Glycolytic capacities depend on developmental stage in the clownfish *Amphiprion ocellaris*

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ABSTRACT: Detailed knowledge about basic metabolism in the early life stages of fishes helps increase our understanding of energetically important life phases. However, little is known about metabolic capacities during ontogenetic development of anemone fishes before and after settlement. This study established activities of 3 key metabolic enzymes — citrate synthase (CS), pyruvate kinase (PK) and lactate dehydrogenase (LDH) — to establish oxidative and glycolytic capacities in eggs, larvae and juveniles of false clownfish *Amphiprion ocellaris* before and after hatch, and during ontogenetic metamorphosis from the pelagic to sedentary mode of life (settlement stage). Oxidative capacity significantly increased after hatching and remained constant throughout development. Compared to just-hatched larvae (1–2 d old), the glycolytic and fermentative capacities on the contrary were about 2.5- and 2.1-fold higher in pre-settlement (7–8 d old) larvae, respectively, and decreased significantly in post-settlement (15 d old) juveniles. Thus, relative glycolytic enzyme activity ratios verified that settlement-stage anemone fish larvae mostly rely on glycolytic capacities for the burst-swimming capabilities needed during settlement before entering into the benthic mode of life. Intraspecific variances in enzyme activities due to ontogenetic transition should be considered when using key metabolic enzymes as biomarkers for analysing larval physiological status.

KEY WORDS: Anemone fish · Ontogenetic transitions · Settlement · Citrate synthase · CS · Pyruvate kinase · PK · Lactate dehydrogenase · LDH

1. INTRODUCTION

Coral reef-associated fishes such as anemone fishes are characterized by a bipartite life history (Leis 1991). Hatched larvae have to pass a pelagic early life phase before late-larval stages develop to pre-settlement juveniles and are competent for metamorphosis and settlement towards a reef-resident life (Allen 1972, Ross 1978). Many studies have investigated larval fish development, focusing on nutrition (e.g. Holt 2003, Sales & Janssens 2003, Olivotto et al. 2003, 2005, 2006, 2011, Liew et al. 2006, Avella et al. 2007, Madhu et al. 2012, Vargas-Abúndez et al. 2019), challenging environmental conditions (e.g. Munday et al. 2009, Hess et al. 2015, Jacob et al. 2017) and settlement mechanisms (e.g. Ochi 1985, Fisher et al. 2000, 2005, Bellwood & Fisher 2001, Wright et al. 2005, Hogan et al. 2007, Dixson et al. 2011). Since adult anemone fishes are demersal and sedentary to their host anemones, larval recruitment to the reefs is essential for their population dynamics (Ochi 1985). The successful transition between pre- and post-settlement, then, is of great importance to ensure the persistence of their populations (Ochi 1985). Offspring of anemone fishes *Amphiprion* sp. experience very short larval stages (Green & McCormick 2001, Madhu et al. 2006, 2012). Newly hatched larvae exhaust their

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yolk material within a few hours after hatching, and their highly differentiated intestinal tract and relatively advanced swimming ability enables them to start exogenous feeding on live prey directly thereafter (Green & McCormick 2001, Madhu et al. 2012). After spending about 1 wk in the pelagic stage (8–10 d post-hatching, dph), anemone fish larvae enter the pre-settlement stage, initiating the metamorphosis to juveniles (Green & McCormick 2001, Madhu et al. 2012). Late-larval anemone fishes have well-developed sensory systems (Job & Bellwood 2000) and become attracted to olfactory cues of suitable habitats for settlement (Dixson et al. 2011). Indeed, settlement-stage anemone fish larvae have outstanding swimming capabilities. Compared to other reef fish larvae, settling anemone fish larvae with a total length of only 7 mm show the highest swimming speeds of up to 49 body lengths s⁻¹, which has been proposed to approach the maximum limit for sustained aquatic vertebrate locomotion (Bellwood & Fisher 2001). Swimming ability appears to increase steadily across the larval phase and decrease rapidly after settlement, and both size and developmental stage are limiting factors of maximum critical swimming speeds in larvae (Bellwood & Fisher 2001).

Several studies have investigated the underlying metabolic capacities during ontogenetic development of fishes (e.g. Segner & Verreth 1995, Desrosiers et al. 2008, Tong et al. 2017). Changes in the metabolic capacities of fish coincide with ontogenetic development of muscle, as different types of muscle fibres have different metabolic capacities (Jebsen 1962, Bass et al. 1969, Meyer-Rochow et al. 1994, Stoiber et al. 1999). Teleost fish generally have higher proportions of white muscle compared to red muscle fibres, leading to overall higher glycolytic than oxidative capacities (Stoiber et al. 1999). Interspecific variations of energetic capacities, however, ultimately depend on the life modes of the fishes (Sullivan & Somero 1980, Childress & Somero 1990, Somero & Childress 1990, Wieser 1995). The energetics of embryos and newly hatched fish larvae further differs from that of adults due to their small body size (Wieser 1995). Early life stages of fishes almost completely rely on aerobic energy metabolism after hatching (El-Fiky et al. 1987), with glycolytic power increasing throughout further development (Wieser 1995). Compared to adult fish, larval stages are capable of conducting much higher tail beat frequencies (Blaxter 1986). The observed changes in swimming ability towards maximum speeds in settlement-stage anemone fish larvae (Fisher et al. 2000, Bellwood & Fisher 2001) therefore indicate changes in locomotion physiology during development, as observed in herring larvae (Batty 1984) and the larvae of cyprinid fish (El-Fiky et al. 1987).

However, metabolic capacities of the early developmental stages of fish have often been examined for routine activity levels (Wieser 1995), and information about the ontogenetic patterns of the central metabolic pathways of anemone fishes during important life phases, such as before and after settlement, is scarce. This study therefore aimed to establish the capacities of key metabolic enzymes in early life stages of *A. ocellaris*. Our main objective was to investigate potential differences in oxidative and glycolytic metabolic enzyme capacities, focusing on important phases of ontogenetic development: before and after hatch, and before and after metamorphosis (settlement stage). Key enzymes from selected organs and tissues are important indicators of activity levels and mode of life (Almeida-Val et al. 2006). Analyses of specific enzyme capacities for aerobic (citric acid cycle) and anaerobic (glycolysis and lactate fermentation) metabolic pathways thus might reveal differences in metabolic capacities during the transition between pre-settlement and post-settlement phases of anemone fish larvae. Citrate synthase (CS) initiates the mitochondrial citric acid cycle (Childress & Somero 1979) and has been shown to correlate well with mass-specific oxygen consumption (Seibel et al. 2000). CS therefore represents a good proxy for overall oxidative capacity. Pyruvate kinase (PK) is one of the key enzymes of the glycolytic pathway which catalyses the last step in the process of pyruvate formation, and is commonly used as a proxy for overall glycolytic capacity. Lactate dehydrogenase (LDH), which converts pyruvate into lactate under anaerobic conditions for fermentative ATP generation, represents a good proxy for anaerobic metabolic capacity (Childress & Somero 1979). The relative enzyme activity ratios for glycolytic, fermentative and oxidative capacities can further reveal the relative reliance on the different metabolic pathways. PK/CS and LDH/CS activity ratios can be used for determining relative overall glycolytic and fermentative vs. oxidative capacities, respectively (Bass et al. 1969, Hochachka et al. 1983, Miller et al. 2014). The PK/LDH activity ratio displays a relative indication of the glycolytic vs. fermentative capacity, particularly when samples of homologous tissues show similar LDH/CS activity ratios (Hochachka et al. 1983).
2. MATERIALS AND METHODS

2.1. Maintenance

The *Amphiprion ocellaris* broodstock was originally purchased (von Wussow, Hamburg) and maintained for several years as mated pairs (1 male, 1 female) in separate 100 l tanks within a recirculating artificial seawater system at the aquarium facility MAREE (Marine Experimental Ecology) of the Leibniz Centre for Tropical Marine Research (ZMT), Bremen, Germany. Fish were fed frozen *Artemia* sp., *Mysis* sp., and baby krill ad libitum twice a day, and terracotta plates were provided for shelter and as a place for egg deposition. A portion (10%) of the tank water was exchanged weekly and parameters were kept constant (photoperiod: 16 h light:8 h dark; temperature: 26 ± 1°C; salinity: 33 ± 1 ppt; nitrite < 0.1 ppm; ammonia: 0 ppm). On the evening before hatching (8 d post-laying), egg clutches were transferred from broodstock tanks to separate 10 l tanks, under the same conditions as described above. After hatching, larvae and juveniles were continuously fed on live feed. Rotifers *Brachionus* sp. were provided for the first 6 d. Enriched brine shrimp *Artemia* sp. nauplii were added from Day 3 onwards, and continued as the sole feed from Day 6. The tank water was additionally enriched with *Nannochloropsis* sp. to provide food for the live feed and avoid light reflections. To ensure good water quality, 50% of water was exchanged each day to remove unconsumed food and faeces.

2.2. Tissue preparation

In total, 4 developmental stages were sampled: eggs right before hatching (8 d post-laying), 2 larval stages (1–2 and 7–8 dph) and 1 juvenile stage (15 dph). Biometric data such as standard length (to the nearest 0.1 mm) and wet weight (Sartorius ME36S, precision d = 0.001 mg) of sampled individuals are given in Table 1. After reaching the determined age, eggs, larvae and juveniles were sampled and stored at −80°C until preparation. Dissection followed a defined constant procedure. Due to minimum tissue requirements, samples had to be pooled (Moyano et al., 1996; Pimentel et al., 2015); 10 eggs, four 1–2 dph larvae, and two 7–8 dph larvae were pooled per sample, respectively (see Table 1). All larvae and juveniles were gutted to avoid confounding factors introduced from the enzymes of the live prey. Additionally, the 7–8 dph larvae and 15 dph juveniles were decapitated to further avoid any potential influence of cerebral enzymes. Samples were homogenized mechanically in ice-cold homogenization buffer (75 mM Tris-HCl, 1 mM EDTA, pH 7.5) using plastic pestles and further lysed via ultrasonication (Bandelin, Sonopuls homogeniser; amplitude: 20%, pulse on: 0.1 s, pulse off: 1.0 s). Preliminary test measurements allowed the assessment of extract dilutions to meet optimal reaction conditions (linear activities over time, independence of substrate concentration). After centrifugation (10 min, 4°C, 2655 × g; Eppendorf, 5804R) supernatants were frozen and stored at −80°C until measurement. Previous tests showed no significant variances in enzyme activities due to freezing and thawing, and the elapsed time between freezing the supernatant and measuring the enzyme activities was not longer than 6 wk.

2.3. Enzyme activity measurements

All enzymatic measurements determined the maximum reaction velocity ($V_{max}$) under substrate-saturated conditions. The homogenization buffer (75 mM Tris-HCl, 1 mM EDTA, pH 7.5) was used as a blank. Enzyme activities of samples were monitored in a thermally controlled spectrophotometer (Lambda 35 UV/VIS Spectrophotometer, Perkin Elmer) at 27°C in technical triplicates, using UV WinLab v.6.0 software (Perkin Elmer). CS activity (EC 4.1.3.7) was determined following the method of Sidell et al. (1987), adjusted by Michalek (2012). Tris-HCl buffer (75 mM, pH 8.2), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB; 0.25 mM), acetyl-coenzyme A (0.2 mM) and homogenate were incubated for 5 min at 27°C. After adding oxaloacetate (0.2 mM), CS activity was monitored by following the increase in absorbance at 412 nm for 4 min. CS activity was calculated using the DTNB moles/mL and error margins.

| Table 1. Biometric data (mean ± SD) from offspring of *Amphiprion ocellaris*. Eggs, 1–2 d post-hatch (dph) and 7–8 dph larvae were pooled to gain sufficient wet weight for enzymatic analyses. SL: standard length; WW: wet weight; n: total number of samples |
|-----------------|---------|---------|---------|---------|
|                 | Eggs    | 1–2 dph| 7–8 dph | 15 dph  |
| Pooled ind. sample$^{-1}$ | 10      | 4       | 2       | 1       |
| Individuals     | 120     | 24      | 22      | 7       |
| No. of samples  | n = 12  | n = 6   | n = 11  | n = 7   |
| SL (mm)         | −2.0    | 3.6 ± 0.4 | 4.8 ± 0.4 | 6.3 ± 0.5 |
| WW (mg)         | 1.18 ± 0.06 | 0.92 ± 0.14 | 3.15 ± 0.79 | 8.30 ± 1.51 |
lar coefficient at 412 nm of $13.6 \times 10^3$ M cm$^{-1}$ and expressed in U g$^{-1}$ wet weight. PK activity (EC 2.7.1.40) was measured following the method of Hickey & Clements (2003), adjusted by Michalek (2012). Reaction buffer (pH 7.6) (containing 50 mM Tris, 50 mM KCl, 5 mM MgSO$_4 \times 7$ H$_2$O), NADH (0.25 mM), PEP (0.5 mM), L-LDH (5.5 mM) and homogenate were incubated for 5 min at 27°C. After adding ADP-K-salt (1 mM), PK activity was monitored by following the decrease in absorbance at 340 nm for 4 min. The PK activity was calculated using the NADH molar extinction coefficient at 340 nm of $6.31 \times 10^3$ M cm$^{-1}$ and expressed in U g$^{-1}$ wet weight. LDH activity (EC 1.1.1.27) was determined following the method of Lushchak et al. (1998, 2001), adjusted by Michalek (2012). Tris-HCl buffer (50 mM, pH 6.8), NADH (0.15 mM) and homogenate were incubated for 5 min at 27°C. After adding sodium pyruvate (1 mM), LDH activity was monitored by following the decrease in absorbance at 340 nm for 4 min. The LDH activity was calculated using the NADH molar extinction coefficient at 340 nm of $6.31 \times 10^3$ M cm$^{-1}$ and expressed in U g$^{-1}$ wet weight.

### 2.4. Statistical analysis

Spectrophotometric measurements of samples were processed in technical triplicates to minimize analytical errors. Final results are given as means ± SD. Statistical analyses were performed using SigmaPlot v.11 (Systat Software) with significance accepted at $p \leq 0.05$. Raw data were ln transformed in order to improve normal distribution and variance homogeneity. When assumptions of normality and homoscedasticity were met, parametric 1-way ANOVA was used to test for significant differences in enzyme activities between developmental stages. When assumptions were not met, the non-parametric Kruskal-Wallis test was used. Post hoc tests (ANOVA: Holm-Sidak method; Kruskal-Wallis test: Dunn’s test) were performed to identify the source of the differences.

### 3. RESULTS

In Fig. 1, the enzymatic capacities of CS, PK and LDH are presented in relation to the developmental stage of *Amphiprion ocellaris*. CS capacity in eggs was significantly lower than in hatched larvae (ANOVA, Holm-Sidak, $p < 0.001$); however, no significant differences were found between different larval stages and juveniles. Glycolytic enzyme capacities

![Fig. 1. Mean (±SD) enzyme activities of *Amphiprion ocellaris* eggs, larvae and juveniles: (a) citrate synthase (CS) activity, (b) pyruvate kinase (PK) activity and (c) lactate dehydrogenase (LDH) activity. Bars not sharing a letter show statistically significant differences in enzyme activities between developmental stages ($p \leq 0.05$); n: number of samples; dph: days post-hatching.](image)


increased significantly between 1−2 and 7−8 dph (Kruskal-Wallis, Dunn’s, PK and LDH: p < 0.05), whereas no differences were observed comparing eggs to 1−2 dph, and 7−8 to 15 dph, respectively (Kruskal-Wallis, Dunn’s, PK and LDH: p > 0.05). This pattern was also reflected in the significantly higher PK/CS and LDH/CS activity ratios in 7−8 dph larvae compared to the other developmental stages (ANOVA, Holm-Sidak, p < 0.001), albeit with an increase in relative glycolytic capacity with age (Fig. 2). However, after reaching the stage of metamorphosis, PK/CS and LDH/CS activity ratios were significantly lower in 15 dph juveniles (ANOVA, Holm-Sidak, p < 0.001; Fig. 2). Looking at the relative glycolytic vs. anaerobic capacity given by PK/LDH activity ratios, the glycolytic capacity was significantly lower after hatch (ANOVA, Holm-Sidak, p < 0.001), with aerobic and anaerobic glycolytic capacities being balanced.

4. DISCUSSION

4.1. Metabolic capacities in early developmental stages

The main objective of this study was to investigate potential differences in oxidative and glycolytic metabolic enzyme capacities in early life stages of *Amphiprion ocellaris*. Oxidative capacity in 1−2 dph larvae increased significantly right after hatching—about 129% compared to eggs—and remained constant throughout ontogenetic development up to 15 dph juveniles (Fig. 1a). This leads to the assumption that basal aerobic metabolism is not directly affected during settlement processes in *A. ocellaris*. In contrast to CS, glycolytic enzyme capacities showed no differences between eggs and 1−2 dph larvae, but both glycolytic and fermentative capacities significantly increased towards the pre-settlement larval stage of 7−8 dph (Fig. 1b,c). Similar observations were made in African catfish *Clarias gariepinus* larvae (Segner & Verreth 1995); investigations of ontogenetic patterns of metabolic enzymes revealed an increase in glycolytic capacities between early and late larval phases, whereas the oxidative capacity remained rather constant (Segner & Verreth 1995). These results conform to literature about larval metabolism. After the yolk sac period, the capacity of anaerobic glycolysis increases successively (Wieser 1995). Similar patterns were reported for larvae and juveniles of red drum *Sciaenops ocellatus* and lane snapper *Lutjanus synagris* (Clarke et al. 1992). In particular, anemone fish are generally known for their sedentary mode of life,

![Fig. 2. Mean (±SD) metabolic enzyme activity ratio in 4 developmental stages of *Amphiprion ocellaris*: (a) pyruvate kinase to citrate synthase (PK/CS) activity ratio, indicating relative glycolytic vs. oxidative capacity; (b) lactate dehydrogenase (LDH) to CS activity ratio, indicating the relative anaerobic vs. oxidative capacity and (c) PK/LDH activity ratio, indicating the relative glycolytic vs. anaerobic capacity. Bars not sharing a letter show statistically significant differences in enzyme activity ratios between developmental stages (p ≤ 0.05); n: number of samples. dph: days post-hatching](image-url)
in which they are usually closely associated with their host anemones. Before settlement, though, larvae have to pass the pelagic larval phase. During the pelagic phase, coral reef fish larvae may be transported off the reef, and in order to ensure their survival, anemone fishes need a way to return to the reef for settlement. Reef fish larvae have shown differences in the development of swimming ability shortly after hatching (Fisher et al. 2000, 2005). Behavioural studies have demonstrated increased reactions towards olfactory cues of anemones during the settlement phase (e.g. Dixson et al. 2011), combined with increased swimming ability (Fisher et al. 2005, Hogan et al. 2007). The development of a critical swimming ability is thought to be linked to both the morphological development of the locomotory system and the ability to use available energy reserves (Fisher et al. 2000, Videler 2011).

These observations are well in line with the enzymatic results of this study. Generally, the relative glycolytic and fermentative capacities increased significantly in 7–8 dph larvae (Fig. 2a,b). Compared to just-hatched larvae (1–2 dph), the relative glycolytic vs. oxidative metabolic capacity indicated by PK/CS activity ratios (Bass et al. 1969) was about 2.5-fold higher in pre-settlement 7–8 dph larvae, which decreased significantly again in post-settlement 15 dph juveniles. Pre-settlement 7–8 dph larvae therefore rely more on glycolytic than oxidative energy production. Similar results were given for the relative fermentative vs. aerobic metabolic capacities represented by LDH/CS activity ratios (Bass et al. 1969). Whereas the LDH/CS ratios of eggs, 1–2 dph larvae and 15 dph juveniles were quite similar (about 6), the value for the LDH/CS ratio was double for 7–8 dph larvae (about 12). These data lead to the conclusion that pre-settlement anemone larvae show a high dependence upon anaerobic glycolysis (Hochachka et al. 1983) compared to the other tested developmental stages. In case of similar LDH/CS ratios, as given for eggs, 1–2 dph larvae and 15 dph juveniles (see Fig. 2b), PK/LDH ratios can provide indications of relative aerobic glycolytic capacities (Hochachka et al. 1983). While 1–2 dph larvae showed a balanced capacity of aerobic glycolytic vs. fermentative metabolism (ratio of 1), both eggs and 15 dph juveniles displayed a trend towards higher capacity for aerobic glycolysis (Fig. 2c) (Hochachka et al. 1983), indicating that ATP generation through lactate fermentation is not as important as for 7–8 dph larvae. These metabolic results are in line with behavioural observations (Dixson et al. 2011). Larvae from the related anemone fishes Amphiprion percula and A. melano-
variances in enzyme activities due to these ontogenetic transitions should be considered when using metabolic key enzymes as biomarkers for analysing larval physiological stress or disorders.

4.2. Methods limitations

It is commonly known that there are differences in enzymatic activities among different tissues (Bass et al. 1969, Somero & Childress 1980); i.e. results of whole-larvae measurements need to be considered differently than measurements taken from pure muscle tissues. Due to the small size of the early developmental stages, however, it was not possible to take pure muscle tissues for our analyses. Whole 1–2 dph larvae were used for enzymatic analyses, while 7–8 dph and 15 dph individuals were decapitated (Michalek 2012, Imam 2015). Special care therefore is needed when examining absolute enzyme activities, as the investigation of ontogenetic development of organs and associated metabolic enzyme activities was not part of this study. This approach does not allow for direct comparisons of raw enzyme activity values, but provides a general proxy for metabolic capacities between the developmental stages.

5. CONCLUSIONS

This study revealed that glycolytic metabolic enzyme capacities significantly differ in pre- and post-settlement offspring of *Amphiprion ocellaris*. The important transition from late larva to settled juvenile can therefore be followed by analysing relative glycolytic capacities, as pre-settlement larval stages appear to highly depend upon fermentative energy production. This can help us to understand the behavioural observations of previous studies regarding swimming ability and settlement processes of anemone fish larvae. Intraspecific variances in metabolic enzyme capacities due to these ontogenetic transitions should be considered when investigating larval physiological stress or disorders.

Acknowledgements. We hereby confirm that all animals used in this research were treated in accordance with the respective animal welfare guidelines and permits (Senate Bremen, AZ 522-27-11/02-00(132)). We are grateful to Prof. Dr. G. Graf for his support during the thesis, and K. Michalek, C. v. Waldthausen, and S. Bröhl are thanked for their support at the ZMT laboratories. We further thank the anonymous reviewers who provided helpful comments to further improve the manuscript.

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Editorial responsibility: Victor Benno Meyer-Rochow, Oulu, Finland

Submitted: May 3, 2019; Accepted: September 2, 2019
Proofs received from author(s): November 17, 2019