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

Nina Paul[1,2,*], Andreas Kunzmann[1]

[1]Leibniz Centre for Tropical Marine Research (ZMT), 28359 Bremen, Germany

[2]Present address: Alfred Wegener Institute, Helmholtz Centre for Polar and Marine Research (AWI), 27570 Bremerhaven, Germany

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

*Corresponding author: nina-paul@gmx.de

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 lar-

vae 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 mo-

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 13.6×10^3 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$_4$ × 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 6.31×10^3 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 6.31×10^3 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
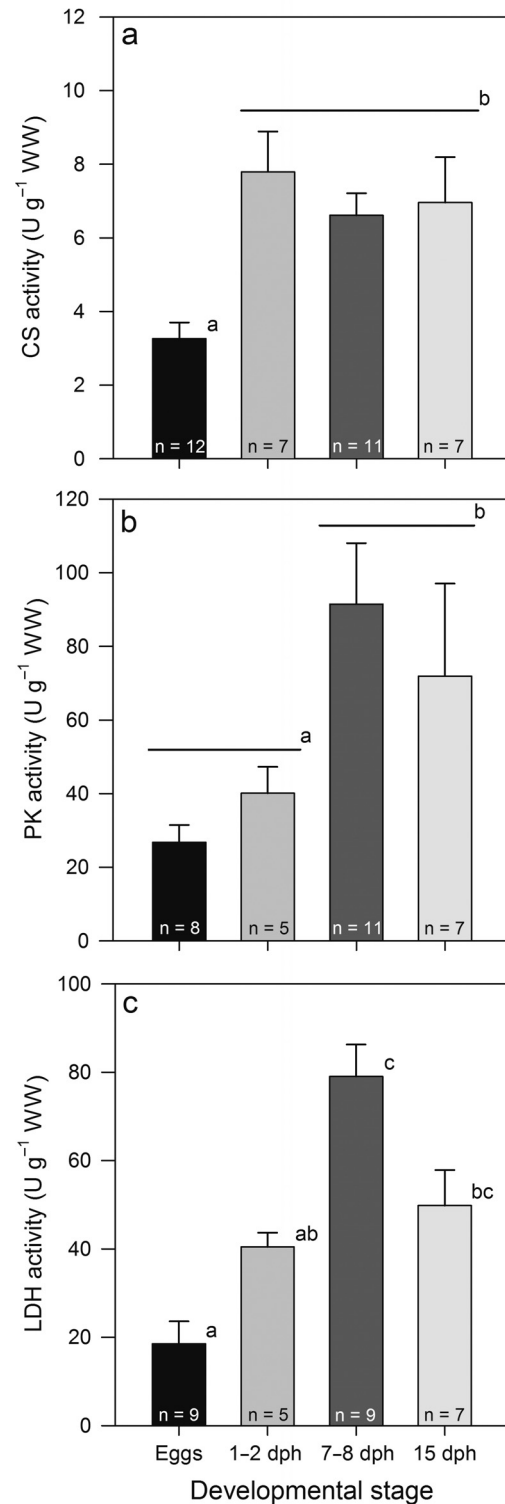

Fig. 1. Mean (±SD) enzyme activities of *Amphiprion ocellaris* eggs, larvae and juveniles: (a) citrate synthase (CS) activity, (b) pyruvate kinase (PK) activity and (c) lactate dehydrogenase (LDH) activity. Bars not sharing a letter show statistically significant differences in enzyme activities between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

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,
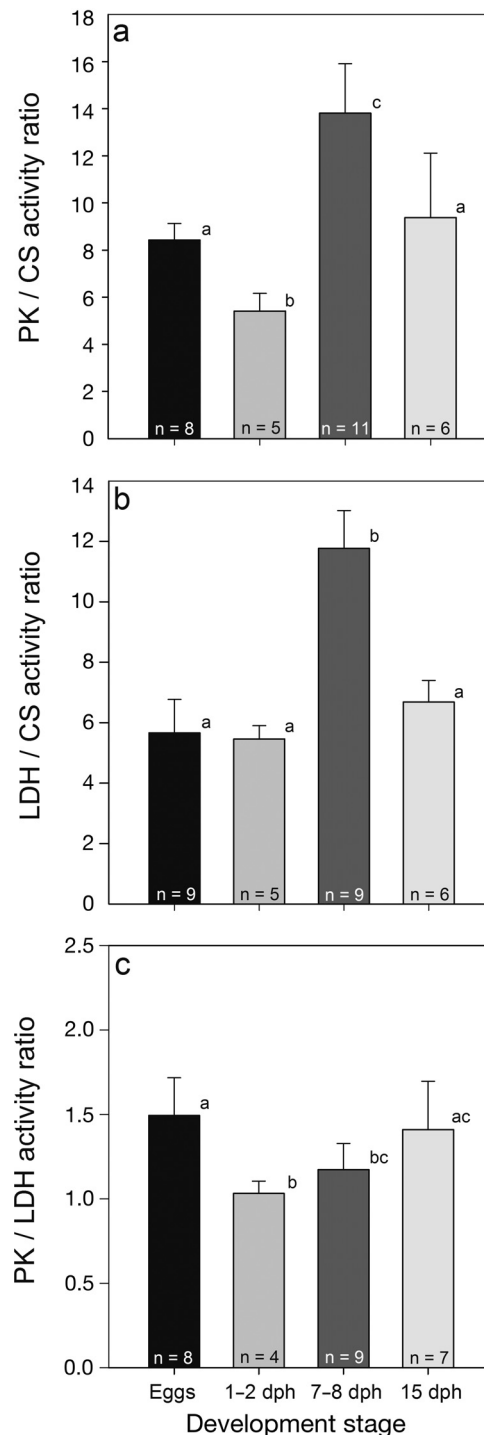

Fig. 2. Mean (±SD) metabolic enzyme activity ratio in 4 developmental stages of *Amphiprion ocellaris*: (a) pyruvate kinase to citrate synthase (PK/CS) activity ratio, indicating relative glycolytic vs. oxidative capacity; (b) lactate dehydrogenase (LDH) to CS activity ratio, indicating the relative 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

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-*

pus were highly attracted to anemone odour at the age of 7 dph (Dixson et al. 2011). The metabolic capability to actively swim towards pre-determined habitats at the age of settlement thus increases the survival probability of anemone fish. The observed burst swimming capacities of pre-settlement anemone fish larvae in previous studies may result from drastically increased glycolytic power, especially through energy generation during lactate fermentation processes (Childress & Somero 1979).

Interestingly, post-settlement 15 dph juveniles showed decreased reliance on relative glycolytic capacities compared to pre-settlement larvae (Fig. 2a,b). These findings may be related to the fact that burst swimming ability is no longer needed after successful settlement. As mentioned above, interspecific variations in metabolic muscle capacities are highly dependent upon the mode of life (Sullivan & Somero 1980, Somero & Childress 1990). With increasing body mass, pelagic fishes such as kelp bass *Paralabrax clathratus* and rainbow trout *Salmo gairdneri* showed increasing anaerobic glycolytic capacities and decreasing oxidative capacities (Somero & Childress 1990). Benthic Dover sole *Microstomus pacificus* on the contrary, showed decreasing capacities of both glycolytic and oxidative metabolism with increasing body size (Somero & Childress 1990). The increasing anaerobic glycolytic capacities in the muscles of pelagic fishes therefore represent the adaptive value of strong burst locomotion that is needed for their way of life — which is not essential for benthic fishes (Childress & Somero 1990). These findings fit well with the observed changes in the glycolytic capacities of anemone fish offspring before and after settlement. After approximately 1 wk in the pelagic phase, *A. ocellaris* larvae start to enter the benthic stage and begin to look for suitable hosts (Miyagawa-Kohshima et al. 2014), which is well presented in the high glycolytic capacities needed for extensive burst swimming capabilities in 7–8 dph larvae. As soon as they successfully settle, anemone fish establish a sedentary life mode close to their chosen host anemone so that extensive burst swimming is no longer needed. Analysing relative glycolytic capacities thus appears to be a good tool for following the ontogenetic transition of pre- and post-settlement anemone offspring as they change their habitat from the pelagic to the benthic zone. The notable variation in PK activities of 15 dph juveniles (Fig. 1) might indicate different stages of the juveniles within the settlement process. Even though they are the same age, some of the juveniles might still be in the on-going settlement procedure and therefore still show increased glycolytic power. Vari-

ances 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.

LITERATURE CITED

Allen GR (1972) The anemonefishes: their classification and biology. TFH Publications, Neptune City, NJ

Almeida-Val VMF, Gomes ARC, Lopes NP (2006) Metabolic and physiological adjustments to low oxygen and high temperature in fishes of the Amazon. In: Val AL, Almeida-Val VMF, Randall DJ (eds) The physiology of tropical fishes, Vol 21: fish physiology. Academic Press, Amsterdam, p 443–500

Avella MA, Olivotto I, Gioacchini G, Maradonna F, Carnevali O (2007) The role of fatty acids enrichments in the larviculture of false percula clownfish *Amphiprion ocellaris.* Aquaculture 273:87–95

Bass A, Brdiczka D, Eyer P, Hofer S, Pette D (1969) Metabolic differentiation of distinct muscle types at the level of enzymatic organization. Eur J Biochem 10:198–206

Batty RS (1984) Development of swimming movements and musculature of larval herring (*Clupea harengus*). J Exp Biol 110:217–229

Bellwood DR, Fisher R (2001) Relative swimming speeds in reef fish larvae. Mar Ecol Prog Ser 211:299–303

Blaxter JHS (1986) Development of sense organs and behaviour of teleost larvae with special reference to feeding and predator avoidance. Proc 9th Larval Fish Conf 115:98–114

Childress JJ, Somero GN (1979) Depth-related enzymatic activities in muscle, brain, and heart of deep-living pelagic teleosts. Mar Biol 52:273–283

Childress JJ, Somero GN (1990) Metabolic scaling: a new perspective based on scaling of glycolytic enzyme activities. Am Zool 30:161–173

Clarke ME, Calvi C, Domeier M, Edmonds M, Walsh PJ (1992) Effects of nutrition and temperature on metabolic enzyme activities in larval and juvenile red drum, *Sciaenops ocellatus*, and lane snapper, *Lutjanus synagris.* Mar Biol 112:31–36

Desrosiers V, Le François NR, Tveiten H, Andreassen I, Blier PU (2008) Ontogenesis of catabolic and energy metabolism capacities during the embryonic development of spotted wolffish (*Anarhichas minor*). Comp Biochem Physiol B 150:200–206

Dixson DL, Munday PL, Pratchett M, Jones GP (2011) Ontogenetic changes in responses to settlement cues by anemonefish. Coral Reefs 30:903–910

El-Fiky N, Hinterleitner S, Wieser W (1987) Differentiation of swimming muscles and gills, and development of anaerobic power in the larvae of cyprinid fish (Pisces, Teleostei). Zoomorphology 107:126–132

Fisher R, Bellwood DR, Job SD (2000) Development of swimming abilities in reef fish larvae. Mar Ecol Prog Ser 202:163–173

Fisher R, Leis JM, Clark DL, Wilson SK (2005) Critical swimming speeds of late-stage coral reef fish larvae: variation within species, among species and between locations. Mar Biol 147:1201–1212

Green BS, McCormick MI (2001) Ontogeny of the digestive and feeding systems in the anemonefish *Amphiprion melanopus.* Environ Biol Fishes 61:73–83

Hess S, Wenger AS, Ainsworth TD, Rummer JL (2015) Exposure of clownfish larvae to suspended sediment levels found on the Great Barrier Reef: impacts on gill structure and microbiome. Sci Rep 5:10561

Hickey AJR, Clements KD (2003) Key metabolic enzymes and muscle structure in triplefin fishes (Tripterygiidae): a phylogenetic comparison. J Comp Physiol B 173:113–123

Hochachka PW, Stanley C, Merkt J, Sumar-Kalinowski J (1983) Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. Respir Physiol 52:303–313

Hogan JD, Fisher R, Nolan C (2007) Critical swimming speed of settlement-stage coral reef fishes from the Caribbean: a methodological and geographical comparison. Bull Mar Sci 80:219–231

Holt GJ (2003) Research on culturing the early life stages of marine ornamental fish. In: Cato JC, Brown CL (eds) Marine ornamental species: collection, culture and conservation. Iowa State Press, Ames, IA, p 251–254

Imam RM (2015) Activity of key metabolic enzymes in early life stages of key fish species from the Northern Benguela Current upwelling system. MSc thesis, University of Bremen

Jacob H, Pouil S, Lecchini D, Oberhänsli F, Swarzenski P, Metian M (2017) Trophic transfer of essential elements in the clownfish *Amphiprion ocellaris* in the context of ocean acidification. PLOS ONE 12:e0174344

Jebsen JW (1962) Proteins in fish muscle. In: Heen E, Kreuzer R (eds) Fish in nutrition. Fishing News, London, p 68–72

Job S, Bellwood DR (2000) Light sensitivity in larval fishes: implications for vertical zonation in the pelagic zone. Limnol Oceanogr 45:362–371

Leis JM (1991) The pelagic stage of reef fishes: the larval biology of coral reef fishes. In: Sale PF (ed) The ecology of fishes on coral reefs. Academic Press, San Diego, CA, p 183–230

Liew HJ, Ambak MA, Abol-Munafi AB, Chuah TS (2006) Embryonic development of clownfish *Amphiprion ocellaris* under laboratory conditions. J Sustain Sci Manag 1: 64–73

Lushchak VI, Bagnyukova TV, Storey KB (1998) Effect of hypoxia on the activity and binding of glycolytic and associated enzymes in sea scorpion tissues. Braz J Med Biol Res 31:1059–1067

Lushchak VI, Bagnyukova TV, Storey JM, Storey KB (2001) Influence of exercise on the activity and the distribution between free and bound forms of glycolytic and associated enzymes in tissues of horse mackerel. Braz J Med Biol Res 34:1055–1064

Madhu K, Madhu R, Krishnan L, Sasidharan CS, Venugopalan KM (2006) Spawning and larval rearing of *Amphiprion ocellaris* under captive condition. Marine Fisheries Information Service, Technical and Extension Series, 188. Central Marine Fisheries Research Institute, Cochin

Madhu R, Madhu K, Retheesh T (2012) Life history pathways in false clownfish *Amphiprion ocellaris* Cuvier, 1830: a journey from egg to adult under captive condition. J Mar Biol Assoc India 54:77–90

Meyer-Rochow VB, Ishihara Y, Ingram JR (1994) Cytochemical and histological details of muscle fibers in the southern smelt *Retropinna retropinna* (Pisces; Galaxioidei). Zool Sci 11:55–62

Michalek K (2012) Activity of metabolic key enzymes in early life stages of Cape horse mackerels, *Trachurus capensis* from the Northern Benguela Current upwelling system. MSc thesis, University of Bremen

Miller NA, Chen X, Stillman JH (2014) Metabolic physiology of the invasive clam, *Potamocorbula amurensis*: the interactive role of temperature, salinity, and food availability. PLOS ONE 9:e91064

Miyagawa-Kohshima K, Odoriba S, Okabe D, Baba Y and others (2014) Embryonic learning of chemical cues via the parents' host in anemonefish (*Amphiprion ocellaris*). J Exp Mar Biol Ecol 457:160–172

Moyano FJ, Díaz M, Alarcón FJ, Sarasquete MC (1996) Characterization of digestive enzyme activity during larval development of gilthead seabream (*Sparus aurata*). Fish Physiol Biochem 15:121–130

Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Døving KB (2009) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc Natl Acad Sci USA 106:1848–1852

Ochi H (1985) Temporal patterns of breeding and larval settlement in a temperate population of the tropical anemonefish, *Amphiprion clarkii.* Jpn J Ichthyol 32: 248–257

Olivotto I, Cardinali M, Barbaresi L, Maradonna F, Carnevali O (2003) Coral reef fish breeding: the secrets of each species. Aquaculture 224:69–78

Olivotto I, Zenobi A, Rollo A, Migliarini B, Avella M, Carnevali O (2005) Breeding, rearing and feeding studies in the cleaner goby *Gobiosoma evelynae.* Aquaculture 250:175–182

Olivotto I, Rollo A, Sulpizio R, Avella M, Tosti L, Carnevali O (2006) Breeding and rearing the sunrise dottyback *Pseudochromis flavivertex*: the importance of live prey enrichment during larval development. Aquaculture 255: 480–487

Olivotto I, Di Stefano M, Rosetti S, Cossignani L, Pugnaloni A, Federica Giantomassi F, Carnevali O (2011) Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larval development and metamorphosis: molecular and biochemical implications. Comp Biochem Physiol A Mol Integr Physiol 159:207–218

Pimentel MS, Faleiro F, Diniz M, Machado J and others (2015) Oxidative stress and digestive enzyme activity of flatfish larvae in a changing ocean. PLOS ONE 10:e0134082

Ross RM (1978) Reproductive behavior of the anemonefish *Amphiprion melanopus* on Guam. Copeia 1978:103–107

Sales J, Janssens GPJ (2003) Nutrient requirements of ornamental fish. Aquat Living Resour 16:533–540

Segner H, Verreth J (1995) Metabolic enzyme activities in larvae of the African catfish, *Clarias gariepinus*: changes in relation to age and nutrition. Fish Physiol Biochem 14: 385–398

Seibel BA, Thuesen EV, Childress JJ (2000) Light-limitation on predator-prey interactions: consequences for metabolism and locomotion of deep-sea cephalopods. Biol Bull (Woods Hole) 198:284–298

Sidell BD, Driedzic WR, Stowe DB, Johnston IA (1987) Biochemical correlations of power development and metabolic fuel preferenda in fish hearts. Physiol Zool 60: 221–232

Somero GN, Childress JJ (1980) A violation of the metabolism-size scaling paradigm: activities of glycolytic enzymes in muscle increase in larger-size fish. Physiol Zool 53:322–337

Somero GN, Childress JJ (1990) Scaling of ATP-supplying enzymes, myofibrillar proteins and buffering capacity in fish muscle: relationship to locomotory habit. J Exp Biol 149:319–333

Stoiber W, Haslett JR, Sänger AM (1999) Myogenic patterns in teleosts: What does the present evidence really suggest? J Fish Biol 55(Suppl A):84–99

Sullivan KM, Somero GN (1980) Enzyme activities of fish skeletal muscle and brain as influenced by depth of occurrence and habits of feeding and locomotion. Mar Biol 60:91−99

Tong X, Yang L, Tang X, Yang X and others (2017) Patterns of catabolic capacities and energy metabolism in developing embryos and yolk-sac larvae of turbot (*Scophthalmus maximus* L.). Aquaculture 481:177−183

Vargas-Abúndez AJ, Basilio Randazzo B, Foddai M, Sanchini L and others (2019) Insect meal based diets for clownfish: biometric, histological, spectroscopic, bio-

chemical and molecular implications. Aquaculture 498: 1−11

Videler JJ (2011) An opinion paper: emphasis on white muscle development and growth to improve farmed fish flesh quality. Fish Physiol Biochem 37:337−343

Wieser W (1995) Energetics of fish larvae, the smallest vertebrates. Acta Physiol Scand 154:279−290

Wright KJ, Higgs DM, Belanger AJ, Leis JM (2005) Auditory and olfactory abilities of pre-settlement larvae and post-settlement juveniles of a coral reef damselfish (Pisces: Pomacentridae). Mar Biol 147:1425−1434