to which I proudly belong.

Dedico esta tésis a Dios, quien me bendice todos los días. A mis padres, Laureano II y Acela María por su esfuerzo y amor. A mi esposo Juan y mi hijo Jerónimo, quien ha venido a llenar mi vida, y a la comunidad Wayuu, a la que orgullosamente pertenezco.

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Abstract

In an effort to determine the optimal temperature for rearing *Centropomus undecimalis* larvae during the yolk resorption period, larval development was measured under four different temperature regimes (23, 25, 28 and 31 °C). The eggs were incubated at 28 °C until hatching, which occurred at about 17 hours post-fertilization. After hatching, temperatures were adjusted to the respective treatment levels. Measurements were collected from 25 individual larvae across rearing temperatures at the following pre-determined time intervals: at hatching, 24 hours post hatch (hph), 48 hph, and 72 hph. Morphometric measurements were obtained from photomicrographs, including yolk sac length and height, oil globule diameter, standard length, body height at anal pore, and eye diameter. Larvae in the 25 °C treatment had longer median standard length, body height, and more energy reserves than those larvae reared at other temperatures. The yolk sac and oil-globule were present up to 72 hph at 23 and 25°C, while these were entirely consumed after 48 hph in treatments at 28 and 31 °C. *Centropomus undecimalis* larvae had the highest growth rates during the first 24 hph, and this period corresponded to the highest energy consumption as determined by the decrease in yolk-sac and oil-globule volume. Survival was assessed during the third trial only. The 31 °C treatment presented the worst

survival percentages, with a maximum survival of 37.2% at 24 hph, and 100% mortality at 72 hph. The 25 °C treatment featured higher survival at the end of the trial than the other treatments with 1.7% survival. Eye diameter didn't vary significantly with time and was not a useful parameter for tracking development during yolk resorption. These results led to the conclusion that 25 °C was the optimal temperature to raise snook larvae during the yolk-resorption period.

1. Introduction

The commercial fishery for common snook, *Centropomus undecimalis* (Bloch), was closed in Florida in 1957 to prevent overharvest (Brennan et al., 2006). Nevertheless, the density of the wild population continued to decrease over the next 50 years. This trend is a consequence of commercial and recreational overfishing (Hill, 2005), habitat degradation and destruction (Bruger and Haddad, 1986; Gilmore et al, 1983), and extreme low winter temperatures such as those experienced during early 2010. Recreational harvest also remain closed in the Gulf of Mexico after the 2010 cold-kill. This situation concerns resource managers (Muller and Taylor 2006), and has prompted research efforts to evaluate snook population enhancement programs (Brennan et al., 2006)

The Mote Marine Laboratory and the Florida Fish and Wildlife Conservation Commission have conducted joint research to advance snook stock enhancement since 1996 by developing large scale aquaculture technology (Brennan et al., 2005; Wittenrich et al., 2009). In 2006, staff at Mote Marine Laboratory achieved reproduction of the species in captivity (Main et al., 2007; Resley et al., 2009; Rhody et al., 2011). Small numbers of snook have been produced for stocking of recreational fisheries using seed from wild broodstrock (Brennan and Leber., 2001;Yanes-Roca et al., 2009).

Cerqueira and Tsuzuki (2008) demonstrated that improvements in spawning induction techniques and larviculture practices enhance survival rates between 10 to 30 % in a closely related species, *Centropomus parallelus*. Nevertheless, several obstacles remain for successful culture of fish larvae (Holt 2003), including efficient use of limited yolk reserves and avoiding premature yolk resorption, understanding live food requirements and transition, and reaching sufficient numbers of fingerlings for stock enhancement (Neidig et al., 2000).

In both cultured and wild marine fish species, the transition from endogenous (yolk sac) to exogenous active feeding is associated with substantial mortality (Ostrowski and Laidley, 2001). According to Blaxter (1969), the most critical processes during early development are the rate and efficiency of yolk-sac resorption and the allocation of yolk for embryo development and metabolic energy.

Adult *C. undecimalis* stop feeding at temperatures near 11°C and die at temperatures <11°C (Howells et al., 1990). There is currently no defined temperature range for larvae, but Gilmore et al. (1983) found that at 20°C, spawning activity was suppressed and larval mortality increased. Gonadal maturation and spawning begins when water temperatures reach 22–23°C (Tucker and Campbell, 1988; Peters et al., 1998). Juveniles are very sensitive, with a lower limit close to 13°C. Adult snook typically inhabit waters between 26 and 29°C. Egg incubation times are strongly affected by egg size and water temperature. Temperature controls the efficiency at which yolk is converted into body tissue (Hunter, 1981; Blaxter 1969) and also the rate of development of the

feeding apparatus and visual system (Wittenrich et al., 2009). Ambient temperatures thus affect general larval development (Green and McCormick, 2001). All of these factors affect the size of larvae at first feeding and the length of the time period between hatching and starvation (Hunter, 1981). Knowing the correct culture temperature during yolk resorption is one of the bottlenecks in snook aquaculture; optimal culture temperatures for larval rearing will improve larval survival rates. Regarding predation, swimming speed is also positively correlated with larval length (Batty and Blaxter, 1992), and hence the ability to catch prey and avoid predators is influenced by larval size as well as by the yolk in reserves required to meet metabolic demands (Blaxter and Hempel, 1963). The eyes are essential to orientation and hunting (Fuiman, 1983), and their development can also be assumed to be important during early larval life.

The research presented here addresses the influence of temperature during the first few hours of larval development. The approach was to conduct morphometric measurements of *C. undecimalis* larvae exposed to different temperature treatments under controlled laboratory conditions. The morphological parameters measured were selected based on their importance to first feeding.

2. Materials and Methods

2.1 Biological material

C. undecimalis eggs and sperm were obtained from captive broodstock maintained in 48 m^3 circular tanks at the Mote Aquaculture Research Park in Sarasota, Florida. Broodstock were strip-spawned following induction with gonadotropine-releasing hormone (GnRHa), in July and September 2010 and in March 2011. Milt was collected using a syringe and a cannula (Fig. 1a) and then stored on ice, while eggs were stripped from female snook (Fig. 1b). Eggs were fertilized with milt from several males; sperm was activated with 35 ppt sea water. Fertilized eggs were poured into 10L hatching cones (Fig. 1c) with aeration and left until they reached the blastula stage. Unfertilized eggs were discarded after they sank, leaving fertilized eggs floating at the surface (Fig. 1d). Incubation took place at 28 ºC water in the microcosm systems (Fig. 1e) until hatching, about 17 hours post-fertilization.

Three temperature experiments (trials) were conducted. In the first two trials, a sample of 0.03 ml of the floating eggs (≈200 eggs) was taken using an insulin syringe once blastula stage was confirmed to stock each of the rearing systems. For the third trial, the same methodology was used to stock the treatments. About 24 hours after hatching, the larvae started to die, indicating a problem with

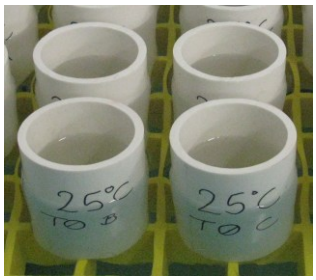
the quality of the eggs. For this reason, these samples were discarded and the microcosms were restocked with newly hatched larvae (9 hours post hatch) from different adults. We used larvae because eggs were not available. The stocking density remained the same (≈200 larvae per microcosm). Measurements for T0 were taken at this time, and were subsequently treated as all the measurements from the other trials.

a.

b.

c.

d.



e.

Fig 1. (a) Collection of the milt, (b) collection of the eggs by stripping, (c) hatching cones with aeration, (d) sample of buoyant fertilized eggs, (e) microcosms where eggs were stocked.

2.2 Treatments

Larvae were exposed to three different temperatures (25, 28 and 31 °C) in the first two trials. These temperatures were selected based on earlier work which suggested that optimal development of the snook larvae occurs within a specific temperature range (Limouzy, 1987). Considering the results of the first two trials, and knowing that spawning can start when temperatures reache 22 to 23 °C (Tucker and Campbell, 1988; Peters et al., 1998), a fourth temperature (23 °C) treatment was added in the third trial to examine response of the larvae to a lower temperature.

Each trial consisted of 25 microcosms per system (Fig. 2a) set in shallow rectangular raceways supplied with re-circulating water and exposed to U.V filtration to minimize bacterial growth. The microcosms were small PVC cylinders, (2.6 cm in diameter); the base was covered with a 95 µm nitex mesh to prevent the escape of larvae and allow circulation of water. The top of the microcosm extended above the surface of the water bath and was exposed to air (Fig. 2b). The microcosms were randomly placed on an elevated platform within the raceway that allowed water to flow around and under each microcosm (Fig. 2). Temperatures were maintained by setting the room air conditioning to a lower temperature and then heating the raceways to 23, 25, 28 or 31 °C. The heaters were controlled using thermostats. To track the stability and accuracy ($\pm$ 0.5 °C), temperature was recorded every 15 minutes by loggers immersed in each system.

Larval development parameters were recorded at four times: at hatching, 24 hours post-hatch (hph), 48 hph, and 72 hph. Approximately 25 individuals from each system where removed each time for photography and measurement.

a.

b.

Fig 2. (a) Water table structures, with recirculating water, and (b) raceways in water table with microcosms and heater.

2.3 Parameters measured and statistical analyses

Larvae were mounted in a Sedgewick Rafter counting slide and photographed using a compound microscope fitted with a digital camera. Measurements were conducted using the photomicrographs. Specific measurements included standard length, body height at anal pore, eye diameter, yolk sac length and height, and oil globule diameter (Fig. 3). These metrics were extracted using the Visilog 6.8® software.

The Kruskall-Wallis test was used to establish the differences between the observations from different treatments at hatch. Simple linear regression was used to assess trends in time and growth rate vs. yolk consumption rate where calculated and illustrated. For convenience, observations were grouped in 0, 24,

48 and 72 hph when tabulated, even though it actually took several hours to complete the collection of photographs of the larvae collected at each observation time.

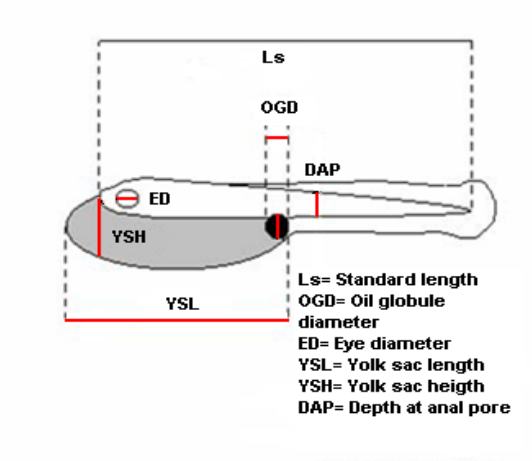


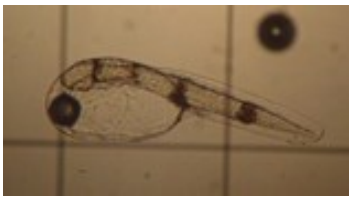
Fig 3. Sketch of a fish larva showing parameters recorded. Ls: standard length, OGD: oil globule diameter, DAP: depth at anal pore, ED: eye diameter, YSH: yolk sac height and YSL: yolk sac length.

2.4 *Survival*

Dead larvae degraded rapidly, especially in the first two trials. This was due to bacterial growth within the microcosms spurred by egg-related substances remaining in suspension after hatching. Unfortunately, these initial trials were not designed to assess larvae survival and it was not possible to determine how many larvae had died.

The third trial did not suffer from bacterial growth due to degradation of egg remains because newly hatched larvae were stocked directly in the microcosms.

In this trial, survival was assessed by pulling one of the microcosms from each treatment and counting the total number of live and dead.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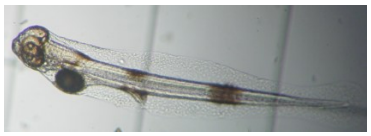
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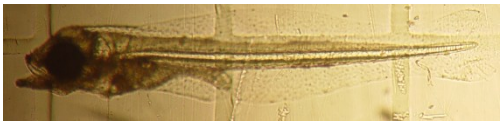
d.

Fig 4. (a) Newly-hatched larva with prominent yolk sac and anterior oil globule; (b) larva 24 hours post hatch; (c) larva 48 hph, with pigmented eyes; (d) larva 72 hph, with open mouth and anal pore visible, and some remaining oil globule.

3. Results

Eye diameter, body height and ratio of body height to standard length didn't show coherent patterns during the trials. Of these, only eye diameter during trial 2 increased over time in the different treatments. However this was observed in a single instance. Therefore, these particular variables were not taken into consideration to follow larval development.

3.1 Morphometry

Initial characteristics of the morphological parameters measured were found to be statistically significant different from one trial to another $p<0$ (Fig. 5). These initial parameters were not used to derive conclusions about the growth rate of larvae nor their efficiency in using energy at different temperatures since the difference is simply a result of using different broodstock for different trials. The variables that were used to follow the development of the larvae were yolk volume, oil globule volume, and standard length of the larvae.

3.1.1 Yolk Volume.

Rapid consumption of yolk volume occurred during the first 24 hours after hatching. During this time larvae used an average of 89 to 94% of the yolk, and

the percentage consumed increased with temperature. Larvae incubated at 23, 25, 28 and 31 °C used 89, 92.8, 93.8 and 94.1% of their yolk during the first 24 hph, respectively (Fig. 5). Even though the percentage of yolk consumed varied from one treatment to another, all treatments consistently showed most of the yolk being absorbed in the first 24 hph. At the end of trials 1 and 2 larvae at 25°C had some yolk left, while those at 28 and 31 °C had depleted it completely. In trial 3, treatments at 23 and 25° had yolk left over at 72 hph, while those at higher temperatures had consumed all yolk, as in the first two trials.

The greatest increase in larval length also occurred during the first 24 hph, with larvae treated at 25, 28 and 31°C increasing 1.04 $\pm$ 0.2 mm/day, 0.9 $\pm$ 0.2 mm/day and 0.7 $\pm$ 0.4 mm/day, respectively, slowing down to ~0.1mm/day after the first 24 hph.

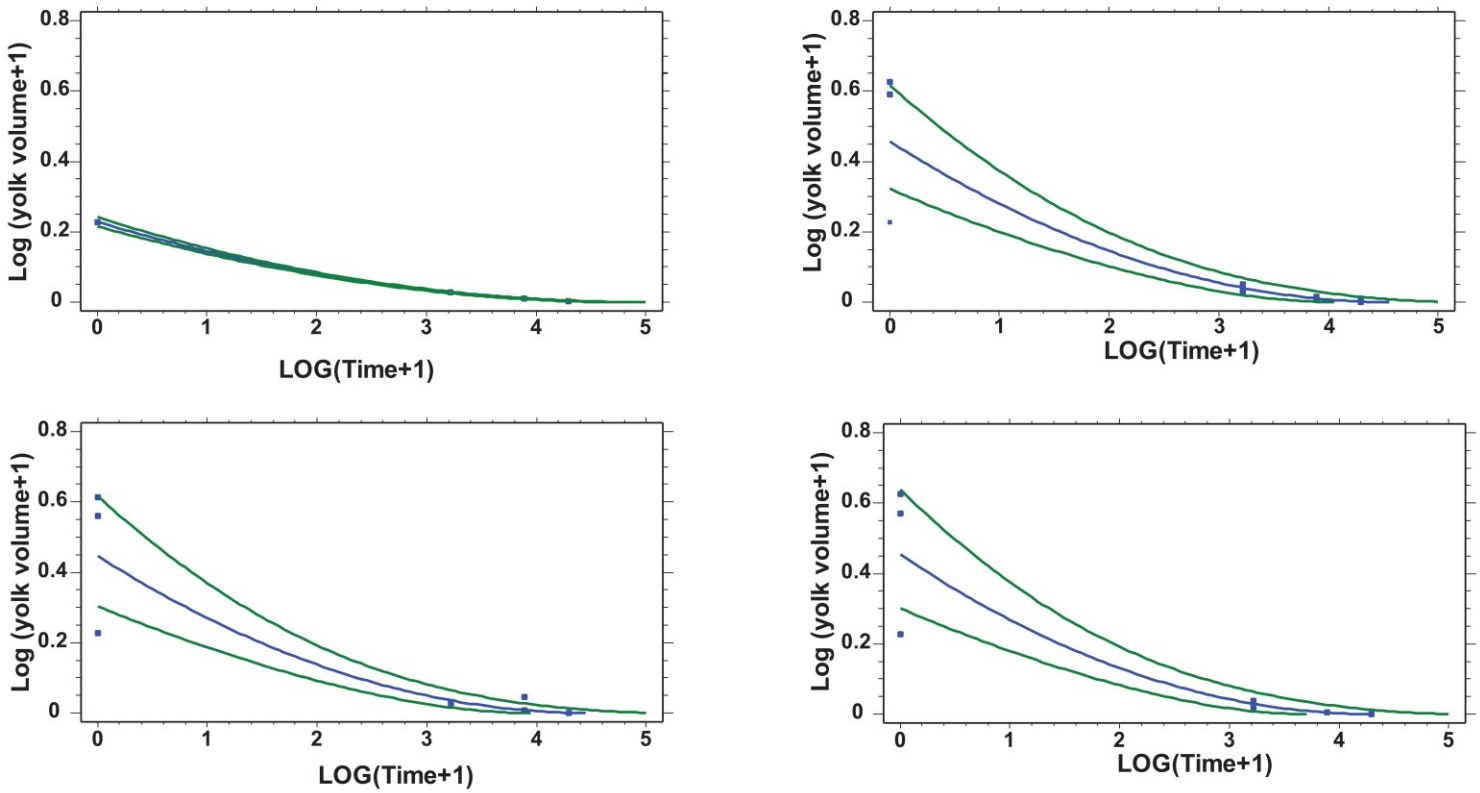

Fig 5. Yolk depletion with time at (a) 23°C, (b) 25 °C, (c) 28 °C and (d) 31 °C. The highest yolk consumption occurred during the first 24 hours after hatching.

3.1.2 Standard length (SL).

The first trial was carried out over a period of 48 hours post hatch (hph), after which there were insufficient larvae to collect any meaningful observations. Similarly, there were no data available for the treatment at 31 °C at 48 hours post hatch due to the deterioration of the samples. In the first trial, larvae in the 25°C treatment showed the highest standard length mean, with 2.5 ± 0.09 mm compared to 2.2± 0.26 mm at 28 °C (Fig. 6). The second trial was conducted to 72 hph. Larvae at 25 °C again showed the highest standard lengths, with a mean of 2.51 ± 0.15 mm compared to 2.42 ± 0.17 and 2.44 ± 0.12 mm at 28 and 31°C, respectively (Fig. 6). Even though in trial 2 larvae all treatments reached 72 hph, it was difficult to obtain the 25 larvae needed from the treatment at 31 °C.

Once again, at the end of the third trial, larvae in the 25°C treatment showed the largest standard length mean, with 2.4 ± 0.18 mm, while larvae at 28°C showed the smallest with 2.16 ± 0.18 mm. Also, larvae in the 31 °C treatment didn't complete the 72 hph of the trial; they died after 48 hph (Fig. 6).

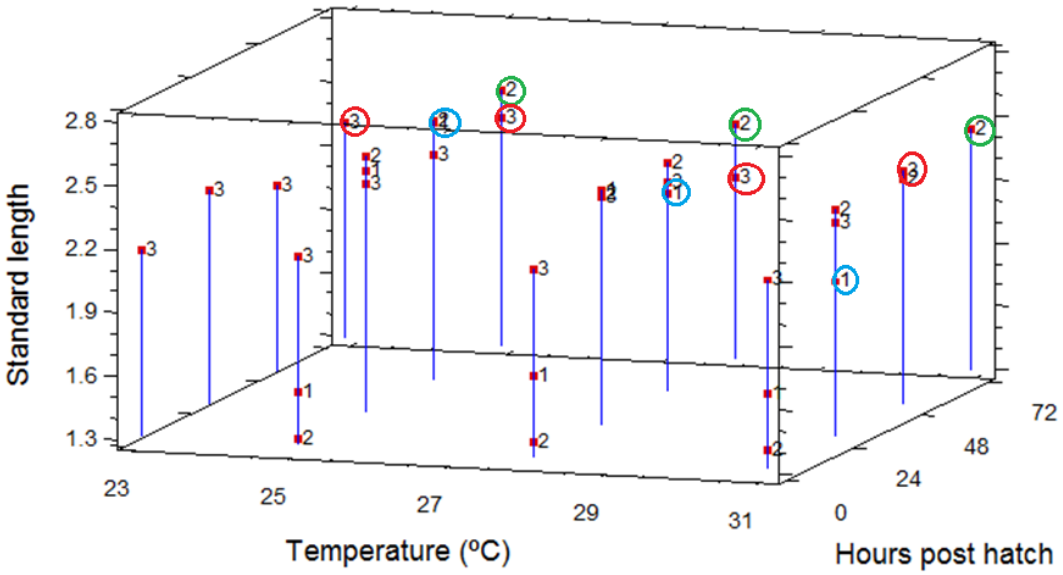

Fig 6. Standard length means for each temperature treatment at the end of each trial. Final results at the end of the first trial are indicated with blue circles, for the second trial with green circles and for the third trial with red circles.

3.1.3 Growth rate and yolk utilization.

The treatments at 25 °C showed the highest growth rate while using less yolk than treatments at 28 and 31 °C (Fig. 7). Larvae at 23 °C used less yolk than those at 25 °C, but they grew at the slowest rate relative to any other treatment and trial. Larvae at 28 °C grew slower than those at 25 and 31 °C. At 28 °C, larvae consumed more yolk than those at 25 °C, but less than those at 31 °C. Larvae at 31 °C grew slightly faster than those at 28 °C, but they also consumed their yolk reserves faster.

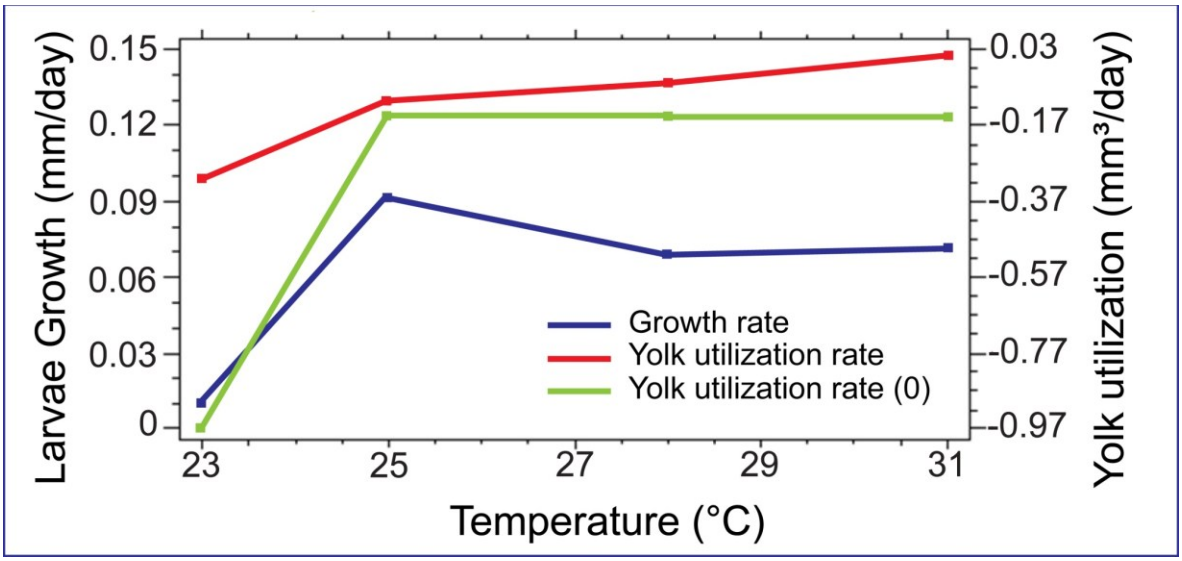

Fig 7. Growth rate and yolk consumption of the larvae at each temperature. Red line represents yolk utilization, with an assumed zero rate at hatch. The green line shows yolk utilization measured after hatch without taking into consideration such an initial condition.

3.2 Survival

At hatching time, survival was assumed to be 100%. Survival in subsequent times was assessed only during the third trial. The 31 °C treatment presented the worst survival percentages, with a maximum survival of 37.2% at 24 hph and 100% mortality at 72 hph. Treatments at 23 °C and 28 °C showed high percentages of survival at 24 hph, i.e. 90.6% and 96.1% survival, respectively. The 25 °C treatment, with 1.7% survival, featured higher survival at the end of the trial than the other treatments (Table 1). We did not expect high survival in any treatment because larvae were not fed during the experiments.

Table 1

Percentage of larvae alive and dead in each of the treatments during the third trial, at 24, 48 and 72 hours post hatched.

	24 hph		48 hph		72 hph	
T°	Alive	Dead	Alive	Dead	Alive	Dead
23	90.6	9.4	13.4	86.6	1	99
25	89.3	10.7	13.4	86.6	1.7	98.3
28	96.1	3.9	2.1	97.9	0.3	99.7
31	37.2	62.8	6.8	93.2	0	100

4. Discussion

The optimal temperature for development and growth during different life stages of fish varies with environmental conditions and time (Gadomski and Caddell, 1991; Imsland et al., 1996). Yanes-Roca (2006) and Yanes-Roca and Main (In Press), found that 28 °C showed the highest hatching rate for common snook. This suggested that this could be the best temperature to incubate snook eggs as well. This was also the temperature used by Stephen and Shafland (1982), who found a hatching standard length range between 1.4 to 1.5 mm. Because of these earlier studies, 28 °C was the incubation temperature used in the present study. The standard length at hatching found in our study ranged from 1.34 to 1.70 mm; this is slightly larger than the range reported by Stephen and Shafland (1982). It is possible that the difference between the sizes of the larvae between the earlier studies and our experiment was related to the use of different broodstock to obtain the eggs. Broodstock variability is a factor known to be critical for egg quality (Yanes-Roca *et al*., 2009; Kjorsvik *et al*. 1990).

Rapid yolk-sac resorption, along with the greatest increase in larval length, occurred immediately following hatching. This is similar to the observations of Williams et al., 2004 with *Lutjanus campechanus.* Our snook larvae grew about 1mm/day in the first 24 hph, slowing to about 0.1 mm/day afterwards. This trend

was observed regardless of the treatment temperature, suggesting that rapid growth is a characteristic of larval development. Such rapid growth likely enables larvae to find food faster and increases the probabilities of survival in nature.

The energy available to each larva in the yolk-sac and oil globule is known to be used for growth of new tissue, for larval development, and also for basic body functions (Gray, 1926). Temperature is a factor that regulates the metabolic requirements for food, governs the rate of food processing (Brett, 1979), food intake, and growth (Peter, 1979). Therefore, when temperature increases, the rates of metabolic requirements also increase (Blaxter and Hempel, 1966).

The three trials show that larvae at 25 °C had greater standard lengths compared with the other temperature treatments. Larvae grown at 25 °C were the only ones left with energy reserves in the first and second trials. In the third trial, larvae at 23 °C also had some reserves left. These results suggest that at these lower temperatures of 23-25 °C, *C. undecimali*s larvae need less energy to satisfy their metabolic demands, leaving energy for conversion into new tissue. These individuals gained in length using the same amount of yolk compared to individuals grown at higher temperatures (Fig. 1). A rise in temperature of 10 °C typically increases the metabolic rate about 2.3 times (Brett and Groves 1979). For the range of temperatures used to rear snook larvae in this research, the temperature effect may have been slightly less (order of factor of 2) because the temperatures were toward the high end of tolerance for this species (J. Torres, University of South Florida, personal communication, Sept 2011). We estimated that snook larvae at 31 °C devoted ~1.2 times more energy to maintain their

metabolic requirements compared to those at 25 °C, and ~0.6 times more energy than those grown at 28 °C

Even though the larvae sampled at 23 °C had standard lengths similar to the larvae at 25 °C, this treatment showed many stunted larvae with remaining energy at the end of the trial; this suggested a very low metabolic rate and low energy consumption. Effectively these larvae did not grow efficiently. Larvae at 28 °C also showed the slowest growth, and while their entire energy reserves were depleted, they didn't present stunted larvae. This suggests that their energy was more efficiently used to satisfy the higher metabolic demands than larvae at 23 °C. Finally, the increased metabolic requirements led larvae grown at 31 °C to exhaust their energy reserves much faster and die of starvation at an earlier time than larvae in the other treatments at lower temperatures. Even though larvae at 31 °C reached the 72 hph during the second trial, it was difficult to obtain the 25 larvae sample because very few larvae were left.

Marine fish larvae tend to increase protein metabolism when yolk-sac resources are depleted (Wright and Fyhn, 2001). Williams *et at.,* 2004 suggested that this may help larvae to digest its first prey, but they also suggested that if a significant portion of proteins used for metabolic needs arises from tissue resoption, this will work in a negative way and reduce the larval length prior to first feeding. Fully developed larvae, in terms of standard length, pigmented eyes, open mouth and anal pore, and with additional energy reserves, might be in an optimal condition to either use the surplus energy to pursue prey, or use the energy to grow further and keep a good size after resorption to gain more advantage to pursue their first

prey. Fish at 23°C won't have the chance to pursue prey because their slow growth would keep them at a sub-optimal level of development. Fish, as all organisms, have an optimal temperature for growth and survival (Brett, 1979; Gadomski and Caddell, 1991). Our conclusion from the experiments conducted at Mote was that larvae grown at 25 °C during the yolk resorption period exhibited many optimal features, such as higher standard lengths, energy reserves left, and higher survival. This is consistent with previous observations that many fish tend to live longer and grow larger in the cooler part of their temperature range (Ricker, 1979).

Although eye diameter has been previously used (Fuiman 1983, Gisbert et al., 2002, Peña and Dumas, 2009) as a useful morphological index of fish larvae development, this was not the case for *C. undecimalis.* Eye diameter didn't follow a consistent pattern during the yolk resorption period in our experiments. Only in trial 2 larvae show a tendency for the eye diameter to increase over time in the three temperature treatments. However, because this result was observed in only one of three trials, we conclude that eye diameter is not a reliable measurement. The ratio of body height to standard length likewise showed no significant differences in two of the three treatments.

The experiments suggested a slower rate of energy utilization by exposing snook larvae to 25 °C during the yolk resorption period, which led to increased survival of the larvae. On the contrary, it was clear that 31°C is too high of a temperature for these larvae because of the high mortalities during the experimental trials.

5. Conclusions

Different factors, including the physiological condition of the broodstock from which the eggs come, may cause statistically significant differences between the initial characteristics of recently hatched larvae. In our experiments, initial characteristics including standard length, yolk volume, oil volume, body height varied from one trial to another. Based on these observations, is not recommended to compare larvae proceeding from two different spawns, but rather to consider trends in the evolution of the parameters measured over time for different treatments.

Taking into account morphological attributes such as standard length, body height, volume of energy reserves, as well as survival, we conclude that 25 °C was the optimal temperature to raise snook larvae during the yolk-resorption period. The larvae grown at 25 °C had more energy reserves than those at other temperatures, with a longer standard length. This represents an advantage at the age of first feeding. While larvae grown at 23°C also showed extended energy reserves, their low metabolic rate led to under-developed (stunted) larvae. These larvae were not likely to be viable and were not ideal for aquaculture. At 28 °C, larvae presented satisfactory development, but they reached shorter standard lengths as they exhausted their energy supplies before the larvae grown at 25

°C. The 28 °C larvae may not survive unless they were able to capture first prey earlier than larvae under other treatments, and their reduced length may put them at a disadvantage. Based on the particularly low survival rate of larvae, 31 °C was conclusively not a good temperature to raise snook larvae for aquaculture or restocking purposes.

The eye diameter and the ratio of body height to standard length didn't show coherent patterns during the experiments. These parameters were not useful to follow larvae development during the yolk resoption periods.

Knowing that embryos and larvae must subsist on yolk and oil reserves until their digestive and feeding systems develop sufficiently for prey capture and digestion (Green and McCormick, 2001), Rhody (2006) showed the importance of tracking the gut development in the specific case of flame angel fish, which undergo rapid gut and sensory development exhibiting the capacity to feed after 72 hph. Therefore, further studies are required to determine when snook larvae may be ready for first feeding. The research of metrics associated with the development of the digestive system of larvae at 25 °C and 28 °C, for example, would be very beneficial to future aquaculture studies.

Given the results outlined above for 25 and 28 °C, it would be useful to extend the experimental phase of this study until after first feeding, but providing the same type of diet and concentration of food for the larvae. The results on survival and further development could determine whether the advantages in terms of standard length and extended energy reserves shown by larvae raised at 25 °C

are significant to help larvae catch their first prey, or whether they have no significance.

We found that conditions that stimulate bacterial growth in the experimental setting can severely impair an experiment. We obtained best results when we isolated the larvae from the materials left over from the eggs upon hatching, rather than allowing fertilized eggs to hatch in the treatment microcosms. The spent egg matter functions as fertile ground for bacterial growth. Bacteria rapidly consume day-old larvae are will render experiments useless.

Appendices

Appendix A. Standard Length Mean and standard deviation at each time frame of the three trials.

Experiment	T°	Hph	Median	Mean	Standard deviation
1	25	0	1.57	1.55	0.08
	28		1.68	1.69	0.10
	31		1.65	1.66	0.11
2	25	0	1.33	1.34	0.11
	28		1.37	1.37	0.13
	31		1.42	1.39	0.09
3	23	0	2.26	2.19	0.14
	25		2.26	2.19	0.14
	28		2.26	2.19	0.14
	31		2.26	2.19	0.14
1	25	24	2.45	2.45	0.14
	28		2.44	2.41	0.21
	31		1.92	2.03	0.35
2	25	24	2.55	2.51	0.17
	28		2.45	2.39	0.14
	31		2.36	2.38	0.13
3	23	24	2.32	2.32	0.18
	25		2.39	2.38	0.11
	28		2.36	2.36	0.14
	31		2.35	2.33	0.18
1	25	48	2.52	2.52	0.09
	28		2.28	2.24	0.26
2	25	48	2.55	2.52	0.13
	28		2.40	2.38	0.20
	31		2.42	2.37	0.22
3	23	48	2.23	2.18	0.23
	25		2.41	2.37	0.15
	28		2.33	2.30	0.21
	31		2.41	2.41	0.13
2	25	72	2.55	2.51	0.15
	28		2.49	2.42	0.17
	31		2.48	2.44	0.12
3	23	72	2.32	2.33	0.19
	25		2.44	2.39	0.18
	28		2.11	2.16	0.18

Appendix B. Yolk-sac volume median, mean and standard deviation at each time frame of the three trials.

Experiment	T°	Hph	Median	Mean	Standard deviation
1	25	0	0.80	8.03E-01	1.51E-01
	28		0.75	7.52E-01	1.22E-01
	31		0.76	7.70E-01	1.26E-01
2	25	0	0.86	8.69E-01	1.66E-01
	28		0.84	8.46E-01	9.86E-02
	31		0.87	8.69E-01	7.53E-02
3	23	0	0.24	2.56E-01	1.02E-01
	25		0.24	2.56E-01	1.02E-01
	28		0.24	2.56E-01	1.02E-01
	31		0.24	2.56E-01	1.02E-01
1	25	24	0.03	2.80E-02	6.70E-03
	28		0.03	2.80E-02	7.15E-03
	31		0.04	3.86E-02	9.76E-03
2	25	24	0.05	5.12E-02	1.14E-02
	28		0.03	3.07E-02	6.49E-03
	31		0.02	2.18E-02	5.78E-03
3	23	24	0.03	2.74E-02	7.50E-03
	25		0.03	3.36E-02	1.05E-02
	28		0.03	2.74E-02	7.50E-03
	31		0.02	2.58E-02	7.59E-03
1	25	48	0.01	1.75E-01	2.98E-02
	28		0.01	1.70E-01	3.20E-02
2	25	48	0.01	1.49E-02	3.25E-03
	28		0.0092	9.08E-03	3.26E-03
	31		0.0063	6.47E-03	1.76E-03
3	23	48	0.01	1.08E-02	4.39E-03
	25		0.0098	9.63E-03	2.94E-03
	28		0.0072	7.21E-03	3.83E-03
	31		0.005	5.55E-03	4.74E-03
2	25	72	0.051	5.44E-03	1.76E-03
	28		0	0.00E+00	0.00E+00
	31		0	0.00E+00	0.00E+00
3	23	72	0.0033	3.52E-03	3.05E-03
	25		0.00	5.38E-04	1.07E-03
	28		0.00	0.00E+00	0.00E+00

Appendix C. Oil volume median, mean and standard deviation at each time frame of the three trials.

Experiment	T°	Hph	Median	Mean	Standard deviation
1	25	0	4.19E-03	4.17E-03	6.21E-04
	28		3.59E-03	3.72E-03	3.72E-04
	31		3.59E-03	3.62E-03	5.47E-04
2	25	0	3.05E-03	3.34E-03	9.43E-04
	28		3.05E-03	3.36E-03	6.14E-04
	31		3.05E-03	3.04E-03	5.37E-04
3	23	0	2.57E-03	2.50E-03	3.94E-04
	25		2.57E-03	2.50E-03	3.94E-04
	28		2.57E-03	2.50E-03	3.94E-04
	31		2.57E-03	2.50E-03	3.94E-04
1	25	24	1.02E-03	1.07E-03	2.77E-04
	28		9.05E-04	9.63E-04	3.07E-04
	31		1.15E-03	1.09E-03	2.62E-04
2	25	24	1.44E-03	1.74E-01	1.00E-02
	28		9.05E-04	1.68E-01	1.20E-02
	31		6.97E-04	1.76E-01	1.26E-02
3	23	24	1.15E-03	1.03E-03	3.50E-04
	25		9.05E-04	9.89E-04	5.33E-04
	28		9.05E-04	9.05E-04	2.60E-04
	31		6.97E-04	7.93E-04	2.75E-04
1	25	48	5.24E-04	4.61E-04	1.13E-04
	28		3.82E-04	4.18E-04	1.12E-04
2	25	48	5.24E-04	5.16E-04	1.04E-04
	28		2.68E-04	2.86E-04	8.10E-05
	31		2.68E-04	2.37E-04	9.74E-05
3	23	48	3.82E-04	3.81E-04	1.94E-04
	25		2.68E-04	3.43E-04	1.39E-04
	28		2.68E-04	2.52E-04	1.32E-04
	31		2.24E-04	2.17E-04	1.45E-04
2	25	72	6.54E-05	1.14E-04	1.40E-04
	28		0.00	0.00E+00	0.00E+00
	31		0.00	0.00E+00	0.00E+00
3	23	72	1.13E-04	1.35E-04	1.02E-04
	25		3.35E-05	3.63E-05	3.73E-05
	28		0.00	3.46E-06	9.11E-06

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