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The influence of diet on the early development of two seahorse species (*H. guttulatus* and *H. reidi*): Traditional and innovative approaches

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ABSTRACT

Larval nutrition plays a key role in the development of a sustainable aquaculture where fish development, health and wellness are of prime importance. For some species, satisfactory growth and survival rates are met providing exclusively enriched rotifers and *Artemia*; however, feeding on copepods during the larval period has been shown to improve growth in both larval and juvenile fish, including seahorses. For the first time, the effects of different diets (*Artemia* and copepods) on the early development of juvenile seahorses (*H. guttulatus* and *H. reidi*) development were analysed by combining biometry, traditional histology and FPA-FTIR Imaging spectroscopy. Survival and growth and biochemical composition on the liver in seahorse were significantly affected by the type of diet offered. The results achieved were related to differences in the digestion of the two types of live preys, mainly dependent on their biochemical composition and permeability of the exoskeleton.

1. Introduction

The high demand of seahorses for the aquarium trade, the traditional Chinese medicine and as souvenirs, in addition to the destruction and degradation of their coastal habitats (seagrasses, coral reefs and mangroves), has raised many concerns over their long-term viability in nature (Vincent, 1996; Lourie et al., 1999; Vincent et al., 2011, Kumaravel et al., 2012). Currently, all seahorses species are included in Appendix II list of endangered species by CITES (Convention for the International Trade in Endangered Species), restricting the legal import and export of dead or alive seahorses.

The ex-situ production of seahorses has the potentiality to represent a valid partial alternative to wild caught specimens but for most species such activity is still a relative new field that need the assessment of some technical challenges (Lourie et al., 1999; Foster and Vincent, 2004; Koldewey and Martin-Smith, 2010; Cohen et al., 2016; Planas et al., 2017). One of the critical bottlenecks that culturists have to face in seahorse rearing is the low survival in early developmental stages (Scaratt, 1995; Payne and Rippingale, 2000; Olivotto et al., 2008a). Such constrain is due to many factors related to environmental and zoothecnical conditions and feeding, among others (Woods, 2000; Chang and Southgate, 2001; Sheng et al., 2006; Olivotto et al., 2008b; Planas et al., 2017). Feeding and nutrition play a key role in the growth and survival of juveniles.

Live prey such as rotifers, *Artemia*, Mysidiacea and copepods are required in the rearing of many seahorse species. In most cases, *Artemia* nauplii and rotifers (*Brachionus* sp.) are the first option because they can be easily cultured in large quantities at high densities (Olivotto et al., 2008c). However, rotifers and *Artemia* do not represent the natural food of seahorses, and do not always provide adequate fatty acid profiles. In addition, these prey do not always provide the sizes required by young seahorse (Faulk and Holt, 2005). Furthermore, difficulties are known regarding their digestion by early stages in some seahorse species (Olivotto et al., 2011; Blanco et al., 2015). Therefore, alternative prey, other than rotifers and *Artemia*, are explored with great interest by the scientific community (Calado et al., 2017). In the wild, adult seahorses feed on a variety of prey (mostly crustaceans) (Teixeira and Musick, 2001; Woods, 2002; Kendrick and Hyndes, 2005; Kitsos et al., 2008; Storero and González, 2008; Gurkan et al., 2011; Valladares et al., 2017), including copepods (Tipton and Bell, 1988; Franzoi et al., 1993) but the natural diet of young seahorses is still unknown. It has been reported that feeding young pipefish and seahorses on cultured copepods may significantly improve survival rates and growth (Payne et al., 1998; Payne and Rippingale, 2000; Olivotto et al., 2008a; Blanco

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and Planas, 2015). Delbare et al. (1996) summarized the advantages of using copepods in larviculture, such as a wider size range, a typical movement, and a high and optimal content in essential highly unsaturated fatty acids (HUFAs). These fatty acids, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are extremely important for fish survival and growth, being essential in larval diets (Sargent et al., 1999).

Any failure or limitation in nutrients or energy uptake may affect the adeguate development of organs and structures, and consequently, further growth and survival. Since the gastrointestinal tract and associated organs may account for up to 40% of an animal's metabolic rate (Cant et al., 1996), it is understandable that problems in digestion or periods of malnutrition could affect the digestive tract resulting in further serious functional disorders (Olivotto et al., 2011; Piccinetti et al., 2012). Therefore, degeneration and composition of tissues in some digestive organs such as intestine and liver are probably the best indicators for the identification of starvation conditions (Diamond and Hammond, 1992; Wang et al., 2006; Olivotto et al., 2011). Currently, there are several well established traditional analytical techniques and methods available for gut/liver analysis, including visual and microscopic observations as well as biochemical, molecular and proteome analysis. Those methodologies play a pivotal role in the analysis of fish gut/liver and some have been used as gold standards and standardized methods serving scientific researches due to their relative validity and accuracy. However, these techniques and methods are normally expensive, time-consuming, laborious and in some cases difficult to apply to seahorses.

The present study investigates the effects of different diets on the composition of seahorses liver combining, for the first time, traditional histology and Focal Plane Array Fourier Transform Infra-Red (FPA-FTIR) Imaging spectroscopy. FTIR spectroscopy is a fast, label-free analytical technique widely applied for investigating functional groups, bonding types, and molecular conformations of the most relevant biological molecules. This technique analyzes the vibrational transitions due to the interaction between matter and the infrared radiation and allows to obtain at the same time and on the same sample important information on the whole molecular composition of the analysed sample. FPA FT-IR Imaging spectroscopy couples the potential of visible microscopy, which permits a visual inspection of the sample, with innovative focal plane array detectors, which let simultaneously acquire small areas with a spatial resolution near to the diffraction. This analytical technique allows to perform the imaging analysis of non-homogeneous biological samples in terms of composition and structure of the most relevant biomolecules (Gioacchini et al., 2014; Giorgini et al., 2015a, 2015b), and, hence, monitor the biochemical modifications, either induced or naturally occurring, in human and animal tissues and cells. In particular, it has been successfully applied to evaluate biochemical changes in liver of several fish species under different feeding conditions (Gioacchini et al., 2014; Carnevali et al., 2017; Forner-Piquer et al., 2017). On continuing to investigate this topic, in the present study, FPA-FTIR Imaging spectroscopy was applied, for the first time, to analyze the effects of different diets (rotifers, *Artemia* and copepods) on seahorses liver samples, with the aim to identify appropriate spectral biomarkers correlatable with the biochemical changes in tissues composition. The spectral data were also complemented with biometry and traditional histology outcomes.

2. Material and methods

2.1. Ethics

Animal capture, handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (Real Decreto 1201/2005, 10th October) and the Regional Government Xunta de Galicia (Ref. REGA ES 36057020 2001/16/FUN/BIOL.AN/MPO02).

2.2. Breeding conditions

Newborn seahorses were obtained from broodstock of the seahores *Hippocampus reidi* (tropical species) and *Hippocampus guttulatus* (temperate species). Breeders were maintained in four aquaria units connected to a recirculation system and under moderate aeration (Planas et al., 2008). Each husbandry aquaria consisted of three subunits of 160 L each (85 height × 75 length × 50 wide cm) working as an autonomous closed system. Water quality was checked periodically for NO_2, NO_3 and NH_4/NH_3 content (0 mg L^{-1}). Salinity and pH levels were 37 ± 2 and 8.0 ± 0.2, respectively for both species. Temperature levels and photoperiods were adapted according to the needs of each species. A water temperature of 19 ± 0.5 °C and a 16L:8D photoperiod regime were applied for *H. guttulatus* during the breeding season (Planas et al., 2013), whereas a temperature of 26 ± 0.5 °C and a constant 14L:10D photoperiod (Olivotto et al., 2008c) were applied to *H. reidi*. Adult seahorses were fed twice daily on a diet consisting on long time enriched adult *Artemia sp.* (EG, AF, MC450; Iberfrost®, Spain; 40–70 *Artemia* seahorse^{-1} dose^{-1}) (Planas et al., 2017) and frozen Mysis (3F®-Frozen Fish Food, Iberfrost, Spain). When available, a single daily dose of wild-captured Mysidacea (15–20 *Leptomysis* sp. and/or *Siriella* sp.) was also provided. Wastes and uneaten food were removed daily by siphoning the bottom of aquaria.

2.3. Rearing system

Seahorses broodstocks were monitored several times a day to check for newborn release from male's pouch. Newly released juveniles were carefully collected by siphoning and transferred (5 juveniles L^{-1}) to 30 L pseudo-Kreisel aquaria connected to a semi-opened recirculation. Temperatures and photoperiod were same as for breeders. The aquaria were filled with seawater filtered by a series of filter-cartridges (20, 10, 5 and 1 μm) and UV treated (76w; 16 L min^{-1}) (JR1/50). The rearing system included a degasifying column and two 50 L chambers including mechanical (up to 20 μm) and biological filters (perforated plastic bioballs) and aerators. From the biofilter unit, the seawater was pumped to 36w UV units (AquaMedic®, Germany) and then to a 50 L reservoir aquarium, being finally routed by gravity towards the rearing aquaria (Illuminati et al., 2010; Planas et al., 2012).

2.4. Live prey culture

Microalgae (*Phaeodactylum tricornutum*, *Rhodomonas lens* and *Isochrysis galbana*) were cultured at 22 ± 1 °C in 80 L plastic bags containing sterilized seawater supplemented with F2P (100 g L^{-1}) media (VarAqua©). Additionally, silicates were added to *P. tricornutum* cultures, and 200 μL F2P media to the *R. lens* culture flasks.

Artemia cysts (AF®, Inve, Spain) were hatched at 28 °C for 20 h in 20 L units, and the hatched nauplii gently rinsed with tap-water, collected on a 125 μm mesh, rinsed and transferred to 5 L buckets for metanauplii production (100 *Artemia* mL^{-1}). Metanauplii of several ages and sizes (24 h, 48 h, 72 h and 96 h; Fig. 3.4) were enriched twice daily on a mixture consisting on live microalgae (*P. tricornutum* 10^7 cells mL^{-1}), Red Pepper (0.015 g L^{-1}), Bernaqua®, Belgium) and dried Spirulina (0.03 g L^{-1}, Iberfrost®, Spain). *Artemia* nauplii and enriched metanauplii were used for feeding seahorse juveniles.

Adult *Artemia* was produced for the feeding of adult seahorses. For that, the nauplii (EG, AF, MC450; Iberfrost®, Spain) were grown in 100 L units, at 26–28 °C with gentle aeration and constant light. A long-time enrichment (3–6 days) was carried out in *Artemia* from day 16 onwards on a mixture consisting on live microalgae *P. tricornutum* and *I. galbana* (10^7 cells mL^{-1}), Red Pepper (0.015 g L^{-1}, Bernaqua®, Belgium) and dried Spirulina (0.03 g L^{-1}, Iberfrost®, Spain) (Planas et al., 2017). Copepods (*Acartia tonsa*) were cultivated for the early feeding of seahorse juveniles in 700 L tanks at 26–27 °C and 38 salinity, at an initial density of 1 copepod mL^{-1}. Copepods were fed every two