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The early life of Seahorses. Development, morphology, and digestive biochemistry

BRUNO NOVELLI

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

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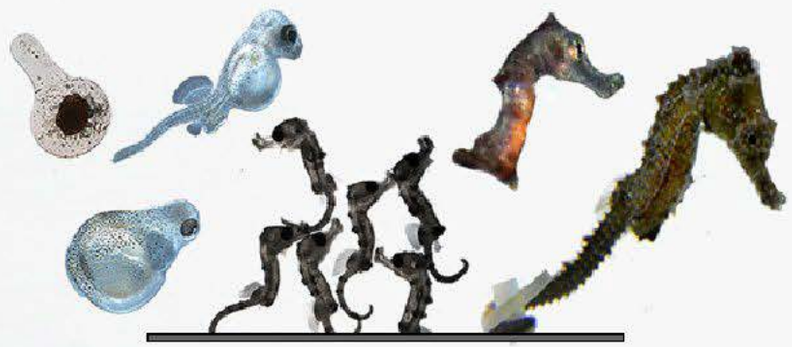

BRUNO NOVELLI

TESIS DOCTORAL
LAS PALMAS DE GRAN CANARIA
2016

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

D Daniel Montero Vítores.....coordinador de programa de doctorado

DE. Acuicultura y Ecosistemas Marinos ..... DE LA

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA,

Hace Constar,

Que el Consejo de Doctores del Programa de doctorado en su sesión de fecha..30 de noviembre de 2016.....tomó el acuerdo de dar el consentimiento

para su tramitación, a la tesis doctoral **titulada "The early life of seahorses. Development, morphology, and digestive biochemistry"** presentada por el doctorando D. Bruno Novelli y dirigida por la Doctora Dª Lucía Molina Domínguez y el Dr. D. Juan Socoro Cruz

Y para que así conste, y a efectos de lo previsto en el Artº 6 del Reglamento para la elaboración, defensa, tribunal y evaluación de tesis doctorales de la Universidad de Las Palmas de Gran Canaria, firmo la presente en Las Palmas de Gran Canaria,

a..30.de.....Noviembre.....de dos mil...dieciseis.....

UNIVERSIDAD DE LAS PALMAS
DE GRAN CANARIA

# The early life of seahorses. Development, morphology, and digestive biochemistry

**Bruno Novelli**

**Doctorado en Acuicultura:**

**Producción Controlada de Animales Acuáticos**

**Escuela de Doctorado**

**ULPGC**

Tesis para optar al Grado de Doctor

Las Palmas de Gran Canaria 2016

Directores:

Ph.D. Lucía Molina-Domínguez

Ph.D. Juan Antonio Socorro-Cruz

*A mamma, papà*

*e nonna*

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# Abreviaturas

# Abreviaturas

(A) atrium
(ABD-PO) abdominal post-dorsal vertebrae
(ABD-PR) abdominal pre-dorsal vertebrae
(AF) anal fin
(AI) anterior intestine
(ANG) angular
(AN) anus
(AOR) antorbital
(APH) apophysis
(ART) *Artemia*
(AT) adipose tissue
(AVV) atrio-ventricular valve
(B) brain
(BA) bulbus arteriosus
(BB) brush border
(BE) basophilic epithelial cells
(BH) basihyial
(BLK) blank
(BO) basoccipital
(BP) basal process
(BR) branchiostegal rays
(C) cornea
(CAD) caudal vertebrae
(CD) coracoid
(CER) cervical vertebrae
(CF) caudal fin
(CH) ceratohyal
(CH) ceratohyal
(CL) chloride cell
(CL) cleithrum
(CNT) coronet
(CR) choroid rete
(CT) Connective tissue
(CY) hyaline cartilage
(D) dentary
(DF) dorsal fin
(DOR) dorsal vertebrae
(DR) distal radial
(DS) dermal substance
(E) eye
(EC) elastic cartilage
(ECT) ectopterygoid
(EE) egg envelope
(EF) eosinophilic cells
(EN) endopterygoid
(EP) epiotics
(ETD) ethmoidal region
(ETP) ethmoid plate
(EX) exoccipitals
(F) frontal
(FC) frontal crest
(FG) foregut
(FU) fusion
(G) gill
(GA) gill arcs
(GB) gall bladder
(GC) goblet cell
(GCV) gill cavity
(GG) gas gland
(GLY) glycogen
(GN1) gonadal lobe 1
(GN2) gonadal lobe 2
(H&E) Hematoxilin and Eosin
(H) heart

(HA) haemal arch
(HDG) headgut
(HG) hindgut
(HH) hipohyal
(HKN) head kidney
(HP) hepatocytes
(HTS) hematopoietic tissue
(HY) hypural bones
(HYC) hyosymplectic cartilage
(HYC) hypural cartilage
(HYD) hyoid region
(HYM) hyomandibular
(HYP) hypural plate
(I) iris
(IH) interhyal
(IO) interopercular
(IOV) valve between midgut and esophagus
(IV) ileocaecal valve
(J) jaw
(JW) jaws region
(KDN) kidney
(L) liver
(LA) lachrymal
(LD) lipid droplets
(LET) lateral-ethmoid
(LF) longitudinal fold
(LI) Langerhans islets
(LIP) lipids
(LM) lamella
(LS) lens
(MC) Meckel's cartilage
(MC) mucous cell
(ME) metapterygoid
(MET) mesethmoid
(MG) midgut
(MR) medial radial
(MVL) microvilli
(MWM) molecular weight marker
(MX) maxillary
(N) notochord
(NA) neural arch
(NA) nutrient absorption
(NC) notochord
(NCL) provitelline nucleolus
(NCP) nasal capsule
(NRC) neurocranium
(NSP) nasal spine
(O) otolith
(OE) esophagus
(ON) optic nerve
(OP) operculum
(OPR) opercular region
(ORB) orbital region
(OS) ovary sac
(OV) optical vesicles
(OY) oocytes
(P) peritoneal membrane
(PA) palatine
(PAS) parasphenoid
(PCS) pre-coronet spine
(PE) pellucid zone
(PF) primordial follicle
(PFR) pre-frontal
(PGM) pigmentation
(PH) pharynx
(PI) posterior intestine
(PM) pre-maxillary
(PN) pancreas
(POP) pre-opercular
(POZ) post-zigapophysis
(PR) proximal radial
(PRZ) pre-zigapophysis
(PSB) pseudobranch
(PTC) pterotic crest

(PTM) post temporal
(PTO) pterotic
(Q) quadrate
(QJ) quadratojugal
(R) retina
(RA) retroarticular
(RC) rostral cartilage
(RCT) rectum
(RG) ring
(RM) rete mirabile
(RY) rays
(S) sclera
(SAV) sinoatrial valve
(SB) swim bladder
(SBO) sub-orbital
(SC) corpuscle of Stannius
(SC) scapula
(SD) supra-dorsal vertebra
(SET) supra-ethmoid
(SF) sphenotic
(SH) shield
(SIB) supranuclear inclusion body
(SLD) supranuclear lipid droplets
(SM) symplectic
(SN) snout
(SNC) notochord sheath
(SO) supra-occipital
(SP) spleen
(SPN) spine
(SUS) suspensoria
(SV) sinus venosus
(TB) taste buds
(TBL) tubules
(THF) thyroid follicle
(THG) thyroid gland
(TM) taenia marginalis
(TP) transverse process
(TY) thymus
(UB) urinary bladder
(UH) urohyal
(UP) urinary pore
(V) vertebra
(VC) vertebral centrum
(VCL) fat vacuoles
(VE) vein
(VLC) vacuolated colloid
(VO) vomeral
(VT) ventricle
(YS) yolk sac
(ZY) zymogen granules

# Agradecimientos

# Agradecimientos

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# Resumen

# Resumen

Los caballitos de mar son especies susceptibles al declive de sus poblaciones naturales, debido tanto a la destrucción de sus hábitats como a su particular biología reproductiva. El volumen de su comercio está provocando una peligrosa reducción del stock natural. Por ello, todas las especies de caballitos de mar se incluyeron en 2002 en el Apéndice II de la Convención sobre el Comercio Internacional de Especies Amenazadas de Fauna y Flora Silvestres (CITES) y en 2003 en la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza (IUCN). La difícil situación de los caballitos de mar se debe tanto a la fuerte presión sobre las poblaciones naturales como a las dificultades de su producción en cautividad, que presenta una baja supervivencia de los recién nacidos, constituyendo el cuello de botella para la producción masiva de estas especies. El cultivo podría ser una de las medidas paliativas para mantener la biodiversidad animal y aliviar la presión ejercida sobre las poblaciones naturales por el sector acuarístico, pesquero y medicinal.

La presente **Tesis Doctoral** incluye el estudio del período que abarca desde la fecundación del huevo a los treinta días de vida fuera de la bolsa incubadora paterna, usando las especies *Hippocampus reidi* y *Hippocampus hippocampus*. Durante este periodo los caballitos de mar muestran una alta mortalidad en cautividad, aunque existen algunas condiciones que favorecen su cultivo, como el alto número de caballitos producidos por puesta y la capacidad depredadora mostrada desde el primer momento de vida libre. La cría en cautividad de estos peces es un aspecto reciente y novedoso. El

éxito del cultivo de una especie pasa por el conocimiento principalmente de la biología, de los cambios morfológicos en sus primeras etapas de vida y de la capacidad de cubrir todos sus requerimientos nutricionales. Con el objetivo de aumentar los conocimientos sobre esta fase crucial en el desarrollo se aplicaron técnicas osteológicas, histológicas, histoquímicas y enzimológicas, n

Por otro lado, se aclara la confusión que existe en bibliografía por la denominación de estos animales en el momento que dejan la bolsa incubadora, que debido a su alto grado de desarrollo al nacer se consideran: "juveniles", "crías", "alevines", "recién nacidos-juveniles", "juveniles de edad temprana".

Con la presente Tesis Doctoral se demuestra la hipótesis de la existencia de metamorfosis en *H. reidi* y *H. hippocampus*. Se describe detalladamente el desarrollo morfológico de sus estructuras y funcionalidad, tanto internas, como es el caso del aparato digestivo, como externas, con la observación de características morfológicas ausentes en los adultos (aleta caudal) y la ausencia de elementos presentes en los adultos (espinas, anillos óseos y placas óseas). Todo se describe en un contexto ya descrito de los caballitos de mar recién nacidos, que se caracteriza por una primera fase planctónica previa a la bentónica y un elevado índice de mortalidad que disminuye después de las primeras semanas de vida.

Con la presente Tesis Doctoral por primera vez se propone considerar los recién nacidos de caballitos de mar como larvas, y ampliar la hipótesis de una fase larvaria a las demás especies. Se abren las puertas al estudio de la nutrición larvaria en caballitos de mar, y se propone el estudio de la bioquímica digestiva como

herramienta eficaz para establecer los requerimientos nutricionales específicos de cada especie.

# Summary

# Summary

The destruction of their natural habitats as well as their peculiar reproductive biology make seahorses as species susceptible to decline. The high volume of their trade is the cause of the dangerous reduction in the natural stock. All species of seahorses were included in 2002 in Appendix II of CITES, and in 2003 in the Red List of the International Union for the Conservation of Nature (IUCN). It is necessary to promote its conservation. The strong pressure on natural populations, and the low newborns survival in captivity, that represent the bottleneck for a massive production of these species, make seahorses situation very delicate. The massive production of seahorses could be one of the palliative measures to maintain the animal biodiversity and could relieve the pressure on natural populations due to the high market demand of aquarist, fisheries, and medicinal sectors.

Using *Hippocampus reidi* and *Hippocampus hippocampus* species, was studied in the present **Doctoral Thesis,** the span of time from the fertilization of the egg to the thirty days of life outside the male broodpouch. During this period, seahorses show a high mortality rate in captivity, although some positive conditions promoted their breeding, as the high number of larvae produced, or the high hunting ability from the first moment of free life. The captive breeding of these species is a recent and novel aspect. The knowledge of their biology, morphological changes during earliest stages, and nutritional requirements, make it possible the success of the mass scale production. Osteological, histological, histochemical and enzymatic

techniques were applied to increase knowledge during this very delicate phase.

On the other hand, this **Doctoral Thesis** clarifies the confusion about the status of newborn seahorses after leave the male brood pouch. Due to the high development of newborns, they are considered and reared as "juveniles", "offspring", “fry”, as well as "newborn-juveniles", or "early-juveniles" in the literature.

With the present **Doctoral Thesis**, the hypothesis of a metamorphic period in *H. reidi* and *H. hippocampus* species was demonstrated. The detailed development of its structures and their functionality, both internal, as in the case of development of the digestive tract, as well as external, with adults missing elements observed (caudal fin), or morphological adult structures present (spines, bony rings and bony plates). Everything is described in a newborn seahorse known framework, that is characterized by a first planktonic phase before the benthic one and by a high rate of mortality that decreases after the first few weeks of life.

For the first time, with the present **Doctoral Thesis,** it is proposed to consider newborn seahorses as larvae and to expand to the others seahorses’ species the hypothesis of a larval phase after the release. The doors are opened to the larval nutrition in seahorse, and their digestive biochemistry was proposed as effective tool to identify the specific nutritional requirements for each species.

# Introducción

## 1.1 Los caballitos de mar de la leyenda a la realidad

Los caballitos de mar son peces óseos que deben su nombre común a la forma particular de la cabeza, dotada de un hocico tubular que se parece mucho a la de un caballo, y al medio donde viven, aguas marinas y salobres. En la literatura existen más de 140 especies (Eschmeyer y Fricke 2016), pero entre estas solo 38 resultan realmente diferentes por morfología, genética y/o geografía y están respaldadas por investigaciones bien detalladas (Lourie *et al.* 2016).

Todos los caballitos de mar pertenecen al género *Hippocampus*, (Rafinensque 1810) procedente de las palabras del griego antiguo "hippos" (caballo) y "kampos" (monstruo marino) (Smith 1870).

Son miembros de la familia *Syngnathidae*, que procede del griego antiguo "syn" (fusionados) y "gnathus", (maxilas) (Ahnesjö y Craig 2011). Esta familia incluye a los peces pipa, caballos pipa, dragones de mar y caballos pipa pigmeos (Wilson y Orr 2011).

Estas criaturas, representadas en la mitología como mitad caballos y mitad peces, servían a los dioses como Poseidón (Scales 2009) (mitología griega) o Manannan mac Lir (mitología celta) (Conway 2001) para atravesar los mares. Símbolo de imaginación, espíritu creativo, coraje, y de respeto por el mar (Conway 2001) son considerados signo de fuerza y poder y han servido como fuente de inspiración a poetas y escritores (Faleiro 2011).

Además, los caballitos de mar son objeto de superstición. Las historias de ninfas cabalgando sobre caballos de mar (Nereidas) que acudían al rescate de marineros náufragos o en apuros (Demetriou 2010; Jurkić 2012), los convirtieron en un signo de buena suerte para los marinos que suelen tatuárselo como protección.

Esta presencia de la figura del caballito de mar a lo largo de la historia es debida a la fascinación que estos pequeños animales causan en el hombre por su morfología y biología reproductiva. Fascinación que sigue intacta hasta la actualidad. De hecho, el interés por su biología y cultivo se ha visto incrementar en los últimos años, debido a razones económicas, culturales, científicas y educacionales (Olivotto et al. 2011).

## 1.2 Características morfológicas

Los caballitos de mar fueron denominados Lophobranchii por Cuvier (1817) debido a que sus branquias tienen un aspecto de pequeños penachos con lóbulos de forma redondeada e irregular. Son peces de talla variable entre un mínimo de 1,1 cm de *Hippocampus satomiae* hasta un máximo de 35 cm de *Hippocampus abdominalis* (Lourie *et al.* 2016).

Como resultado de la evolución, las especies pertenecientes a esta familia han adoptado una posición vertical y todos tienen como base un mismo modelo morfológico y funcional (Figura 1.1). Muestran dimorfismo sexual (Foster y Vincent 2004; Koldewey y Martin-Smith 2010) y es fácil distinguir los dos sexos tras la madurez sexual por la presencia de la bolsa incubadora en la parte ventral del tronco de los machos y el abdomen más curvo de las hembras (Lourie et al 2004).

Los caballitos de mar son peces marinos inusuales, debido a la presencia de un cuello relativamente largo que permite que tengan la cabeza en ángulo recto respecto al cuerpo (Koldewey y Martin-Smith 2010).

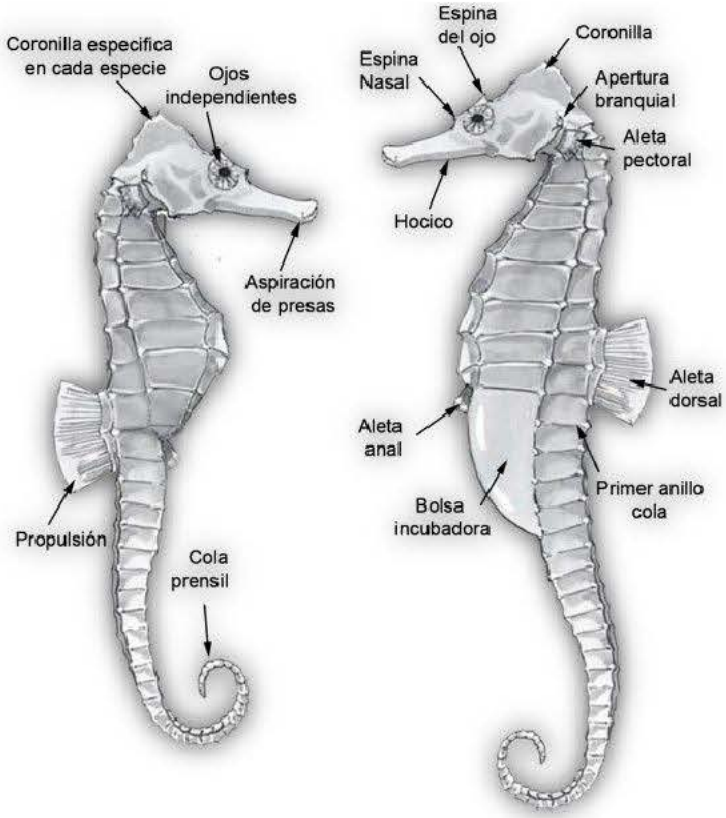



**Figura 1.1** Morfología de caballito de mar (modificada de Indiviglio 2001).

El hocico, sin dientes, (Foster y Vincent 2004; Keivany 2014) varía de tamaño en función de la especie, es tubular y relativamente largo respecto a la medida de la cabeza. Se trata de un aparato sofisticado y especializado en la captura de presas vivas a través de una tipología de alimentación llamada "pivot feeding", que se caracteriza por la succión que el caballito de mar es capaz de crear en el interior de su hocico, donde la presa resulta aspirada gracias a la compresión y distensión de algunos músculos (Van Wassenbergh *et al.* 2013). Los ojos se pueden mover de forma independiente, habilitando a estos animales a optimizar el área de búsqueda de la presa (Land 2014). En adultos se

ha observado presencia de fóvea en la retina (Mosk *et al.* 2007; Lee y O'Brien 2011), consiste en un área especializada asociada al desarrollo de una visión aguda y precisa. Los caballitos de mar se consideran depredadores visuales (Lee y O'Brien 2011; Storero y Gonzales 2009), que se alimentan de pequeños crustáceos (Kanou y Kohno 2001; Teixeira y Musick 2001; Woods 2002; Kitsos *et al.* 2008) anfípodos y principalmente misidáceos (Woods 2002; Foster y Vincent 2004).

En la parte apical de la cabeza poseen una cresta característica para cada especie (Lourie *et al.* 2016), que constituye un elemento para diferenciar caballitos de especies similares (Lourie y Randall, 2003; Lourie y Kuiter 2008) e incluso, poblaciones diferentes de la misma especie como *Hippocampus hippocampus* europeo y norteafricano (Lourie *et al.* 2004) o ejemplares de la misma especie (Freret-Meurer *et al.* 2013).

No presentan estómago y poseen un largo intestino que va desde el esófago hasta el recto (Wardley 2006; Segade *et al.* 2015). A diferencia de otros teleósteos, no poseen escamas, sino una fina lámina de piel sobre una serie de placas óseas que se visualizan como anillos en torno al tronco y a la cola (Lourie *et al.* 2004). Estos anillos óseos varían según las especies, y las placas forman un dermoesqueleto continuo que confiere flexibilidad a la estructura. Además, se añaden muchas espinas, sobre las cuales pueden crecer filamentos de piel que le ayudan a camuflarse con el medio natural (Lourie *et al.* 2004; Curtis 2006).

Poseen un sistema vertebral altamente especializado. Está constituido por elementos vertebrales que presentan cambios morfológicos específicos. Se trata de estructuras metaméricas organizadas que permiten la natación en posición vertical (Bruner y Bartolino 2008). La

cola es una estructura prensil que usan para mantenerse anclados al sustrato, y así poder cazar y camuflarse (Porter 2013). También resulta fundamental para el macho en el momento del nacimiento de las crías (Lipton y Thangaraj 2014). El esqueleto apendicular está formado por las aletas pares, que corresponden a las aletas pectorales, que tienen función estabilizadora y de ascenso en la columna de agua (Breder y Edgerton 1942), y por las aletas impares: la dorsal con función locomotora, y la anal con tamaño reducido para facilitar la expulsión de heces, huevos y crías (Gardner 2003). Como consecuencia de la evolución las aletas pélvicas están ausentes (Foster y Vincent, 2004), y la aleta caudal sólo aparece en la cría de algunas especies (Kanou y Kohno 2001; Choo y Liew 2006; Franz-Odendaal y Adriaens 2014).

## 1.3 Características reproductivas

Generalmente en el reino animal los machos compiten por elegir la hembra mejor según las exigencias reproductivas.

En singnátidos al quedarse embarazado el macho, se pensaba que también los roles sexuales podrían estar invertidos, es decir, que fueran las hembras las que compitieran por el macho. Se ha observado que en especies monógamas no se registra esta inversión, mientras que especies polígamas sí (Rosenqvist y Berglund 2011), por lo que si se presenta una inversión de roles sexuales es eventualmente debida a la mayor densidad de ejemplares. En un primer momento se pensaba además que todas las especies de caballitos de mar podrían ser monógamas, con machos que aceptan los huevos de solo una hembra durante toda la estación, o el periodo reproductivo (Wilson y Martin-Smith 2007; Woodall *et al.* 2011), aunque en algunos estudios se ha

observado poligamia (Wilson y Martin-Smith 2007; Naud *et al.* 2009; López *et al.* 2015).

Probablemente la monogamia es una estrategia reproductiva resultante del aumento de éxito reproductivo en animales que se encuentran a baja densidad y con un estilo de vida sedentario, dependientes del mimetismo para combatir la depredación (Foster y Vincent 2004). Los factores que favorecen la poligamia se ven reducidos por otros factores que fomentan la monogamia, como son el cuidado paternal (Wilson y Martin-Smith 2007) y la sincronización de reproducción entre macho y hembra, que mejora con la familiaridad entre la pareja y reduce las oportunidades de apareamientos múltiple (Vincent *et al.* 2004).

Otra peculiaridad de esta familia es el cortejo. Estas especies muestran saludos diarios, "daily greetings", durante los cuales cambian de color para comunicar con la pareja (Vincent y Sadler 1995; Otero-Ferrer 2012). Estos saludos diarios resultan necesarios en este proceso de sincronización (Vincent y Sadler 1995). Con falta de sincronización, en el momento de la transferencia los huevos pueden caer fuera de la bolsa, ocasionando puestas perdidas (Foster y Vincent 2004).

Los caballitos de mar llevan a cabo un cortejo muy específico, ascendiendo y descendiendo en espiral por la columna de agua, hasta que la hembra a través del ovopositor (Gonçlaves y Oliveira 2010), una estructura retráctil que aparece a través de la apertura urogenital (Silveira y Fontoura 2010), deposita los huevos en la bolsa incubadora del macho. Los movimientos están sincronizados y la transferencia de los huevos y su fecundación se realizan al mismo tiempo (Figura 1.2).

El estudio de Van Look *et al.,* (2007) ha demostrado la imposibilidad física del macho para fertilizar los huevos en el interior de la bolsa incubadora a través de los conductos espermáticos y que los espermatozoides deben salir de la bolsa incubadora para poder fertilizar los huevos. La fecundación debe ser considerada, por lo tanto, como externa, ya que tiene lugar en el agua y no en el interior de la bolsa del macho como se creía anteriormente.

En una pareja bien sincronizada, mientras que el macho lleva a cabo la maduración de los embriones dentro de la bolsa incubadora la hembra madura nuevos ovocitos; generalmente el mismo día que el macho expulsa los recién nacidos, la hembra deposita nuevos huevos en la bolsa incubadora del macho.

**Figura 1.2** Deposición de huevos en la bolsa incubadora del macho en *Hippocampus whitey* (b), *Hippocampus breviceps* (b) y expulsión de la cría en *Hippocampus spp.* (oceanwildeimages.com)

## 1.4 La difícil situación de las poblaciones naturales de caballitos de mar

Debido a su atractivo aspecto y a su particular biología, los caballitos de mar consiguen atraer del público en general. Actualmente son una de las especies bandera presentadas como símbolo de protección de la

naturaleza, que favorecen proyectos para su estudio y conservación e implementan programas de conservación.

Una de las consecuencias más significativas de la sobreexplotación de los recursos marinos y de la inadecuada gestión de los espacios naturales marinos, es la reducción progresiva de su biodiversidad. En las zonas costeras, donde la actividad humana es más frecuente, los caballitos de mar constituyen un grupo en el que se manifiestan perfectamente las consecuencias de la sobreexplotación y de la degradación del medio ambiente (Foster y Vincent 2004; Martin-Smith *et al.* 2004; Vincent *et al.* 2011).

Los principales problemas a los que se enfrentan son: la eutrofización que afecta a los hábitats naturales de estas poblaciones (Sundin *et al.* 2011), la contaminación (Scobell y Mac Kenzie 2011), y el cambio de temperatura de los océanos que afecta toda la biología reproductiva (Ahnesjö *et al.* 2008).

Además, el comercio de estos animales se caracteriza por su utilización en algunas medicinas tradicionales, especialmente la china y sus derivadas (medicina tradicional japonesa y coreana), en las cuales son utilizados en tratamientos contra el asma, la disfunción sexual, la depresión y otras dolencias (Rosa *et al.* 2013).

China provocó un aumento de la demanda de estos animales al ritmo de su economía, gracias también al reconocimiento de su medicina tradicional como una opción válida de asistencia sanitaria por parte de la Organización Mundial de la Salud (Kumaravel *et al.* 2012). En menor medida la sobreexplotación se debe también al comercio de ejemplares vivos para acuarios marinos, la venta de individuos secos como suvenires, e incluso como alimento (Martin-Smith y Vincent

2006; Rosa *et al.* 2011). A esta problemática, se le añaden los casos de captura accidental, especialmente por el efecto causado por la pesca de arrastre (Lawson 2014, Gristina *et al.* 2015).

La difícil situación de las poblaciones naturales de los caballitos de mar fue reconocida oficialmente por los países miembros de la CITES, el 13 de noviembre 2002, cuando decidieron votar a favor de la inclusión de todas las especies de caballitos de mar en el en el Apéndice II del Convenio (con entrada en vigor a partir de mayo 2004). Las especies catalogadas en el Apéndice II de CITES, son las que muestran poblaciones naturales consideradas en riesgo, o que podrían estarlo a causa del comercio internacional. Además, en 2003 las especies de caballitos de mar fueron incluidas también en la Lista Roja de la UICN (Unión Internacional para la Conservación de la Naturaleza). Esta lista constituye el inventario mundialmente reconocido sobre el estado de amenaza de las especies.

## 1.5 Medidas de conservación de los caballitos de mar

Para la conservación de los caballitos de mar es necesario plantear un enfoque interdisciplinar, que incluya la protección de los ecosistemas, la gestión de la pesca y la regularización comercial (Vincent *et al.* 2011). Además, el cultivo puede ser considerado un método alternativo y necesario para reducir la presión sobre poblaciones naturales (Payne y Rippingale 2000; Lin *et al.* 2008; Faleiro 2011; Murugan *et al.* 2011).

Entre los objetivos de la acuicultura también se incluye el cultivo de especies con un destino diferente al consumo humano, apoyando actividades como la pesca deportiva, la acuariología y la repoblación.

Gracias a la inversión en investigación y una mayor presión sobre la sostenibilidad del comercio de peces ornamentales, se están desarrollando métodos para mejorar el cultivo en cautividad de muchas especies de peces marinos de arrecife (Rhyne y Tlusty 2012). Actualmente el comercio de peces ornamentales mueve más de dos billones de peces vivos en todo el mundo. En el caso de los peces de agua dulce más del 90% son criados en cautividad, mientras que la mayoría de las especies marinas procede de captura (Molina y Segade 2014; Olivotto et al. 2016). La acuicultura de peces ornamentales persigue un producto final de lujo, dirigido al consumo en países industrializados (Leal et al. 2015; Rhyne et al. 2012), pero mantiene la finalidad de evitar la sobreexplotación de los recursos naturales, especialmente de aquellas especies consideradas amenazadas.

Actualmente la mayoría de la acuicultura dedicada a los caballitos de mar implica operaciones a pequeña escala en países desarrollados, empleando poco personal y vendiendo animales vivos para el mercado de la acuariología (Koldewey y Martin-Smith 2010). La demanda de mercado que tienen estos animales es mayor que la oferta, y las consecuencias son la sobreexplotación de los stocks silvestres (Murugan *et al.* 2009) y su declive, principalmente en las aguas del Atlántico occidental e Indo Pacificas (Molina y Segade 2014).

El protocolo de cultivo de individuos adultos de caballito de mar está estandarizado, se han mantenido en cautividad y con éxito distintas especies de *Hippocampus*, tanto tropicales como templadas, y de distinta procedencia (Australia, Europa, África y América). Además, se ha conseguido cerrar el ciclo en cautividad de varias especies

(Wilson y Vincent 2000; Hora y Joyeux 2009; Murugan *et al.* 2009; Otero-Ferrer 2012; Blanco 2014).

La producción en cautividad de caballitos de mar es una actividad reciente (Molina y Segade 2014; Planas 2008) y todavía se necesita tiempo para resolver problemas de cultivo (Olivotto *et al.* 2011). Por la estrategia reproductiva y el elevado desarrollo de sus crías podría ser relativamente fácil cultivar caballitos de mar, pero estos sufren cambios ontogénicos que todavía se desconocen, y que requieren una cadena de alimento vivo en las primeras semanas de vida (Koldewey y Martin-Smith 2010). Esta falta de conocimiento se presenta principalmente en la biología de los recién nacidos, y en particular en el desarrollo durante las primeras fases de vida y en su bioquímica digestiva. Se ha demostrado que cultivar recién nacidos en pequeña escala con alimentos de más calidad, y probablemente más biodigeribles, lleva a una menor tasa de mortalidad (Hora y Joyeux 2009; Payne y Rippingale 2000; Zhang *et al.* 2015; Sales *et al.* 2016). Sin embargo, los resultados son diferentes cuando se alimentan con prensas vivas (con enriquecedores) comúnmente utilizadas en acuicultura para una producción a gran escala como son *Artemia* y rotífero. En el último caso, sus primeras etapas de vida están caracterizadas por una alta tasa de mortalidad (Planas *et al.* 2008; Olivotto *et al.* 2008; Murugan *et al.* 2009; Otero-Ferrer *et al.* 2010; Lin *et al.* 2012). Existen varios trabajos que se centran en el cultivo de los recién nacidos, testando diferentes dietas (Olivotto *et al.* 2008; Otero-Ferrer *et al.* 2010; Zhang *et al.* 2015), densidades de cultivo, temperaturas (Lin *et al.* 2010), concentraciones de oxígeno, intensidades luminosas, frecuencias de alimentación, fotoperiodos

(Shubert *et al.* 2016), salinidades (Hora *et al.* 2016). Pero son pocos los trabajos que intentan explicar los cambios en la ontogenia durante las primeras fases de vida, y que probablemente son responsables al menos en parte de la elevada tasa de mortalidad observada, aunque se han descrito sus características morfológicas (Silveira 2000; Azzarello 1990; Wardley 2006; Choo y Liew 2006; Kanou y Kohno 2001), y se ha intentado analizar los requerimientos digestivos (Blanco *et al.* 2015). Esta falta de información sobre las primeras fases de vida de los caballitos de mar es probablemente debida a que, al ser considerados juveniles teóricamente presentan las mismas capacidades digestivas de los adultos. En general, las mayores dificultades para la producción de peces marinos se centran en las primeras fases de vida debido a la vulnerabilidad y a los estrictos requerimientos bióticos y abióticos de las larvas para sobrevivir, desarrollar y crecer adecuadamente (Hamre et al. 2013) y el caballito de mar, en este sentido no resulta ser una excepción.

## 1.6 Importancia del conocimiento de las primeras fases de desarrollo en la producción de peces

El ciclo de vida en los peces incluye varios periodos en los que los animales generalmente pueden presentar morfología o hábitos distintos. Debido a la diversidad y complejidad del mundo marino existe una gran diferencia en la terminología referente a las etapas, fases o intervalos del primer periodo de vida de los peces marinos (Helfman *et al.* 2009). Dichas fases se caracterizan por las mayores tasas de crecimiento y mortalidad y por los mayores cambios morfológicos y celulares que tienen lugar durante las mismas

(Zambonino Infante y Cahu 2001). Hasta el momento, las fases del ciclo de vida se han clasificado mediante subdivisiones que muestren eventos generales reconocibles (Figura 1.3).

En la clasificación más simple a estos periodos se atribuyen los nombres de huevo (que después de la fertilización o la activación contiene el desarrollo del embrión), periodo embrionario (después del cual eclosiona la larva), periodo larvario (en el cual el animal se transforma en juvenil), juvenil (con fenotipo de adulto, pero sin capacidad para reproducirse) y adulto (Balon 1999).

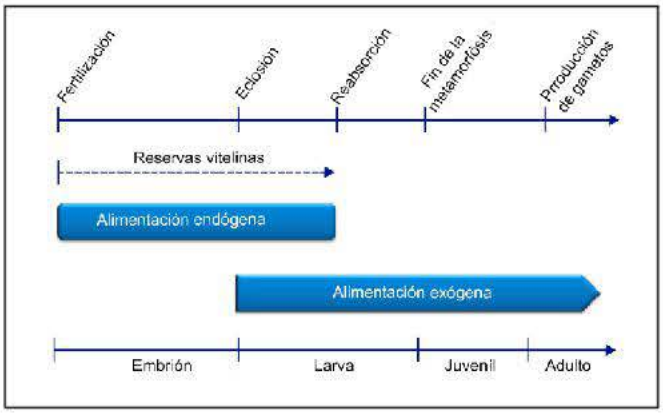



**Figura 1.3** Estadios de desarrollo en peces marinos (modificado de Betancor 2012)

Los tipos de producción en acuicultura se pueden clasificar de acuerdo con la serie de etapas del ciclo biológico que abarque el sistema productivo y de manera general consta de las siguientes fases: reproducción y producción de huevos; fase de larva y post-larva o alevín; fase juvenil y adulto (Orvay 1993).

En cautividad, para cerrar el ciclo de vida de una especie, es necesario tener el control de su biología reproductiva y conseguir que los

ejemplares nacidos en cautividad se reproduzcan bajo condiciones controladas (Hora y Joyeux 2009). Sin embargo, la obtención de la reproducción estable y controlada de una especie para consumo humano, o como apoyo de actividades como la pesca deportiva, la acuariología y la repoblación, requiere de un conocimiento mucho más profundo de la especie. Para tener éxito en un cultivo marino a gran escala es necesario controlar no solo la biología reproductiva, sino también conocer la biología y ontogenia de las primeras fases de vida, con el objetivo de conseguir la máxima supervivencia de la cría (Qin 2013). En condiciones de cultivo, debido al aporte regulado de alimentos y a la ausencia de depredadores la tasa de supervivencia en la cría de peces es más alto respecto al medio marino (Helvik *et al.* 2009), donde de los millones y millones de larvas producidas más del 99% muere durante el primer año de vida por el efecto combinado de inanición y depredación (Helfman *et al.* 2009). A pesar de ello se registra una alta tasa de mortalidad en especies donde no se conoce la biología de su desarrollo fisiológico y las necesidades derivadas de éste, tanto para crear un verdadero cuello de botella en la reproducción a grande escala de algunas especies marinas, entre las cuales todas las especies de caballito de mar (Olivotto *et al.* 2008, 2011).

## 1.7 El periodo larvario o "periodo crítico"

En la transición desde la fase embrionaria hasta la fase adulta se diferencian tres modelos de desarrollo, que puede ser: indirecto, transitorio y directo (Balon 1999).

El desarrollo indirecto constituye el modelo mayoritario en la naturaleza, en el cual, existe un periodo larvario en el que el individuo

(o larva) se transforma gradualmente en juvenil mediante una metamorfosis. Durante el desarrollo transitorio, se observa un alevín con vestigios de larva (salmónidos), mientras que el desarrollo se considera directo cuando no existe una fase larvaria propiamente dicha (*Cyphotilapia frontosa* y *Gambusia affinis,* Flegler-Balon 1989; *Labeotropbeus,* Balon 1977; *Pterapogon kauderni* Vagelli 2004).

**Tabla 1.1** Modelos de desarrollo (modificada de Balon 1999)

| Indirecto | Transitorio | Directo |
|---|---|---|
| **Embrión** | **Embrión** | **Embrión** |
| *segmentación* | *segmentación* | *segmentación* |
| *embrión* | *embrión* | *embrión* |
| *embrión libre* | *embrión libre* | *embrión libre* |
| **Larva** | **Alevín** | |
| *aleta primordial* | *(larva vestigial)* | **Juvenil** |
| *aletas formadas* | *alevín* | |
| **Juvenil** | **Juvenil** | **Adulto** |
| *Transición juvenil* | *Parr* | |
| **Adulto** | *Smolt* | |
| **Senescencia** | *Juvenil* | **Senescencia** |
| | **Adulto** | |
| | **Senescencia** | |

El termino larva procede del latín y significa espectro o mascara. No fue hasta 1735, gracias a Linneo, cuando se le atribuyó un significado zoológico a este término, debido al hecho de que la oruga es solo la máscara del insecto perfecto. Con este término se indica algo "irreconocible" (Flegler-Balon 1989), y se usa en ictiología debido a que las larvas tienen una morfología y fisiología muy diferentes en comparación con los adultos.

El periodo larvario en acuicultura ha sido siempre considerado como un "periodo crítico" (Fabre-Domergue y Biétrix 1897; Hjiort 1914). El periodo larvario corresponde al periodo de vida que transcurre entre la eclosión y el fin de la metamorfosis, en el cual se produce

progresivamente la aparición y diferenciación de las características de los animales adultos (Power *et al.* 2008).

Los factores que influyen en las fluctuaciones de supervivencia de las larvas que pueden ser 1) externos abióticos (físicos: temperatura, iluminación. flujo de agua; químicos: amonio, salinidad, pH) y bióticos (presencia de zooplancton, fitoplancton o microrganismos como virus y bacterias), o 2) internos (genéticos, etológicos, biológicos nutricionales). De todos los factores ante mencionados, la alimentación (proceso de captura e ingesta) y la nutrición (proceso para la obtención de energía necesaria a crecer, mantener y reparar los tejidos) son los que más afectan la supervivencia y el crecimiento de las larvas de peces marinos (Civera-Cercedo et al. 2004). La falta de conocimientos acerca de los requerimientos necesarios durante este periodo tan delicado, tras haber consumido las reservas vitelinas, se traduce en una alta mortalidad al comienzo de la alimentación exógena. Además, es importante considerar que las larvas tras la eclosión requieren un alimento adaptado a sus exigencias biológicas desde el primer momento, debido a que fisiológicamente el sistema digestivo no está completamente desarrollado y posee una actividad enzimática menor.

En las especies de peces marinos cultivados comercialmente estudios sobre la ontogenia temprana de enzimas digestivas han sido realizados para aproximar y determinar la capacidad digestiva (Civera-Cercedo et al. 2004). La capacidad digestiva depende de la maquinaria enzimática con la que cuentan, y aun estando disponibles alimentos, si estos no cubren todos los requerimientos nutricionales de las larvas, desde el primer momento, estas estarán destinadas a debilitarse y morir. En este sentido Blaxter y Hempel en 1963 acuñaron el término

"punto de no retorno" para describir el momento en el que las larvas, como resultado de la inanición, están demasiado debilitadas como para poder aprovechar en un segundo momento de alimentos adecuados aun estando disponibles, y por eso Cushing (1975) propuso la hipótesis "Match-Mismatch", que sugiere que el momento de reproducción en muchos peces marinos ha evolucionado para que la eclosión de las larvas se produzca en lugares donde la comida está disponible.

## 1.8 La metamorfosis en peces

La metamorfosis es un concepto inherente e integrador, que afecta tanto a la biología del desarrollo como a la ecología, a la evolución de la vida, la fisiología, la biología celular y la conservación (Bishop *et al.* 2006). Se trata de una serie de eventos superpuestos impulsados por una regulación endocrina que tienen como resultado cambios fisiológicos, morfológicos, alimentarios y de hábitat (Pinto 2013). Si analizamos la etimología, el termino metamorfosis procede del griego "*meta*" (cambio) y "*morphe*" (forma), por ello, los cambios morfológicos acompañados por cambios de hábitat o de comportamiento tendrían que ser los criterios principales para definir la metamorfosis en larvas de peces (Rousseau y Dufur 2012).

La metamorfosis se manifiesta cuando los animales gradual o drásticamente dejan las características larvarias y adquieren las características de los adultos. En algunos casos es un proceso muy marcado y es sencillo establecer el periodo en el cual se manifiesta la metamorfosis, como en el caso de los peces planos, sin embargo, en otras especies es un proceso más dificil de identificar. Además, aparte de la dificultad de identificar cuándo se produce la metamorfosis, hay

que sumar la cantidad de variables que pueden considerarse parte o no de este proceso, como el cambio de hábitat, los cambios morfológicos, hormonales y de alimentación (Bishop *et al.* 2006).

En el caso de los caballitos de mar, la particular biología reproductiva de la especie da lugar a que no se le se identifique un periodo larvario en su desarrollo; la cría se suele definir juvenil debido al elevado grado de desarrollo que presenta en el momento de salir de la bolsa paterna (Koldewey y Martin-Smith 2010).

## 1.9 Los caballitos de mar, ¿larvas o juveniles?

Aunque los tres modelos de desarrollo parecen ser claros, la gran diversidad de los animales marinos y sus diferentes estrategias de reproducción pueden resultar, a veces, motivo de confusión. Este es el caso de los caballitos de mar, animales que han adoptado una estrategia de reproducción muy particular entre los vertebrados.

Los caballitos de mar son los representantes de la única familia de vertebrados en la que el desarrollo embrionario tiene lugar en una bolsa incubadora paterna (Whittington 2015). La bolsa incubadora tiene las funciones de proteger, osmorregular, nutrir y transmitir compuestos antibacterianos a embriones y larvas (Melamed *et al.* 2005) tras el cortejo y la deposición de los ovocitos por parte de la hembra (Partridge *et al.* 2007; Ripley 2009). Al final de la gestación el macho generalmente de noche o en las primeras luces del día, expulsa fuera de su bolsa incubadora a las crías, agarrándose con la cola al sustrato y realizando movimientos para facilitar su salida (Hale 1996). El macho de caballito de mar libera un número elevado de recién nacidos, que parecen perfectamente formados, capaces de depredar desde el

momento del nacimiento. En la literatura no son considerados como larvas, los recién nacidos se consideran principalmente "juveniles" (Payne y Rippingale 2000; Kanou y Kohno 2001; Woods 2003; Murugan *et al.* 2009; Sommer *et al.* 2012; Shubert *et al.* 2016) o se denominan como "crías" (Stölting y Wilson 2007; Lin *et al.* 2012; Faleiro *et al.* 2016), "alevines" (Chang y Southgate 2001), "recién nacidos-juveniles" (Falerio y Narciso 2011; Sales *et al.* 2016) "juveniles de edad temprana" (Martinez-Cardenas y Purser 2012, 2016).

Sin embargo, se observan algunas características larvarias, como una primera fase planctónica que puede durar desde las dos semanas hasta un mes (Woods 2003; Job et al. 2006), la presencia de elementos ausentes en los adultos, como la aleta caudal (Choo y Liew 2006; Franz-Odendaal y Adriaens 2014) o la ausencia de estructuras morfológicas muy importantes presentes en los adultos, como las espinas, los anillos óseos y las placas óseas (Silveira 2000; Franz-Odendaal y Adriaens 2014). Además, el índice de mortalidad en caballitos de mar cultivados en cautividad disminuye después de las primeras dos semanas de vida en el medio marino (Wilson and Vincent 2000; Woods 2000; Otero-Ferrer *et al.* 2010; Hora y Joyeux 2009) como se observa en otros teleósteos al llegar al periodo juvenil.

## 1.10 Técnicas para el estudio del desarrollo en larvas

La cría en masa de grandes cantidades de larvas es uno de los hitos para el cultivo a escala comercial de cualquier pez marino. En la actualidad, los esfuerzos de investigación se dirigen a determinar las condiciones que incrementen la sobrevivencia y viabilidad de las

larvas, así como a caracterizar la evolución fisiológica de las mismas (Alarcón-López y Martínez-Díaz 1998). En peces generalmente el desarrollo embrionario y la eclosión ocurren en el medio marino donde las larvas dependen completamente de sus reservas vitelinas hasta la apertura de la boca. Conocer los estadíos de desarrollo y determinar cuál se sitúa el animal, sobretodo en animales vivíparos como el caballito de mar, es el primer paso para planear el régimen alimenticio e intentar así cubrir los requerimientos fisiológicos necesarios para su desarrollo. **La observación y la medida** de los caballitos de mar dentro de la bolsa incubadora, aportan datos básicos para determinar el estadío de desarrollo de estos animales al nacer. El estudio de la morfología y funcionalidad del tracto digestivo en larvas de peces es muy relevante (Govoni, 1986), debido a que de esta forma se establecen indicios sobre la posibilidad de ofrecer alimento (vivo o inerte), para lograr el inicio de la alimentación exógena (Gisbert *et al.* 2004; Yúfera y Darias 2007) y establecer si la alimentación empleada es la adecuada. Una de las herramientas disponibles para este fin son los **estudios histológico e histoquímico,** utilizados para describir el desarrollo de los órganos y los cambios celulares que se presentan en los primeros días de vida de un pez, durante y posteriormente a la absorción del saco vitelino (Sarasquete *et al.* 1995; Ribeiro *et al.* 1999; Zambonino Infante y Cahu 2001; Sanchez-Amaya *et al.* 2007). Estas herramientas han sido utilizadas en un gran número de estudios, y en particular en todas las especies cultivadas a gran escala. Para incrementar el cultivo y la producción de juveniles también es necesario conocer el desarrollo osteológico de las primeras etapas de vida. El **estudio del desarrollo osteológico** se utiliza principalmente para controlar el correcto

desarrollo morfológico y corregir eventuales errores de alimentación, o métodos de cultivo larvario, que puedan provocar deformidades (Koumoundouros *et al.* 1997; Gavaia *et al.* 2002; Sfakianakis *et al.* 2004). Además, la morfología externa estudiada detalladamente puede confirmar o rechazar la presencia de metamorfosis en larvas de peces. En cuanto al **estudio enzimático**, se basa en el conocimiento detallado de la fisiología digestiva en las distintas etapas de vida larvaria mediante la determinación de las actividades enzimáticas digestivas como son las actividades proteasa, amilasa, fosfatasa, etc. Estas actividades resultan tan importantes ya que se ha comprobado que el nivel de actividad de algunas enzimas actúa como un buen indicador del estado nutricional de la larva, de manera que los datos obtenidos pueden ser relevantes para establecer el momento óptimo para realizar el destete (Moyano *et al.* 1996; Kolkovski 2001; Cara *et al.* 2003) .

## 1.11 Objetivos

El caballito de mar se posiciona como una nueva especie con interés para la acuicultura por su creciente demanda de mercado y la sobreexplotación de los stocks naturales, sin embargo, el cultivo en cautividad afronta importantes dificultades para su desarrollo a gran escala, entre las que destaca la baja supervivencia en las etapas iniciales de desarrollo. Precisamente este periodo es el menos estudiado de su desarrollo y en el que aparecen menos trabajos publicados por la dificultad de su estudio en el medio marino. Por ello, la presente tesis doctoral aborda el estudio de las primeras etapas de vida en dos especies de caballito de mar, la especie presente en las costas de las islas Canarias, *H. hippocampus,* y una de las más comercializadas,

*Hippocampus reidi*. Debido a la falta de consenso en la denominación de los caballitos de mar en las primeras fases de vida, se planteó como objetivo general de la presente Tesis Doctoral el estudio de los recién nacidos de las especies citadas de caballito de mar para dilucidar si poseen una fase larvaria fuera de la bolsa incubadora del macho. Además, se planteó también analizar la ontogenia de la bioquímica digestiva para aportar nuevos elementos con el fin de colaborar en la mejora del diseño de los protocolos de alimentación para estas especies, y como consecuencia, conseguir la optimización del cultivo. Para poder alcanzar el objetivo general anteriormente expuesto, se plantearon los siguientes objetivos específicos:

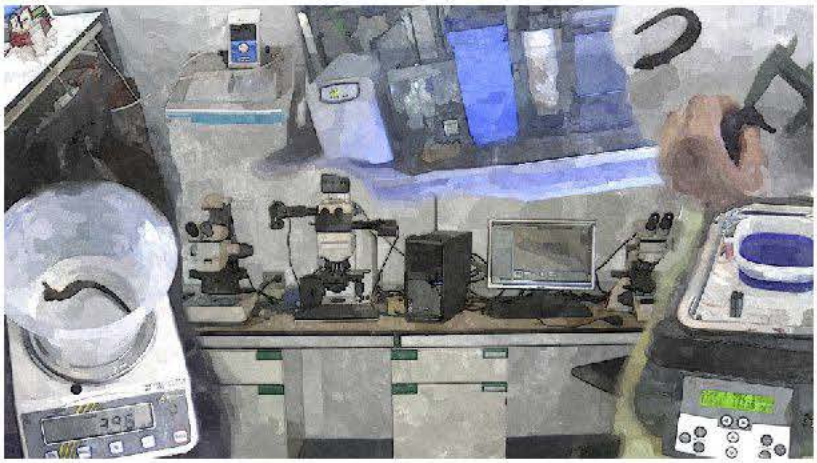
1 Estudiar detalladamente el desarrollo de *H. reidi* dentro de la bolsa incubadora y comparar las características morfológicas de los recién nacidos en el momento de salir de la bolsa incubadora con las características morfológicas de los adultos.

2 Estudiar detalladamente las primeras fases de vida de *H. reidi* y *H. hippocampus* desde los puntos de vista histológico y osteológico para aclarar controversias de denominación.

3 Aumentar los conocimientos sobre la ontogénesis osteológica de *H. hippocampus* y la ontogénesis histológica de *H. reidi* estableciendo los puntos críticos durante el desarrollo, tanto a nivel osteológico como a nivel histológico.

4 Analizar la ontogenia y caracterización enzimática del sistema digestivo durante los primeros 20 días de vida de *H. reidi*.

5 Utilizar por primera vez la bioquímica digestiva como herramienta indicadora del estado nutricional de los caballitos de mar.



# Material y métodos

## 2.1 Organización de la tesis

De acuerdo a los objetivos propuestos, la presente Tesis Doctoral se desarrolla sobre cuatro estudios que analizan el periodo de vida de los caballitos de mar que abarca desde la fertilización del huevo hasta los primeros 30 días de vida después de la expulsión de la bolsa incubadora paterna. Un diagrama esquemático de la tesis es presente en la figura 2.1.

**Con el primer estudio** se describe el desarrollo indirecto de los caballitos de mar y los cambios morfológicos que ocurren en el interior de la bolsa incubadora paterna hasta la expulsión. El desarrollo del embrión y de la larva de edad tempana (que generalmente no se puede observar en caballitos de mar) nos ayuda a comprender las diferencias en el desarrollo entre animales ovíparos y vivíparos, permitiendo comparar los caballitos de mar con larvas de otras especies de teleósteos marinos, además facilita la observación de las diferencias entre recién nacidos y adultos.

**Con el segundo estudio** se observa el desarrollo de las estructuras internas para evaluar cuál era el grado de organogénesis de los recién nacidos y se describen los cambios morfológicos internos hasta el trigésimo día de vida fuera de la bolsa incubadora. Este estudio nos permite relacionar caballitos de mar los recién nacidos con las larvas de otros teleósteos marinos, comparando el grado de desarrollo y estableciendo la presencia o la ausencia de características larvarias en esta fase tan delicada.

**Con el tercer estudio** se observa el desarrollo de las estructuras externas evaluando el grado de osificación de los recién nacidos y

describiendo los cambios morfológicos externos hasta el trigésimo día de vida fuera de la bolsa incubadora. Este estudio nos permite relacionar caballitos de mar los recién nacidos con las larvas de otros teleósteos marinos. Además, se describe detalladamente el fenotipo de los recién nacidos de caballitos de mar y se compara con el fenotipo de los adultos.

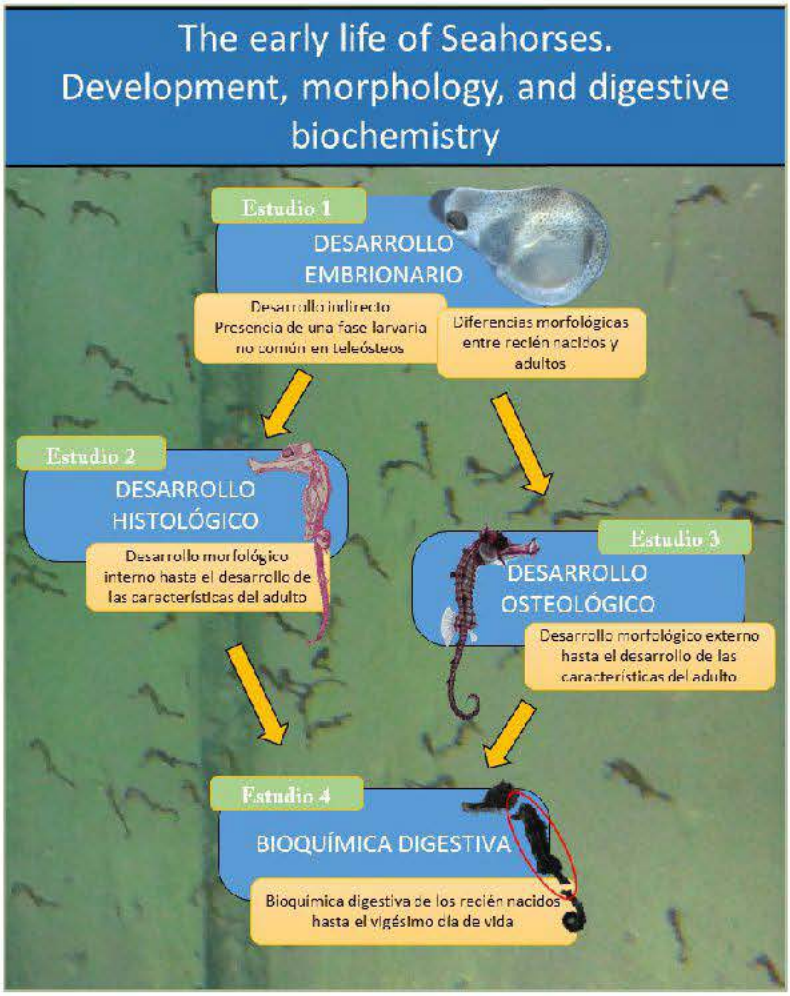



**Figure 2.1** Diagrama esquemático de los estudios incluidos en la tesis

**Con el cuarto estudio** se describe la bioquímica digestiva para evaluar las capacidades del sistema digestivo de los recién nacidos hasta el vigésimo día de vida, y se proponen parámetros bioquímicos para establecer el estatus nutricional de los caballitos de mar durante su desarrollo. Este es un periodo en el cual se debe hacer énfasis en los requerimientos mínimos necesarios para asegurar la supervivencia y mantener el crecimiento de las larvas. El estudio de la bioquímica digestiva permite describir la fisiología digestiva y las actividades enzimáticas y permite aportar conocimientos con el fin de optimizar la dieta según las exigencias de los recién nacidos.

## 2.2 Instalaciones para el cultivo de caballitos de mar

El cultivo de los caballitos se llevó a cabo en las instalaciones del Instituto Universitario Ecoaqua en la sala de acuarios. El sistema de cultivo permitía gran flexibilidad de trabajo teniendo la capacidad de suportar condiciones de cultivo con circulación abierta, recirculación parcial, o recirculación total, por medio de una entrada de agua procedente de una toma de la mar que tras la decantación y la filtración podía ser regulada según las necesidades. Para el mantenimiento de los animales se adoptó un sistema de recirculación parcial que permitía mantener una calidad del agua muy alta. La instalación donde se mantuvieron los caballitos de mar se compone de cuatro estructuras separadas, dos módulos A1 y A2 para los recién nacidos y dos módulos B1 y B2 para los reproductores (Figura 2.2). Cada módulo A alberga 18 acuarios de cristal, 12 de 34,96 litros y 6 de 16,1 litros, con un tanque de filtración de 153,9 litros, por un volumen total del agua en la estructura de 1072,98 litros. Cada módulo B poseía 17 acuarios de

cristal, de los cuales 5 tenían un volumen de 114 litros, 6 de 50,56 litros, y otros 6 de 75,84 litros, con un tanque de filtración de 500 litros, por un volumen total de 1828,4 litros.


**Figura 2.2** Instalaciones para caballitos de mar (IU Ecoaqua)

## 2.2.1 Mantenimiento de los reproductores

El agua en la instalación para los reproductores se obtenía por una bomba sumergible (Ocean Runner OR6500, AquaMedic, Bissendorf, Alemania), colocada en el filtro de 6500 l/h. La filtración mecánica se llevaba a cabo por dos unidades de prefiltración en columna (AquaMedic, Bissendorf, Alemania) con foamex, mientras que la filtración biológica se realizaba mediante roca viva. También se utilizó un separador de proteínas Turboflotor 5000 Shorty II (AquaMedic, Bissendorf, Alemania), con bomba tipo Venturi de 3500 l/h (Ocean Runner OR 3500), que incluía una rueda de agujas. La temperatura se regulaba con un enfriador dotado de lámpara ultravioleta (UV-C)

(Teco TR-60, Ravenna, Italia) y dos calentadores de 300W (Eheim, Alemania), y se controlaba automáticamente mediante Powerbox AT Control (AquaMedic, Bissendorf, Alemania). A esta unidad estaban conectadas otras sondas que registraban el nivel del agua, el pH y la salinidad. Los datos se enviaban y almacenaban en un ordenador para su visualización. Las parejas de reproductores se mantuvieron por separado en acuarios idénticos situados en línea. Estos estaban provistos de objetos plásticos en su interior como plantas y redes para la sujeción de los animales (Figura 2.3).

**Figura 2.3** Batería de acuarios para los reproductores, módulo B1

En los tanques la temperatura osciló entre los 25,5 °C y los 26,5 °C para las parejas de *Hippocampus reidi*, y entre los 20,5 °C y los 21,5 °C para las parejas de *H. hippocampus*. El fotoperiodo era de 14D:10N (T5, 24w y 10000K, AquaMedic of North América) y el oxígeno varió entre 6,3 hasta 6,7 mg/L, con un caudal de 25 l/h que aportaba una renovación de agua aproximadamente de 8 veces al día. Estos parámetros se mantuvieron durante todo el periodo experimental.

Los reproductores se mantuvieron con una cantidad controlada de alimento, suministrado en dos tomas diarias, utilizando productos

comerciales: *Artemia* y Mysis congelados (Ocean Nutrition™, Essen, Belgium) al 12% del peso húmedo de los animales. Durante el experimento de desarrollo osteológico la alimentación de los reproductores se realizó con misidáceos vivos, (*Siriella armata*, *Leptomysis lingvura* y *Paramysis nouveli*) capturados en la zona de Risco Verde (27° 51' N, 15° 23' W), Gran Canaria (España), que ya habían demostrado su efectividad como alimento para estas especies (Otero-Ferrer 2012)

## 2.2.2 Cultivo de los recién nacidos

La circulación del agua en cada módulo A se realizaba mediante una bomba sumergible de 6500 l/h (Ocean Runner OR6500, Alemania). El sistema de filtración (AquaMedic Riff 500, Bissendorf, Alemania) es una unidad compacta formada por tres elementos: un módulo de pre-filtración relleno con foamex para filtración mecánica, un separador de proteínas (Turboflotor 1000) con bomba tipo Venturi y rueda de agujas (Ocean Runner PH 2500), y un filtro de goteo relleno con biobolas para filtración biológica.

La temperatura se regulaba por un enfriador/calentador dotado de lámpara UV-C (Teco TR20, Ravenna, Italia) y controlaba siempre por un Powerbox AT Control (AquaMedic©, Bissendorf, Alemania). También a esta unidad estaban conectadas las sondas para controlar el nivel, el pH, y la salinidad, y los datos registrados eran enviados y almacenados en el ordenador para su visualización.

Los recién nacidos se cultivaron a una densidad de 80 individuos/acuario con una temperatura que oscilaba entre los 25,5 °C y los 26,5 °C para *H. reidi*, y entre los 20,5 °C y los 21,5 °C para *H.*

*hippocampus* (Figura 2.4). El fotoperiodo fue de 12D:12N (T5, 24W, y 10000K, AquaMedic of North América) y el oxígeno varió entre 6,3 y 6,7 mg/L, con un caudal de 16,2 l/h (25-30 ml/6 sec.) que renovaba el agua aproximadamente 12 veces al día. Estos parámetros se mantuvieron durante todo el periodo experimental. Los recién nacidos fueron alimentados cada día con dos tomas de *Artemia* enriquecida a razón de 0,7 unidades/ml, calculada mediante conteo volumétrico por triplicado bajo una lupa (Leica S6E L2, Alemania).

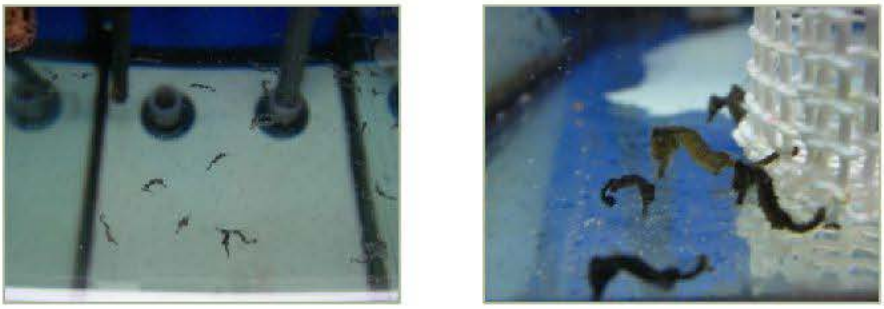


**Figura 2.4** Cultivo de recién nacidos, módulo A1

## 2.3 Preparación de presa viva

Para los experimentos se utilizaron dos tipos de quistes de *Artemia*: inicialmente se utilizó *Artemia* tipo EG 850 mm (Inves, Acuazul s.c. Cádiz, España) decapsulada, mientras que durante los experimentos finales de utilizó Sep-Art Gsl Magnetic *Artemia* Cysts (Inves, Acuazul s.c. Cádiz, España), contando con un separador de quistes SEP-Art Technology Separator (Inve, Dendermonde, Bélgica) capaz de separar quistes (previamente tratadas y recubiertas con una capa magnética no tóxica) de nauplios, por atracción magnética.

### 2.3.1 Descapsulación de *Artemia*

La *Artemia* tipo EG 850 mm se decapsuló previamente para aprovechar toda la energía contenida en los nauplios, según el protocolo adoptado del GIA, Grupo de Investigación en Acuicultura. Inicialmente se ponían a hidratar los quistes y después se decapsulaban con lejía e hidróxido de sodio, en agua de mar. Al disolver los coriones de los quistes, se manifestaba una variación de color entre el marrón y el naranja. Antes que la solución entrara en contacto con los nauplios se neutralizaba lavando bien con agua de mar. Los quistes se conservaron a 4 °C en una solución hipersalina (salmuera), preparada previamente disolviendo sal marina en agua de mar.

### 2.3.2 Eclosión de *Artemia*

El procedimiento se iniciaba con el llenado con agua de mar filtrada de los recipientes tronco-cónicos de 5 litros. A los recipientes se le colocaba un calentador para mantener la temperatura constante entre 28-29 °C, y aireación fuerte, para obtener un buen nivel oxígeno y una adecuada agitación de los quistes en el recipiente. Se aplicaba una iluminación continua durante 24 horas para conseguir una mayor eficacia en el proceso de eclosión de los quistes.

### 2.3.3 Separación de *Artemia*

Tras 24h, cuando se usaba Sep-Art Gsl Magnetic *Artemia* Cysts, los nauplios eclosionados estaban mezclados con los quistes, y para separarlos se utilizó el separador de *Artemia*. El separador consistía en un tubo con un ángulo de 90° que contenía imanes (Figura 2.5). Una

vez que se ponían los 5 litros del recipiente tronco-cónico (que contenía *Artemia* eclosionada y quistes) dentro del tubo se añadía agua, a un caudal de 8-10 l/min. Los quistes se veían atraídos por los imanes debido a la capa magnética y los nauplios de *Artemia* se recolectaban al final del tubo con un cubo y una malla.

**Figura 2.5** *Artemia* **recién eclosionada con tecnología Sep-Art**

## 2.3.4 Enriquecimiento de *Artemia*

Se usaron recipientes tronco-cónicos de 5 litros, en los que se introducía un calentador para mantener la temperatura constante entre 28-29 °C y un aireador para facilitar una buena oxigenación del agua y el mantenimiento de los nauplios en suspensión. Durante el periodo experimental de recién nacidos de caballitos de mar en las instalaciones del Instituto Universitario Ecoaqua se utilizaron tres enriquecedores comerciales:

- EASY DHA SELCO (INVE, Dendermonde, Belgium)
- DHA Protein Selco (INVE, Dendermonde, Belgium)
- MULTIGAIN (Biomar, España)

El enriquecimiento se realizaba durante 24h según la proporción establecida por cada fabricante.

## 2.4 Mantenimiento diario

La rutina diaria se dividía principalmente en dos partes, la preparación del alimento y la limpieza de las instalaciones. Todos los días se preparaba y contaba la *Artemia* enriquecida, se ponía la que ya había eclosionado a enriquecer, y los nuevos quistes, a eclosionar. Para la alimentación de los adultos era necesario descongelar los alimentos comerciales previamente pesados.

Las instalaciones se sifonaban diariamente para limpiar el fondo de los tanques, aspirando los residuos del día anterior y desinfectado con lejía cuando se consideraba necesario (presencia de algas o anémonas). Se controlaba el buen funcionamiento de las instalaciones (iluminación, temperatura y oxígeno), y cuando resultaba necesario, se procedía a la limpieza de los vasos de los separadores de proteínas.

## 2.5 Recogida de muestras biológicas

Los experimentos fueron realizados siguiendo las líneas señaladas en la directiva 2010/63/EU aprobada por el Parlamento y el Consejo de la Unión Europea relativa al uso de animales con fines científicos. El sacrificio de los animales cumplió los requisitos recogidos en la normativa vigente (Real Decreto 1201/2005) y el procedimiento fue aceptado por el Comité Ético del Bienestar Animal de la Universidad de Las Palmas de Gran Canaria (ULPGC) en 2010 (Referencia 002/2011 CEBA ULPGC).

La recogida de caballitos de mar se realizaba previamente a la primera alimentación. Las muestras procedentes de puestas pérdidas o abortos naturales venían recolectadas rápidamente observadas y fotografiadas en agua de mar. Las muestras de caballitos de mar recogidas del tanque, se ponían en pequeños vasos de precipitado plástico donde se añadía anestésico (Clove Natural Essential Oil, Guinama, Alboraya, Valencia, España), evaluado como el más adecuado y eficaz en caballitos de mar (Pawar *et al.* 2011; Qin *et al.* 2016). Los vasos se colocaban en una bandeja con hielo hasta la muerte de los caballitos de mar.

Las muestras procedentes de puestas pérdidas/abortos naturales (después ser observadas y fotografiadas) y las muestras para los análisis histológicos y osteológicos, tras el sacrificio con la metodología antes descrita, se conservaron en formalina tamponada 10% (Sigma Chemical Co. España). Aquellas usadas para analizar la bioquímica digestiva fueron inmediatamente sumergidas en nitrógeno líquido, y mantenidas a una temperatura de –80°C hasta la liofilización.

## 2.6 Parámetros biológicos

Los parámetros biológicos evaluados en reproductores fueron el peso húmedo, longitud del hocico, de la cabeza, y la longitud total. En embriones longitud total. En los recién nacidos fueron tomados peso seco, peso húmedo, longitud del hocico, de la cabeza y longitud total.

Los parámetros biológicos de los recién nacidos fueron usados principalmente para establecer el crecimiento y la cuantificar la actividad de las enzimas digestivas. Los parámetros biológicos de los reproductores se utilizaron para formar las parejas, establecer la

cantidad de alimento diario a suministrar, y controlar su posible variación (Tabla 2.1).

**Tabla 2.1** Parámetros biológicos muestreados

| | Reproductores | Recién nacidos | | | Embriones |
|---|---|---|---|---|---|
| | | H | O | E | |
| Peso húmedo | ■ | ■ | ■ | | |
| Peso seco | | | | ■ | |
| Longitud total | ■ | ■ | ■ | | ■ |
| Longitud cabeza | ■ | | | | |
| AGR | | ■ | ■ | ■ | |
| SGR | | ■ | ■ | ■ | |
| DWI | | ■ | | ■ | |
| WG | | | | ■ | |

(AGR) tasa de crecimiento absoluto; (DWI) incremento del peso diario; (E) Enzimología; (H) Histología; (O) Osteología; (SGR) tasa diaria de crecimiento; (WG) Peso ganado.

La metodología de trabajo se diferenciaba según el material biológico muestreado; en el caso de toma de parámetros biológicos de recién nacidos, los animales que se medían eran previamente sacrificados, en el caso de reproductores, se medían parámetros biológicos a partir de animales vivos. En este último caso el interés era conseguir el menor estrés posible favoreciendo su rápida recuperación.

## 2.6.1 Peso de los reproductores

Los reproductores fueron ligeramente anestesiados previamente con aceite de clavo (Guinama, Alboraya, Valencia) en solución al 5% en alcohol absoluto a una dosis de 40 ppm (Otero-Ferrer 2012).

Una vez hizo efecto, se secaron brevemente con papel y se pesaron en un vaso de precipitado tarado con agua, en una balanza de precisión

(Kern, EW 150 3M, Alemania). Los reproductores, tras ser pesados, se colocaron en un recipiente de recuperación con aireación antes de ser devueltos a los acuarios de cultivo (Figura 2.6).


**Figura 2.6** Toma de peso y medidas de reproductores

## 2.6.2 Medidas de los reproductores

Los reproductores fueron anestesiados previamente con aceite de clavo (Guinama, Alboraya, Valencia) en solución al 5% en alcohol absoluto a una dosis de 40 ppm (40 ml en 5 litros de agua). Las medidas tomadas de los reproductores fueron aquellas comúnmente utilizadas en la taxonomía de caballitos de mar (Lourie *et al.* 2003). La longitud total, desde la parte apical de la coronilla hasta la parte final de la cola con la cabeza del caballito de mar puesta en ángulo de 90° respeto al cuerpo, la longitud del hocico, desde la parte apical del hocico hasta la espina nasal, la

longitud de la cabeza, desde la parte apical del hocico hasta el cleitro (Figura 2.7).

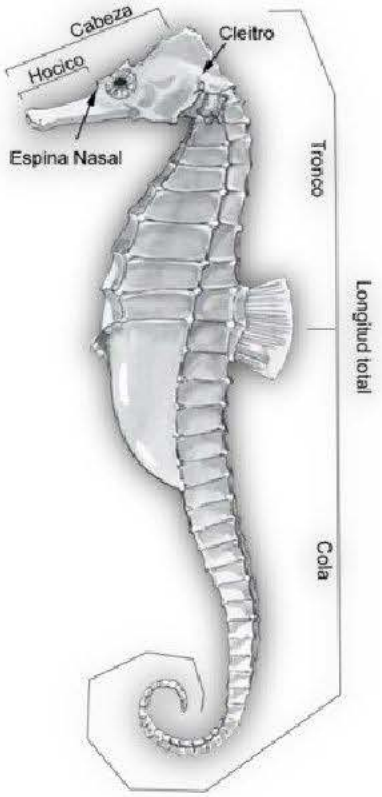



**Figura 2.7** Medidas tomadas en reproductores
(Modificado de Indiviglio 2001)

## 2.6.3 Peso de los recién nacidos

Los recién nacidos se pesaron en función de la necesidad de recoger muestras biológicas. En el caso de los análisis en los que las muestras biológicas se conservaron en formalina tamponada se registró el peso húmedo; cuando las muestras biológicas tenían que ser congeladas inmediatamente, los pesos secos fueron tomados después de la liofilización. Para obtener el peso húmedo se utilizó una balanza de

precisión (Mettler Toledo AG204), los animales sacrificados se secaron rápidamente con papel y se colocaron individualmente en la balanza. La operación se repitió con cada una de los recién nacidos (Figura 2.8).

**Figura 2.8** Balanzas para medir peso húmedo y peso seco de los recién nacidos

El peso seco de los recién nacidos se realizó después del proceso de liofilización. Los caballitos se pesaron individualmente en una microbalanza de precisión (XP6, Mettler Toledo, Greifensee, Switzerland). Se pesaron seis animales por cada punto de muestreo de todos los experimentos.

## 2.6.4 Medidas de los recién nacidos

Las mismas medidas tomadas en los reproductores fueron tomadas para los recién nacidos, se midieron las longitudes de hocico (hasta la espina nasal), cabeza (hasta el cleitro), y total. Además, en los recién nacidos se tomó la medida del tronco y de la cola. Las medidas se tomaron con un proyector de perfiles de alta precisión (Mitutoyo PJ-R 3000, U.S.A.), según el protocolo establecido por Lourie *et al.* (2003).

Los caballitos de mar después del sacrificio se apoyaron lateralmente en un cristal porta objeto, donde se añadía una gota de agua para obtener un mejor contraste en el aparato de medición, y así obtener medidas exactas (Figura 2.9).


**Figura 2.9** Muestreo de recién nacidos, medidor de perfiles

## 2.6.5 Tasa de crecimiento absoluto (AGR)

La tasa de crecimiento absoluto (Absolute Growth Rate) entendido como mm/mg ganados diariamente se calculó en acuerdo con Hopkins (1992) utilizando la ecuación:

$$\mathbf{AGR} = \frac{(\mathbf{Lf} - \mathbf{Li})}{(\mathbf{T2} - \mathbf{T1})}$$

dónde Lf es la longitud total/peso húmedo (mm/mg) del animal al final de cada fase de desarrollo T2, y Li es la longitud total/peso húmedo (mm/mg) del animal al inicio de cada fase de desarrollo T1.

## 2.6.6 Tasa diaria de crecimiento (SGR)

La tasa diaria de crecimiento (Specific/Instantaneous Growth Rate) indica el incremento de peso en relación a los días transcurridos desde el comienzo del periodo de alimentación. Estos datos se calcularon con la ecuación:

$$\mathbf{SGR} = \frac{(\mathbf{ln\,W2 - ln\,W1})}{(\mathbf{T2 - T1})}$$

dónde W1 y W2 representan el peso húmedo al tiempo t1 y t2 respectivamente.

## 2.6.7 Incremento del peso diario (DWI)

El incremento del peso diario (Daily Weight Increase) en porcentaje (%DWI) de los recién nacidos se calculó de acuerdo con Ricker (1958). El modelo exponencial usado fue:

$$\mathbf{DWI\ \%} = (e^{g} - 1) \times 100$$

dónde g representa el crecimiento específico diario SGR = (lnW2 - lnW1) / (T2 - T1). La unidad 1 es la longitud total/peso húmedo inicial y la longitud total/peso húmedo eg incrementan $e^{g} - 1$ al final de la unidad de tiempo.

## 2.6.8 Peso Ganado (WG)

Este parámetro (Weight Gained) fue empleado para evaluar el efecto de diferentes regímenes alimenticios en el crecimiento de los recién nacidos. Se define como la relación entre el incremento de biomasa

comparado con el peso inicial. El peso ganado fue calculado usando la ecuación:

$$\mathbf{WG} = \frac{(\mathbf{W2} - \mathbf{W1})}{(\mathbf{W1})} \times 100$$

## 2.7 Periodo de vida en caballitos de mar recién nacidos

Generalmente para establecer la edad en larvas de peces, el punto de referencia es la eclosión. El término utilizado es DAH en referencia a este momento (procedente del inglés: day after hatching, día después de la eclosión). Los caballitos de mar son animales vivíparos que han evolucionado una estrategia reproductiva en la cual los machos llevan a cabo una gestación. Los huevos vienen protegidos osmorregulados y nutridos dentro de una bolsa incubadora o "marsupium" donde tiene lugar la eclosión.

Para establecer el periodo de vida, no siendo visible la eclosión, el punto de referencia fue el nacimiento y se utilizó el término DAB (procedente del inglés: day after birth, día después del nacimiento) (Murugan *et al.* 2009; Otero-Ferrer *et al.* 2010; Pawar *et al.* 2011). El nacimiento se considera el momento en el cual los caballitos de mar salían de la bolsa incubadora.

## 2.8 Análisis bioquímicos

La composición bioquímica de los nauplios de *Artemia,* de los metanauplios enriquecidos y de los caballitos de mar se analizó en el Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA-Arucas Islas Canarias, España).

Todas las muestras se lavaron con agua destilada, se congelaron a (-80 °C, y se liofilizaron previamente a la realización de los análisis bioquímicos. Los lípidos totales fueron extraídos usando la metodología cloroformo-metanol descrita por Folk y colaboradores (1957), mientras que el contenido de proteínas se calculó usando el método de Kjeldhal (AOAC 2010).

## 2.9 Estudio embrionario

Los huevos fueron obtenidos de hembras que venían monitoreadas visualmente. Huevos expulsados no fertilizados caídos durante el apareamiento fuera de la bolsa incubadora del macho fueron cuidadosamente recogidos en el fondo del acuario para dos veces (371 y 296 huevos), huevos fertilizados solo una vez (46 huevos intactos). Durante el período de gestación que dura dos semanas en *H reidi*, los embriones fueron obtenidos a partir de varios abortos naturales durante el embarazo masculino (cuatro abortos: 346, 297, 312 y 318 embriones/larvas). En cada aborto se encontraban caballitos de mar pertenecientes a una o dos fases diferentes de desarrollo. Estas muestras se combinaron para componer una serie que representa las principales fases del desarrollo embrionario.

Todas las muestras fueron observadas con una lupa Leica (Leica M 125 Microsystems Wetzlar, Alemania), y fotografiados digitalmente con objetivos Leica (Leica DFC 420, Microsystems Wetzlar, Alemania). Medir fueron obtenidos usando un software de procesamiento de imágenes (Image Pro Plus, MEDIA Cybernethics, EE.UU.).

## 2.10 Estudio histológico e histoquímico

La finalidad del estudio histológico fue la de analizar la morfología y algunas funcionalidades de las estructuras internas. Se plantearon varias tinciones, después del corte en parafina la tinción de Hematoxilina y Eosina (Martoja y Martoja-Pierson 1970) fue la base para describir la organogénesis de los caballitos de mar, con la tinción PAS (Martoja y Martoja-Pierson 1970) se detectaron el glucógeno y las glucoproteínas neutras, y con el corte en congelación y la tinción Negro Sudán B y Hematoxilina de Mayer (Oliveira *et al.* 2007), la presencia de gotas lípidicas.

Se utilizaron seis parejas reproductoras. La organogénesis fue estudiada empleando 360 caballitos de mar durante un período de 30 días. Los recién nacidos fueron criados por triplicado dividiendo 180 caballitos de mar de cada parto en tres acuarios (60 recién nacidos por acuario). Seis caballitos por día (dos por acuario) se muestrearon aleatoriamente para realizar el análisis morfológico (H&E, PAS y negro Sudán). El triplicado incluía un grupo de 18 recién nacidos procedentes de tres diferentes parejas reproductoras (seis por cada pareja reproductora). Cada grupo se subdividió por triplicado, empleando seis ejemplares de caballitos de mar por cada análisis y cada triplicado se componía de seis caballitos de mar procedentes de tres parejas reproductoras distintas.

### 2.10.1 Cortes en parafina, preparación muestras y material

Tras varios ensayos, los cristales portaobjetos se trataron con la sustancia Poli-L lisina (Sigma®) para aumentar la fijación, dada la

escasa adherencia del tejido, que se despegaba al momento de montar los cubreobjetos.

### 2.10.2 Tratamiento de los cristales portaobjetos

Se prepararon tres baños, uno con la solución de Poli-L y dos de agua destilada. La solución de Poli-L se preparó disolviendo 0,01 gr de Poli-L lisina en 100 ml de agua destilada. Los cristales portaobjetos permanecieron durante cinco minutos en cada baño, empezando con el baño conteniente Poli-L, y posteriormente se dejaron secar en posición horizontal a temperatura ambiente.

### 2.10.3 Inclusión y corte en parafina

El procedimiento para embeber los tejidos en parafina (punto de fusión 56 °C), se ha mantenido sin cambios desde hace más de un siglo. Su objetivo es conseguir que el agua de los tejidos sea sustituida por este tipo especial de cera.

Después del sacrificio y la conservación en formalina, las muestras de tamaño más pequeño se colocaron en pequeñas redes metálicas antes ser puestas en las clásicas cubetas de plástico (Figura 2.10).

Se utilizaron estas pequeñas redes para evitar la pérdida de las muestras durante el proceso de inclusión en parafina. Las muestras de tamaño mayor se colocaron directamente en las cubetas. Las muestras se lavaron en agua corriente durante 30 minutos, y a continuación, se efectuó la inclusión en parafina con un procesador automático de tejidos Jung Histokinete 2000 (Leica, Nussloch, Alemania).

**Figura 2.10** Redes para la inclusión en parafina

El procesador automático gracias a una escala ascendente de alcoholes deshidrataba la muestra y, a continuación, tras clarificarla con dos pasadas por xilol (disolvente en el que es soluble la parafina) la incluye en parafina caliente evitando la retracción del tejido (Tabla 2.2).

**Tabla 2.2** Deshidratación (alcoholes), clarificado (xilol) e inclusión en parafina

| | | | | | |
|---|---|---|---|---|---|
| 1 | Alcohol 70% | 3 h | 7 | Alcohol 100% | 2 h |
| 2 | Alcohol 90-96% | 2,5 h | 8 | Xilol | 30 min. |
| 3 | Alcohol 90-96% | 2,5 h | 9 | Xilol | 30 min |
| 4 | Alcohol 90-96% | 2,5 h | 10 | Xilol | 30 min |
| 5 | Alcohol 100% | 2 h | 11 | Parafina | 3 h |
| 6 | Alcohol 100% | 2 h | 12 | Parafina | 6 h |

Tras el último paso por parafina, las muestras se dejaron durante una hora en una bomba de vacío. A continuación, se confeccionaron los bloques de parafina, que al enfriarse confieren a la muestra la dureza necesaria para ser seccionada. Se utilizó el dispensador de parafina

MEDITE TES 99 (Leica, Alemania) (Figura 2.11). El corte de los bloques de parafina (por lo menos 24h después de su preparación) se realizó con un micrótomo Jung Autocut 2055 a un espesor de 4µm.

**Figura 2.11** Dispensador de parafina y preparación de bloques

Los cortes fueron colocados en un contenedor de agua caliente (46-48 °C) durante un tiempo aproximado de 8-15 segundos con el fin de distender la parafina y el tejido, gracias a las fuerzas de tensión superficial que se generaban en la lámina de parafina. Los cortes se recogieron con los portaobjetos tratados con Poli-L sobre los cuales se adherían dejando drenar el agua por gravedad.

## 2.10.4 Tinción con Hematoxilina y Eosina

Para la descripción ontogénica de los caballitos de mar, se decidió adoptar la tinción de Hematoxilina y Eosina (H&E). Es una de las tinciones más comúnmente usada, en la que un colorante básico y otro ácido van a teñir de diferente color las estructuras ácidas y básicas de la célula, y permiten estudiar y distinguir claramente los diferentes tejidos que componen las estructuras internas.

El primer colorante, la hematoxilina de Harris, tiene una afinidad con los tejidos básicos, que al microscopio aparecen de color violeta. La eosina por el contrario presenta afinidad con estructuras ácidas que aparecen de color rosáceo.

El protocolo tuvo que modificarse por el tono de la hematoxilina en el resultado final que resultaba demasiado fuerte, reduciendo el tiempo de contacto de las muestras con este colorante. Además, fueron eliminadas dos etapas: el paso de la muestra por HCl y por agua amoniacal.

Los cristales portaobjetos se dejaban secar a temperatura ambiente por 24 horas, o se ponían 30 min en una estufa a una temperatura de 100 °C cuando se deseaba trabajar en el mismo día. Las muestras, antes de pasar por la batería de tinción, se trataban con xilol para eliminar la parafina de los tejidos y con una batería descendente de alcoholes para hidratarla (Tabla 2.3).

**Tabla 2.3** Desparafinado (xilol) e hidratación (Agua destilada) (H&E)

| | | | | | |
|---|---|---|---|---|---|
| 1 | Xilol | 2 min. | 5 | Alcohol 70% | 2 min. |
| 2 | Xilol | 2 min. | 6 | Agua destilada | 2 min. |
| 3 | Alcohol 100% | 2 min. | 7 | Agua destilada | 2 min. |
| 4 | Alcohol 100% | 2 min. | 8 | Agua destilada | 2 min. |

A continuación, las muestras venían teñidas, deshidratadas en alcoholes, y clarificadas en xilol (Tabla 2.4).

**Tabla 2.4** Tinción (H&E), deshidratación (alcoholes) y clarificación (xilol)

| | | | | | |
|---|---|---|---|---|---|
| 9 | Hematoxilina | 10 min | 15 | Alcohol 100% | 2 min |
| 10 | Agua grifo | 12 min | 16 | Alcohol 100% | 2 min |
| 11 | Eosina | 4 min | 17 | Xilol | 2 min |
| 12 | Alcohol 96% | 2 min | 18 | Xilol | 2 min |
| 13 | Alcohol 96% | 2 min | 19 | Xilol | 2 min |
| 14 | Alcohol 96% | 2 min | 20 | Montaje cristales | |

A continuación, se montaron los cristales portaobjetos con los cristales cubreobjetos, preparado una resina Eukitt® (pegamento) conteniente xilol (Panreac, Barcelona, España). Tras 24h los cristales portaobjetos estaban listos para la observación al microscopio. Tras la tinción (H&E), se observaron grandes vacuolas en el interior del intestino de los caballitos de mar el día del nacimiento, (en la parte inferior a la válvula íleo-cecal), se realizaron las siguientes tinciones para establecer si estas vacuolas eran de procedencia lipídicas, proteica, o acuosa. El fin era obtener resultados útiles que ayudaban a entender requerimientos alimenticios específicos de los recién nacidos.

## 2.10.5 Reacción del ácido periódico de Schiff (PAS)

La técnica histoquímica empleada para la detección de hidratos de carbono es la tinción de PAS (Periodic Acid Schiff). El ácido periódico (0,5 g en 100 ml agua destilada) y el reactivo de Schiff confieren a estas sustancias un color rojizo brillante. Se utilizaron muestras fijadas en formaldehido y procesadas en parafina. Los cristales portaobjetos se dejaron secar a temperatura ambiente 24 horas, o se ponían 30

minutos en la estufa a 100 °C. Las muestras antes pasar por la batería de tinción se trataban con xilol para eliminar la parafina de los tejidos y con una batería descendente de alcoholes para hidratarla (Tabla 2.5).

**Tabla 2.5** Desparafinado (xilol) e hidratación (Agua destilada) (PAS)

| | | | | | |
|---|---|---|---|---|---|
| 1 | Xilol | 2 min. | 5 | Alcohol 70% | 2 min. |
| 2 | Xilol | 2 min. | 6 | Agua destilada | 2 min. |
| 3 | Alcohol 100% | 2 min. | 7 | Agua destilada | 2 min. |
| 4 | Alcohol 100% | 2 min. | 8 | Agua destilada | 2 min. |

A continuación, las muestras pasaban a la batería de tinción, se deshidrataban en una batería de alcoholes y se clarificaban con la batería de xilol (Tabla 2.6).

**Tabla 2.6** Tinción (PAS) deshidratación (alcoholes) y clarificación (xilol)

| | | | | | |
|---|---|---|---|---|---|
| 9 | Ácido Periódico | 5 min | 15 | Alcohol 96% | 2 min |
| 10 | Lavados agua dest. | Varios | 16 | Alcohol 96% | 2 min |
| 11 | Reactivo Schiff | 10 min | 17 | Alcohol 100% | 2 min |
| 12 | Agua corriente | 10 min | 18 | Alcohol 100% | 2 min |
| 13 | Hematoxilina Harris | 6 min | 19 | Xilol | 2 min |
| 14 | Agua corriente 96% | 2 min | 20 | Xilol | 2 min |

El montaje de los cristales portaobjetos con los cubreobjetos ha sido realizado con una resina Eukitt® que contiene xilol (Panreac, Barcelona, España). Como en la tinción H&E, tras 24h los cristales portaobjetos estaban listos para la observación al microscopio.

### 2.10.6 Corte en congelación, preparación muestras y material

Para establecer la presencia de lípidos se decidió utilizar el Negro Sudán (2g en 100 ml de alcohol), contrastándolo con la Hematoxilina de Mayer con el objetivo de acentuar las gotas lipídicas que aparecían de color negro. El corte en congelación era debido a la necesidad de conservar íntegros los lípidos.

### 2.10.7 Tratamiento de cristales portaobjetos

Los cristales portaobjetos con los cuales se ha recogido las muestras fueron gelatinizados previamente. Se disolvió gelatina en polvo (4,5 g), cromo III y potasio sulfato (0,44 g) en agua destilada calentada a 80°C (900 ml). Se introdujeron los portaobjetos en la solución a 70 °C filtrada durante 5 minutos, se dejaron secar en la estufa toda la noche (50 °C), y se guardaron en un lugar seco.

### 2.10.8 Inclusión en agar y cortes en congelación

Las muestras conservadas en formaldehido 10% se lavaron tres veces en un tampón fosfato de solución salina (PBS, Phosphate buffered solution) durante 5 min. Tras los lavados, los caballitos de mar se prepararon en bloques de agar (medio constituido por agar al 1,5% y sacarosa al 5% p/v) en PBS para el corte en congelación. La solución de PBS fue preparada con 2 litros de agua destilada, 2,96 g de fosfato sódico bibásico, 0,86 g de fosfato potásico anhidro $K_2HPO_4$ y 14,4g de cloruro sódico. La solución preparada (0,75g agar, 5g sacarosa, PBS hasta 50 ml), tras ser calentada hasta ebullición, y enfriada a temperatura ambiente, solidifica. Con la ayuda de una lupa y pequeños

contenedores circulares de plástico, se prepararon los bloques en los cuales se insertó la muestra. Estos bloques, cortados con el criostato necesitaban ser criopreservados. El baño durante 24 h a temperatura ambiente en una solución de sacarosa al 30% (p/v) y PBS tenía esta finalidad (12g sacarosa, 50 ml PBS 30%). Los bloques se incluyeron en un medio OCT 4583 (Tissue-tek, Miles) y fueron cortados en criostato (Leica Jung CM 3000, Nussloch, Alemania) a 6 µm de espesor y a una temperatura de -24°C (Figura 2.12).

**Figura 2.12** Preparación de bloques de agar y corte en congelación

## 2.10.9 Tinción con Negro Sudán y Hematoxilina de Mayer

Una vez cortada la muestra, se dejaba secar al aire durante 20 minutos, a continuación, se pasaba por la batería de tinción (Tabla 2.7).

**Tabla 2.7** Tinción Negro Sudan y Hematoxilina de Mayer

| | | | | | |
|---|---|---|---|---|---|
| 1 | PBS | 2 min. | 5 | Alcohol 70° | 2 min. |
| 2 | PBS | 2 min. | 6 | Hematoxilina Mayer | 5 min. |
| 3 | PBS | 2 min. | 7 | Agua corriente | 5 min. |
| 4 | Negro Sudán | 90 min. | 8 | Montaje glicerina | |

Se preparó una solución saturada de Negro Sudán en alcohol 70° para la tinción de los lípidos, que fue contrastada con Hematoxilina de Mayer. La muestra, una vez teñida, fue montada en glicerina (Dako Glicergel mounting médium, USA).

### 2.10.10 Descripción histológica e histoquímica

El orden de la descripción histológica y la división sistémica se realizó según Genten y colaboradores (2009). El intestino se dividió en tres zonas, bucofaríngea, anterior y posterior. Las dos últimas regiones se dividieron con respecto a su localización: en la parte anterior o posterior a la válvula íleocecal.

## 2.11 Estudio osteológico

El proceso de osificación fue estudiado en 120 caballitos de mar. Seis caballitos fueron muestreados diariamente hasta el décimo día después del nacimiento, cada dos días hasta el vigésimo cuarto día, y cada tres hasta el trigésimo día. El protocolo adoptado para teñir huesos y cartílagos ha sido una combinación de los protocolos de los de Taylor y Van Dyke (1985), y de Dingerkus y Uhler (1977). En el protocolo (Tabla 2.8) no se modificaron los pasos a seguir, pero se añadió un lavado previo debido a una fuerte pigmentación de los caballitos de mar recién nacidos. Las muestras (conservadas en formalina) se lavaron con una solución de agua oxigenada y peróxido de hidrógeno hasta la desaparición de los cromatóforos. Se trató de una solución muy agresiva, por la cual fue necesario observar su efecto bajo lupa durante 5 a 10 minutos.

**Tabla 2.8** Protocolo de tinción de huesos y cartílagos (Modificado de Socorro 2006)

| Paso | Tratamiento | Tiempo | Finalidad | Soluciones (cada 100 ml) |
|---|---|---|---|---|
| 1 | Lavado | 5-10 min | Clarificación cromatóforos | 1g KOH<br>10 ml agua oxigenada (110V)<br>hasta 100 ml de agua destilada |
| 2 | Alcían Blue (8GX) | 2 h | Tinción cartílagos | 10 mg de azul Alcían BGX<br>80 ml alcohol 95%<br>20 ml ácido acético glacial |
| 3 | Alcohol 95% | 1 h | Hidratación | 95 ml alcohol 100%<br>5 ml agua destilada |
| 4 | Alcohol 95% | 1 h | | |
| 5 | Alcohol 95% | 1 h | | |
| 6 | Alcohol 75% | 1 h | | 75 ml alcohol 100%<br>15 ml agua destilada |
| 7 | Alcohol 40% | 1 h | | 75 ml alcohol 100%<br>15 ml agua destilada |
| 8 | Alcohol 15% | 1 h | | 15 ml alcohol 100%<br>75 ml agua destilada |
| 9 | Agua destilada | 1 h o toda la noche | | |
| 10 | Solución de tripsina (conservar a la obscuridad 4°C, utilizar a T. ambiente) | 1h | Digestión tejidos (controlar al microscopio las muestras) | 90 mg de tripsina<br>70 ml agua destilada<br>30 ml solución saturada de Na B4O7 |
| 11 | Rojo Alizarina | 90 min | Tinción huesos | 1g/L rojo alizarina en una solución de KOH 0,5% |
| 12 | Glicerina KOH 1:3 | 12-24 h | Clarificación | 25 ml glicerina<br>75 ml KOH 0,5% |
| 13 | Glicerina KOH 1:1 | 12-24 h | | 50 ml glicerina<br>50 ml KOH 0,5% |
| 14 | Glicerina KOH 3:1 | 12-24 h | | 75 ml glicerina<br>25 ml KOH 0,5% |
| 15 | Glicerina pura + timol | | Conservación | |

Al término de este proceso se podían observar los cartílagos de color azul mientras que las partes osificadas quedaron en color rojo. Las observaciones se hicieron bajo lupa (Leica, M125, Alemania) y

microscopio (Olympus CX41, Japón) y las fotografías con objetivos Leica DFC 420 y Olympus XC30.

### 2.11.1 Denominación huesos y cartílagos

La denominación de huesos y cartílagos seguían diferentes trabajos: Gregory (1959) para el cráneo, Matsuoka (1985) y Jardim y Santos (1994) parar las primeras estructuras neurocraneales. Azzarello (1990), Silveira (2000), y Leysen *et al.* (2011) para la adaptación de las estructuras osteológicas de los otros teleósteos a las especies de caballitos de mar. El concepto de cartílago hiosimpléctico procedía de Kadam (1958), y el de hueso jugal de De Beer (1937).

El estudio de Arratia y colaboradores (2001), así como el de Bruner y Bartolino (2008), fueron utilizados por la descripción del sistema vertebral. Para describir la osificación, se subdividió el esqueleto en tres partes principales: el esqueleto craneal, el esqueleto axial, que incluía la columna vertebral hasta la aleta caudal, y el esqueleto apendicular, que comprendía las aletas y sus soportes.

## 2.12 Estudio enzimático

Las actividades enzimáticas de las principales enzimas digestivas se analizaron para evaluar la respuesta nutricional respeto a diferentes enriquecedores de *Artemia*, elegida como presa viva. Se usaron 6 parejas reproductoras y se muestrearon un total de 375 caballitos de mar para evaluar la actividad enzimática durante el desarrollo. Se sacrificaron 15 caballitos de mar recién nacidos y 45 por cada punto de muestreo (1, 2, 4, 6, 8, 10, 15 y 20 DAB). El triplicado se componía de

45 caballitos, 15 por tratamiento. Las 15 larvas de cada tratamiento formaban el triplicado de cada tratamiento y procedían de tres diferentes parejas reproductoras, cinco caballitos por pareja. Las actividades enzimáticas, bajo específicas condiciones, se expresaron como número de micromoles de producto producidas por minuto, y por mg de proteínas. El coeficiente de extinción (diferencia entre producto y sustrato) se obtuvo usando una curva estándar de 4-Methylumbelliferone (Sigma M1381).

## 2.12.1 Preparación de las muestras

Los extractos enzimáticos, usados para evaluar la actividad de las enzimas digestivas se prepararon homogeneizando individualmente el intestino de cada caballito de mar en agua destilada (200 µL) después la remoción de cabeza y cola (Figura 2.13).

**Figura 2.13** Preparación de muestra y lector de micro placas

Los homogenizados se centrifugaron por 5 min a 13000g a 4 °C y los extractos se alicuotaron y conservaron (-20 °C) hasta ser analizados. Las actividades enzimáticas se analizaron usando varios sustratos fluorescentes en un lector de micro placas (Cytation 3, Cell Imaging Multi-Mode Reader, USA). Los sustratos y las condiciones analíticas fueron aquellas descritas por Rotllant *et al.* (2008) con algunas modificaciones.

## 2.12.2 Tripsina

El sustrato para la tripsina (Boc-Gln-Ala-Arg-7-amino-4-methylcoumarin hydrochloride; B4153; Sigma-Aldrich, Madrid, España) se diluyó en dimetil sulfóxido (DMSO) hasta obtener una concentración final de 20 µM. Cada pocillo de la micro placa se preparó con 10 µL de homogenizado diluido, mezclado a 190 µL de tampón (Tris-HCl, 0.1 M CaCl2 pH 8.0), y a 5 µL de sustrato. La fluorescencia se medió a 380/440 nm EX/EM.

## 2.12.3 Proteasa total

Para analizar la proteasa total se utilizó una macromolécula como la caseína que hace más compleja la digestión. La caseína fluorescente 0.5% w/v (C0528; Casein fluorescein isothiocyanate from bovine milk, type III, Sigma-Aldrich, Madrid, España) se utilizó para medir la actividad enzimática. En cada pocillo se mezclaron 150 µL de tampón Borato (pH 8.0), y 40 µL de homogenizado. Para empezar la reacción 6 µL de caseína fluorescente 0.5% (w/v) se añadieron en cada pocillo. La fluorescencia fue medida a nm 485/538 EX/EM.

## 2.12.4 Esterasas no específicas

La actividad de las esterasas se analizó utilizando tres sustratos con una diferencia entre ellos de longitud de cadena. Fueron empleados el butirato, (4-methylumbelliferyl butyrate 19362; MUB-Fluka), el heptanoato (4-methylumbelliferyl heptanoate M2514; Sigma-Aldrich, Madrid, España) y el oleato (4-methylumbelliferyl oleate 75164; Sigma-Aldrich, Madrid, España). Los sustratos fueron disueltos en tampón (pH 8.0) a una concentración final de 0.4 mmol/L. Cada 250 µL de sustrato 0.4M, se mezcló en la microplaca con 15µL de homogeneizado alicuotado de los recién nacidos. La fluorescencia fue medida a 355/460 nm EX/EM.

## 2.12.5 Fosfatasas

Los sustratos para analizar las fosfatasas ácida y alcalina fueron respectivamente el MUP (4-methylumbelliferyl phosphate, M-6491) y el DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate D-6567;). Una solución stock de 10mmol/L de MUP/ DiFMUP fue preparada disolviendo sustratos en tampón borato (pH 8.0). 20 µL de homogeneizado fue mezclado y añadido en la microplaca. La fluorescencia fue medida a 360/ 440 nm EX/EM.

## 2.12.6 Zimograma de enzimas digestivas

Se realizó un zimograma para observar la capacidad caseinolítica de las enzimas digestivas, técnica que evita la pérdida de actividad de las enzimas estudiadas. El zimograma de proteasas alcalinas se realizó usando las técnicas descriptas por García-Carreño y colaboradores

(1993). El gel de poliacrilamida con dodecilsulfato sódico (SDS-PAGE) ha sido preparado de acuerdo con la descripción de Laemmli (1970), usando acrilamida (Figura 2.14). El gel (8 × 10 × 0.075 cm) constaba de dos partes: el gel de almacenamiento, (4% T-2.6% C) y el gel de separación (11.5% T-2.6% C), donde T era la concentración total de los monómeros y C la parte de los monómeros totales que representa la bis-acrilamida. En la placa se añadían 5 mL de Marcador de Peso Molecular (Molecular Weight Marker). El extracto enzimático (20 µL) se mezclaba en partes iguales a la del tampón (0.125 MTris–Cl, 2% SDS, 20% v/v glicerol, 0.04% bromofenol blue, pH 6.8; sin 2-mercaptoetanol) sin calentar.


**Figura 2.14** Preparación gel de poliacrilamida (SDS PAGE) y tinción

El volumen por cada muestra contenía 5.22 ± 1.65 µg de proteína soluble determinada a través del método de Bradford (Bradford, 1976), y se colocaba en pocillos individuales a 0°C. Las condiciones de la electroforesis fueron: 120 min a corriente constante de 220V, 15 mA, 2W. A continuación, el gel venía lavado e incubado a 50 mmol/L tampón TRIS-HCl (pH 9), conteniente el 3% de caseína (Sigma) por 30 min a 2 °C. El paso siguiente era elevar la temperatura a 37 °C por

90 min sin agitación. El gel se lavó, se fijó en 12% TCA y se tiño toda la noche (0,025% p/v Coomassie Brilliant Blue BBC R-250, 40% v/v metanol, 7% v/v ácido acético glacial). El lavado fue realizado usando una solución (40:7:53) de metanol, ácido acético y agua destilada.

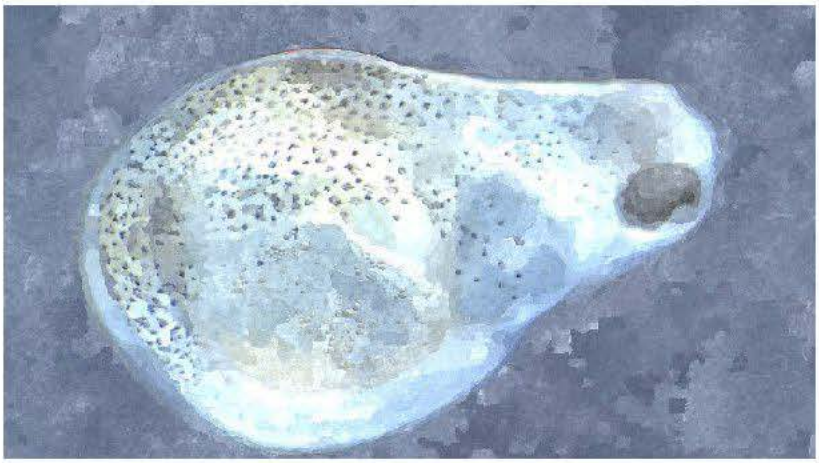
## 2.13 Análisis estadístico

Todos los análisis estadísticos se realizaron con el paquete estadístico SPSS (SPSS versión 21, Inc., Chicago, IL, USA).

**La relación longitud-peso** se estimó como una regresión potencial que relaciona una medida lineal (talla) con una de volumen (peso) de acuerdo con la ecuación: $WT = a L^b$ (Ricker 1975; Gulland 1983; Pauly 1984), en donde WT es el peso total del pez en miligramos, a es una constante de regresión, equivalente al factor de condición (Fc), L es la longitud total (LT) en milímetros y b es el coeficiente de regresión.

El efecto de los tratamientos sobre el crecimiento de los caballitos de mar, se evaluó usando un **Análisis de la Varianza Anidado**, aplicando el modelo general lineal. La diferencia significativa (HSD) se usó para comparar el valor de la actividad enzimática entre tratamientos a la misma edad, con un nivel de confidencialidad del 95%.

Un **Análisis Multivariante** (cluster) combinando todos los datos procedentes de todos los análisis enzimáticos se utilizó para investigar la similitud entre individuos de la misma edad y con diferentes regímenes alimenticios. Este análisis fue repetido en varias edades para evaluar la similitud/diferencia entre los tratamientos durante el crecimiento. Se utilizó un enlazado único de amalgamación, y las distancias euclideas se utilizaron para establecer las distancias entre objetos en el espacio multidimensional.



# Estudio I

## *The early life of seahorse, Hippocampus reidi (Syngnathidae). Development into the male broodpouch*

In preparation

B Novelli, JA Socorro, F Otero-Ferrer ,and L Molina Domínguez

## 3.1 Resumen

Machos y hembras de caballitos de mar *Hippocampus reidi* se colocaron por pares con el fin de establecer parejas reproductoras. Durante el primer período de cohabitación la escasa sincronización causó puestas perdidas y abortos naturales. Se recogieron cuidadosamente del fondo de los acuario huevos fertilizados y no fertilizados, así como embriones vivos en diferentes etapas de desarrollo. Con estas muestras, se describió en detalle el desarrollo morfológico de *H. reidi* desde la fecundación de los huevos, hasta la salida de la bolsa paterna. Se muestran por primera vez imágenes de huevos fertilizados, así como su ruptura después de pocos minutos en agua marina. La masa del saco vitelino inicialmente ovoide, adquiría una forma esférica, y se reabsorbía progresivamente hasta la expulsión de los caballitos de mar. La cola comenzó a diferenciarse y levantarse desde la superficie del deuteroplasma cuando los caballitos están todavía dentro del huevo. Carecen de aleta primordial y desarrollan algunas de las características de la especie antes de la reabsorción del saco vitelino, como la aleta dorsal incluyendo los radios. Una vez expulsados, los caballitos de mar muestran una etapa avanzada de desarrollo, aunque carecen de importantes características morfológicas presentes en los adultos como las placas óseas, los anillos óseos y la coronilla. La cola no es prensil y se observa una pequeña aleta caudal formada, que no aparece en adultos.

## 3.2 Abstract

Males and females' seahorses of *Hippocampus reidi* were maintained in pairs in order to stablish breeding pairs. During the first period of seahorse's cohabitation the scarce synchronization lead to eggs lose and natural male abortions. Fertilized and unfertilized eggs were carefully recollected on the aquarium bottom, as well as the embryos at different stages of development. Using these samples, the morphological development of *H. reidi* from the fertilization of the eggs until the release from paternal pouch was described in detail. Images of fertilized eggs, as well as its rupture after few minutes in marine seawater were reported for the first time. The vitelline mass from ovoid acquired a spherical shape and was reabsorbed progressively until the seahorses' release. Seahorses tail started to rise from the surface of the deuteroplasm when are into the egg envelope. They lack of primordial fin fold and develop some characteristics of the species, like the dorsal fin including the rays before the reabsorption of yolk sac. At release seahorses present an advanced stage of development even if lack of important morphological characteristics of the adults like rings plates and coronet. The tail was not prehensile and a little caudal fin was present, fin that lacks in adults.

## 3.3 Introduction

Seahorses are one of most fascinating fishes in the marine environment due to their specialized anatomical features. Present a tubular snout (Leysen *et al.* 2011) that allows a peculiar way to catch prey named suction feeding (Van Wassenberg *et al.* 2013), the orientation of the body axis during the swim is vertical, and the propulsion is given by the dorsal fin instead of the caudal fin (Breder and Ergenton 1942). The whole body is composed by an armor of bony plates instead of scales (Lourie *et al.* 2004), are provided of a prehensile tail to attach the substrate (Porter *et al.* 2013), and are able to camouflage developing skin filaments (Curtis 2006). Furthermore, seahorses are distinguished for the peculiar reproductive strategy. They are viviparous fishes where the males receive telolecithal eggs from their female partners that fertilize (Van look *et al.* 2007) and incubate in a specialized pouch localized in their abdomen (Carcupino *et al.* 2002). The brood pouch is a skin pocket, with a small pore situated anteriorly able to receive eggs and to isolates the pear-shaped eggs and embryos from the external environment sealing it (Carcupino *et al.* 2002). As well as most teleost fish with parental care, they produce larger eggs with a small brood and investing much energy in their offspring development. Seahorses' pouch penetrated by blood vessels, with structures that were altered during gestation (Laksanawimol *et al.* 2006). In the normal stage, the pouch consists of two layers of epithelium, a pseudostratified columnar epithelium (inner) and a stratified cuboidal epithelium (outer). When the embryo develops within the pouch, they are embedded in the inner epithelial layer that is extended to enclose each developing embryo and

separating per compartments (Laksanawimol *et al.* 2006). The brood pouch works as a "pseudoplacenta" during the gestation (Carcupino *et al.* 2002) with nutritional, respiratory and osmoregulatory capacity. The role of the brood pouch in viviparous animals is to protect eggs and embryos. In seahorses was also demonstrated the interchange between pouch and embryo (Linton and Soloff 1967; Carcupino *et al.* 2002; Melamed *et al.* 2005). Supplementary nutrition to embryos was initially proposed (Gill 1905) and successively rejected due to the survival and development of the embryo once extracted from the brood pouch (Azzarello 1991), but upregulated nutrient transporter genes discovered during the gestation, relaunch this hypothesis (Whittington *et al.* 2015). In fact, male seahorses invest in its young with a cost to parental fitness before their release, that is reflected in the quality of the offspring (Otero-Ferrer *et al.* 2014). The impossibility to study the development of the embryo in seahorses without the sacrifice of the animal (that interrupt natural embryo development) is due to the sealed pouch. There are some morphological descriptions on newborns seahorses after the release (Novelli *et al.* 2015; Choo and Liew 2006), and scarce and not recent studies on the description of their embryos (Ryder 1881; Brunelli 1922; D'Ancona 1932; Wetzel and Worums 2004). The knowledge increase about seahorses' embryogenesis makes possible to compare seahorse and others fish larvae. For the first time, fertilized eggs of seahorses were illustrated with images, and the major stages of embryonic development into the *Hippocampus reidi* brood pouch were described.

## 3.4 Material and methods

### 3.4.1 Culture facilities

The experiment was performed at the facilities of the Parque Científico Tecnológico Marino (PCTM) of the Las Palmas of Gran Canary University. Two autonomous experimental units were used to rear broodstock. Seawater was purified using a 5-mm sand filter (S500; Jazzi, Guangzhou, China). The filtration performed in a separate 500l rectangular fiberglass tank using physical (blue tower, and skimmer Turboflotor 5000 Shorty Compact; Aqua Medic, Bissendorf, Germany), and biological (live rocks) elements.

The seawater was heated and sterilized by ultraviolet rays (UV) using aquarium chillers (TC60, Teco, Ravenna, Italy). All the aquaria were illuminated with T5 tubes (24W, 10 000K; Aqua Medic) and aerated (6.3-6.7 mg/l). Values of temperature, pH, and salinity were monitored by PC (AT-Control System; AquaMedic) as well as aquaria water levels.

### 3.4.2 Broodstock and conditions

New couples of broodstock were formed and held in rectangular 90l glass aquariums (one pair per tank) provided of plastic seagrass for the attachment. The animals were fed on frozen mysids (Ocean Nutrition, Essen, Belgium) twice a day, 6 days per week. The temperature ranged between 25-27 °C, with a summer photoperiod (14L:10D).

### 3.4.3 Biologic material, eggs, embryos and larvae

Eggs were obtained from female broodstock, that were visually monitored. Freshly released and unfertilized and dropped eggs were carefully recollected on the aquarium bottom for two times (371 and 296 eggs), fertilized eggs one time (46 intact eggs). During the gestation period that takes two weeks in this species, seahorses' embryos were obtained from various natural abortions during male pregnancy (four abortions: 346, 297, 312 and 318 embryo/larvae). Samples in each abortion include one or two different stages of development. These samples were combined to assemble a series that represent the major stages of embryonic development. All the samples were observed with Leica stereo microscope (Leica Microsystems, Wetzlar, Germany), and digitally photographed with Leica DFC 420 camera (Leica Microsystems, Wetzlar, Germany). Measure were obtained using an image processing software, (Image pro-Plus, Media Cybernethics, USA).

## 3.5 Results

### 3.5.1 Embryogenesis

Unfertilized eggs of *H. reidi* were demersal, lack of chorion, and characterized by and fragile external membrane. Eggs were rod-shaped, sometime pear-shaped (1.21mm±0.11mm/ 0,56mm±0,05mm), and containing numerous oil droplets with a distinctive orange/yellow colour along its body. It was present a perivitelline space (Figure 3.1a). Early fertilized eggs were pear-shaped (1.28mm±0.09mm/0.84±0.07), opaque, with a central nucleus.

The eggs were smaller in its proximal pole and roundest around the nucleus; were provided by abundant lipid droplets with orange/yellow colour (Figure 3.1b). The envelope is a thin and permeable lamina that in seawater medium swelled, and acquired progressively a rounded shape (Figure 3.1c) until the egg rupture after few minutes (Figure 3.1d).

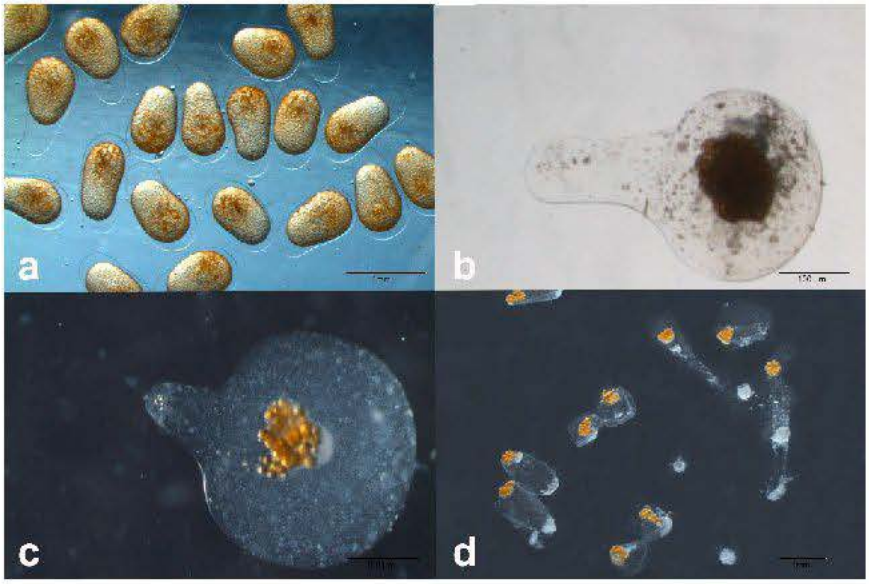



**Figure 3.1** Seahorses eggs (*H. reidi*) a: unfertilized eggs; b: fertilized egg c: rounded fertilized egg in seawater; d: rupture of fertilized eggs in after few minutes in seawater

The morphological changes from fertilized eggs until the release were progressive, but the hatch lead to divide their development into pre- and post-hatching stage. For this reason, the description of the development into the brood pouch of the longsnout seahorses, *H. reidi*, in this study is based on 2 developmental stage, early embryogenesis composed by three phases, and yolk sac larva formed by two phases (Table 3.1).

**Table 3.1** Summary of the major developmental events into the brood pouch. (The measure represents the total length of the embryos and larvae)

| Early embriogenesis | | | Yolk sac larva | |
|---|---|---|---|---|
| Zygote<br>Early cleavages<br>Embrionic shield<br>Primitive streak | Optic vesicles<br>Caudal region raised<br>Pigmentation | Tail growth<br>Spherical vitelline<br>Thin egg envelope<br>Eye formation | Head orietated at 90°<br>Fins formation<br>Pigmented eye | Yolk sac reabsorption<br>Snout and tail development<br>Spines formation |
| 1A | 1B | 1C | 2A | 2B |
| 1280-1370 µm | 1370-2200 µm | 2200-3300 µm | 3800-4090 µm | 4090-8320 µm |
| Pre-hatch period | | | Post-hatch period | |
| Egg (1280 µm) | | Hatch (3300-3800 µm) | | Release (8320 µm) |

### 3.5.2 Stage 1: early embryogenesis

The early embryogenesis comprising: zygote, early cleavages, embryonic shield, primitive streak, and the embryo outlines appeared around the vitelline curvature encircling the yolk (phase 1a). The head region started to differentiate itself (Figure 3.2a). The head was positioned ventrally, and was aligned along the midline of the embryonic shield (1370 µm). The anterior part of the neural tube could be distinguished from the ovoid vitelline mass and the optic vesicles appeared towards the end of epiboly as semispherical structures. The caudal region is raised from the surface of the deuteroplasm. The embryo showed pigmentation along the dorsal zone of the animals and the vitelline mass showed an ovoid shape. Numerous lipid droplets were present in in the vitelline sac (phase 1b). As embryogenesis proceeded, the melanophores migrated along the head and the tail gathering in two point (head arrows) along the dorsal line of the animals (Figure 3.2b). The tail was extended backwards, as a simple cylindrical prolongation of the hind portion of

the body. The vitelline mass acquired a spherical shape (phase 1c), the head started to rise and the eye was more pigmented (2200 μm). The fins were absent, the embryo continued to increase in size along the yolk mass remained coiled around it until the hatching (3330 μm) it was possible to appreciate the thin egg envelope (Figure 3.2c)

### 3.5.3 Stage 2: yolk sac larva

*H reidi* showed an indirect development. The embryo hatched into the brood pouch and the development followed in that place until the release. The embryo remained nestled within the stromal tissue of the brood pouch (Figure 3.2d).

The yolk sac 'larva (3800 μm) showed characteristic posture of the seahorse head, with the orientation at 90° respect to the body axis (phase 2a). In the eye is possible to observe the retina, and started the morphogenesis of the jaw. The yolk sac present lipid droplets. Seahorses lack of primordial fin while dorsal fin is well defined. Pectorals, and anal fin appeared and the lepidotrichia of the fins were differentiated. The body was very pigmented at this phase with melanophores absent only in the ventral side of the yolk sac.

The yolk sac larvae increased in size and the yolk sac is being progressively reabsorbed in the thoracic-abdominal region. During this phase (4090 μm) until the birth, is possible to observe two principal characteristics. The first one was the peculiar elongation of the snout (with relative formation of the jaws), that begins before the reabsorption of the vitelline sac, and the second one was progressive reabsorption of the yolk sac. The lipid droplets also disappeared progressively (Figure 3.2e).

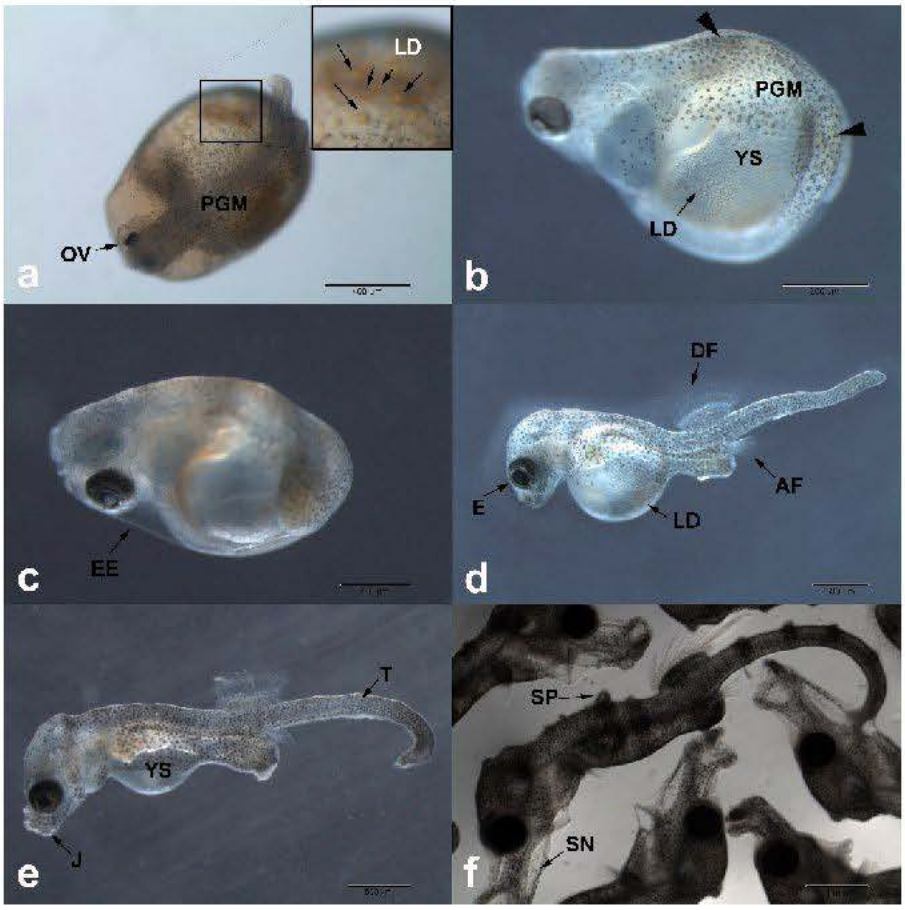



**Figure 3.2** Development of seahorses (*H. reidi*) into the brood pouch. a: embryo formation in the animal pole of the egg; b: embryo growth; c: embryo after the hatching; d: yolk sac larva; e: reabsorption of the yolk sac; f: seahorses at release. (AF) anal fin; (DF) dorsal fin; (E) eye; (EE) egg envelope; (J) jaw; (LD) lipid droplets; (OV) optical vesicles; (PGM) pigmentation; (SN) snout; (SP) spine; (YS) yolk sac.

Before to be released in the marine medium, while develops proceeded, seahorses showed the prolongation of the tail (that at this stage was no prehensile), and snout. Furthermore, together with the snout and tail elongation, the pigmentation increase with melanophores migrating until the ventral part of the body, and metameric little spine were formed along whole body (phase 2b).

When newborn seahorses were released, (8320 μm ± 340 μm) lack of coronet, nose spine, body plate, and body ring. They showed little spines along the body, one in the upper portion of the head. The tail was no prehensile and the caudal fin is very reduced (Figure 3.2f).

## 3.6 Discussion

From the fertilization to the release morphological changes in embryo and larvae happen progressively into the brood pouch. Some authors used these changes to classify the pre-release development in period and stages that differing in names and times (Wetzel an Worums 2004, Sommer *et al.* 2012, Brunelli 1922). Due to the complexity and quantity of the changes observed in this study two principal stages were distinguishes divided by the hatch moment: early embryogenesis and yolk sac larvae.

Unfertilized and fertilized eggs presented morphological difference in *H. reidi* and their shape is unusual among teleost fishes. In the first case were rod-shaped and sometime pear-shaped with oil droplets along all the eggs body, as observed also for others authors. Planas *et al.* (2010) propose the different degrees of ripening/hydration or to compression by adjacent eggs as the cause of the morphological difference between unfertilized eggs. The presence of permeable and thin membrane and the lack of chorion make it a valid hypothesis. The cause of the numerous lipid droplets along the whole eggs body could be due at the rupture of the nucleus as described in *H. guttulatus* (Brunelli 1922) (not observed) that release the lipid droplets into the yolk. Unfertilized eggs were more resistant in marine water respect to the fertilized eggs that morphologically were pear-

shaped, opaque, and showed a more marked asymmetry. Fertilized eggs in seawater showed changes in shape (from pear to round quickly and progressively, until its rupture. This fact was also observed by Brunelli (1922), that in *H. guttulatus* formulated the hypothesis of a difference in molecular concentration between egg and seawater. In according with Brunelli (1922), the high permeability of the thin envelope (see Stölting and Wilson 2007) of the eggs together with the different density could be the cause of the rupture. Azzarello (1990) demonstrates that the brood pouch was not essential for nutritional survival of the embryos and yolk sac is the primary energy source in *Syngnathus scovelli*. Stölting and Wilson (2007) explain that the pouch epithelium of pipefishes species generally have a rudimentary brooding structures similar to normal skin tissue, like in *Nerophis spp.* while seahorses provided of more complex pouches. Others authors highlighted the pseudoplacenta function of seahorses sealed pouch, that gives protection, oxygen, and nutriments (Carcupino *et al.* 2002; Melamed *et al.* 2005; Whittington *et al.* 2015). Due to the observations carried out in this work it can be affirmed that a fertilized egg in seawater swelled it, break it, and not leading to the formation of the embryo.

The eggs size registered in the present study are in accordance with the results reported previously for this specie (Monteiro et al. 2003), confirming that *H. reidi* despite its mayor size, produce smallest eggs respect to smallest species of seahorses. According with Foster and Vincent (2004), and Planas *et al.* (2010) differences in egg size within seahorse species depend on latitude rather than the size of the animals

and seahorses with a bigger pouch no produced bigger eggs but a higher number of eggs.

In seahorses was described a thin egg envelope and was detected the high choriolytic enzyme (HCE) as the only hatching enzyme that seems to have only a swelling effect (Kawaguchi *et al.* 2016). Furthermore, was described a softening of the egg envelope during the development broke with the subsequent movement of embryos. After the formation of the embryo, the tail was the first element that appeared separated from the yolk sac. This fact was also observed in *Syngnathus fuscus* (Hardy, 1978) and in *Hippocampus zosterae* where this phase was named tail-bud period (Weetzel and Worums 2004). Probably the early raise of caudal region, and its development, play a key role in the mechanical egg rupture during hatch moment.

Differences between seahorses' species were observed in the pigmentation pattern, in *Hippocampus erectus* seems to form bands while in *H. reidi* was uniform along the head and dorsal zone of the animals. The pigmentation in *H. reidi* started during the early embryogenesis, while in *Hippocampus guttulatus* and *H. erectus* (Brunelli 1922; Hardy, 1978) after the hatch. It could be due to the different period of gestation, larger in *H. guttulatus* and *H. erectus* (Foster and Vincent, 2004) respect to *H. reidi*.

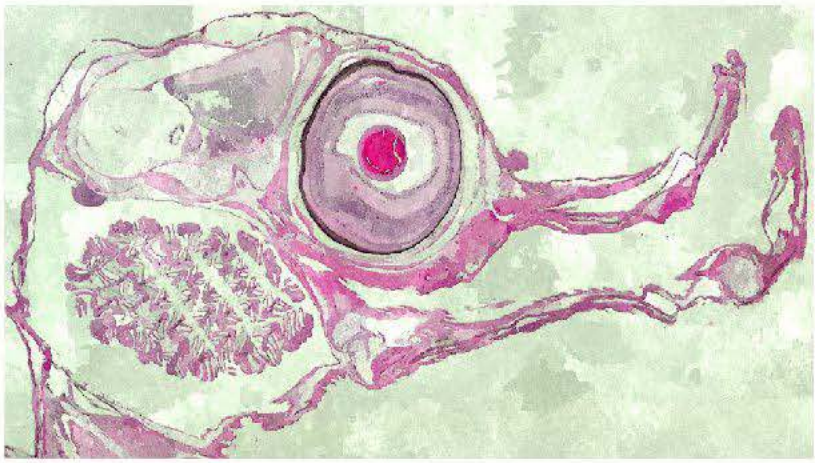
The development of the eye starts in the pre-hatching phase, and follow in the post hatching phase, the same was descripted in *H. guttulatus* by Brunelli (1922) and visible in Faleiro and Narciso images (2010). The lens was distinguishable when seahorses were into the egg, this rapid development of the eye allow newborns to have an acuity vision and facilitate the catch, even if at birth and

during the first 30 days of free life lack of fovea (Novelli *et al.* 2015), a specialized area in the retina associated with acuity vision and described in adults' seahorses (Lee and O'Brien 2011; Mosk *et al.* 2007).

Brunelli point out on the difference between seahorses and the most of teleost fish larvae, where the primordial fin is absent and the fin rays formed before yolk sac reabsorption. The functional significance of the larval median finfold remain unresolved, several hypotheses have been proposed, between them, the undulatory swimming and the respiration in pre-hatched larvae and free-swimming stages (van den Boogaart et al. 2012). Probably, seahorses loosed this firstly phase during the evolution respect to the pipefishes, in fact some morphological peculiarities of the adults are reflected in the early development of embryo and larvae. Primordial fin-fold was absent, like observed also in others seahorses (*H. zosterae*, Wetzel and Worums 2004) and pipefish (*Syngnathus acusimilis*, Drozov *et al.* 1995; *Syngnathoides biaculeatus*, Dhanya *et al.* 2005), while the development of dorsal fin and rays happen quickly. Peculiar morphological characters of the species were developed also during the early life of others fish, like the pectoral fins in scorpaeniformes (Richards 2006).

A difference between seahorses and pipefish was the orientation of the head after the hatch. In seahorses the head showed an angle of 90 degrees' respect to the body axis while in pipefishes continues to develop in line with the body axis (Silva *et al.* 2006). This difference is related with the characteristics of each species as well as the formation of the caudal fin.

Seahorses lack of caudal fin after hatch and during the later development was imperceptible. In pipefish appeared after hatch and showed a larger size (Silva *et al.* 2006). The presence of a reduced caudal fin in newborns, and the absence in adults, were also observed also in others seahorses' species (Choo and Liew 2006; Franz-Odendaal and Adriaens 2014). It seems to be a sort of adaptation during the pelagic phase, where the tail was no prehensile and seahorses swimming in the water column. This paper not only give new knowledge about seahorses' early life, but in contrast with previous studies, (Wetzel and Wourms 2004; Choo and Liew 2006) also confirm that newborns are not replicates of adults, supporting the study of Novelli *et al.* (2015) where it was demonstrated that newborn seahorses of *H. reidi* when released are larvae instead of juveniles. The external morphological description highlights the absence at birth of morphological structure present in the adults like coronet, spines, bony plates, and bony rings. Furthermore, newborns avoid of an important physiological tract of the juveniles and adults, like the prehensility of the tail, that not allow newborn to present the same behavior than adults.



# Estudio II

## *Development of seahorse (Hippocampus reidi, Ginsburg 1933): histological and histochemical study*

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B Novelli, JA Socorro, MJ Caballero, F Otero-Ferrer, A Segade-Botella, and L. Molina Domínguez
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## 4.1 Resumen

Tanto los aspectos biológicos como la demanda mundial para acuarios, convierte a los caballitos de mar en nuevas especies con alto potencial para su producción comercial; sin embargo, la baja tasa de supervivencia de los recién nacidos representa el principal cuello de botella en su cultivo. En este estudio, la organogénesis de Hippocampus reidi fue analizada desde la expulsión de la bolsa incubadora hasta el trigésimo día después de su nacimiento, utilizando métodos histológicos e histoquímicos. Para el estudio de las etapas tempranas de su vida, 360 caballitos fueron sacrificados, seccionados, y teñidos con las técnicas de hematoxilina y eosina, acido periódico de Schiff, y negro Sudán B. En el momento del nacimiento, la boca y el ano están abiertos, la vejiga natatoria inflada, y el sistema visual altamente desarrollado. Entre los resultados, destaca la presencia del saco vitelino hasta el 2° día después del nacimiento, los bucles del intestino para acomodar su alargamiento, y la capacidad de las larvas para absorber los lípidos en el tracto anterior y posterior del intestino. Transcurrió un corto espacio de tiempo (7-8 días) entre la reabsorción del saco vitelino y la formación de las gónadas, con los folículos primordiales visibles desde el décimo día después del nacimiento. Por primera vez, se describe en detalle la organogénesis en H. reidi; los caballitos de mar sufren una marcada metamorfosis y el desarrollo indirecto observado en esta especie conducen a reconsiderar el término "juvenil" utilizado para H. reidi durante este período.

## 4.2 Abstract

Biological aspects and global demand for aquarium promote seahorses as new species with high potential for commercial purposes; however, the low newborn survival rate represents the main bottleneck of seahorses farming. In this study, the organogenesis of the *Hippocampus reidi* was analysed from release until the 30th day after birth, using histological and histochemical approaches. To study the stages of their early life, 360 individuals were killed, sectioned, and stained with haematoxylin and eosin, periodic Acid-Schiff, and Sudan Black B techniques. At birth, mouth and anus were open, the swim bladder inflated, and the visual system highly developed. Among the results, it was emphasized the presence of the yolk sac until the 2nd day after birth, the loops of the intestine to accommodate its elongation, and the ability of the larvae to absorb lipids in the anterior and posterior tract of the intestine. A short time (7/8 days) between reabsorption of yolk sac and formation of gonads was registered, with primordial follicles visible from the 10th day after birth. For the first time, organogenesis in *H. reidi* was described in detail; seahorses underwent a marked metamorphosis, and the indirect development observed in this species lead up to reconsider the term ''juvenile'' used for *H. reidi* during this period.

## 4.3 Introduction

The vast majority of adult and juvenile seahorses extracted from the wild are traded alive as ornamental fish or dried for traditional Chinese medicine (CITES 2002; Rosa et al. 2011; Vincent 1996). For this reason, the Convention on International Trade of Endangered Species (CITES) in November 2002 lists in the Appendix II all the species of seahorses.

Rearing seahorses in captivity offers not only a great contribution to stop overexploitation of wild individuals, but also new farming opportunities, and is sometimes a way of combining aquaculture and tourism (Ingram 2005). Success in larval rearing requires to encompass the larval stages and to apply selective weaning and rearing techniques. With this in mind, several histological and enzymatic studies on the larval period for aquaculture-promising species have been carried out (Hachero-Cruzado et al. 2009; Ortiz-Delgado et al. 2011; Papadakis et al. 2013).

The Western Atlantic seahorse *Hippocampus reidi* (Ginsburg 1933) has several characteristics favourable to success in aquaculture, such as the high number of young released (Foster and Vincent 2004), and the ability of the newly released to successfully feed since birth (Van Wassenbergh et al. 2009). However, the main bottleneck for seahorse aquaculture is the low newborn survival rate (Lin et al. 2012; Otero-Ferrer et al. 2010; Willadino et al. 2012) and the capacity to find adequate nutrient requirements during their early life.

Nowadays, the organogenesis in seahorses and the type of development after release (direct or indirect) are still unclear. Ontogeny in fish can be subdivided into intervals: embryo, larva,

juvenile, adult, and senescent periods, and each period may be divided into phases. Fish present two forms of development: direct and indirect. In direct development, the embryo leads directly to the juvenile or adult period, while indirect development involves a larval stage in which a metamorphic phase leads to the juvenile period (Balon 1999). In the juvenile period, fish are already similar in form, behaviour, and physiology to adults (see Bishop et al. 2006). Although newborn seahorses are considered already metamorphosed (Choo and Liew 2006) and reared as juveniles (Hora and Joyeux 2009; Palma et al. 2014; Pham and Lin 2013), previous studies described both external morphological changes (Silveira 2000) and the first planktonic stage before the benthic one (Hora and Joyeux 2009; Mai and Velasco 2012; Otero-Ferrer et al. 2010).

For these reasons, the aims of this study were to observe the internal morphology during the first month of life of *H. reidi* and to describe morphological changes, in order to establish direct or indirect development in their offspring.

## 4.4 Materials and methods

### 4.4.1 Seahorse facilities

Animals were maintained at the University of Las Palmas facilities (ULPGC, Spain) (Parque Científico Tecnológico) and reared on semi-closed recirculating seawater system (20 % new water turnover per day) in four autonomous experimental units. Two units were used for broodstock breeding and the others for rearing purposes. *H. reidi* broodstock were held in 90-l glass tanks provided of plastic plants and nets for attachment, separated in breeding pairs (one aquarium per

pair), under natural ranges of temperature and photoperiod (25 °C; 12D:12N). In two experimental units, broodstock seawater treatment happened in separate compartmentalized rectangular tanks of 500 l per unit. The seawater treatment per unit consisted on mechanical (Aqua Medic Riff 2000, Bissendorf, Germany) and biological (live rocks) filter system. An aquarium chiller (Teco TR60, Ravenna, Italy), provided also with UV-C light, regulated the water temperature. The offspring were reared in 30-l glass aquariums, at density of two newborn per litre. Each aquarium was provided of oxygen tube, and water renewal oscillated around 54 %/h. In the other two experimental units, offspring seawater treatment was located at the bottom of each unit. It consisted on mechanical (2 per Aqua Medic Riff 500, Bissendorf, Germany, each unit) and biological (live rocks) filter system. An aquarium chiller (Teco TR20, Ravenna, Italy), provided also with UV-C light, regulated the water temperature.

### 4.4.2 Water quality

In broodstock and offspring aquaria, the oxygen range remained between 6.3 and 6.7 mg/l. Ammonia, nitrites, nitrates, and phosphates concentrations were maintained below detection levels.

### 4.4.3 Broodstock breeding

Animals were separated in breeding pairs (one aquarium per pair), under natural ranges of temperature and photoperiod (25 °C; 12D:12N). The glass tanks were provided of plastic plants and nets for attachment. The standard length of broodstock employed during this study ranges from 10 to 12 cm. Animals were fed with frozen mysids

(Ocean Nutrition™, Essen, Belgium) twice a day, 6 days per week, and approximately 12 % of their wet body weight. Aquaria bottom was cleaned every day.

### 4.4.4 Newborn rearing conditions

The offspring remained under natural temperature and photoperiod ranges (25 °C; 12D:12N) and fed with *Artemia* salina nauplii (EG type 850 mm, INVE Aquaculture) enriched for 24 h with EASY SELCO DHA (INVE Aquaculture, Dendermonde, Belgium) twice per day, 7 days per week. The prey density was 0.7 nauplii/ml, and aquaria bottom was cleaned every day.

### 4.4.5 Sampling and sample processing

The organogenesis was studied employing 360 seahorses from various breeding pairs and during a 30-day period. Newborns were reared per triplicate; 180 seahorses coming from each broodstock release were separated in three aquaria (60 newborns per aquarium). From each release, six individuals per day (two per aquarium) were sampled randomly to perform morphological analyses (H&E, PAS and Black Sudan). The triplicates for the morphological analyses included a pool of 18 newborns coming from three different breeding pairs (six for each broodstock pair). Each pool was subdivided in triplicate, six seahorses were used for each analysis, and each triplicate was composed by seahorses (two seahorses per analysis) coming from each pair of broodstock. Seahorses were killed using clove oil anaesthetic overdose (natural clove essential, Guinama, Alboraya, Valencia, Spain) and conserved in 10 % buffered formalin (Sigma Chemical

Co.). Individuals were killed daily until the 10th DAB, every other day until the 24th DAB, and every 3 days until the 30th DAB.

### 4.4.6 Chronological description

The unusual reproductive strategy employed by seahorses with the incubation of the embryos inside the male brood pouch does not allow determining the hatching moment. Therefore, the terms ''day of birth'' (DOB) and ''day after birth'' (DAB) were used to establish the time when offspring were expelled from the pouch.

### 4.4.7 Measurements

All seahorses sampled were used for growth analyses; the total length (TL) was measured following Lourie (2003) with a profile projector (Mitutoyo PJ-R 3000, USA). The wet body weight was measured with a precision balance (Mettler Toledo AG204, Greifensee, Switzerland). Size and weight measurements were used to estimate offspring growth. The absolute growth rate (AGR), as the mm or mg gained per day, was calculated using the equation (Lf - Li)/t2 - t1 (Hopkins 1992). The Lf was the mean length/wet weight (mm/mg) of the sample at the end of each developmental stage at time t2; Li was the mean length/wet weight at the end of the previous stage at the time t1. The ratio daily weight increase (%DWI) to specific growth rate (SGR) was calculated according to Ricker (1958). The exponential model used was SGR = (lnW2 - lnW1)/t2 - t1, and %DWI = 100 9 ($e^{SGR}$ - 1), whereW1 and W2 were the length/wet weight at t1 and t2, respectively. The unit 1 is the initial length/wet weight; and the length/wet weight eg increased eg - 1 at the end of a unit of time.

### 4.4.8 Seahorses' developmental stages

Seahorses' development until the 30th DAB was subdivided into four main developmental stages, based on internal/external morphological observations and behavioural changes. In the first stage, mouth and anus were open and yolk sac was located around the tubular intestine, while in the second stage, yolk sac and supranuclear vesicles in the hindgut were progressively reabsorbed, and the intestine maintains a rectilinear structure. In the third stage was included the major change, at the end of this stage, seahorses showed all the elements of a juvenile, including the geometrical disposition of the intestine with the progressive formation of the loops. Seahorses started to change its behaviour from planktonic to benthic. In the last sage, seahorses increased in size, completed the formation of the coronet, and improved tail prehensile abilities maintaining the same internal morphological characteristics of the third stage.

### 4.4.9 Histological and histochemical evaluation

Paraffin blocks (each containing one single newborn) were sectioned at 5 lm, cut with sagittal and transversal sections, and stained following the haematoxylin and eosin (H&E) protocol for histological evaluation (Martoja et al. 1970). In order to detect the polysaccharides such as glycogen, the sections were further stained using the periodic Acid-Schiff (PAS) method. For the identification of lipids, agarose blocks (each containing one single newborn) were placed in a cryoprotective embedding medium (OCT 4583, Tissue - Tek, Miles) and sectioned at 6 lm in a freezing cryostat (Leica Jung CM 3000, Nussloch, Germany). Sections were incubated in a Sudan Black B solution for 90 min. The

staining was then differentiated in 70 % ethanol, counterstained in Mayer's haematoxylin, and mounted in a glycerine medium (Dako Glycergel mounting medium, USA). The mounted sections were examined under an

Olympus CX41 light microscope (Olympus, Hamburg, Germany) and photographed with an Olympus XC30 camera (Olympus, Hamburg, Germany), linked to a computer using image-capturing software (Cell B®, Olympus, Hamburg, Germany).

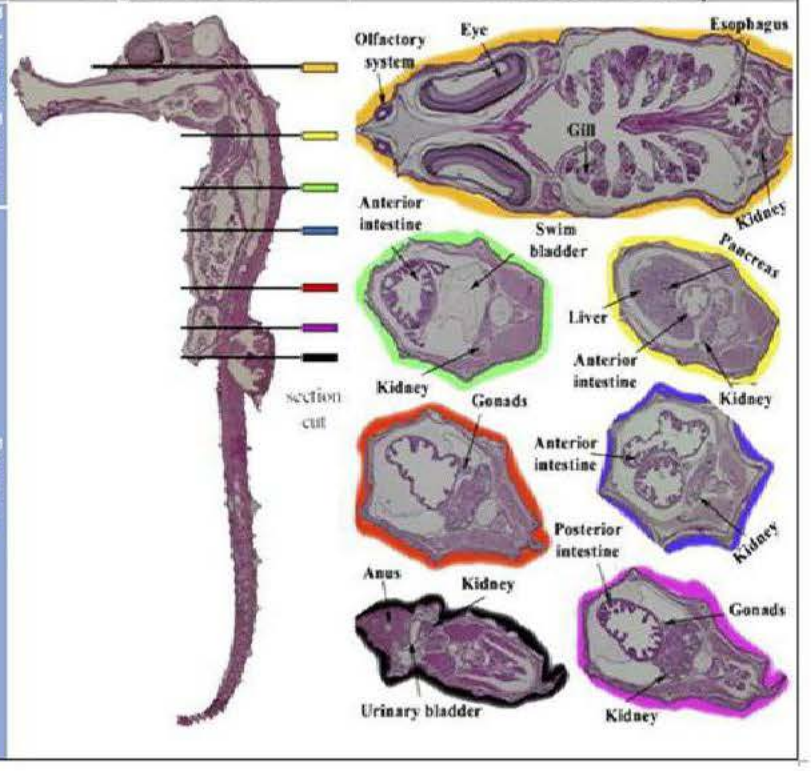
## 4.5 Results

### 4.5.1 General observations

At birth, *H. reidi* newborns showed several physiological systems highly functional, such as the visual or locomotor system; others gradually increase the functionality, such as digestive, circulatory, respiratory, immune or urinary system, while others were visible later, like the reproductive system or some endocrine glands. The morphological changes in the cellular architectures observed during the histological development underline some important physiological changes, in particular the increase in the digestive capacity. The summary of the major developmental events occurred during the organogenesis are included in Table 4.1.

**a le** Summary of the major developmental events of the organogenesis, and general morphology of *H. reidi*

| Cronology | | Morphological observation | A |
|---|---|---|---|
| DOB | Stage 1 | Visual system completed, heart compartmentalized, yolk sac, mouth and anus opened, taste buds, gut differentiated, esophagus with mucous cells, gut with four chambers, PAS-positive brush border, supranuclear inclusion bodies, swim bladder inflated, acidophilic zymogen granules, some thyroid follicles | Endogenous Exogenous |
| DAB 2 | | Trabeculae in the atrium, yolk sac reabsorption | |
| DAB 3 | Stage 2 | Atrioventricular valve formation, hepatocytes placed in cords, first spleen observation, supranuclear lipid droplets in the midgut | Exogenous |
| DAB 4 | | Glycogen presence and lipid storage in the liver | |
| DAB 6 | | Mucous and chloride cells (gills) | |
| DAB 8 | | Reabsorption of the supranuclear vesicles in the hindgut | |
| DAB 9 | Stage 3 | Female reproductive system | |
| DAB 10 | | Corpuscles of Stannius | |
| DAB 12 | | Loops in the intestine, some oocytes in at the second stage | |
| DAB 16 | | Perivisceral adipose tissue, lipid droplets in the posterior intestine, some oocytes at the third stage | |
| DAB 18 | | Langerhans islets | |
| DAB 20 | Stage 4 | More than ten thyroid follicles | |
| DAB 30 | | Fat vacuoles in some oocytes | |




Illustrative microsections of 8 DAB seahorse (sagittal) and 20 DAB seahorse (transversal). A aliment events of the organogenesis, and general morphology of *H. reidi*

### 4.5.2 Growth

Seahorses showed a progressive increase in weight and length until the shift from planktonic to benthic phase, in the 1st, 2nd and 3rd stage. The highest AGR and SGR and DWI values were observed at 3rd stage and decreased at the 4th (Table 4.2).

**Table 4.2** Growth of *H. reidi* during the first month of life

| DAB | GROWTH (mm/mg) | | AGR (mm/mg day) | | SGR (mm/mg day) | | DWI (%) | |
|---|---|---|---|---|---|---|---|---|
| | TL±SD | W±SD | TL | W | TL | W | TL | W |
| 0–2 | 6.93 ± 0.36 | 2.31 ± 0.6 | 0.16 | 0.2 | 0.02 | 0.09 | 3.12 | 8.71 |
| 2–8 | 8.78 ± 1.37 | 3.55 ± 1.25 | 0.49 | 0.4 | 0.04 | 0.11 | 4.95 | 19.87 |
| 8–18 | 13.43 ± 3.18 | 12.93 ± 8.75 | 0.93 | 2.4 | 0.05 | 0.18 | 6.92 | 84.37 |
| 18–30 | 20.61 ± 3.92 | 33.56 ± 15.75 | 0.51 | 2.1 | 0.02 | 0.05 | 2.45 | 23.02 |

*AGR* absolute growth rate, *DAB* day after birth, *DWI* daily weight increase, *SD* standard deviation, *SGR* specific growth rate, *TL* total length, *W* weight

### 4.5.3 Endogenous reserves

At birth, seahorses presented an advanced stage of development; externally, there was no sign of the round-shape yolk sac, but internal morphology con- firmed its presence. Located around the tubular intestine (Figure 4.1a), the homogeneous and acidophilic yolk sac showed reduced volume, no presented the oil globule, and provided with small lipid droplets (Sudan Black positive; Figure 4.1b). The yolk sac was completely reabsorbed at the end of the 2nd DAB.

### 4.5.4 Digestive system

When seahorses left the brood pouch, the mouth and anus were open, and the gut, which was morphologically differentiated, could be divided principally into four parts: head gut (oral and gill cavities), foregut (oesophagus), midgut (anterior intestine), and hindgut (posterior intestine and rectum; Figure 4.1a). The tubular mouth, with

no teeth, evolved into a pharynx with a wealth of taste buds (Figure 4.1c), located between four pairs of gill arcs (Fig. 1a). The foregut comprised an external layer of striated muscle and a stratified epithelium, which was rich in mucous cells and containing neutral mucosubstances (stained positively with PAS; Fig. 1d). This species is stomachless, a valve separated oesophagus from midgut (Figure 4.1a). Midgut and hindgut extended linearly from oesophagus to the rectum presenting four chambers (Figure 4.1a). A monostratified layer of cubic and columnar cells with an evident nucleus an apical microvilli characterized the midgut. It could be divided into two regions: cubic simple epithelial cells composed the first and shorter tract (after the valve between oesophagus and midgut), and longitudinal folds were present in the second region (Figure 4.1a). The ileocaecal valve divided the midgut from the hindgut, and in the posterior tract, the lumen had a tendency to widen (Figure 4.1a). In the epithelial cells of the hindgut, supranuclear inclusion bodies (PAS positive) were observed (Figure 4.1e). The whole gut showed a PAS-positive brush border (Figure 4.1e). Until the 2nd/3rd DAB, the pharynx and oesophagus showed no changes. Inside the midgut and hindgut, seahorses presented almost intact *Artemia* nauplii, and there was no lipid absorption in the midgut enterocytes (Figure 4.1f). More folds both in the midgut and in the hindgut were observed, and goblet cells started to secrete neutral glycoproteins (PAS positive).

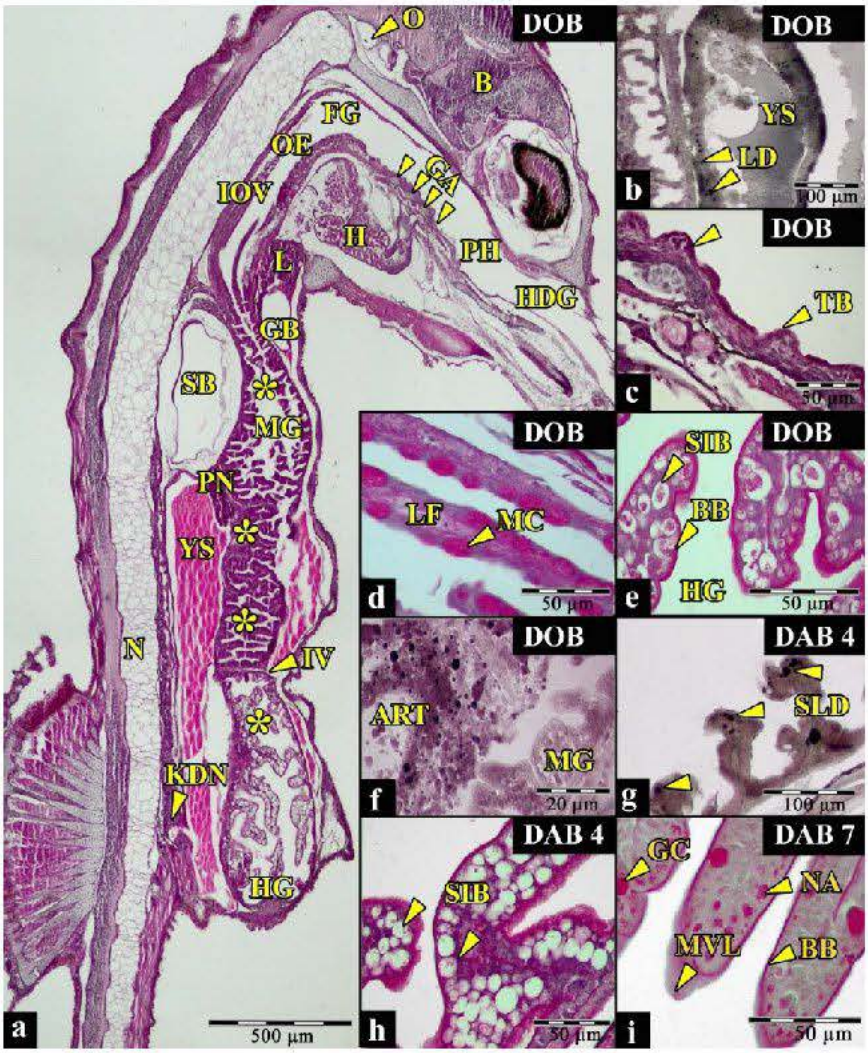
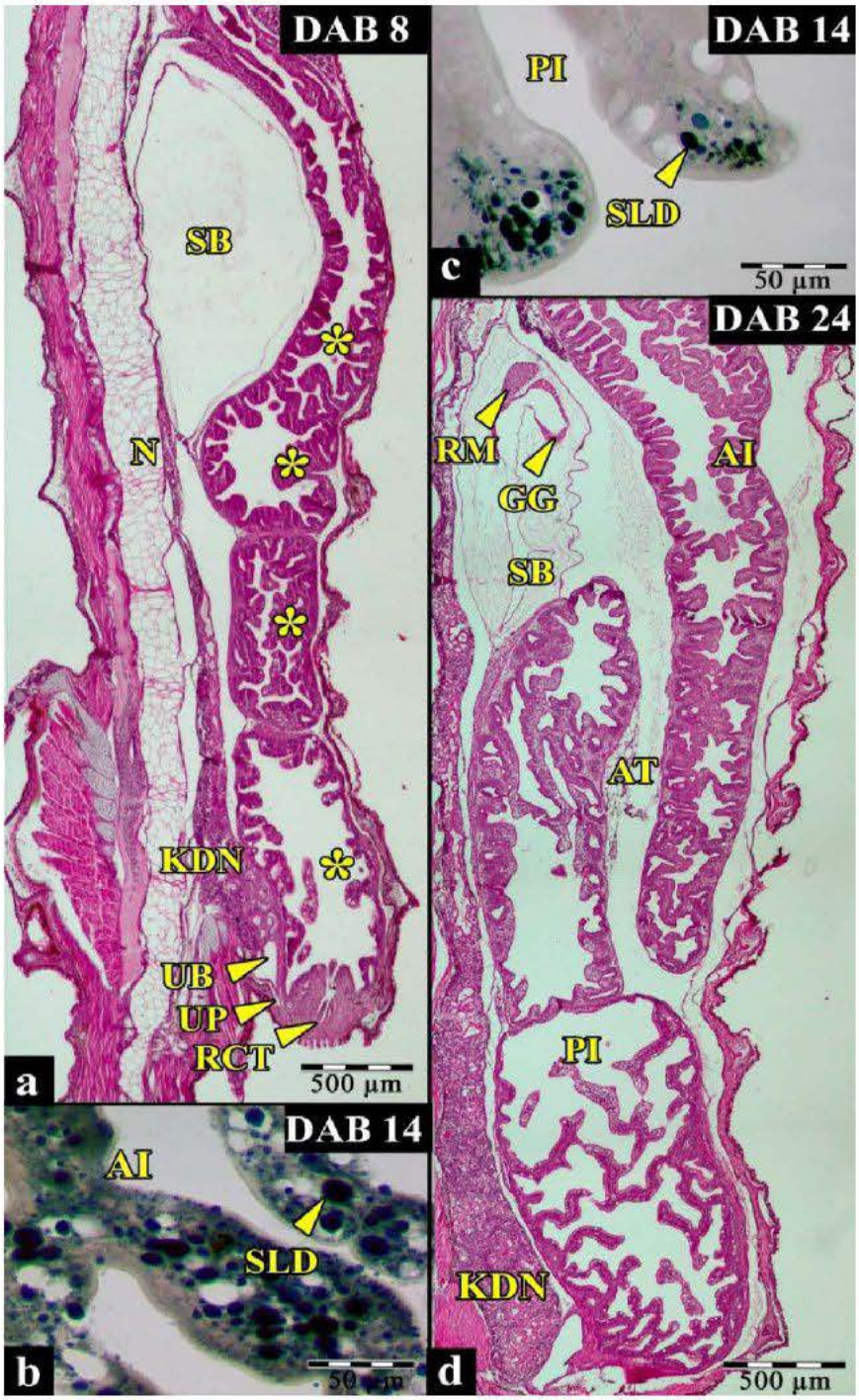



**Figure 4.1** Sagittal microsections of *H. reidi* larvae; digestive system DAB 0-7. (a) Internal morphological aspects of larvae at birth (H&E); (b) lipid droplets in the yolk sac (Black Sudan); (c) taste buds in the pharynx (H&E); (d) mucous cells in the esophagus (PAS); (e) detail of glucose presence in the hindgut (PAS); (f) *Artemia salina* nauplii in the midgut (Black Sudan); (g) Supranuclear lipid droplets in the midgut (Black Sudan); (h) supranuclear vacuoles in the hindgut (PAS); (i) goblet cells in the midgut (PAS). (*) Gut chamber, (ART) Artemia, (B) brain, (BB) brush border, (FG) foregut (GA) gill arcs, (GB) gall bladder, (GC) goblet cell, (H) heart, (HDG) headgut, (HG) hindgut, (IOV) valve between midgut and esophagus, (IV) ileocaecal valve, (KDN) kidney, (L) liver, (LD) lipid droplets, (LF) longitudinal fold, (MC) mucous cell, (MG) midgut (MVL) microvilli, (N) notochord, (NA) nutrient absorption, (O) otolith, (OE) esophagus, (PH) pharynx, (PN) pancreas, (SB) swim bladder, (SIB) supranuclear inclusion body, (SLD) supranuclear lipid droplets, (TB) taste buds, (YS) yolk sac.

At the 3rd/4th DAB, in the central tract of the midgut, the first presence of supranuclear lipid droplets (Black Sudan positive; Figure 4.1g) was recorded. At the same time, goblet cells (unreactive to the specific stain: PAS) in the last tract of the midgut were observed. Just as at birth, at the 4th/5th DAB supranuclear vesicles in the hindgut were present (PAS positive stain; Figure 4.1h). At the 7th DAB, the goblet cells increased in number (Figure 4.1i). Until the 9th DAB, the oesophagus showed prominent longitudinal folds with a more intensive presence of mucous cells.

There was a general increase in intestine length up until the 8th/9$^{th}$ DAB, and the incipient structure divided into four chambers continued to be tubular and rectilinear in shape (Figure 4.2a). In the hindgut, the supranuclear vesicles were progressively reabsorbed. At the 9$^{th}$ DAB, the formation of the loop and perivisceral adipose tissue were observed for the first time. Until the 10th DAB, smaller vacuoles in the intracellular spaces replaced gradually larger lipid supranuclear vacuoles. At the 12th DAB, the gut completed the torsion inside the abdominal cavity and formed two intestinal loops to accommodate the growth of the digestive tract. At this point, the intestine of newborn showed the same shape observed in the adults. At the 14th DAB, there were lipid droplets not only in the anterior intestine (Figure 4.2b), but also in the posterior (Figure 4.2c).

**Figure 4.2** Sagittal microsections of *H. reidi* larvae, digestive system DAB 8-30. (a) morphological aspect of digestive system before the metamorphosis (H&E); (b) lipid absorption in the anterior intestine (Black Sudan); (c) lipid absorption in the posterior intestine (Black Sudan); (d) morphological aspect of digestive system after the metamorphosis. (*) Gut chamber, (AI) anterior intestine, (AT) adipose tissue, (GG) gas gland, (KDN) kidney, (N) notochord, (PI) posterior intestine, (RCT) rectum, (RM) rete mirabile, (SB) swim bladder, (SLD) supranuclear lipid droplets, (UB) urinary bladder, (UP) urinary pore.

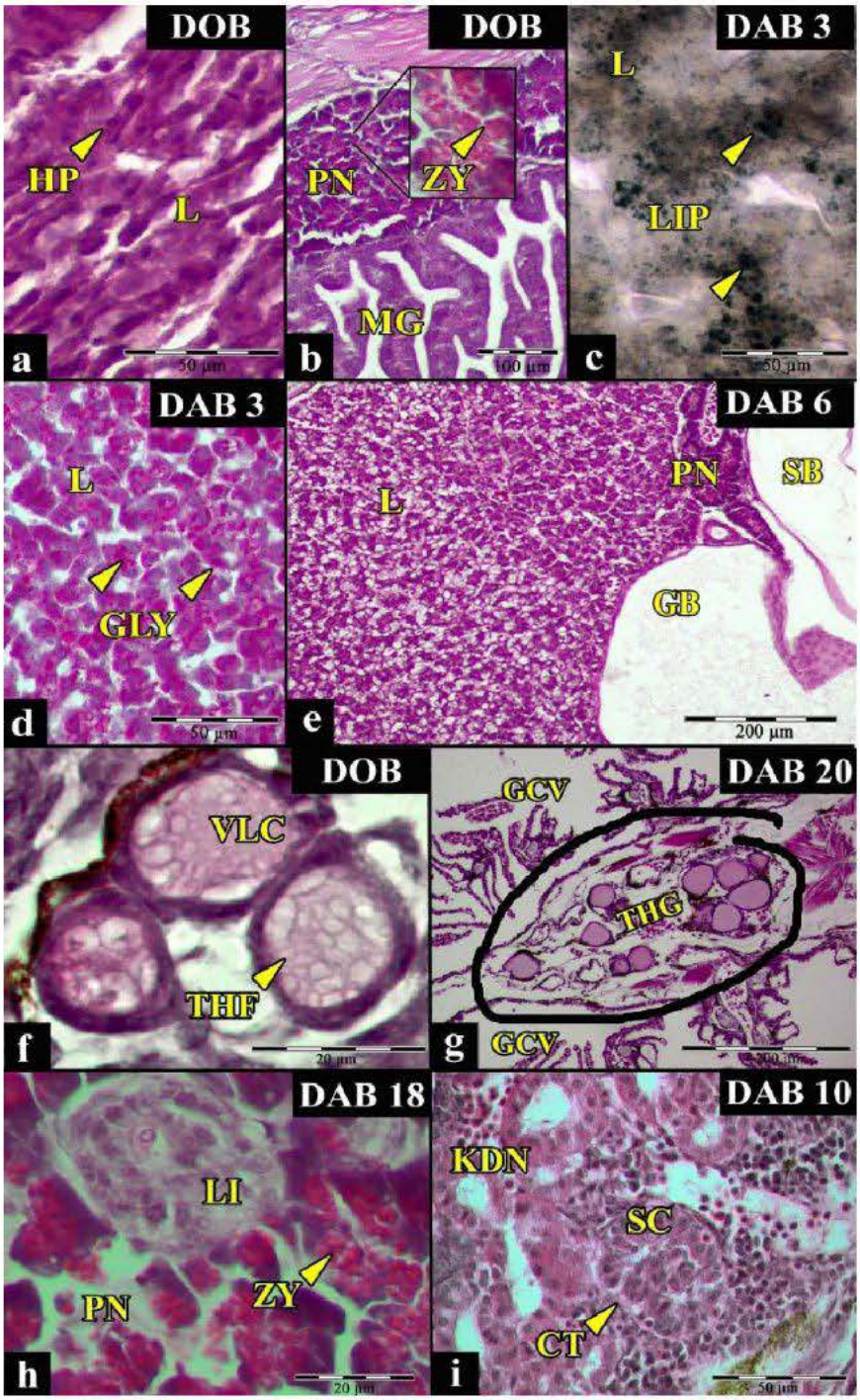

DAB 8
SB
N
KDN
UB
UP
RCT
a
500 µm
DAB 14
AI
SLD
b
50 µm
DAB 14
PI
SLD
c
50 µm
DAB 24
RM
GG
SB
AI
AT
PI
KDN
d
500 µm

From the 20th to the 30th DAB, the digestive system maintained the same characteristics; in the stratified squamous epithelium lining the oral cavity and pharynx, a progressive increase in the number of mucus-secreting cells and sensory cells (taste buds) was observed. In addition, in the esophagus the major presence of mucus-secreting cells caused a release of large amounts of neutral and acidic glycoconjugates. On the last day of the study, the complex and advanced structure of the intestine, with two loops, major presence of perivisceral adipose tissue, and very rich in longitudinal folds and goblet cells was observed. The posterior intestine and the rectum maintained a rectilinear structure (Fig. 2d).

### 4.5.5 Glands associated with the digestive tract

At birth, the liver occupied a large area in the peritoneal cavity, and the hepatic parenchyma appeared as compact basophilic tissue with rounded hepatocytes (Figure 4.3a) that accumulated glycogen (GLY; PAS positive).

**Figure 4.3** Sagittal (a-f, h, i) and transversal (g) microsections of *H. reidi* larvae, glands associated at the digestive system and endocrine glands DAB 0-30. (a) Hepatic parenchyma, compact basophilic tissue (H&E); (b) exocrine pancreatic tissue with acidophilic zymogen granules (H&E); (c) lipid storage in the hepatic parenchyma (Black Sudan); (d) glycogen storage in the hepatic parenchyma (PAS); (e) hepatic parenchyma, cells with polymeric shape, a central nucleus and a vacuolated cytoplasm, and gall bladder (H&E) (f) Ovoid structures of thyroid follicles with colloid filled cavities (H&E); (g) thyroid gland (H&E); (h) Langerhans islets, endocrine pancreatic cells with small and poorly stained cytoplasm (H&E); (i) corpuscles of Stannius, parenchyma surrounded by connective tissue in the thoracic kidney (H&E).; (CT) Connective tissue, (GB) gall bladder, (GCV) gill cavity, (GLY) glycogen, (HP) hepatocytes, (KDN) kidney, (L) liver, (LI) Langerhans islets, (LIP) lipids, (MG) midgut, (PN) pancreas, (SB) swim bladder, (SC) corpuscle of Stannius, (THF) thyroid follicle, (THG) thyroid gland, (VLC) vacuolated colloid,(ZY) zymogen granules


DOB
HP
L
a
50 µm
DOB
PN
ZY
MG
b
100 µm
DAB 3
L
LIP
c
50 µm
DAB 3
L
GLY
d
50 µm
DAB 6
L
PN
SB
GB
e
200 µm
DOB
VLC
THF
f
20 µm
DAB 20
GCV
THG
GCV
g
200 µm
DAB 18
LI
PN
ZY
h
20 µm
DAB 10
KDN
SC
CT
i
50 µm

The pancreas was located in the internal face of the liver, around the bloodstream. Acidophilic zymogen granules were detectable in exocrine pancreatic tissue (Figure 4.3b).

The gall bladder wall consisted of a simple columnar epithelium, supported by an underlying fibrovascular lamina propria, located in the upper part of the swim bladder, and connected with the intestine (Figure 4.1a). At the 3rd DAB, the hepatocytes were placed in cords and exhibited a considerable nucleus; the parenchyma showed lipid storage (Figure 4.3c) and a major presence of glycogen (Figure 4.3d). At the 3rd/4th DAB, the pancreas was extra-hepatic and joined to the liver, extending linearly in the dorsal place of the anterior intestine.

Until the 6th DAB, the hepatic parenchyma underwent important morphological changes; the more efficient sinusoidal system supported polymeric hepatic cells, with a central nucleus and a large vacuolated cytoplasm (Figure 4.3e). Liver and gall bladder gradually increased in size (Figure 4.3e).

In the liver, proliferation of sinusoids and a more evident vacuolization of hepatocytes were observed. The columnar epithelium of the gall bladder with basally located nuclei appeared cuboidal when the organ was distended. Until the last day of study, no relevant morphological changes were observed. In line with age, the liver was able to store higher quantities of lipid and glycogen.

### 4.5.6 Endocrine glands

The thyroid follicles constituted the functional unit of this gland, congregated into several clusters located near the zones where the afferent gill arteries leave the ventral aorta. At birth, three follicles

were visible as spherical or ovoid structures distended by a large amount of vacuolated colloid (Figure 4.3f). Several seahorses at the 6th DAB showed more than five follicles. On the 20th DAB, more than ten follicles were observed (Figure 4.3g). The follicles increased progressively in diameter and number until the 30th DAB. At the 18th DAB, endocrine pancreatic tissue was observed for the first time; the Langerhans islets were composed of cells with small and poorly stained cytoplasm (Figure 4.3h). At the 10th DAB, the corpuscles of Stannius (Figure 4.3i) were seen for the first time and are surrounded by connective tissue in the thoracic kidney.

### 4.5.7 Visual system

At birth, the pigmentation pattern of the eyes was complete, and the photoreceptors (cones and rods) were present between the pigment epithelium and the outer limiting membrane. The retina showed all ten cell layers that make it up (Figure 4.4a). Lens cells were organized into two distinct cell sub-populations: a monolayer of basophilic epithelial cells and a layer of eosinophilic cells elongated into fibres (Figure 4.4a). An external sheath of hyaline cartilage in the posterior portion of the eyes was observed (Figure 4.4a). The morphological structure of the visual system was complete: cornea, lens, iris, retina, optic nerve, choroid body, sclera (Figure 4.4b, c). From the 3rd DAB, the choroid rete offered a better vascularization of the retina, and the thin layer of capillaries alternating with rows of slender fibroblast (like cells) developed into a pluristratified layer (Figure 4.4c). Until the last day of study, the eyes grew progressively in size without evident modification. There was no presence of fovea.

### 4.5.8 Swim bladder and locomotion

At birth, a very functional swim bladder and five developed and cartilaginous fins (dorsal, anal, caudal, and paired pectoral) allowed comfortable swimming and buoyancy. *Hippocampus reidi* is a physoclist species; the pneumatic duct is lost during embryonic development. The swim bladder, rapidly inflated after the newborns release, was located dorsally in relation to the digestive tract (Figure 4.1a). At birth, the gas gland and rete mirabile were present (Figure 4.4d).

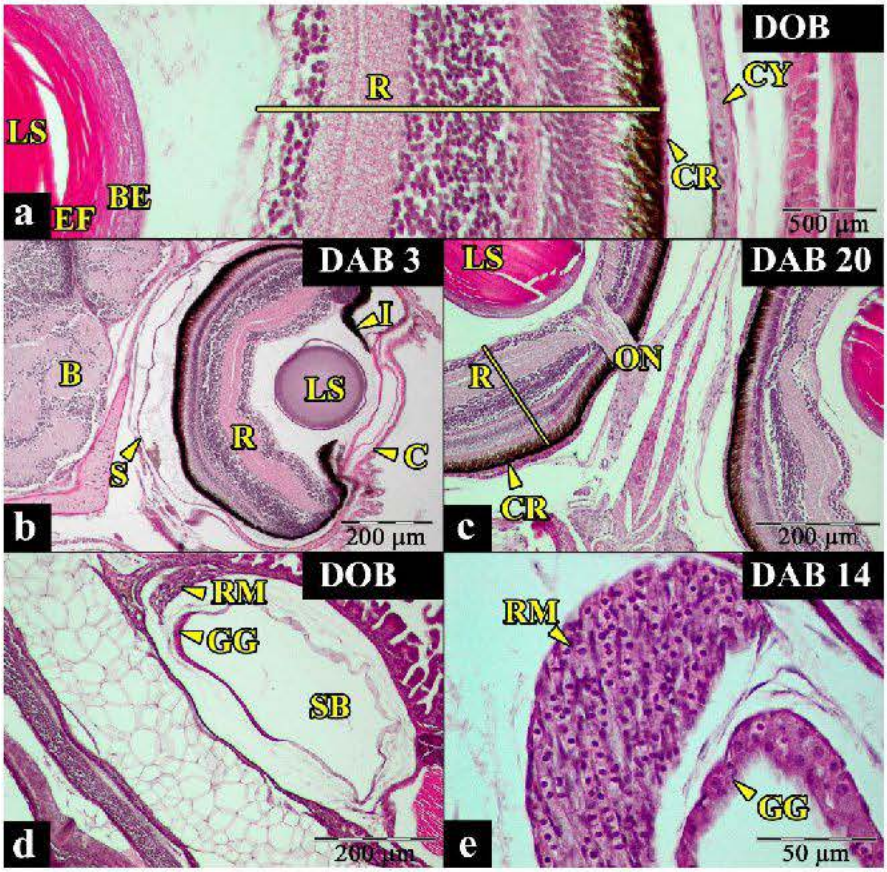


**Figure 4.4** Sagittal (a, b, d, e) and transversal (c) microsections of *H. reidi* larvae, visual system and swim bladder DAB 0-30. (a-c) Morphological structure of the eye (H&E); (d) Swim bladder located dorsally respect to the digestive tract (H&E); (e) detail of rete mirabile and gas gland (H&E). (B) Brain, (BE) basophilic epithelial cells, (C) cornea (CR) choroid rete, (CY) hyaline cartilage, (EF) eosinophilic cells, (GG) gas gland, (I) iris, (LS) lens, (ON) Optic nerve, (R) retina, (RM) rete mirabile, (S) sclera, (SB) swim bladder.

Blood supply of the rete mirabile increased in proportion with animal growth, observing numerous blood capillaries with abundant blood cells closely placed. Arterial and venous capillaries were not distinguishable (Figure 4.4e). Until the 30th DAB, both gas gland and rete mirabile increased in size.

### 4.5.9 Heart

The heart observed in the top part of the peritoneal cavity presented four chambers: sinus venosus, atrium, ventricle, and bulbus arteriosus. The sinoatrial valve is also formed (Figure 4.5a). The atrium was narrow towards the ventricle where the atrioventricular valve was not present. The basophilic aspects highlighted the elastic connective tissue and smooth muscle forming the walls of the pyriform bulbus arteriosus. At the 2nd DAB, the first trabeculae were seen in the outer wall of the atrium, and at the 3rd/4th DAB the formation of the atrioventricular valve led to the complete compartmentalization of the heart. The sinoatrial valve consisted of cardiac muscle and connective tissue core, while the atrioventricular valve was not muscular, but connective tissue structures were lined with endocardium. From the 6th DAB, the trabeculae proliferation in the atrium was observed; in the ventricle, the trabeculae extended across the lumen. In relation to seahorse growth, the heart increased in size and functionality

(chambers that are more capacious) until the last day of the study (Figure 4.5b).

### 4.5.10 Gill and pseudobranch

At birth, four cartilaginous gill arches were present. The cartilaginous axis of the primary lamellae, where the chondrocytes were bound in an extracellular cartilaginous matrix, sustained lamellae. The secondary lamellae proliferated towards the pharyngeal cavity (Figure 4.5c), forming some small gill filaments. On the 4th DAB, epithelial and pillar cells making up the lamellae could be differentiated. At the 6th DAB, some mucous cells and chloride cells were seen.

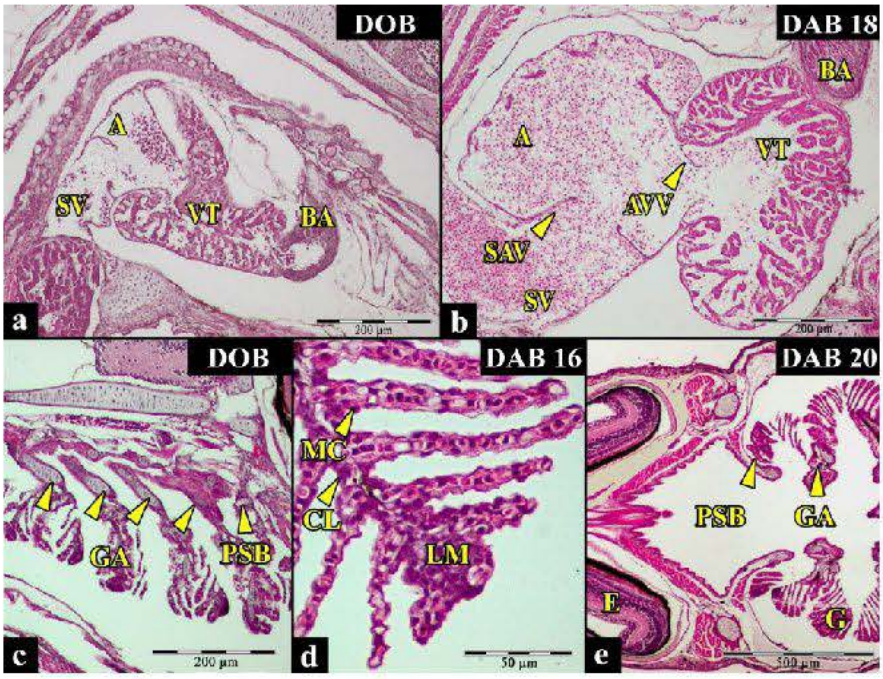


**Figure 4.5** Sagittal (a-d) and transversal (e) microsections of *H. reidi* larvae, heart and respiratory system DAB 0-30. (a) Heart located in the top part of the peritoneal cavity (H&E); (b) complete compartmentalization of the heart (H&E); (c) Cartilaginous gill arches and pseudobranch (H&E); (d) morphological detail of gill lamellae (H&E); (e) pseudobranch (H&E). (A) Atrium, (AVV) atrio-ventricular valve, (BA) bulbus arteriosus, (CL) chloride cell, (E) eye, (G) gill, (GA) gill arc, (LM) lamella (MC) mucus cell, (PSB) pseudobranch, (SAV) sinoatrial valve, (SV) sinus venosus (VT) ventricle.

From the 7th DAB to the 30th DAB, the gill lamellae became noticeably longer and had better vascularization by larger sanguineous capillaries (Figure 4.5d). The presence of chloride and mucus cells increased progressively with age. The pseudobranch (PSB) is a paired structure derived from the first gill arch and attached to the underside of the operculum. At birth, it was visible as undifferentiated cells (Figure 4.5c). At the 4th DAB, a cluster of filament was observed. The pseudobranch enlarged progressively until the 30th DAB and improving vascularization (Fig. Figure 4.5e).

### 4.5.11 Kidney and urinary bladder

Two thin layer of hemopoietic tissue along the dorsal side of the oesophagus formed the head kidney; it was composed by rounded basophilic cells without nephritic tubules (Figure 4.6a). Abundance of proximal and distal tubules, located up to the rear part of the swim bladder, characterized the excretory kidney (Figure 4.1a). Until the 6th DAB, the morphological aspect of the kidney remained unchanged, successively revealing a higher vascularization (sinusoids and blood cells); proximal and distal tubules were also located in the pre-cephalic kidney at this moment. The organ grew progressively, and at the 20th DAB the tubules were bounded in a large amount of hemopoietic tissue until the thoracic portion (Figure 4.6b). During the studied period, there was no presence of glomerulus. The urinary bladder was located

between the posterior portions of the intestine and the kidney and, at birth, was lined by a pseudo-stratified epithelium consisting of dense smooth muscle bundles and connective tissue. The urinary bladder no showed morphological changes (Figure 4.2a) and emptied outside through a urogenital pore connected to the anus (Figure 4.6c).

### 4.5.12 Spleen and thymus

At the 3rd DAB the spleen was observed for the first time, it was located near the liver in the superior portion of the peritoneal cavity and was surrounded by pancreatic tissue.

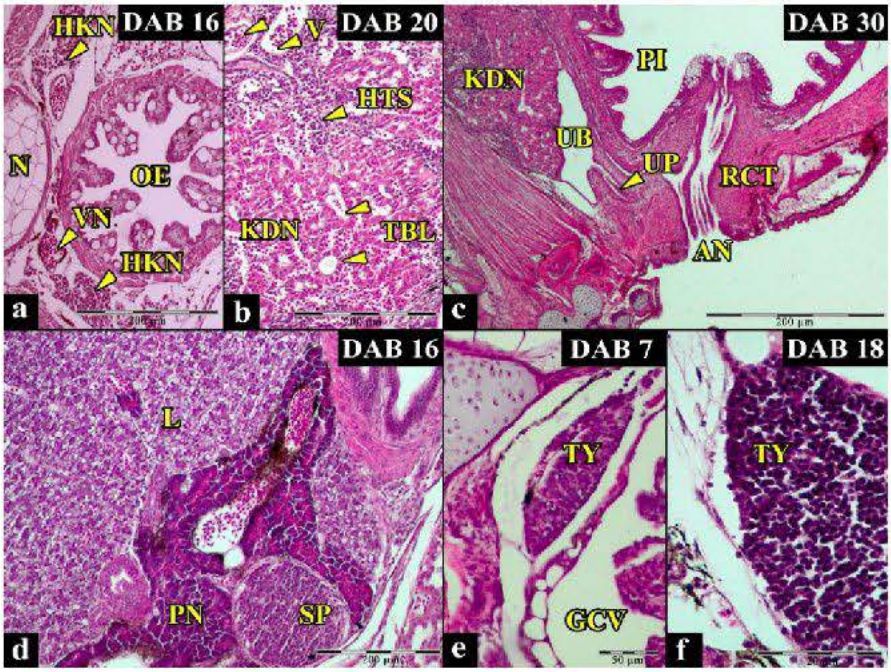


**Figure 4.6** Sagittal (c-f) and transversal (a-b) microsections of *H. reidi* larvae, kidney, urinary bladder, spleen and thymus DAB 0-30. (a) Neurocranial section, head kidney composed by rounded basophilic cells behind an esophagus rich of longitudinal folds (H&E); (b) kidney, thoracic portion with proximal and distal tubules (H&E); (c) excretory system, urinary pore and anus (H&E); (d) spleen in the superior portion of the peritoneal cavity surrounded by pancreatic tissue (H&E); (e) thymus, agglomerate of undifferentiated cells contained in a thin thymic capsule (H&E); (f) thymus, basophilic tissue formed by rounded cells. (AN) Anus, (GCV) gill cavity, (HTS) hematopoietic tissue, (KDN) kidney, (HKN) head kidney, (L) liver, (N) notochord, (OE) esophagus, (PI) posterior intestine, (PN) pancreas, (RCT) rectum, (SP) spleen, (TBL) tubules, (TY) thymus, (UB) urinary bladder, (UP) urinary pore, (VN) vein

The morphological aspect of the spleen comprised a compact tissue covered by a fibrous capsule (Figure 4.6d).

At the 7th DAB, the splenic sinusoids were evident. The spleen gradually increased its size, and the progressive development of the organ was observed until the 30th DAB. In the splenic parenchyma, there were no traces of lymphoid follicle or melanomacrophage centres.

Until the 6th/7th DAB, the thymic parenchyma remained visible as an agglomerate of undifferentiated cells contained in a thin thymic capsule. At birth, the thymus showed a high basophilic aspect and was located in the dorsal region of each gill cavity (Figure 4.6e). Successively, the thymus increased in size occupying progressively a larger area. Until the 18th DAB, the thymic parenchyma was composed of basophilic rounded cells (thymocytes) showing undifferentiated cortex and medulla (Figure 4.6f). Until the 30th DAB, relevant morphological changes were not observed.

### 4.5.13 Reproductive system

On the 9th/10th DAB, in the dorsolateral part of the body cavity an ovary sac with some oocytes in formation stage was found, it was the first trace of female reproductive system. Some primordial follicles

consisting of non-growing oocytes were surrounded by epithelial cells (Figure 4.7a). At the 12th DAB, some oocytes were at the second stage and showed basophilic cytoplasm with diffuse chromatin. They presented one small nucleolus in the ooplasm (Figure 4.7b), and the pellucid zone is not visible. From the 16th DAB, the gonads consisted of two lobes located on the dorsal peritoneal wall on each side of the body cavity (Figure 4.7c, d). The lobes were covered with a layer of the peritoneal membrane (Figure 4.7d) beneath which was the thin tunica albuginea ovary. They contained oocytes in different stages of cytomorphosis located in a spiral pattern. Some young oocytes were at the third stage, showing a simple squamous follicular primary envelope and provitelline nucleoli becoming evident in the karyoplasm.

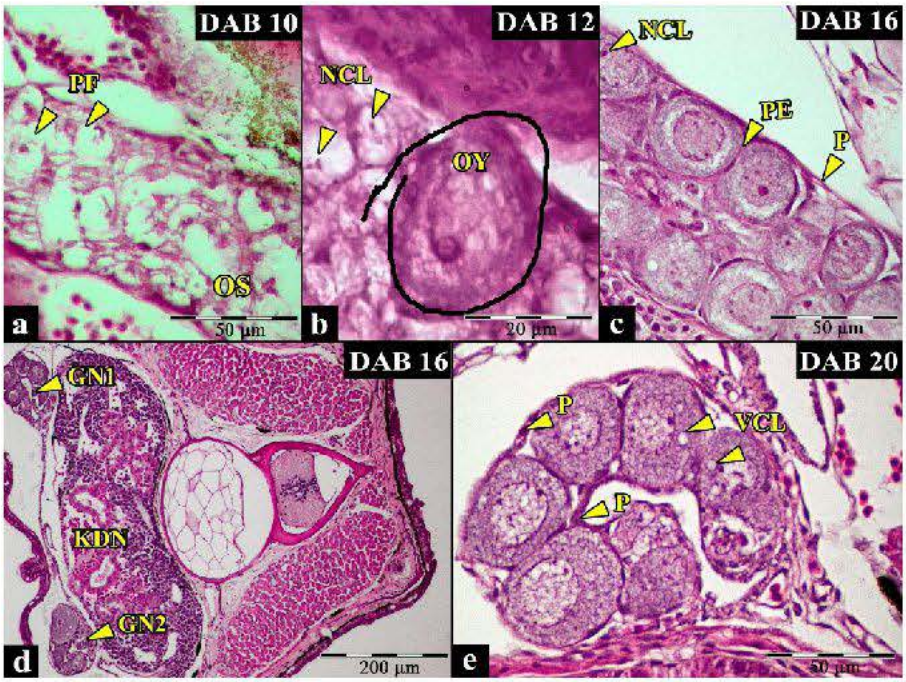


**Figure 4.7** Sagittal (a-c) and transversal (d-e) microsections of *H. reidi* larvae, reproductive system DAB 0-30. (a) Some primordial follicles consisted of non-growing oocyte surrounded by a few epithelial cells (H&E); (b) oocytes at second stage, basophilic cytoplasm and a conspicuous central nucleus with diffuse chromatin (H&E); (c) Detail of gonads with some young oocytes at the third stage of development (H&E); (d) oocytes at the third stage of development; (e) gonadal lobes, oocytes principally at the third stage with some oocytes provide of fat vacuoles in the oviplasm indicated the early development of fourth stage (H&E). (GN1) gonadal lobe 1, (GN2) gonadal lobe 2, (KDN) kidney, (P) peritoneal membrane. (PE) pellucid zone, (PF) primordial follicle, (NCL) provitelline nucleolus, (OS) ovary sac, (OY) oocytes, (VC) fat vacuoles.

At the 20th DAB, the oocytes were at the second previtellogenic stage (third stage), with a large central nucleus surrounded by a granular cytoplasm. Oocytes presented many nucleoli and traces of reserve material. Until the 30th DAB, oocytes were at the third stage of development with an increase in size of the gonads.

In some oocytes, fat vacuoles (VC) were distinguishable in the ooplasm, indicating the early onset of the fourth stage (Figure 4.7e). At the end of the study, only the female reproductive system was observed in all samples analysed.

## 4.6 Discussion

Due to the scarce literature published about the morphology and the physiology during seahorses' early life, this study will help to understand the processes of change in captive-reared newborns of *H. reidi* feed with *Artemia* nauplii. Newborn seahorses showed intense changes in the first three of the four stages of development. This is the period associated with the planktonic phase. The total length/weight increased progressively since birth until the third stage (18 days), when seahorses needed to use energy also to develop physiological systems.

Unlike happen in pelagic fish (Santamaría et al. 2004; Wold et al. 2007), higher rates of growth were observed in the planktonic stage of

*Solea Senegalensis* (Cañavate et al. 2006), with values that decreasing after the metamorphosis. This fact occurs also in *H. reidi* during the 4th stage, mainly characterized by the growth of the pre-existing structures. Unlike happen in *H. guttulatus* (Palma et al. 2014), in *H. reidi* is present a long period between eggs fertilization and yolk sac reabsorption (16–18 days), highlighted by the presence of some reminiscence of yolk sac when seahorses left the brood pouch. It could be due to the capacity of embryo and larvae to use energetic resources coming from the brood pouch in this species of seahorse (Otero-Ferrer et al. 2013, 2014).

The transition from endogenous to exogenous food happened after release, when newborn of *H. reidi* had the pharynx rich in taste buds and the oesophagus provided of abundant mucous cells. The high prey selectivity described in newborn (Hilomen-García et al. 2003) is due not only to the size and the energy consumption of the catch (Celino et al. 2012; Hora and Joyeux 2009), but principally to the presence of numerous taste buds observed in this study. As it has been described in other species, the number of taste buds of specimens studied increased with larval development (Fishelson and Delarea 2004; Hansen et al. 2002) and consequently also increased the gustatory efficiency of juveniles and adults (Govoni et al. 1986; Storero and González 2009).

Newborn seahorses showed an alimentary canal with simpler structures and lower functionality in comparison with the adults, structural and functional development continuously improved with age, as described in other marine fish larvae (Govoni et al. 1986; Jaroszewska and Dabrowski 2011). The absence of teeth associated with the lack of morphological and functional stomach implies that

prey digestion happens entirely in the intestine (midgut and hindgut). In the present study, larval feeding based on larger size prey such as *Artemia*, which presents a more resistant exoskeleton, not allowed to digest and absorb completely the exogenous food in the first days after the release. The proteolytic digestion seemed to be offset by the process of pinocytosis present in the hindgut, displayed by supranuclear acidophilic vacuoles that are then reabsorbed during the development. In accordance with Socorro (2006), the absorption by pinocytosis compensates the poor activity of extracellular protease, as observed in other fish larvae (Govoni et al. 1986; Kjørsvik et al. 1991; Sarasquete et al. 1993; Watanabe 1982).

A sign of larval development is also the gut elongation lead up to the loops formation reported also for others finfish larvae (Santamaría et al. 2004; Yúfera and Darias 2007). The morphology and functionality of midgut and hindgut are simpler in larvae with respect to the complexity of anterior and posterior intestine in juveniles; midgut and hindgut are incipient structure present in larvae before the metamorphosis (Govoni et al. 1986). In the present study, the rectilinear midgut and hindgut became progressively anterior and posterior intestine around the 12th– 18th day after the release. Unlike, in recent study newborns of *H. guttulatus* with the same incipient rectilinear structure were defined as juveniles (Palma et al. 2014). During seahorses' rearing, mass mortality occurs mainly during the pelagic phase (5–7 days) and it decreases after benthic settlement (Hora and Joyeux 2009; Murugan et al. 2009). As observed in the present study, the high newborn mortality seems to be due to the incomplete maturation of the digestive system before the

metamorphosis; thus, research of the optimal diet for newborn seahorses should focus in relation to the inability of newborns to digest the same type of food than juveniles or adults (Yúfera and Darias 2007).

In the present study, we found that newborns fed with *Artemia* nauplii were able to assimilate lipids coming from exogenous alimentation at the 3rd/4$^{th}$ DAB. The lipidic compounds coming from yolk sac are not observed in the enterocytes, while hepatocytes showed stored glycogen after few hours seahorses were released. The quantity of stored glycogen increased during the exogenous alimentation. The rapid development of intestinal enterocytes was combined with an increase in synthesis of lipoproteins accompanied by a considerable change in lipid morphology; this physiological change was described by different authors in others marine fish larvae (Deplano et al. 1991; Gisbert et al. 2004; Sarasquete et al. 1995). Initially, lipids are present as large supranuclear vacuoles and subsequently are replaced by a greater number of smaller vacuoles, located in the intracellular spaces. The lipid compounds observed in young seahorses (vacuoles Sudan Black positive) had a tendency to be adsorbed in both anterior and posterior intestine. It was described also in *Pagrus major* and *Acanthopagrus schlegeli* larvae (Miyazaki and Fujiwara 1988), while most of finfish larvae are able to absorb lipids only in the anterior intestine (García-Hernández et al. 2001; Kjørsvik et al. 1991; Sarasquete et al. 1995).

After seahorses leave the brood pouch, in the thyroid gland the vacuolated colloid indicated the high developmental stage and functionality of the follicles. The high level of development and functionality of the thyroid follicles and the metamorphosis process

could be firmly correlated; the central role of thyroid hormones (THs) in early development and metamorphosis is well established not only in mammals and amphibians but also in flatfish (Power et al. 2001). The follicles morphology observed in *H. reidi* at birth is similar to that described in Atlantic halibut (*Hippoglossus hippoglossus*) during the last stage of metamorphosis (Einarsdóttir et al. 2006).

At the day of birth, the morphology of visual system of newborns was in agreement with the literature, in where they are described as excellent hunters (Van Wassenbergh et al. 2009). Cones and rods in the retina allowed them accurately to identify prey, and mouth and anus open made it possible to pass immediately from endogenous to exogenous feeding. The fovea, a specialized area in the retina associated with acuity vision and found in adults (*H. abdominalis*, *H. barbouri*, *H. subelongatus*, and *H. taeniopterus*, Lee and O'Brien 2011; Mosk et al. 2007), is absent in young specimens. The retina cells throughout the life cycle are renewed, and the only way to preserve this small depression is through asymmetrical cell growth (Easter 1992). The fovea in seahorses arises when renewal of retinal cells begins, and thirty days of study were not enough to observe it.

Once they left the brood pouch, offspring showed an advanced stage of development regarding their locomotor apparatus, with a physoclistous swim bladder (no pneumatic duct) composed of functional gas bladder, rete mirabile, and gas gland. The correct development of swim bladder, inflated immediately after release (Woods 2000), took place in the pouch. In other teleost larvae, the swim bladder formation and inflation constitute a very delicate stage (Roo et al. 2010). Swim bladder hyperinflation was not observed in

this study, although this problem has been cited for seahorse's larvae (Palma et al. 2014; Woods 2000), and histologically the organ was described as functioning properly. The inability of the swim bladder to inflate has been observed exclusively in the larvae of other species and may be the cause of osteological deformities (Chatain 1994), reduction in growth (Chatain 1986), and peaks in mortality (Chatain and Dewavrin 1989).

The gills had structure resembling small tufts, which refers to the first name given to this species "*lophobranchii*" (Cuvier 1817). This organ plays a key role in larval osmoregulation, and histologically seahorse's gill showed numerous chloride cells. This fact could explain the ability of adults of some seahorse species such as *H. reidi* (Rosa et al. 2002), *Hippocampus capensis* (Whitfield 1995), or *Hippocampus kuda* (Hilomen-García et al. 2003), to tolerate a wide range of salinities when living in the proximity of estuaries.

Inside the excretory system, the kidney of young seahorses showed abundant distal and proximal tubules, with glomeruli absent until the last day of study. In the literature, these structures were described in adults (*H. reidi*, Martins et al. 2010); despite other studies classifying seahorses (*Hippocampus spp.*) as fish characterized by a kidney with no glomeruli (Hickman and Trump 1969; Hilomen-García et al. 2003). A first study by Edwards and Condorelli (1928) included the genre *Hippocampus* as a fish with a completely aglomerular kidney and did not find a reduction in functionality in kidneys without glomeruli. The effects of this absence are still unclear and needs further research.

In the present study was observed the early appearance of lymphoid organs, despite this, during larval development the full maturation of

immunological competence develops quite late (Zapata et al. 2006). In *H. reidi* newborns, as in others fish larvae, the absence of cellular structure involved in nonspecific defence mechanisms, such as the melanomacrophage centres and the lymphoid follicle in the spleen, is normal and is not index of reduced defence against disease; the larval spleen is more erythropoietic than lymphopoietic (Nakanishi 1986; Schrøder et al. 1998). This is another characteristic of larval development.

Concerning the reproductive system, the most important difference between the seahorses and other fish larvae was the presence of the gonadal sac from the 10th DAB. While the male reproductive system was not observed, oocytes were detected in all samples. It could be that the *H. reidi* female reproduction system developed earlier than its male counterpart or perhaps, like in zebrafish (Danio rerio) this species could be a hermaphrodite protogynic one (Maack and Segner 2003). Until the 30th DAB, all newborn seahorses sampled were females, and therefore, further studies related to their sex characterization would be interesting to clarify this point.

Finally, we would like to define metamorphosis in seahorses as all the morphological, physiological, and behavioural modifications that happen while a larva transforms to a juvenile. The larva is a post-natal seahorse that differs in morphology, physiology, and habitat from the adult, while the juvenile is a little seahorse with most of the morphological, physiological, and ecological traits of an adult, but not reproductive. It emphasizes the importance to change the point of view on newborns of *H. reidi*; they were neither small adult replicates nor juveniles and showed indirect development (see Balon 1999). After the

embryonic phase, the larval phase started inside the brood pouch and continued outside. Doubtless, newborns seahorse must overcome several critical points before lead up the juvenile phase, and all internal and external changes are related to a metamorphic phase. The metamorphosis is present in the morphological changes of enterocytes and hepatocytes, in the shift from pinocytic to exogenous adsorption, in the intestine loops and the gonads formation, in the shift from planktonic to benthic behaviour, in the development of the tail prehensile abilities or in the coronet growth.

The new aspects of development that explain the high mortality rates were in correlation with the internal morphological changes of larvae. If used inadequate live prey like *Artemia*, the digestive system is not able to adsorb lipids during the first days, one of the most important nutritional factors for larval growth and survival (Watanabe et al. 1983).

At birth, newborn seahorses of *H. reidi* should be considered as larvae in an advanced stage that undergo a delicate metamorphosis process. The term juvenile, adopted principally by the literature to define newborn seahorses, can be used for *H. reidi* approximately 18/20 days after the release, a period during which seahorses completely shed larval characteristics and show most of morphological, physiological, and ecological adult traits.

# Estudio III

## *Development of short snouted seahorse (Hippocampus hippocampus, L. 1758): osteological and morphological aspects*

In revision

Fish Physiology and Biochemistry 2015

B Novelli, F Otero-Ferrer, JA Socorro, MJ Caballero, A Segade-Botella and L Molina Domínguez

## 5.1 Resumen

Existen pocos estudios sobre el desarrollo temprano de los caballitos de mar después de la expulsión de la bolsa incubadora con respecto a los estudios publicados sobre juveniles y adultos. Los recién nacidos, tienen una forma similar a la de los adultos, y por eso son considerados juveniles o alevines. Sin embargo, durante las primeras fases de vida H. hippocampus muestra presenta cambios tanto de comportamiento (paso de hábitat, planctónico a bentónico) como morfológicos. El objetivo de este estudio es definir el estado de desarrollo de H. hippocampus tras su expulsión de la bolsa incubadora paterna y establecer la existencia de desarrollo directo o indirecto mediante un estudio osteológico. El proceso de osificación, se estudió en 120 individuos, desde su liberación hasta los 30 días después del nacimiento. Para analizar el desarrollo osteológico de esta especie se adaptó la técnica de la doble tinción Azul Alcian y Rojo Alizarina, para teñir respectivamente cartílago y hueso. Al nacer, H. hippocampus presenta una estructura principalmente cartilaginosa que osifica en un mes aproximadamente. La armadura ósea compuesta por anillos óseos y placas óseas se desarrolla en 10 días. La aleta caudal, una estructura ausente en caballitos de mar juveniles y adultos, está presente al nacer, y desaparece progresivamente con la edad. La ausencia de estructuras óseas características del caballito de mar adulto en los recién nacidos, como la coronilla, los anillos y placas óseas, las espinas de la cabeza y la falta de prensilidad de la cola, sugieren la existencia de metamorfosis previa a la fase juvenil. Se trata del desarrollo indirecto, la metamorfosis comienza dentro de bolsa incubadora paterna y sigue una vez expulsados. Este hecho nos lleva a reconsiderar la fase de

desarrollo que se suele utilizar para clasificar los recién nacidos de *H. hippocampus*.

## 5.2 Abstract

Information about early development after male release lags behind studies of juveniles and adults' seahorses, and newborns seahorses, similar in shape to adults, are considered juveniles or fry. During *H. hippocampus* early life are present behavioural (shift in habitat, from planktonic to benthic) and morphological changes, for this reasons, the aim of this study is to define the stage of development of *H. hippocampus* after they are expelled from the male brood pouch, and to establish direct or indirect development through an osteological analysis. The ossification process was studied in 120 individuals, from their release to 30 days after birth. To analyse the osteological development, Alcian Blue-Alizarin Red double staining technique for bone and cartilage was adapted to this species. At birth, *H. hippocampus* presents a mainly cartilaginous structure that ossifies in approximately one month. The bony armor composed by bony rings and plates develops in 10 days. The caudal fin, a structure absent in juveniles and adult seahorses, is present at birth, and progressively disappear with age. The absence of adult osteological structure in newborns, like coronet, bony rings and plates, head spines, and components allowing tail prehensile abilities, suggests a metamorphosis before the juvenile stage. During the indirect development, the metamorphic stage started inside brood pouch followed outside, and lead up to reconsider the status of *H. hippocampus* newborns.

## 5.3 Introduction

During evolution, to maximize their survival, fish have developed specialized body forms, distinctive hunting systems, and specialized strategies of reproduction. Seahorses are prime examples of this process. They have developed an elaborately ornamented body, vertical posture, a prehensile tail lack of caudal fin, and a long tubular snout, in order to live camouflaged attached to algae and corals while ambushing copepods, mysids, and other zooplankton substrate (Koldewey and Martin-Smith 2010), and camouflaged among algae and corals are excellent hunters (Foster and Vincent 2004). In addition, seahorses have crowns (the coronet), a bony projection on the top of the head), with different shape according to the specie, and have no scales, but have a body composed of bony rings and bony plates completely covered by dermis (Lourie et al. 1999, 2004).

The reproductive strategy of seahorses consists of eggs incubated in a unique male pouch that promotes growth of highly advanced newborns. Early development is the less studied period of the seahorse's life cycle, and the associated lack of knowledge makes it difficult to know when a young seahorse transforms from newborn to juvenile (Balon 1999).

During indirect development, metamorphosis is a particular life history transition from a larval to a juvenile stage (Balon 1999), accompanied by dramatic changes. Such changes typically include a major restructuring of morphology, and often involve dispersal and settlement to a new habitat, and a shift in nutrition and feeding behavior (Bishop et al. 2006). A recent study demonstrates as the term larva can be used for *Hippocampus reidi* newborn after their release due to

several factors, like the yolk sac presence at birth, the morphological changes of enterocytes and hepatocytes, or the formation of intestine loops during early development (Novelli et al. 2015).

Newborns are considered principally as "juveniles" (Payne and Rippingale 2000; Kanou and Kohno 2001; Woods 2003; Murugan et al. 2009; Sommer et al. 2012), "offspring" (Lockyear et al. 1997; Stölting and Wilson 2007; Lin et al. 2012), "fry" (Chang and Southgate 2001), as well as "newborn-juveniles" (Falerio and Narciso 2011), or "early-juveniles" (Martinez-Cardenas and Purser, 2012).

The aim of this study is (1) to describe the ossification and the morphological changes that happen in *Hippocampus hippocampus*, during the first 30 days after their release, and (2) to evaluate the existence of a metamorphosis and consequently a larval phase. The hypothesis is that the terms juvenile or fry currently used for newborn seahorses of *H. hippocampus* should be modified. In the case of indirect development, the inclusion of this phase as a new step in the life cycle of *H. hippocampus* is proposed.

## 5.4 Material and Methods

Experimental conditions. Animals were maintained at the IU-Ecoaqua University Institute facilities (ULPGC, Spain), and reared in a semi-closed recirculating seawater system (see Novelli et al., 2015). In broodstock and offspring aquaria, the oxygen was daily measured and the range remained between 6.3 and 6.7 mg/l, ammonia, nitrites, nitrates, and phosphates concentrations were maintained below kit detection levels (Macherey-Nagel, Viscolor® Eco, Düren, Germany).

### 5.4.1 Broodstock and offspring culture

Captive-reared *H. hippocampus* broodstock were separated in breeding pairs, and held in 90-l glass tanks provided with plastic plants and nets for attachment. The offspring were reared in triplicate in 30-l glass aquariums, at a density of 4 newborns per liter. Broodstock and newborns were reared under natural summer ranges of temperature and photoperiod (23°C; 12D:12N). Aquaria bottoms were cleaned daily.

### 5.4.2 Broodstock and offspring alimentation

Broodstock feeding was based on live mysids, (*Siriella armata, Leptomysis lingvura, Paramysis nouveli*) caught in the Risco Verde area (27° 51' N, 15° 23' W), Gran Canaria (Spain), reared *Artemia salina* enriched with the ORI-culture (Skretting®, Vervins, France), and frozen mysids (Ocean NutritionTM, Essen, Belgium). Offspring feeding was based on *Artemia salina* nauplii, (EG type 850 mm, INVE Aquaculture), enriched 24 hours with Easy Selco DHA (INVE Aquaculture, Dendermonde, Belgium), twice per day. The prey density was 1 nauplii/ml.

### 5.4.3 Developmental timing

Male seahorses have a brood pouch. When mating, the female seahorse deposits her eggs into his pouch while the male fertilizes them. During the male gestation, the eggs hatch and the embryo develops into his pouch. Successively, young seahorses are released from the male brood pouch.

Due to the impossibility of using the time of hatching as a reference point to establish development stages, the release was considered as the day of birth (DOB) and the time during the development was determinate considering the day after birth (DAB).

### 5.4.4 Data collection and sample processing

The ossification process was studied in 120 individuals. Six individuals were sampled daily until DAB 10, every other day until DAB 24 and every three days until DAB 30. Newborns seahorses were sacrificed with a clove oil anaesthetic overdose (natural clove essential, Guinama, Alboraya, Valencia), and conserved in 10 % buffered formalin (Sigma Chemical Co.) until analysis.

For osteological analysis, cartilage and bones were processed in accordance with the staining techniques established by Taylor and Van Dyke (1985) and Digerkus and Uhler (1977), adapted to this species by the authors due to the strong seahorse pigment. The first step was to wash samples with a potassium hydroxide and hydrogen peroxide solution 1:10 until the pigment disappeared. Skeletal formation was observed using Leica stereo and light microscopes (Leica Microsystems, Wetzlar, Germany), and photographed (after 48 hours) with Leica DFC 420 and Leica DFC 295 cameras (Leica Microsystems, Wetzlar, Germany).

### 5.4.5 Measurement

Total length (TL) and head length (HL) were measured according to Lourie (2003) with a profile projector (Mitutoyo PJ-R 3000, USA) (n= 120). Time spans used to determine the developmental stages for

seahorse newborns, was maintained the same established for Novelli et al. (2015) in another species of seahorse as well as AGR and SGR calculation methods.

### 5.4.6 Nomenclature

The definition of bones and cartilages followed several authors: Gregory (1959) for the skull, Azzarello (1989, 1990), Silveira (2000), and Leysen et al. (2011) for the general osteological structure. We drew the concept of hyosymplectic cartilage from Kadam (1958). Circumorbital bones' homology follow the terminology adopted by other authors for newborn and adult seahorses: the antorbital bone from Leysen et al. (2010), the lachrymal bone (or infraorbital 1) from Azzarello (1989) and Leysen et al. (2010), and the sub-orbital bone (infraorbital 2) from Azzarello (1989), Bergert and Wainwright (1997), and Colson et al. (1998). The studies of Arratia et al. (2001) and Bruner and Bartolino's (2008) were used for the vertebral system description.

## 5.5 Results

### 5.5.1 Growth

During their first month of life, fish growth was uniform in total length, (from 7,9 ± 0,42 mm at birth to 26,64 ± 2,21at DAB 30) and head length (from 2,02 ± 0,1 mm at birth to 5,31 ± 0,41 at DAB 30) (Figure 5.1).

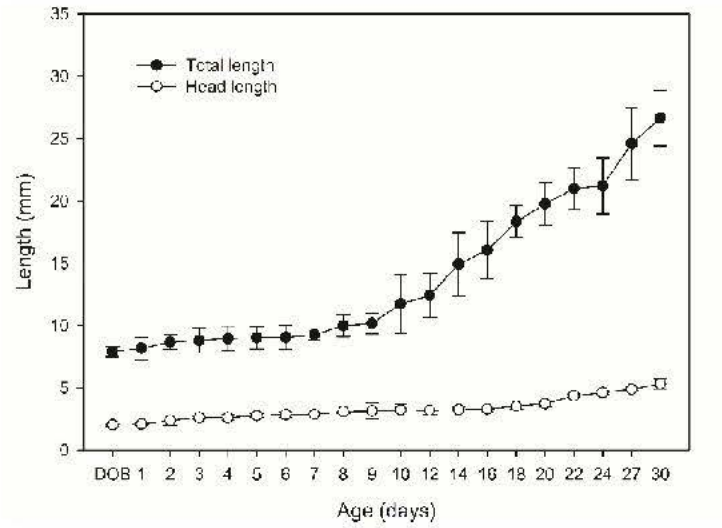



**Figure 5.1** Growth of *H. hippocampus* during first month of life. Total length and head length. DOB, day of birth

While SGR values increased progressively, AGR values decreased progressively until the DAB 18. The highest AGR and SGR values were obtained during stage 4 (Table 5.1).

**Table 5.1** Growth of *H. hippocampus* (wet weight) during the development

| | GROWTH | AGR(mm/day) | SGR(mm/day) |
|---|---|---|---|
| DAB | TL SD | TL | TL |
| 0-2 | 7,9±0,42 – 8,6±0,57 | 0,38 | 1,12 |
| 2-8 | 9,9±0,86 | 0,22 | 1,94 |
| 8-18 | 18,3±1,28 | 0,19 | 2,67 |
| 18-30 | 26,64±2,21 | 0,69 | 3,04 |

AGR Absolute Growth Rate; DAB Day After Birth; SD Standard deviation; SGR Specific Growth Rate; TL Total Length

### 5.5.2 Head skeleton (Figure 5.2a-f, 3a-f)

Seahorses present a cranium characterized by a long tubular snout, where some bones have an elongated shape. The cranium was subdivided in branchiocranium (jaws, suspensoria, ethmoidal, orbital, hyoid and opercular zone) and neurocranium (Figure 5.2a). It was noted for changes, not only in the geometric disposition of the circumorbital series: bones present around the eye in most of teleost and located in the lateral face of the snout in seahorses, but also in the morphology of the jaw, where the dentary bones close to the mouth can rotate upwards approximately 90°. The presence of the coronet was preceded by growth of the pre-coronet spine, a structure located above the supra-occipital bone. This is one of six prominent spines that *H. hippocampus* possess on their heads.

**DOB** (HL 2.02 ± 0,1 mm; Figure 5.2a). When newborns leave the brood pouch lack of head spines and coronet. Signs of ossification were present at four focal points: the upper and lower jaw (maxillo-mandibular apparatus); the suspensoria; and the anterior portion of the neurocranium. In the upper jaw, the pre-maxillary and maxillary were partially ossified; these are two intramembranous and paired bones connected with the rostral cartilage, which extended from the rostral snout to the dentary bone. The palatine bone is another element connected to the rostral cartilage and located in the anterolateral face of the snout; it was still cartilaginous and extended to the cartilage of quadrate located at the forefront of the snout. The ossification process also started in the ectopterygoid bone, the first of three bones that form the pterygoid series. It showed signs of mineralization, had a boomerang shape and was linked to the palatine and quadrate bones.

The intramembranous ossification of the dentary, large paired bones joined by the Meckel's cartilage, began in the lower part of the jaw. In the suspensoria, signs of ossification in the posterior part of hyosymplectic cartilage, a structure that covers the lateral side of the snout, were observed. The quadratojugal, somewhat ossified and located in the dorsal portion of hyosymplectic cartilage, was a flat dermal bone which originated from the quadrate and extended linearly in the upper portion of the hyosymplectic cartilage until the lower area of the eye. There were no traces of ossification in the ethmoidal, orbital, opercular and hyoid regions. The taenia marginalis, or orbital cartilage, surrounds the eye. At birth, the larva's neurocranium showed otolith and a dermal substance in the upper part and the pterotic shape without signs of ossification that characterized the irregular lateral projection of the neurocranium. In the tectum synoticum, the anterior region of the neurocranium, the frontal bone ossification began.

**DAB 1** (HL 2.09 ± 0,1 mm; Figure 5.2b). There was an increase in size of ectopterygoid bones. The metapterygoid bone chondrified. The hyosymplectic cartilage was subjected to mineralization, and the substitution of cartilaginous tissue formed two bones: the hyomandibular and symplectic in its posterior and anterior portion respectively. In the hyoid region, the ceratohyal bone showed signs of ossification; this bone carries out a lever function allowing suction feeding. There were signs of ossification in the parasphenoid bone, a median unpaired bone.

**DAB 2-3** (HL 2.38 ± 0,3 - 2.61 ± 0,1 mm; Figure 5.2c). Was present an increase in size of the maxillary, pre-maxillary bones. In the lower jaw, the dentary bone was in an advanced stage of ossification, as was

the retroarticular bone. The retroarticular bone is a paired and dermal bone located in the posterior face of the dentary and articulated with Meckel's cartilage with a semicircular shape in its upper portion. The Meckel's cartilage was covered by a dermal ossification. The quadrate ossified through an ascending perichondral process. The symplectic and the hyomandibular bones formed one sole plate, and the metapterygoid bone ossified in the lateral/posterior part of the tubular snout. In the ethmoidal region, the ethmoidal plate and the endochondral mesethmoid bone ossified. The prefrontal bone, a small bone located between the eye socket and nasal capsule, was partially ossified. The mineralization in the parasphenoid increased from the orbital zone, reaching jaw and basoccipital directions. In the hyoid region, the ceratohyal bone became completely ossified, while the urohyal bone located in the hyoid area behind the ceratohyal bone and articulated with it, presented partial ossification. Signs of ossification were present in the hypohyal and basihyial bones. The branchiostegal rays remained cartilaginous, while in the neurocranium the pterotic and basoccipital bones showed signs of ossification.

**DAB 4** (HL 2.61 ± 0,2 mm; Figure 5.2d). The palatine bone starting to ossify, as well as the angular bone, on the back of the Meckel's cartilage. It was fused to the dentary in the lateral portion of the lower jaw. Also the endopterygoid bone starting to ossify; the nasal capsule was clearly visible. The ethmoid plate and the pre-frontal were almost completely formed, while the quadrate, symplectic and hyomandibular bones increased their mineralization.

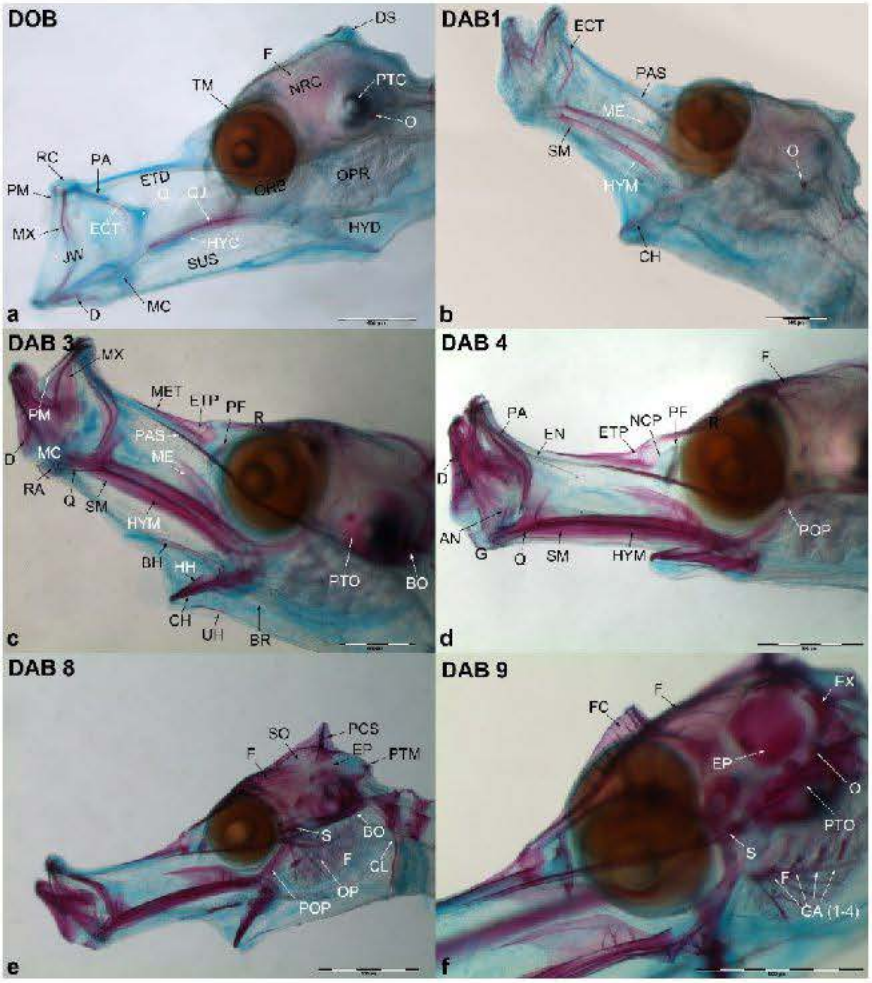



**Figure 5.2** Osteological development of *H. hippocampus* cranium (DOB-DAB 10). Head, lateral view, a-f. (ANG) Angular, (BH) basihyial, (BO) basoccipital, (BR) branchiostegal rays, (CH) ceratohyal, (CL) cleithrum, (D) dentary, (DS) dermal substance, (ECT) ectopterygoid, (EN) endopterygoid, (EP) epiotics, (ETD) ethmoidal region, (ETP) ethmoid plate, (EX) exoccipitals, (F) frontal, (FC) frontal crest, (GA) gill arc, (HH) hipohyal, (HYC) hyosymplectic cartilage, (HYD) hyoid region, (HYM) hyomandibular, (JW) jaws region (MC) Meckel's cartilage, (ME) metapterygoid, (MET) mesethmoid, (MX) maxillary, (NCP) nasal capsule, (NRC) neurocranium, (O) otolith, (OP) operculum, (OPR) opercular region, (ORB) orbital region, (PA) palatine, (PAS) parasphenoid, (PCS) pre-coronet spine, (PFR) pre-frontal, (PM) pre-maxillary, (POP) pre-opercular, (PTC) pterotic crest, (PTM) post-temporal bone, (PTO) pterotic, (Q) quadrate, (QJ) quadratojugal, (RA) retroarticular, (RC) rostral cartilage, (SF) sphenotic, (SM) symplectic, (SO) supraoccipital, (SUS) suspensoria (TM) taenia marginalis, (UH) urohyal.

The pre-opercular bone was located in the anterior portion of the operculum outside the hyomandibular bone and revealed a soft ossification. In the neurocranium ossification increase in the frontal bone.

**DAB 5-9** (HL 2.78 ± 0,1 - 3.13 ± 0,6 mm; Figure 5.2e, f). The supra-occipital bone covered the upper portion of the neurocranium with a thin bone lamina. The pre-coronet spine grew over the supra-occipital bone. (Figure 5.2e). The post-temporal bone as well as the basoccipital and cleithrum starting to ossify. The frontal bone expanded and extended above the optic capsule creating two crests, denominated, frontal crests (Figure 5.2f). Laterally, the pterotic bones were significantly ossified. The formation of the first element and the ossification on the second one reduce the morphological differences in the cranium between adults and newborns. The pre-opercular and sphenotic bones showed ossification as well as the epiotic bones. At DAB 9, triangular exoccipital bones appeared, located behind the supra- occipital bone and over the otolith. Ossification in the operculum, which covers four already ossified gill arches, was visible.

**DAB 10** (HL 3.22 ± 0,4 mm; Figure 5.3a). Dentary, maxillary and pre-maxillary bones presented a strong mineralization. In the latero-dorsal area of the snout, the toothless palatine and the ectopterygoid bones were completely ossified, while the vomeral bone remained cartilaginous. The mesethmoid bone, in the dorsal area of the snout, ossifies together with its cephalic extension, denominated supra-ethmoid bone. The zone standing over the quadratojugal, in *H. hippocampus*, was composed of three bones, the antorbital, the lachrymal and the sub-orbital. The interopercular bone ossified in the

lower part of the suspensoria. The interhyal bone, located in the upper portion of the ceratohyal, was visible and ossified. Other elements showing signs of ossification were located in the opercular region: the branchiostegal rays, and opercular bone.

**DAB 11-20** (HL 3.18 ± 0,3 - 3.72 ± 0,2 mm; Figure 5.3b-d). Almost all skull elements present signs of ossification in the first ten days; until DAB 18 in the jaws region dentary, retroarticular, and angular bones, completed the ossification process. The ossification in the endopterygoid bone increased as well as in the ectopterygoid and palatine bones (Figure 5.3b). The ossified portion of the parasphenoid bone extended to the rostral cartilage (Figure 5.3b). In the suspensoria, antorbital, lachrymal and sub-orbital bones were more pronounced. The cleithrum bone that began to ossify at DAB 7 as a thin bone lamina, at DAB 18 joined cephalic and thoracic regions together. It was connected to the first vertebra and extended from the nape to the ventral portion of the neck. The cleithrum acts as a link between the scapula and coracoid (Figure 5.3b). These two bones were partially ossified, joined to one another and supported the pectoral fins. At DAB 20 the mesethmoid bone was completely ossified, while the vomeral bone was still partially cartilaginous (Figure 5.3c). The lateral ethmoid bones ossified and created a triangular roof sustaining the highest part of the tubular snout (Figure 5.3c). In the upper part of the jaws it is possible to observe as rostral cartilage articulated maxillary and pre-maxillary bones jointed them to the palatine bones (Figure 5.3c). In the neurocranium, the frontal, supra-occipital, pterotics, exoccipitals, epiotics, and post-temporal bones were completely ossified (Figure 5.3d).

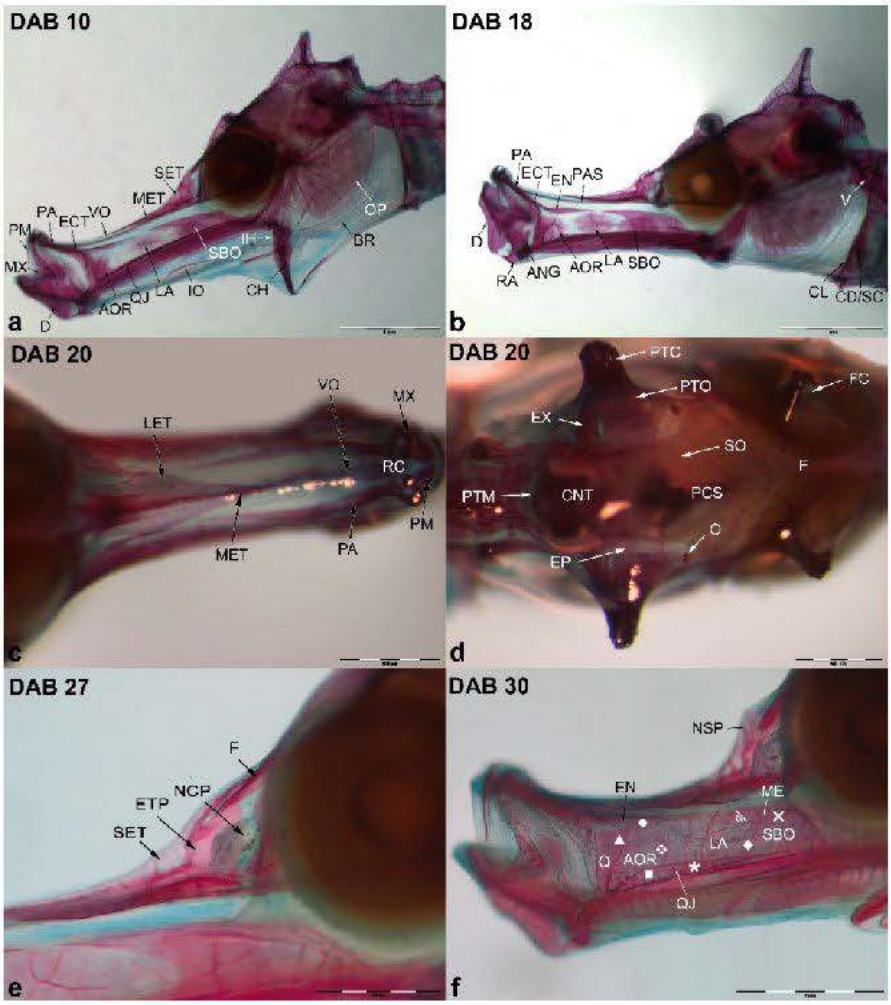



**Figure 5.3** Osteological development of *H. hippocampus* cranium (DAB 10-30). Head, lateral view, a,b,f; head, top view, c,d; nasal capsule, lateral view, e. (ANG) angular, (AOR) antorbital, (BR) branchiostegal rays, (CD) coracoid, (CH) ceratohyal, (CL) cleithrum, (CNT) coronet, (D) dentary, (ECT) ectopterygoid, (EN) endopterygoid, (EP) epiotics, (ETP) ethmoid plate, (EX) exoccipitals, (F) frontal, (FC) frontal crest, (IH) interhyal, (IO) interopercular, (LA) lachrymal, (LET) lateral-ethmoid, (ME) metapterygoid, (MET) mesethmoid, (MX) maxillary, (NCP) nasal capsule, (NSP) nasal spine, (OP) operculum, (PA) palatine, (PAS) parasphenoid, (PCS) pre-coronet spine, (PM) premaxillary, (PTC) pterotic crest, (PTM) post-temporal bone, (PTO) pterotic, (Q) quadrate, (QJ) quadratojugal, (RA) retroarticular, (RC) rostral cartilage, (SBO) sub-orbital, (SC) scapula, (SET) supra-ethmoid, (SO) supraoccipital, (V) vertebra, (VO) vomeral, (X) Metapterygoid and sub-orbital fusion, (◆) sub-orbital and lachrymal fusion, (◺) metapterygoid and lachrymal fusion, (✱) quadratojugal and lachrymal fusion, (❖) antorbital and lachrymal fusion, (●) endopterygoid and antorbital fusion, (■) quadratojugal and antorbital fusion, (▲) antorbital and quadrate fusion.

On the DAB 20 seahorses' cranium presented most of adult morphological characteristics with the increase in size of the pterotic spine and the ossification of frontal and pterotic crests (Figure 5.3d).

**DAB 21-30** (HL 3.86 ± 0,15 - 5.3 ± 0,4 mm; Figure 5.3e, f). The ossification process increased progressively. At DAB 27, supra-ethmoid bone was also completely ossified. At DAB 27, supra-ethmoid bone was also completely ossified. Under the ethmoid plate and lower part of the frontal bone located over the orbit, nasal capsule could be observed but it was also absent the nose spine that developed in the next three days (Figure 5.3f). Until DAB 30, all the bones comprising the lateral face of the snout fused together forming a laminar ossified area. From the suspensoria to the jaws, the sub-orbital bone fused with metapterigoid (X) and lachrymal bones (◆), the lachrymal bone with metapterigoid (◺), quadratojugal (*) and antorbital (❖) bones. The antorbital bone with endopterygoid (•), quadratojugal (■) and quadrate (▲) bones. On the last day of the study, with the formation of the nose spine, seahorse's cranium presented all adult morphological characteristics.

### 5.5.3 Coronet (Figure 5.4a-f)

After release, a cluster of dermal substance was present in the upper part of the head, pre-coronet spine and coronet were absent (Figure 5.4a). Progressively, in the upper part of the supra-occipital bone, from the dermal substance the spine had developed as a robust pointed structure below the dermis with intramembranous ossification, it was the pre-coronet spine.

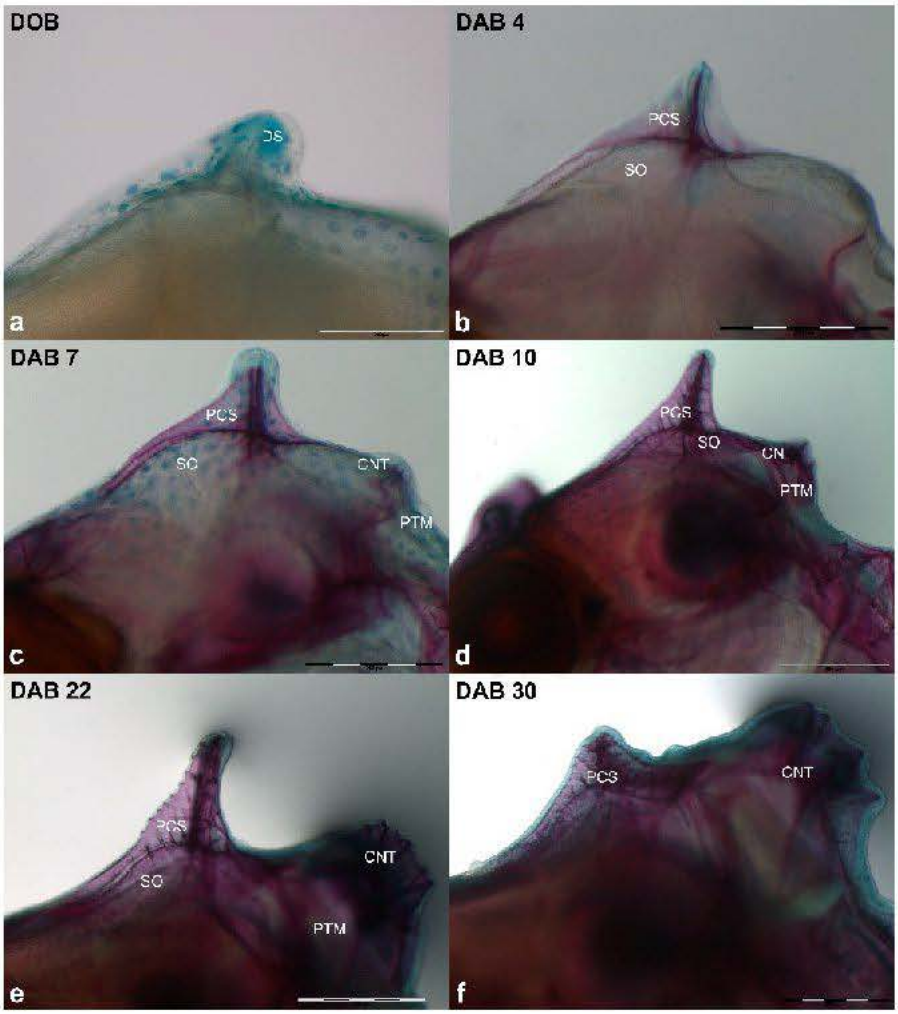



**Figure 5.4** Osteological development of *H. hippocampus* neurocranium and coronet (DOB-DAB 30). Head, lateral view, a-f. (CNT) Coronet, (DS) dermal substance, (PCS) pre-coronet spine, (PTM) post temporal, (SO) supra-occipital.

On DAB 4 (Figure 5.4b), this pointed structure showed more mineralization. For the first time a structure on the post-temporal bone was visible on DAB 7 (Figure 5.4c). On DAB 10 the pre-coronet spine had a triangular pointed shape and was totally ossified, while increase

the ossification on post temporal bone, where the primitive structure of the coronet was just visible (Figure 5.4d).

The coronet and pre-coronet spine increased in size (Figure 5.4e) until the last day of observation, when the shape of the characteristic coronet of this species was clearly visible (Figure 5.4f).

### 5.5.4 Vertebral system (Figure 5.5a-d, 6a-c)

Newborns showed 51±1 vertebrae before the hypural plate (with little variation), which could be subdivided into five classes: cervical (3, V1-V3), abdominal (13: pre-dorsal V4-V9, post-dorsal V13 V19), supra-dorsal (1, V10), dorsal (2 V11-V12) and caudal (32±1, V20-V52±1), by vertebral shape and position (Figure 5.5).

On the DOB, vertebral system was no present, as well as the spine, shield, and plate forming the armor in adult seahorses. After newborns clarification, with the potassium hydroxide and hydrogen peroxide solution, the pigment disappeared and it is possible to observe a distinct notochord that extended from the base of the brain to the end of the tail.

At birth, signs of ossification were present in the neural arch of V1 and V2; on the DAB 1, were also present in the neural arch of V3 (Figure 5.5a). On the DAB 3, chondrogenesis took place progressively starting to the centro-dorsal place of the V13. Signs of ossification were present in cranial, abdominal, supra-dorsal, dorsal and caudal vertebrae (Figure 5.5b). The centrum of V1 showed a mineralization in its dorsal portion. In V2, V3, and V4 chordacentra ossification began in the basal portion.

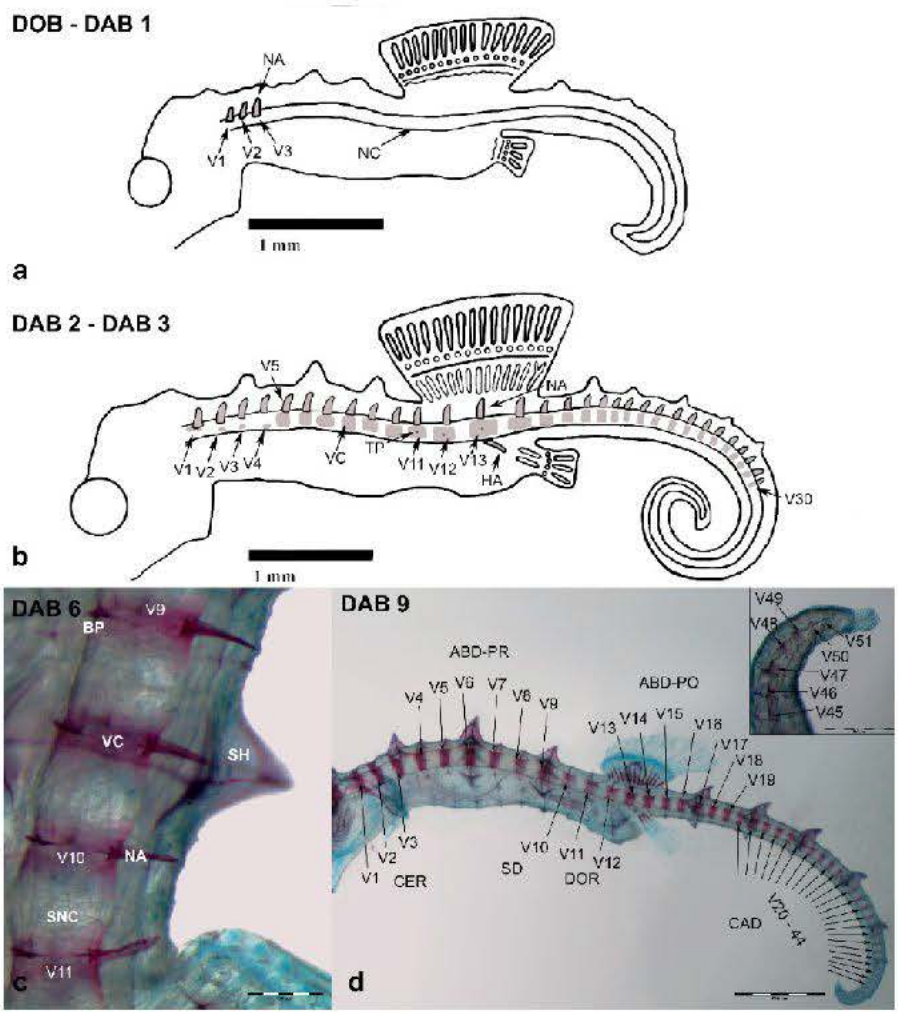



**Figure 5.5** Osteological development of *H. hippocampus* axial skeleton (DOB-DAB 9). Vertebral system, lateral view, a-d. (ABD-PR) Abdominal pre-dorsal vertebrae, (ABD-PO) abdominal post-dorsal vertebrae, (BP) basal process, (CAD) caudal vertebrae, (CER) cervical vertebrae, (DOR) dorsal vertebrae (HA) haemal arch, (NA) neural arch, (NC) notochord, (SD) supra-dorsal vertebra, (SH) shield, (SNC) notochord sheath, (TP) transverse process, (V) vertebra, (VC) vertebral centrum.

Ossification of the centrum took place in twenty-five vertebrae from V5 to V30 and a higher level of mineralised tissue was seen in the abdominal vertebrae (Figure 5.5b). The dorsal vertebrae (V11, V12)

and the first abdominal vertebra (V13) formed the transverse process. The haemal arc, was present in V13. On DAB 6, the vertebral system showed basal processes from V3 to V11; lateral and dorsal shields started to ossify (Figure 5.5c). On the DAB 9, it was possible to carry out a complete vertebra count (Figure 5.5c).

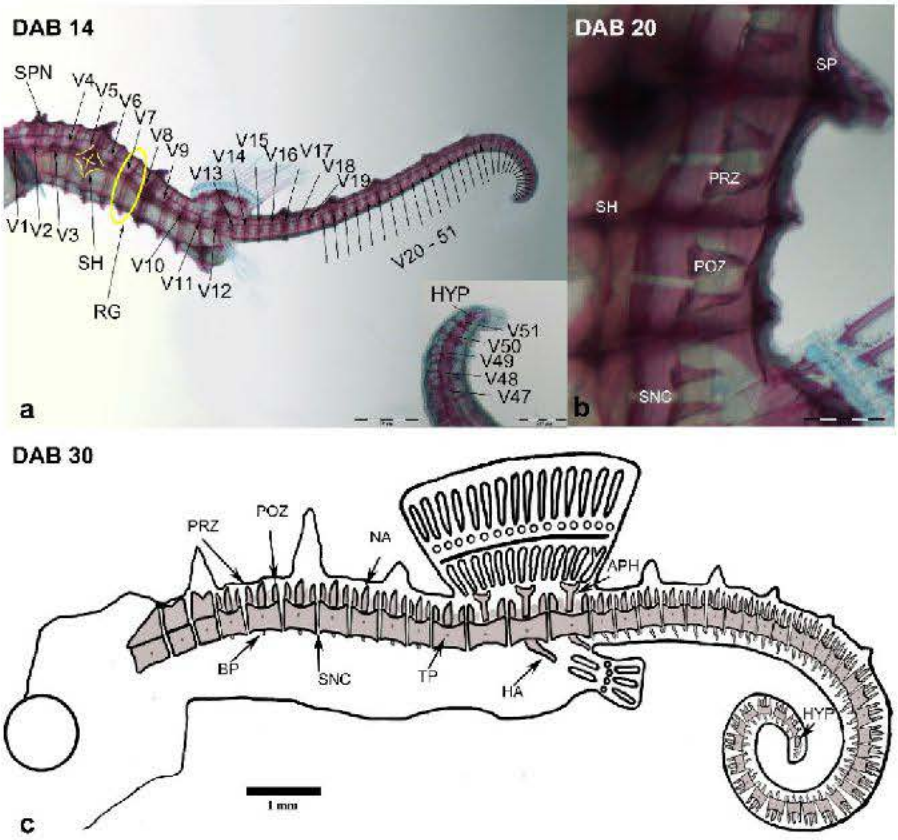



**Figure 5.6** Osteological development of *H. hippocampus* axial skeleton (DAB 10-30). Vertebral system, lateral view, a, b; vertebral system without rings, lateral view, c. (APH) Apophysis, (BP) basal process, (HA) haemal arch, (HYP) hypural plate, (NA) neural arch, (POZ) post-zigapophysis, (PRZ) pre-zigapophysis, (RG) ring, (SH) shield, (SNC) notochord sheath, (SPN) spine, (TP) transverse process, (V) vertebra.

Neural arches were present in 45 vertebrae, right up to the last part of the tail. Haemal arches were present from V13 to V36. Until the DAB

14 shields ossification increases along the whole body, the ossified extremities of shields joint between them and formed rings (Figure 5.6a). A paired pointed apophysis to the extremity of each vertebral body up to the hypural plate appeared (Figure 5.6b).

The vertebral pointed structures before and after the neural arches were pre-zigapophysis and post-zigapophysis (Figure 5.6b); the presence indicated the beginning of the neural tube ossification.

The sheath of the notochord was progressively reduced with the ossification of the vertebrae. Around DAB 20, the external structure comprising rings and spines was similar to that of adults. On DAB 30, the neural tube was not completely ossified while the seahorses' body was completely covered by bony rings and spines until the hypural plate. The elements composing the vertebral system at DAB 30 were represented in the Figure 5.6c.

### 5.5.5 Caudal fin (Figure 5.7a, b)

The last segment of the axial skeleton is the hypural plate. This plate ossified from the hypural cartilage and sustained small segments that could be considered as rays. Until DAB 12, the hypural plate remained cartilaginous while at DAB 14 it was clearly ossified, with little rays included in a dermal matrix. The vertebral system in the final tract showed a flexion in the ventral direction. On DAB 24, the hypural plate was completely ossified, and the dermal matrix was reduced in size. Two or three rays were visible, and the hypural cartilage had been replaced with bone tissue. The caudal fin disappeared progressively with age reducing the morphological difference of newborns respect to

the adult seahorses. However, a small structure at the end of the tail was present until DAB 30.

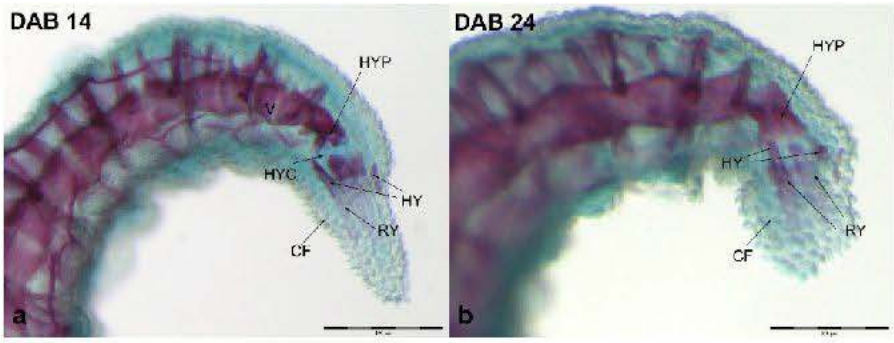



**Figure 5.7** Osteological development of *H. hippocampus* appendicular skeleton (DOB-DAB 30). Caudal fin, lateral view a, b. (CF) caudal fin, (HY) hypural bones, (HYC) hypural cartilage (HYP) hypural plate (POZ), (RY) rays, (V) vertebra.

### 5.5.6 Dorsal fin (Figure 5.8a-f)

The dorsal fin (16-17 rays) is a structure located in the dorso-cental part of the vertebral column that ossifies gradually. At birth, due to the chondrogenesis the morphology of rays, medial radials and distal radials were visible. The medial radials formed a cartilaginous semicircular line in the central part of the fin, while the spherical distal radials formed another semicircular structure that appeared as a subsequence of point (Figure 5.8a). On DAB 3, the proximal radial presented cartilaginous epiphysis and ossified diaphyses (Figure 5.8b). From DAB 3 to DAB 8, the vertebrae developed neural arches and ossified in the centrum (Figure 5.8b). Cellular hypertrophy in the vertebral body leading to the formation of the neural arches' apophyses (Figure 5.8c). Specimens showed a fusion of the two last proximal radials. On DAB 18 (Figure 5.8c), the apophysis of the V12, V13, and V14, forked and formed a basal support for the proximal radials. Three

body rings were involved in the development of the dorsal fin (Figure 5.8d). On DAB 14, the rays and the distal radials were partially ossified (Figure 5.8d), and on DAB 18 the clear ossification of the apophysis was observed (Figure 5.8e).

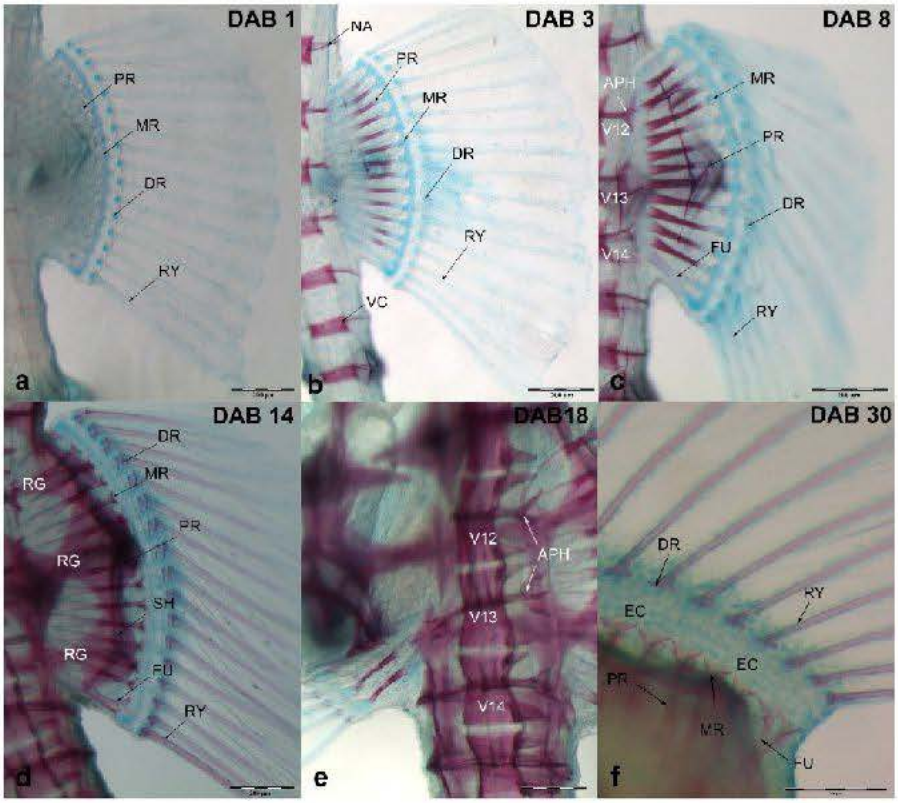



**Figure 5.8** Osteological development of *H. hippocampus* appendicular skeleton (DOB-DAB 30). Dorsal fin, lateral view, a-f. (APH) Apophysis, (EC) elastic cartilage, (HA) haemal arch, (FU) fusion, (DR) distal radial, (MR) medial radial, (NA) neural arch, (PR) proximal radial, (RG) ring, (RY) rays, (SH) shield, (V) vertebra, (VC) vertebral centrum

The distal radials connected the ossified medial ones with an elastic cartilage. On DAB 30, the dorsal fin (proximal, medial, distal radials and rays) was completely ossified. An elastic cartilage remained between medial and distal radials (Figure 5.8f).

### 5.5.7 Anal fin (Figure 5.9a, b)

The anal fin presented the same morphological structure as the dorsal fin and was smaller: three proximal radials, four distal radials and four rays. The haemal apophysis of V13 sustains this fin (Figure 5.9a). The haemal arch was present DAB 4. Until DAB 6, the whole structure remained cartilaginous and the ossification of proximal radials began at DAB 8 (Figure 5.9a). Until DAB 20, the ossification increases progressively (Figure 5.9b), medial and distal radials showed bone tissue. The rays completed fin ossification in the following two or three days.

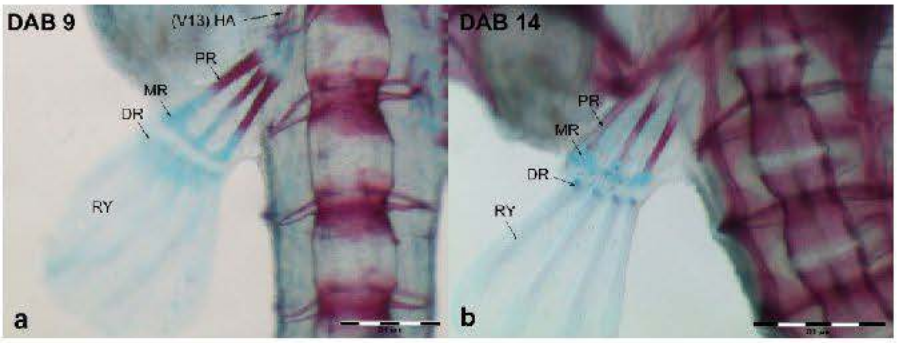



**Figure 5.9** Osteological development of *H. hippocampus* appendicular skeleton (DOB-DAB 30). Anal fin, lateral view, a-b. (DR) distal radial, (HA) haemal arch, (MR) medial radial, (PR) proximal radial, (RY) rays, (V) vertebra.

### 5.5.8 Pectoral fins (Figure 5.10a-e)

The pectoral fins (14 rays) were located just behind the opercula, presented a similar structure to the other fins and were sustained by the pectoral girdle. At birth, cartilaginous distal radial and rays were present (Figure 5.10a). On DAB 7, the external layer of the pectoral fin plate chondrified, cleithrum and coracoid bones were subjected to

mineralization (Figure 5.10b). Distal radial and rays showed a completely cartilaginous structure until DAB 10, (Figure 5.10c).

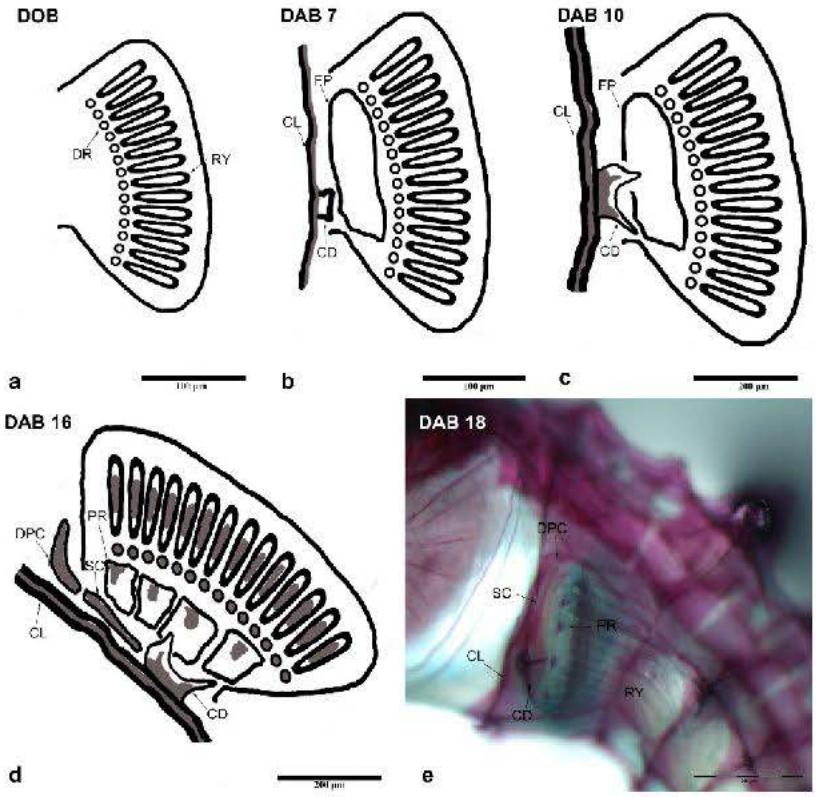



**Figure 5.10** Osteological development of *H. hippocampus* appendicular skeleton (DOB-DAB 30). Pectoral fin, lateral view, a-e. (CD) coracoid, (CL) cleithrum, (DPC) post cleithrum, (DR) distal radial, (FP) pectoral fin plate, (PR) proximal radial, (RY) rays, (SC) scapula.

On DAB 16, four internal proximal radials started to ossify. Bone tissue was present also in distal radials and rays (Figure 5.10d). On DAB 18 increased in size cleithrum coracoid and scapula bones, ossified the post cleithrum bone (Figure 5.10e). On DAB 30, proximal and distal radials and rays were completely ossified, while the rays were partially cartilaginous.

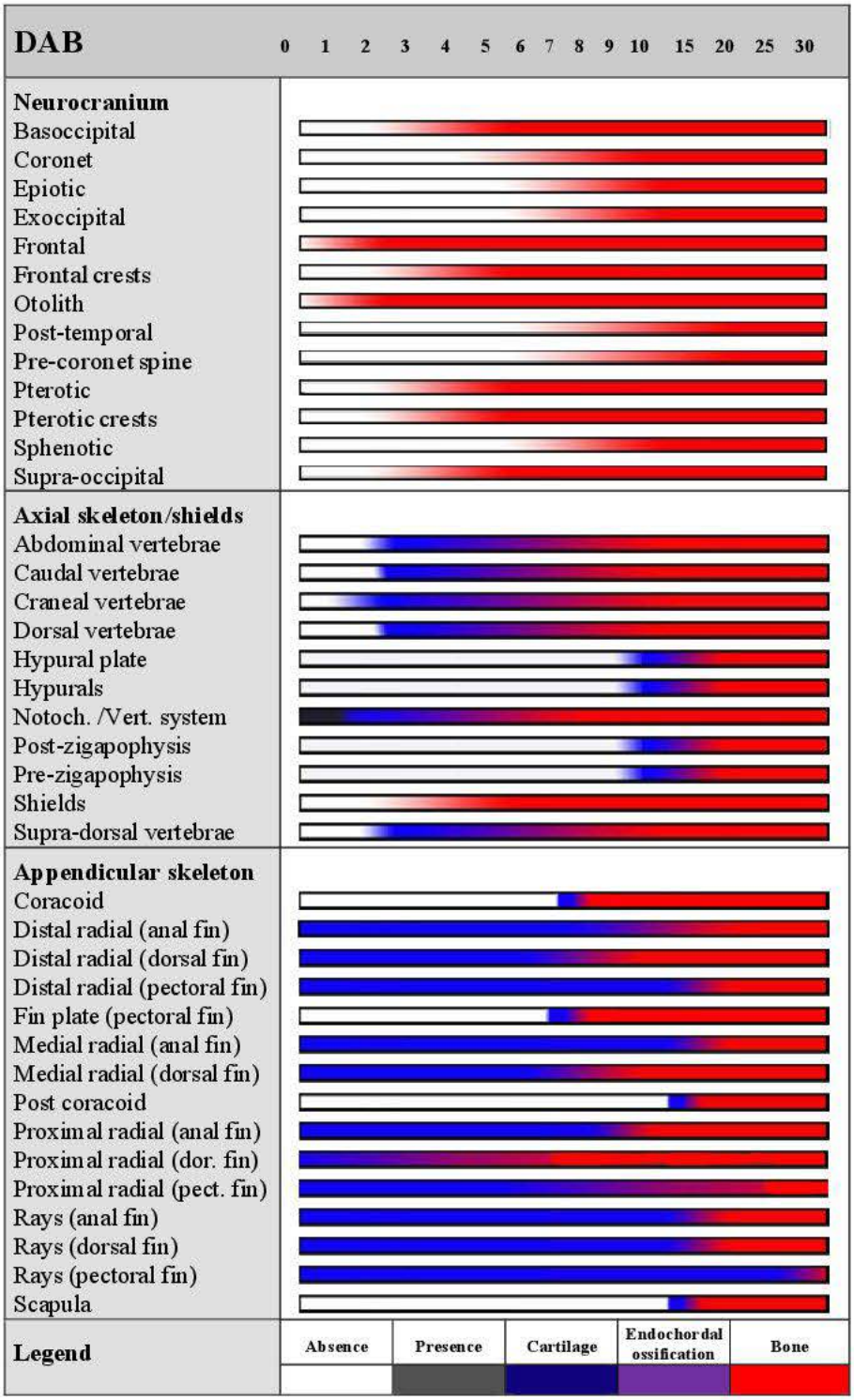
Ossification process of *H. hippocampus* is resumed in the table 5.2.

Table 5.2 Osteological development of *H. hippocampus* during 30 days after their release.

| DAB | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 15 | 20 | 25 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Branchiocranium** | | | | | | | | | | | | | | | |
| Angular | | | | | | | | | | | | | | | |
| Antorbital | | | | | | | | | | | | | | | |
| Basihyial | | | | | | | | | | | | | | | |
| Branchiostegal rays | | | | | | | | | | | | | | | |
| Ceratohyal | | | | | | | | | | | | | | | |
| Cleithrum | | | | | | | | | | | | | | | |
| Dentary | | | | | | | | | | | | | | | |
| Ectopterygoid | | | | | | | | | | | | | | | |
| Endopterygoid | | | | | | | | | | | | | | | |
| Ethmoidal plate | | | | | | | | | | | | | | | |
| Hyomandibular | | | | | | | | | | | | | | | |
| Hyosymplect cartilage | | | | | | | | | | | | | | | |
| Hypohyal | | | | | | | | | | | | | | | |
| Interopercular | | | | | | | | | | | | | | | |
| Lachrymal | | | | | | | | | | | | | | | |
| Lateral-ethmoid | | | | | | | | | | | | | | | |
| Maxillary | | | | | | | | | | | | | | | |
| Meckel's cartilage | | | | | | | | | | | | | | | |
| Mesethmoid | | | | | | | | | | | | | | | |
| Metapterygoid | | | | | | | | | | | | | | | |
| Nasal spine | | | | | | | | | | | | | | | |
| Operculum | | | | | | | | | | | | | | | |
| Palatine | | | | | | | | | | | | | | | |
| Parasphenoid | | | | | | | | | | | | | | | |
| Pre-frontal | | | | | | | | | | | | | | | |
| Pre-maxillary | | | | | | | | | | | | | | | |
| Pre-opercle | | | | | | | | | | | | | | | |
| Quadrate | | | | | | | | | | | | | | | |
| Quadratojugal | | | | | | | | | | | | | | | |
| Retroarticular | | | | | | | | | | | | | | | |
| Rostral cartilage | | | | | | | | | | | | | | | |
| Sub-orbital | | | | | | | | | | | | | | | |
| Supra-ethmoid | | | | | | | | | | | | | | | |
| Symplectic | | | | | | | | | | | | | | | |
| Urohyal | | | | | | | | | | | | | | | |
| Vomeral | | | | | | | | | | | | | | | |

| Legend | Absence | Presence | Cartilage | Endochordal ossification | Bone |
|---|---|---|---|---|---|

Table contents continue on next page


DAB
0 1 2 3 4 5 6 7 8 9 10 15 20 25 30
Neurocranium
Basoccipital
Coronet
Epiotic
Exoccipital
Frontal
Frontal crests
Otolith
Post-temporal
Pre-coronet spine
Pterotic
Pterotic crests
Sphenotic
Supra-occipital
Axial skeleton/shields
Abdominal vertebrae
Caudal vertebrae
Craneal vertebrae
Dorsal vertebrae
Hypural plate
Hypurals
Notoch. /Vert. system
Post-zigapophysis
Pre-zigapophysis
Shields
Supra-dorsal vertebrae
Appendicular skeleton
Coracoid
Distal radial (anal fin)
Distal radial (dorsal fin)
Distal radial (pectoral fin)
Fin plate (pectoral fin)
Medial radial (anal fin)
Medial radial (dorsal fin)
Post coracoid
Proximal radial (anal fin)
Proximal radial (dor. fin)
Proximal radial (pect. fin)
Rays (anal fin)
Rays (dorsal fin)
Rays (pectoral fin)
Scapula
Legend
Absence
Presence
Cartilage
Endochordal ossification
Bone

## 5.6 Discussion

In some species of seahorse, the ossification process starts while the young are in the brood pouch (Azzarello 1990) while in others species the process starts when they are outside (Franz-Odendaal and Adriaens 2014). In *H. hippocampus* the ossification is a process that happens outside the brood pouch, and it is characterized not only by changes in shape, and morphology, but also by the growth of osteological structures absent at birth, such as coronet, head spines, bony rings and bony plates. Franz-Odendaal and Adriaens (2014) indicated that the different timing of ossification was due probably to the different osteogenic strategies during seahorse evolution, but understanding the reasons of these differences is a complex task. The timing of ossification may be influenced by the size of the specie, by the water temperature, and in captive reared seahorses, the broodstock diet is another factor that affects embryonic development (Otero-Ferrer et al. 2014).

Seahorses are considered extreme suction feeders, and according to Van Wassenberg et al. (2009), after release are equipped with a fully functional prey-capture system. When newborns are released, the presence of mineralised tissue in pre-maxillary, maxillary and dentary bones and the ossification of the first skull elements in the branchiocranium, prepare animals during the planktonic phase to catch a wide variety of zooplankton. This happens also in others fish (Gluckman et al. 1999; Liu 2001); there is a link between the development of the feeding apparatus and feeding performance (Wittenrich et al. 2009).

The bones involved in the hyostylic suspension are responsible for their ability to move the entire jaw apparatus forward, separately from the brain case. The jaws are connected to the brain case by the hyomandibular and symplectic, bones originated in the suspensoria by hyosymplectic cartilage, a structure that Kadam (1958) described as just one plate. Even if *H. hippocampus* presented just one cartilaginous plate initially, the ossification highlights two focal points, where two different bones (symplectic and hyomandibular) develop. This observation confirmed neither the description of Silveira (2000) for *H. reidi* nor the description of Leysen et al. (2010) for *Hippocampus capensis*. Silveira (2000) considered one structure divided into two parts in order to describe it in more detail; Leysen et al. (2010) described the symplectic and hyomandibular bones as part of the hyosymplectic cartilage, but affirmed that they were perpendicular structures. This latter difference is due to the controversy about the presence or absence of endochondral metapterygoid in syngnathoid fish, which was well analysed by Azzarello (1989). The presence or absence of endochondral metapterygoid has been a subject of dispute until recent studies and the osteocranium homology adopted caused controversy among Syngnathidae and others vertebrates. In adult animals, due to the fusion of endopterygoid and ectopterygoid bones in a single structure defined as "pterygoid", as occur in *Gasterosteus aculeatus* (De Beer 1937) and probably in *Syngnathus rostellatus* and *H. capensis* (Leysen et al. 2010), is impossible to establish the presence of the pterygoid series. The complete pterygoid series, as described during the development of *H. zosterae*, (Azzarello 1989, 1990) and *H.*

*reidi* (Silveira 2000), was also observed in *H. hippocampus* during the present study.

Other differences regarding osteocranium homology between Syngnathidae and other teleosts, are principally due to the elongation of the tubular snout and to the differences regarding the location of circumorbital bones in front of the orbit instead of around it. Gosline (1965) included the sub-orbital series in the circumorbital bones, as a series of bones that surround and protect the margin of the orbit; in seahorses, some bones included in the sub-orbital series are located along the lateral side of the snout. The largest of this series is the lachrymal bone that Azzarello (1989) and Silveira (2000) located it in the lateral face of *H. zosterae* and *H. reidi* snout, respectively. In the smaller *H. zosterae*, a single sub-orbital and the lachrymal bones have been described (Azzarello, 1989), while in the longsnout seahorse, *H. reidi*, 5 sub-orbitals and the lachrymal bones (Silveira 2000). In this study, three bones were observed in *H. hippocampus*: the antorbital, the lachrymal and the sub-orbital, the same result was observed in *H. capensis* (Leysen et al. 2010). The results, here, suggest the existence of a relation between the size of the snout and the number of the sub-orbital bones that composed it.

According to Lourie et al. (2004) who described the West African population of *H. hippocampus* with a different shape of coronet, the short-snouted seahorse of Canary Islands observed in this study is characterized for the progressive growth of this different type of coronet compared to northern specimens.

Adult seahorses have peculiar armor body formed by rings and plates. Lourie et al., (2004) describe 11 trunk rings and 34 (32-37) tail rings

in *H. hippocampus*, and highlight a difference between Mediterranean and eastern Atlantic seahorses in number of tail rings. In this study, seahorses show 11 trunk rings and 39 (38-40) with body rings starting from the second vertebra and one ring for each vertebra. According to Lourie et al. (2004), *H. hippocampus* found in the eastern Atlantic has more tail rings,

As also observed in other seahorse species (Silveira, 2000; Cho and Liew, 2006; Odendaal and Adriaens, 2014), when newborn are released from the male pouch, they have not yet developed rings and plates. Porter et al. (2013) explained that the bony-plated armor in the tail of seahorses is a multifunctional device that provides structural support, protection and prehension. The formation of the bony plates is preceded by the ossification of the vertebral system, and the absence of these two elements in *H. hippocampus* coincide with the planktonic phase of newborns. There is a relation between the ossification of the tail and its prehensile ability. In fish, the metamorphosis is associated, not only with a shift in allometric growth, shape, morphology and colour, but also with ecological factors that produce changes in larvae trophic status and/or habitat, as occurs in flatfish larvae (Geffen et al. 2007). Shift from planktonic to benthic status occurs in newborn seahorses (Foster and Vincent 2004; Otero-Ferrer et al. 2010). Both research teams observed that newborns of *H. hippocampus* started to bend the substrate after approximately two weeks. In agreement with our results, the remodelled tail structure gradually ossified and after approximately two weeks permitted the animals to bend the substrate.

Generally, the principal function of the caudal fin is the propulsion for swimming, and in the last part of the notochord, fish larvae carried

out a deviation or "flexion" in dorsal direction (urostilo) with the formation of the hypural bones as happens in Scombroid or Sparids (Potthoff 1986; Koumoundouros et al. 2001; Sfakianakis et al. 2004; Socorro 2006). Because of a remodelled tail structure in *H. hippocampus*, there is no dorsal flexion, but a hypural plate at the end of the tail, articulated with some little bones (hypurals or rays). The caudal fin, absent in adult seahorses, is present, as a remnant, in newborns. In *H. hippocampus*, as observed in *H. kuda* (Choo and Liew 2006), *H. reidi*, and *H. subelongatus* (Odendaal and Adriaens 2014), the caudal fin shows a progressive involution. This regression could be related to seahorse life traits, for example, living attached to seabed structures (Foster and Vincent 2004).

In accordance with Balon (1999), teleosts with specialized larval forms (like seahorses) have a transitional stage where the "transformation" from larva to juvenile takes place. It is difficult to determine the exact onset of juvenile development; this study not only highlights the absence of morphological structures in newborns such as coronets, prominent spines, body rings and plates, but also relates the shift from planktonic to benthic status with the ossification of the tail and the development of its prehensile capacity. The juvenile is a fish with definitive characters that differs from the adult in terms of size and reproductive capacity, while newborns of *H. hippocampus* are larvae in its metamorphic stage. The results are in contrast with previous studies, where newborns seahorses were defined perfectly formed in comparison with the adult (Brunelli 1922), or like juvenile metamorphosed (Choo and Liew 2006). The particular strategy of reproduction of *H. hippocampus* would divide the larval period (from

embryo to juvenile) into two phases: the first occurs inside the brood pouch and the second outside, and newborn seahorses should be considered as larvae after the male release. The metamorphic process may suggest that the term "larvae" is the most appropriate, and it is for this reason that we propose the inclusion of this new stage in *H. hippocampus* life cycle.

# Estudio IV

## *Digestive biochemistry as indicator of the nutritional status during early development of the long snouted seahorse (Hippocampus reidi)*

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B Novelli, F Otero-Ferrer, M Diaz, JA Socorro, MJ Caballero, L Molina Domínguez, and FJ Moyano

## 6.1 Resumen

Se utilizó el nivel de actividad de algunas de las principales enzimas digestivas del caballito de mar (*Hippocampus reidi*; Ginsburg, 1933) medidas durante los primeros 20 días después del nacimiento, para evaluar la respuesta nutricional a diferentes enriquecedores de la *Artemia* salina utilizada como alimento vivo. Se observaron diferencias significativas en las actividades de la tripsina, esterasa, y proteasa total, entre los tratamientos experimentales. Los cambios en las actividades de las fosfatasas alcalina y ácida, así como en la ratio tripsina/esterasa, estaban vinculados a los diferentes enriquecedores utilizados. Esto proporciona información sobre algunas de las características del proceso de digestión en los recién nacidos y la influencia de la dieta sobre la maduración del epitelio intestinal durante su desarrollo temprano. El uso de un análisis multivariante que combina la información de todas las actividades enzimáticas destacó las diferencias entre tratamientos. Los resultados obtenidos confirman el interés de la utilización de las enzimas digestivas como indicadores adecuados del estado nutricional de larvas de esta especie y puede ayudar a optimizar los protocolos de alimentación necesarios para la producción a gran escala de los caballitos de mar en cautividad.

Declaración de interés: la sobreexplotación, pérdida de hábitat, y la captura accidental durante operaciones de pesca comercial, hacen que las poblaciones de caballitos de mar sean susceptibles al declive. La alta demanda mundial de caballitos de mar supera la oferta de ejemplares producidos en cautividad, y en esta situación insostenible, *H. reidi*, es una de las especies más comercializadas. La producción de las crías sigue siendo el cuello de botella para el cultivo exitoso de

caballitos de mar. En este sentido, el diseño de un adecuado régimen de alimentación basada en alimentos adaptados a las necesidades nutricionales de la especie es de gran importancia. Debido a su papel clave en la digestión y asimilación de nutrientes, los cambios en los patrones de producción de las principales enzimas digestivas pueden ser utilizados como indicadores del estado nutricional, ayudando a evaluar la adaptación a los alimentos con diferente contenido de macronutrientes y, por tanto, contribuyen a optimizar las estrategias de alimentación para esta especie. Los parámetros bioquímicos propuestos como indicadores del estado nutricional, podrían ser buenas herramientas analíticas, no sólo para establecer el momento adecuado para el cambio de las presas vivas durante el destete, sino también para formular una composición bioquímica más digerible en las presas vivas. Las actividades de las fosfatasas, la ratio tripsina/esterasa y las agrupaciones de actividades enzimáticas, fueron consistentes en los resultados y podrían ser considerados como buenos indicadores del estado nutricional de esta especie.

## 6.2 Abstract

The activities of some of the main digestive enzymes of long snouted seahorse (*Hippocampus reidi*, Ginsburg, 1933) measured during the initial 20 days after birth were used to assess the nutritional response to different enrichment of the *Artemia* salina used as live food. Some significant differences in the activities of trypsin total protease and esterases were observed among the experimental treatments, changes in the activities of acid and alkaline phosphatases, as well as in the trypsin/estaerase ratio, were linked to the different dietary treatments.

This provided information both on some characteristics of the digestion process in newborns and the influence of the diet on the maturation of the intestinal epithelium during their early development. The use of a multivariate analysis combining the information of all the enzyme activities highlighted differences between treatments. Results obtained confirm the interest of using digestive enzymes as valuable indicators of nutritional condition in larvae of this species and may help to optimize feeding protocols required for mass-scale production of seahorses in captivity.

*Statement of relevance*: Overexploitation, habitat loss, and incidental bycatch during commercial fishing operations, make seahorses susceptible to decline. The high global demand of seahorses exceeds supply from specimens breeding in captivity, and in this unsustainable situation, the long snouted seahorse, *H. reidi*, is one of the most frequently commercialized species. Larval rearing remains the bottleneck for successful cultivation of seahorses. In this sense, the design of a suitable feeding regime based on food adapted to the nutritional needs of the species is highly desirable. Due to their key role in nutrient digestion and assimilation, changes in the production patterns of the main digestive enzymes can be used as indicators of the nutritional status helping to assess the adaptation to foods with different content in macronutrients and therefore contributing to optimize feeding strategies for this species. The biochemical parameters proposed as indicators of nutritional status might be good analytic tools, not only to establish the time of change of live prey during the weaning but also to formulate the most digestible biochemical composition of live prey. Phosphatases activities,

trypsin/esterase ratio and the clusters of enzyme activities, were consistent in the results and might be considered as good indicators of nutritional status in this species.

## 6.3 Introduction

Seahorses are widely distributed around the world, principally in tropical and subtropical zones (Kuiter 2009), and due to their attractive appearance and peculiar ecology are often used as flagship species to promote marine conservation. In fact, seahorses have unusual morphological and biological characteristics: they present a tubular snout (Leysen *et al.* 2011), the orientation of the body axis during swimming is vertical, and the propulsion is achieved mainly by the dorsal fin (Breder and Edgerton 1942). In addition, the whole body of seahorses is composed by an armour of bony plates instead of scales (Lourie *et al.* 2004), many species are easily camouflaged developing skin filaments (Curtis 2006) and all them are provided with a prehensile tail to attach to the substrate (Porter *et al.* 2013). Furthermore, seahorses are distinguished by their particular reproductive strategy; the incubation of eggs by the male within a specific brood pouch in which embryos and larvae are developed (Novelli *et al.* 2015; Stölting and Wilson 2007) this being a long process that requires a high input of nutrients and energy coming from egg reserves (Planas *et al.* 2010). There are many problems that make populations of seahorses susceptible to decline: not only due to habitat loss and incidental bycatch during commercial fishing operations (Koldewey and Martin-Smith 2010), but also to the direct overexploitation of adult and juvenile seahorses that are extracted from

the wild and traded alive as ornamental fish, or dried to be used in Traditional Chinese Medicine (Rosa *et al.* 2011; Vincent 1996). For these reasons, the *Convention on International Trade of Endangered Species* (CITES) in November 2002 listed all the species of seahorses in the Appendix II. In this unsustainable situation, the long snouted seahorse, *Hippocampus reidi*, is one of the most frequently commercialized species (Rosa *et al.* 2011). Conservation and management of seahorses depends on interdisciplinary approaches such as ecosystem protection, fisheries management and trade control (Vincent *et al.* 2011). Furthermore, a way to reduce pressure on wild populations is their rearing in captivity (Murugan *et al.* 2011) that is used to repopulate marine areas (Otero-Ferrer 2012) and also contributes to improve the knowledge about their biology and physiology (Olivotto *et al.* 2011).

Breeding techniques developed for culture of some seahorse species at small scale allowed closing their life cycles (Hora and Joyeux 2009), but high mortality rates at early stages of development are still recorded, mainly due to inadequate nutrition and disease (Murugan *et al.* 2009; Truong 1998). Nutritional requirements and suitable feeding patterns are still important gaps in current knowledge that remain as a bottleneck for successful cultivation of seahorses on mass scale (Olivotto *et al.* 2011). In this sense, the design of a suitable feeding pattern based on food adapted to the nutritional needs of the species is highly desirable. It has been demonstrated that feeding larvae of *H. reidi* with rotifers and *Artemia* results in high mortalities (Otero-Ferrer *et al.* 2010), while the use of captured zooplankton or copepod nauplii results in lower mortality and a more successful rearing (Hora and

Joyeux 2009; Payne and Rippingale 2000; Zhang *et al.* 2015b). This suggests that the modification in the fatty acid profile and protein contents of *Artemia* through the use of enrichers may be a good way to adapt the nutritional profile of the live food to the specific requirements of the species.

As occurs in most marine fish larvae, the digestive system of seahorses is not yet fully developed during their initial stages (Novelli *et al.* 2015; Wardley 2006), being unable to digest complex macromolecules at the start of exogenous feeding. Related to this, the evaluation of the type, activity and time sequence of appearance of the main digestive enzymes may be used to characterize the pace of development and maturation of their digestive system during the initial stages, as it has been carried out in many other species of fish (Alvarez-González *et al.* 2008; Martinez *et al.* 1999; Moyano *et al.* 1996), crustaceans (Andrés *et al.* 2010; Rotllant *et al.* 2010), cephalopods (Perrin *et al.* 2004), or bivalves (Albentosa and Moyano 2008). Similarly, due to their key role in nutrient digestion and assimilation, changes in the production patterns of the main enzymes can be used as indicators of the nutritional status of the individuals, this helping to assess their adaptation to foods with different content in macronutrients and therefore contributing to optimize its formulation.

Considering all the above mentioned, the objective of the present work was, in one hand, to provide information on the main features of the digestive biochemistry during the initial developmental stages of *H. reidi* an issue already described in other species like *Hippocampus abdominalis* or *Hippocampus guttulatus* (Blanco *et al.* 2015; Wardley 2006). On the other hand, to assess to what extent the use of living food

with a different content in macronutrients may be reflected in the digestive biochemistry of *H. reidi* offspring, this helping to validate enzyme activities as indicators of differences in the nutritional use of the diet by this species.

## 6.4 Material and methods

### 6.4.1 Rearing and feeding of seahorses

Broodstock and larvae of *H. reidi* were maintained at the Ecoaqua Universitary Institute facilities (ULPGC, Spain). The equipment maintained the water quality with concentration of ammonia, nitrites, nitrates, and phosphates below detection levels (Macherey-Nagel, Viscolor® Eco, Düren, Germany). Broodstock breeding and feeding and larval rearing were carried out following Novelli *et al.* (2015). Newborns were fed twice a day, 7 days per week with *Artemia salina* (0.7 metanauplii/mL) enriched 24 h with three types of commercial products differing in their lipid/protein contents: DHA Protein Selco (27/29%, T1), Biomar Multigain (13/44%, T2) and Easy Selco DHA (0/67%, T3). The enrichment of *A. salina* metanauplii was carried out for 24 h as reported in commercial packaging following the standard protocols used by the GIA (Grupo de Investigación en Acuicultura).

### 6.4.2 Experimental design

The progeny of six broodstock pairs, held separately, was reared to evaluate enzyme activities during the development. A total of 375 seahorses, 15 on the day of release and 45 per each sampling point, were collected on days 0, 1, 2, 4, 6, 8, 10, 15 and 20 after birth (DAB), For each broodstock release, newborns were collected in the

broodstock aquaria, counted, transferred to the larval experimental unit and distributed equally: 100 individuals per aquarium composed a triplicate. Each triplicate was fed using one of the three treatments and five individuals per day were randomly sampled (2+2+1). The 5 seahorses randomly sampled, composed 1/3 of each point of sample. The triplicate for each point of sample was composed of a pool of 15 larvae, coming from three different broodstock pairs, five specimens per pair. The experiment was maintained until all the samples were obtained. Individuals were anaesthetized using natural clove essential (Guinama, Alboraya, Valencia, Spain) in ice-cold water, killed immediately using liquid nitrogen and maintained deep frozen at -80 °C until freeze-dried.

The experiments were carried out in compliance with the Guidelines of the European Union Council (2010/63/EU) and Spanish legislation for the use of laboratory animals. All required permissions have been obtained (Reference 002/2011 CEBA, ULPGC).

### 6.4.3 Growth

Time spans used to determine the developmental stages were based on internal morphological changes of newborns established for Novelli *et al.* (2015) for this species. Growth in dry weight was assessed with a microbalance (XP6, Mettler Toledo, Greifensee, Switzerland), and the absolute growth rate (AGR), was calculated using the equation (Lf − Li) / t2 − t1 (Hopkins, 1992), being Lf he mean dry weight (mg) of the sample at the end of each developmental stage at time t2 and Li the mean dry weight at the end of the previous stage at the time t1. The

ratio daily weight increase (%DWI) to specific growth rate (SGR) was calculated according to Ricker (1958). The exponential model used was SGR = (lnW2 − lnW1) / t2 − t1, and %DWI = 100 × ($e^{SGR}$ − 1), where W1 and W2 were dry weight at t1 and t2, respectively. The unit 1 is the initial dry weight; and the dry weight eg increased eg−1 at the end of a unit of time. The effect of different life prey regimes on growth was calculated as the percentage of biomass increased (mg) in relation to initial weight (mg). The weight gained (WG) was calculated using the equation (W2 − W1) / (W1 × 100).

### 6.4.4 Biochemical analysis

Biochemical composition of *A. salina* nauplii, enriched metanauplii, and seahorses was analysed at the Instituto Universitario de Sanidad Animal (IUSA-Arucas, Canary Islands, Spain). All samples were washed with distilled water and freeze-dried (−80 °C) prior to analysis. Lipids were extracted using the method described by Folch *et al.* (1957). Protein content was calculated using the Kjeldhal method (AOAC, 2010). Analyses were carried out in triplicate.

### 6.4.5 Evaluation of enzyme activities

Enzyme extracts used to evaluate the activity of digestive enzymes were prepared by homogenization of each separate individual in distilled water (200 µL) after removal of the head and tail. The homogenates were then centrifuged for 5 min at 13.000 g at 4 °C and the extracts aliquoted and stored (-20 °C) until being analysed. Enzymatic activities were analysed using different fluorescent substrates in a Fluoroskan reader (Cytation 3 Cell Imaging Multi-Mode

Reader, USA). Substrates and analytical conditions used were those described in Rotllant *et al.* (2008) with some modifications:

**6.4.5.1 Trypsin.**

The substrate Boc-Gln-Ala-Arg-7-amido-4 methylcoumarin hydrochloride (B4153; Sigma-Aldrich, Madrid, Spain) was diluted in dimethyl sulfoxide (DMSO) to a final concentration of 20 µM. 10 µL of the diluted homogenate was mixed to 190 µL of buffer (Tris-HCl, 0.1 M CaCl2 pH 8.0) and 5 µL of the substrate were added to the microplate. Fluorescence was measured at 380/440 nm EX/EM.

**6.4.5.2 Total protease.**

Fluorescent casein 0.5%, (C0528; Casein fluorescein isothiocyanate from bovine milk, type III, Sigma-Aldrich, Madrid, Spain) was used as a substrate. 150 µL of buffer (Borate pH 8.0) and 40 µL of the diluted homogenate were mixed to the microplate. 6 µL of the substrate were added. Fluorescence was measured at nm 485/538 EX/EM.

**6.4.5.3 Non-specific esterases.**

The activity was assayed using three substrates with a different chain length, 4-methylumbelliferyl butyrate (19362; MUB-Fluka), 4-methylumbelliferyl heptanoate (M2514; Sigma-Aldrich, Madrid, Spain) and 4-methylumbelliferyl oleate (75164; Sigma-Aldrich, Madrid, Spain). The substrates were dissolved in phosphate buffer pH 8.0 to a final concentration of 0.4 mmol/L. For each 250 µL of 0.4Msubstrate, 15µL of the aliquoted larvae homogenate was added to

the microplate, and mixed. Fluorescence was measured at 355/460 nm EX/EM.

#### 6.4.5.4 Phosphatases

Alkaline and acid phosphatase substrates used were 4-methylumbelliferyl phosphate, (M-6491; MUP) and 6,8-difluoro-4-methylumbelliferyl phosphate (D-6567; DiFMUP) respectively. A 10mmol/L stock solution of MUPwas prepared by dissolving substrates in pH 8 borate buffer. 20 µL of the diluted homogenate were mixed to the microplate, and mixed. Fluorescence was measured at 360/ 440 nm EX/EM.

The specific activity was expressed as the number of micromoles of product produced per minute per mg of protein under a specified set of conditions. The extinction coefficient (difference between product and substrate) was obtained using a standard of 4-Methylumbelliferone (Sigma M1381).

### 6.4.6 Zymograms of digestive enzymes

Zymograms of alkaline proteinases were prepared using the technique described by García-Carreño *et al.* (1993). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was prepared according to Laemmli (1970), using acrylamide semi-denatured conditions and discontinuous system. The gel (8 × 10 × 0.075 cm) consisted of two parts: the stocking gel 4% T-2.6% C and the separating gel 11.5% T- 2.6% C, where T was total monomer concentration and C was the part of total monomer that is bis-

acrylamide. In the plate, 5 mL of Molecular Weight Marker was added. Enzyme extracts (20 µL) were mixed with equal parts of sample buffer (0.125 MTris–Cl, 2% SDS, 20% v/v glycerol, 0.04% bromophenol blue, pH 6.8; without 2-mercaptoethanol) and were not heated. Volumes of each sample, containing 5.22 ± 1.65 µg of soluble protein determined by Bradford method (Bradford 1976), were loaded into individual gel wells at 0 °C. Electrophoresis running conditions and measurement were: 120 min at constant current of 220 V, 15mA, 2W. After electrophoresis, gels were washed and incubated in 50mmol/L TRIS-HCl buffer, pH 9, containing 3% casein (Sigma) for 30 min at 2 °C. The temperature was raised to 37 °C for 90 min without agitation. Thereafter, gels were washed, fixed in 12% TCA and stained overnight (0,025% p/v Coomassie Brilliant Blue BBC R-250, 40% v/v methanol, 7% v/v glacial acetic acid). Distaining was carried out in a methanol-acetic acid–water solution (40:7:53).

### 6.4.7 Statistical analysis

The effect of different treatments on the growth of seahorses was evaluated using a Nested Analysis of Variance using the General Linear Model, while Tukey's Honestly Significant difference (HSD) test was used to compare mean values of enzyme activities between treatments at the same age and between ages for each treatment, at a confidence level of 95%. In addition, a multivariate analysis (cluster) combining the data obtained from the all the enzyme analysis was used to assess the similarity among individuals receiving each dietary treatment at different ages. Single linkage was selected as the amalgamation rule and Euclidean distances were used for computing

distances between objects in the multidimensional space. All statistical analyses were carried out using the SPSS software package (SPSS version 21, Inc., Chicago, IL, USA).

## 6.5 Results

### 6.5.1 Growth

The use of three different commercial enrichment products for *Artemia* did not affect significantly daily growth of seahorses. Until the 8th DAB the growth of larvae was very similar, but a loss of weight was observed in treatments T1 and T2. From the 8th DAB until the 15th DAB better values of growth were observed in T3, and from the 15th to the 20th DAB in T1. In this latter treatment, the significantly higher growth ($p \leq 0.05$) was evidenced by better values of absolute growth rate, specific growth rate, and gained weight during such period (Table 6.1).

**Table 6.1** Growth of *H. reidi* (dry weight) during the development

| | GROWTH (mg±SD) | | | AGR (mg/day) | | | SGR (mg/day) | | | DWI (%) | | | WG (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **DAB** | **P** | **M** | **D** | **P** | **M** | **D** | **P** | **M** | **D** | **P** | **M** | **D** | **P** | **M** | **D** |
| **0** | 0,29±0,07 | 0,29±0,07 | 0,29±0,07 | - | - | - | - | - | - | - | - | - | - | - | - |
| **0–2** | 0,23±0,05 | 0,24±0,05 | 0,35±0.08 | - | - | 0,03 | - | - | 0,07 | - | - | 63,63 | - | - | 0,01 |
| **2–8** | 0,61±0,16 | 0,62±0,17 | 0,73±0,16 | 0,06 | 0,06 | 0,06 | 0,16 | 0,15 | 0,13 | 68,71 | 68,43 | 62,58 | 0,02 | 0,01 | 0,01 |
| **8–15** | 0,99±0,19 | 1,14±0,45 | 0,85±0,15 | 0,05 | 0,07 | 0,14 | 0,07 | 0,09 | 0,12 | 38,82 | 39,52 | 32,67 | 0,01 | 0,01 | 0,01 |
| **15–20** | 2,61±1,23 | 1,54±0,52 | 1,69±0,69 | **0,33** | 0,08 | 0,08 | **0,20** | 0,06 | 0,04 | **48,14** | 27,46 | 19,17 | **0,02** | 0,01 | 0,01 |

AGR, Absolute Growth Rate; D, Easy Selco DHA; DAB, Day After Birth; DWI, Daily Weight Increase; M, Biomar Multigain; P, Protein Selco; SD, Standard Deviation; SGR, Specific Growth Rate; TL, total length; W, weight; WG, Weight Gain. Bold numbers indicate that the difference was statistical significant ($p \leq 0.05$).

### 6.5.2 Proximate composition

The contents in total protein and lipids of *Artemia*, freshly hatched and enriched, as well as of seahorses at birth is summarized in Table 6.2.

**Table 6.2** Percentage of total protein and lipid content (dry weight) of freshly hatched *Artemia*, and enriched *Artemia* from the three experimental treatments DHA Protein Selco (T1); Biomar Multigain (T2) and Easy Selco DHA (T3)

| | Seahorses DAB 0 | Hatched *Artemia* | DHA Protein Selco (T1) | Biomar Multigain (T2) | Easy Selco DHA (T3) |
|---|---|---|---|---|---|
| **Protein** | 75, 07±0,6 | 49,05$^{a}$±1,18 | 48,9$^{a}$±0,9 | 47,9$^{a}$±0,41 | 43,1$^{b}$±0,04 |
| **Lipids** | 18,41±0,5 | 13,54$^{c}$ ±0,07 | 15,3$^{b}$ ±0,27 | 13,6$^{c}$ ±0,03 | 21,7$^{a}$±0,38 |

Biochemical analysis reflected how the addition of the different products significantly modified the proximate composition of the enriched *Artemia*. The results highlight a significant ($p \leq 0.05$) decrease in the protein content when *Artemia* was enriched with Easy Selco DHA. The values of lipid content in *Artemia* freshly hatched and treated with Biomar Multigain were significantly lower ($p \leq 0.05$) than those in *Artemia* enriched with Protein Selco or Easy Selco DHA. Those latter were also significantly different ($p \leq 0.05$) between them with a higher percentage in the T3 treatment.

### 6.5.3 Evaluation of enzyme activities

Trypsin activity showed a similar trend in all the treatments, with higher values especially during the initial days after the release (Figure 6.1a). The peak of activity between the first and second day was followed by a decrease until the 10th DAB ($p \geq 0.05$), and a further increase from the 10th DAB to the last day of the experiment.

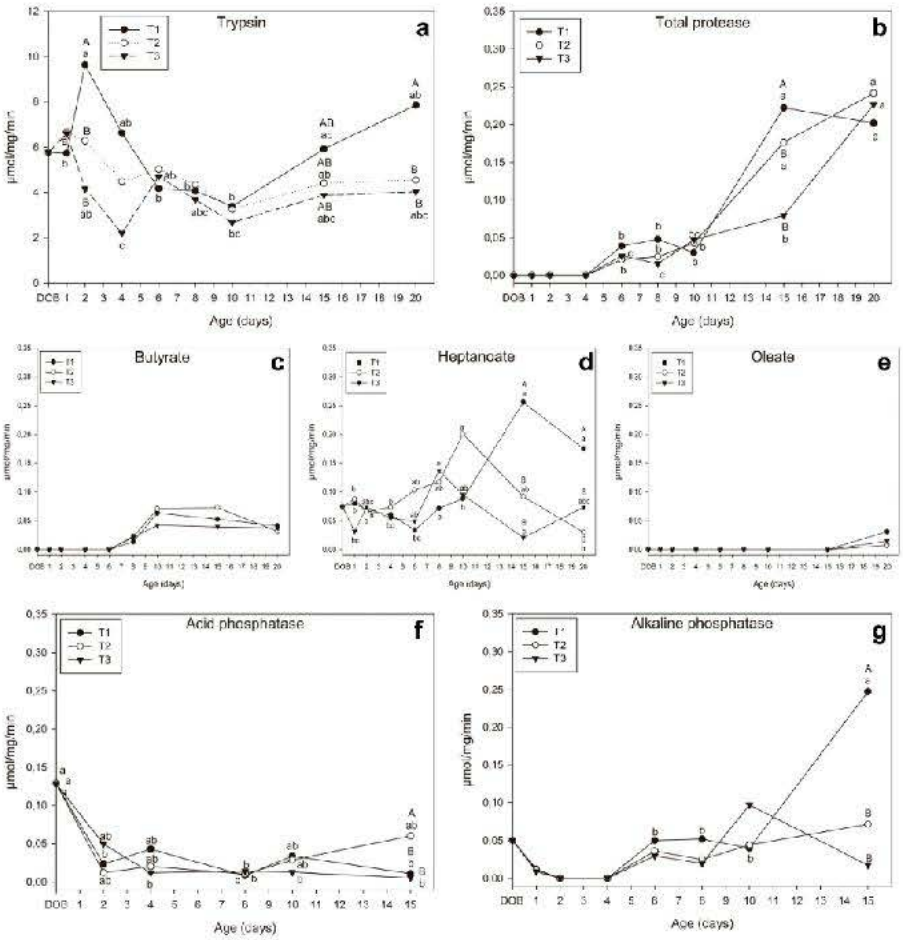


**Figure 6.1** Specific enzymatic activities (a. Trypsin; b. Total protease; c. Butyrate; d. Heptanoate; e. Oleate; f. Acid Phosphatase; g Alkaline phosphatase) during the development of long snouted seahorses (*H. reidi*) offspring fed on different enrichment products: DHA Protein Selco (T1); Biomar Multigain (T2) and Easy Selco DHA (T3).

Significant differences between values of trypsin activity measured at different ages were measured in seahorses in treatments T1 and T3, but not in T2. On the other hand, the higher values of trypsin activity were recorded in seahorses fed on the diet with a higher protein content

(T1), showing statistical differences to the other treatments ($p \leq 0.05$) at the 2nd DAB and at the 20th DAB.

The activity of total protease was detected for the first time at the 6th DAB and increased with age, showing much lower values than those of trypsin (Figure 6.1b). This activity presented the same trend in all the experimental groups, being characterized by an increase of values with age that was statistically significant by 15th DAB in T1 and T2, and by the 20th DAB in all the treatments. A significantly lower activity in seahorses fed on T3 was observed at the 15th DAB.

These results were confirmed by the protease zymogram (Figure 6.2).

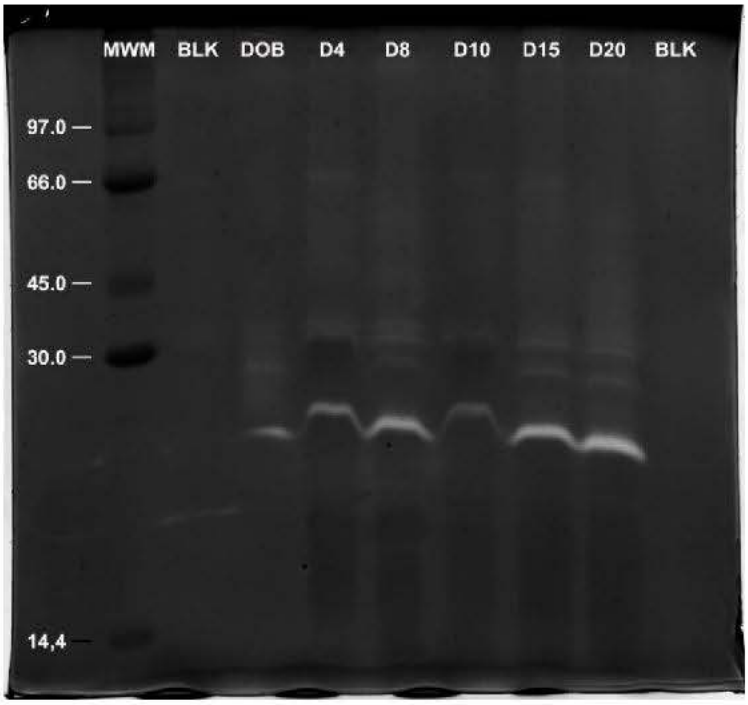


**Figure 6.2** Substrate-SDS-PAGE of extracts obtained from long snouted seahorses (*H. reidi*) at different days after releasing. BLK, blank; MWM, molecular weight marker.

During the development, bands with caseinolytic activity followed the same pattern but became more intense as age of larvae progressed. The bands showing b30 kDa were classified as trypsin. Esterase activity towards any of the three substrates evaluated increased with age in all the experimental treatments. The activity was detectable just in newborns when the medium chain heptanoate was used as substrate (Figure 6.1d), but it was measured later, at 6th DAB and 20th DAB, when using butyrate (Figure 6.1c) and oleate (Figure 6.1e), respectively.

The esterase activity towards heptanoate showed statistical difference ($p \leq 0.05$) between ages, (starting from the 10th DAB until the end of the experiment). The activity towards butyrate and oleate was significantly lower when compared to that obtained with heptanoate and also significantly different between treatments. The ratio protease:esterase, calculated using the activity of trypsin and esterase towards heptanoate presented a similar profile in the three experimental treatments characterized by high values at early ages followed by a decrease until the second week, and a further increase (Figure 6.3). This protease:esterase ratio showed a different pattern depending on the dietary treatment: the continuous variation until the 10th DAB in T2 determined no statistical differences between values, but these were observed at the 20th DAB in T1 and in days 1st and 15th DAB in T3. Also, significant differences between treatments were observe at the 2nd and 15th DAB.

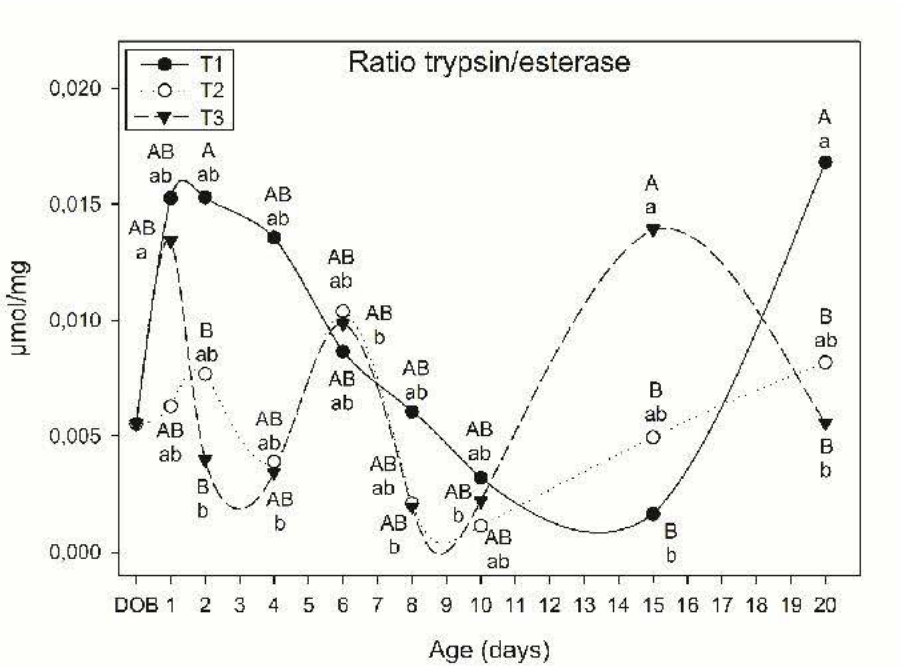


**Figure 6.3** Specific activity of acid and alkaline phosphatases during the development of long snouted seahorses (*H. reidi*) along the three experimental treatments DHA Protein Selco (T1); Biomar Multigain (T2) and Easy Selco DHA (T3).

Higher values of acid phosphatase activity were measured in samples from all treatments at birth ($p \leq 0.05$), this being followed by a significant decrease maintained until the 8th DAB. After this moment an increase was recorded in T1 and T2 by 10th DAB that was maintained until the last day of the study only in T2. Statistical difference ($p \leq 0.05$) between values in this treatment and the rest was registered by 15th DAB (Figure 6.1f).

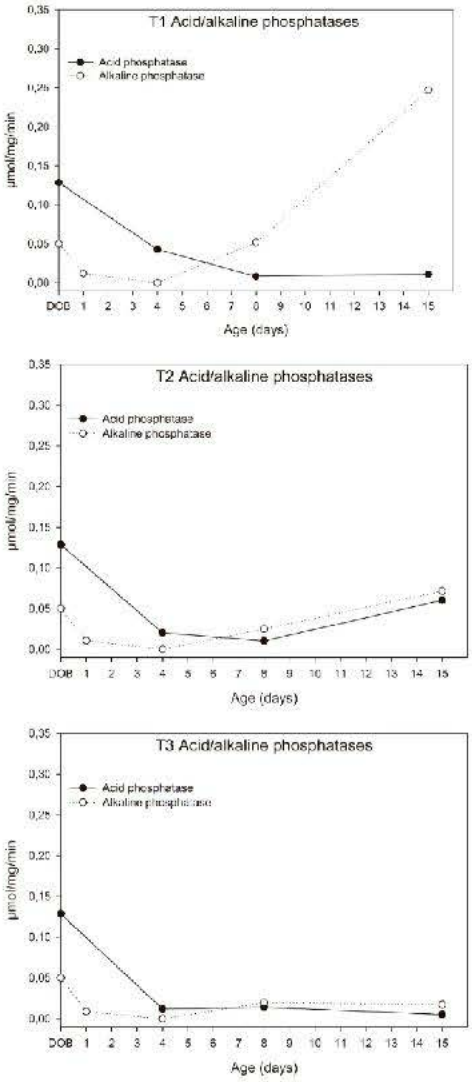


**Figure 6.4** Specific activity of acid and alkaline phosphatases during the development of long snouted seahorses (*H. reidi*) along the three experimental treatments DHA Protein Selco (T1);

Low values of alkaline phosphatase were measured at birth in samples from the three treatments, followed by a decrease and a practical disappearance by 4/5th DAB (Figure 6.4). Nevertheless, after this date, the activity of alkaline phosphatase increased progressively with age in samples from T1, this resulting in statistical difference ($p \geq 0.05$) registered by 15th DAB when compared to values measured in samples from T2 and T3 (Fig. 6.1g). In the three treatments the activities of acid and alkaline phosphatases during larval development showed a clear pattern characterized by the presence of acid phosphatases and absence of alkaline phosphatase in the early days, followed by a progressive increase of the latter with age (Fig. 6.4).

This evolution showed in all cases a critical point characterized by lower values of both activities around the 4–6 days of age. The difference between the treatments was found at the moment on which the activity of alkaline phosphatase reached and exceeded that of acid phosphatase (day 6 in T1, 6–7 in T2, and 7 in T3). In addition, significantly higher levels of activity were measured for this enzyme by the end of the experimental period in T1.

The use of a cluster multivariate analysis to combine results obtained with all the enzyme activities offered a better picture on the similarities and differences between treatments at different moments (Fig. 6.5).

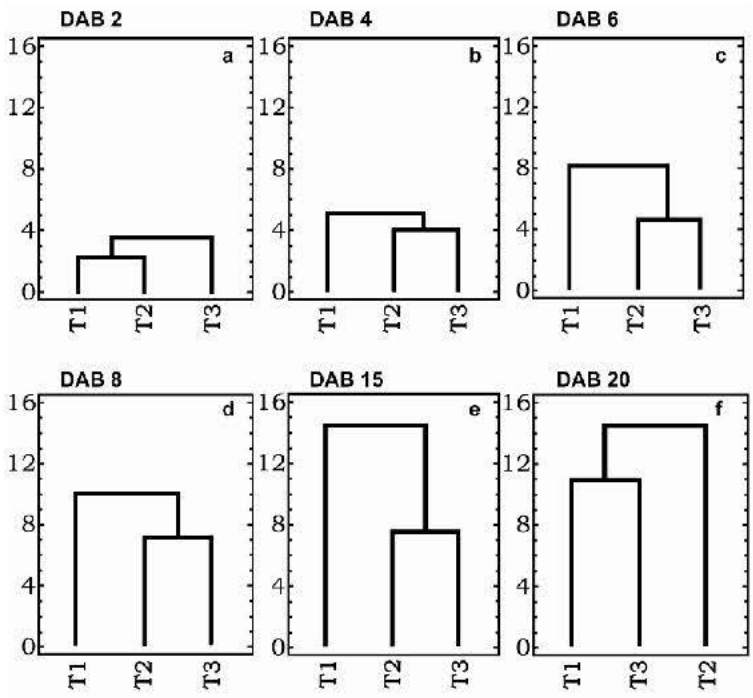


**Figure 6.5** Relationships among 3 treatment groups during larval development of long snouted seahorses (*H. reidi*) based on the similarity of their enzymatic activities performance (7 variables) derived from hierarchical cluster analysis.

## 6.6 Discussion

The present paper studies in detail the activities of digestive enzymes during the early stages of development of long-snouted seahorses (*H. reidi*) receiving different types of live food and correlates them to their nutritional status. Larval rearing remains as a critical bottleneck in the production of marine ornamental fish (Olivotto *et al.* 2016).

In this period, final survival is not only dependent on physical factors (types of aquaria, photoperiod, temperature, aeration) as demonstrated in other species of seahorses (Blanco *et al.* 2014; Planas *et al.* 2012), but also on the selection of a suitable diet. This latter determines the difference between the success and the failure in larval rearing, this being supported by results of growth and survival of newborn seahorses when they are fed on adequate live preys in terms of nutritional quality and digestibility (Blanco *et al.* 2015; Hora and Joyeux 2009; Zhang *et al.* 2015a).

As occurs with other marine fish, important anatomical and physiological changes take place during the larval development of seahorses, although take a comparatively short time; *H. abdominalis* presents a completely functional digestive tract 21 DAB (Wardley 2006) while *H. reidi* could be considered juvenile after 18/20 days (Novelli *et al.* 2015). The activities of enzymes detected few hours after the release of *H. reidi* larvae could be related to the activation of pancreatic zymogen granules observed in their early days (Novelli *et al.* 2015), this being in line with results observed in other marine fish (Cara *et al.* 2003) as well as in other species of seahorses (Blanco *et al.* 2015; Wardley 2006). The high activity of trypsin and low of esterase

observed in the present study during the initial days may suggest that newborns of long-snouted seahorse rely to a great extent on proteins (Oozeki and Bailey 1995). The increase in activity of non-specific esterases with age was correlated to the development of gut epithelium, also evidenced by the increase in the number and size of microvilli (Cara *et al.* 2003) or the intestinal loops (Novelli *et al.* 2015; Wardley 2006).

The variations in the activity of acid phosphatase (enzyme involved in protein pinocytosis and intracellular digestion) during the first two weeks should be related to the utilization of endogenous reserves provided by the yolk sac observed in this species during the early days (Novelli *et al.* 2015). Hence, the progressive decrease of this activity until the 4th/5th DAB should indicate depletion such reserves, suggesting that larvae had a short time span after their release from the male brood pouch to adapt to the marine environment. In this species, larvae suffer important changes in their digestive physiology, passing from endogenous to exogenous alimentation during this phase (Novelli *et al.* 2015). This should explain the loss of weight observed in two of the treatments until the 2nd DAB. While acid phosphatase is linked with the supranuclear inclusion bodies and pinocytic absorption followed by intracellular digestion (Govoni *et al.* 1986), the activity of alkaline phosphatase is related to transport mechanisms of the extracellular digestion and hence, to the emergence of a fully functional intestinal epithelium (Toledo-Cuevas *et al.* 2011; Zambonino Infante and Cahu 2001).

In the present work enrichment of *A. salina* with different commercial products resulted in no significant differences in growth and survival

between the treatments, this being in line with results obtained in other studies (García-Manchón *et al.* 2013; Wardley 2006). Significant differences in absolute growth rate, specific growth rate, and weight gain were only detected in treatment T1 during the last period of the experiment, from the 15th to the 20th DAB and were also reflected in in the patterns of activity measured in several enzymes like trypsin, esterase and alkaline phosphatase.

Different parameters measuring welfare, health or physical strength of larvae in marine species that are used to provide information about their potential for growth and survival are known as larval condition indicators (LCI) (Pope and Kruse 2001). Within them, the activities of digestive enzymes have been extensively used and in the present work three different indicators based in the digestive biochemistry have been applied to assess the nutritional status of larvae:

– The changes in the time pattern of both acid and alkaline phosphatases, related to a proper development of the intestine, (Govoni *et al.* 1986; Moyano *et al.* 1996).

– The protease/esterase ratio, connected to changes in the main orientation of metabolism (Cara *et al.* 2003).

– The use of a multivariate analysis (cluster),which has been used to evaluate other aspects in fish larvae like behavioural variables or morphological characters (Fuiman *et al.* 2005; Wittenrich and Turingan 2011).

In this case, variations in all the enzyme activities were combined into a juvenile. In the present study the proportion between trypsin and

esterase decreased with age until 10 days of age and offered a different response from this moment onwards, this illustrating differences in the relative use of either protein and lipids as substrates in each treatment.

This changing trend for esterases and protases was also observed in other species of seahorses (Wardley 2006). The high relative activity of protease in relation to esterase found in *H. reidi* may be induced by the composition of *Artemia*, which presents a low content or a reduced bioavailability of the HUFA incorporated through the enrichment.

In fact, a recent study observing the higher content of essential fatty acid in copepods respect to the enriched *Artemia*, suggests that HUFA content should be improved with alternative methods of enriching *Artemia* nauplii to feed seahorses (Zhang *et al.* 2015a).

Multivariate analysis has been applied in different types of studies with fish larvae to evaluate behavioural aspects (Fuiman *et al.* 2005) or morphological characters (Wittenrich and Turingan 2011). In the present study it was used to combine the information of all the enzyme activities to detect similarities between individuals in each dietary treatment at different moments of development. During early days (3rd/4$^{th}$ DAB) the cluster analysis showed a great similarity between treatments that may be explained considering the main use of the nutritional reserves remaining in the yolk sac. This is in contrast to the marked differences in terms of survival and ingestion rate after 3 days described in newborns of *H. reidi* and other species of seahorses when fed with different quality of live preys (Murugan *et al.* 2009; Olivotto *et al.* 2008; Otero-Ferrer *et al.* 2010).

Once the digestive system is the main supplier of dietary nutrients to the organism, the effect of exogenous alimentation became more

evident and the difference measured by the Euclidean distance between treatment T1 and the rest increased. This points to a different effect of such diet on the whole digestive process also reflected by the significant differences in AGR, SGR, DWI (%), and WG (%) recorded in this treatment from the 15th to the 20th DAB. The more critical stage for newborn seahorses is the first week after release, when they showed a pelagic behaviour before settling to the bottom (Choo and Liew, 2006; Otero-Ferrer *et al.* 2010). In general, larval mortality in seahorses decreased slightly until the end of the second or third week depending of species, (Martinez-Cardenas and Purser 2011; Otero-Ferrer *et al.* 2010; Palma *et al.* 2016; Payne and Rippingale 2000; Pham and Lin 2013). As a whole, global differences in the digestive biochemistry decreased with age, this being probably due to the increased functionality resulting from the development of the intestinal epithelium. This study highlights new aspects on larval digestion of *H. reidi*, suggesting new methods to evaluate the development of the digestive system during this stage and helping to optimize the rearing protocol from a nutritional perspective. Also confirms the poor digestion of enriched *Artemia* metanauplii hypothesized in others studies (Novelli *et al.* 2015; Olivotto *et al.* 2008; Payne and Rippingale 2000) and suggests this type of live prey should not be used to fed larvae of seahorses during early days, but replaced with a more digestible live prey like rotifers (*Brachionus spp.*) or copepods. Rotifers have offered good results both in *H. reidi* (Souza-Santos *et al.* 2013) and in *H. trimaculatus* (Murugan *et al.* 2009), while copepods resulted in better growth in larvae of *H. guttulatus* (Blanco *et al.* 2015).

## 6.7 Conclusions

The biochemical parameters evaluated were useful not only to establish the more suitable moment for changing the type of live prey during weaning, but also to adapt its biochemical composition to enhance digestibility. The activities of phosphatases, the ratio trypsin/esterase, and the combined evaluation of enzyme activities were consistent with the results of growth and may be considered as good indicators of nutritional status in this species.

# Discusión general

La biología reproductiva de los teleósteos presenta una gran diversidad, mucho mayor que otros grupos de vertebrados, y una de las formas más extremas se presenta entre los miembros de la familia *Syngnathidae* en la que se incluyen los caballitos de mar en los que los huevos se desarrollan en los machos mostrando un caso excepcional de embarazo masculino. De hecho, Brunelli (1922) explica como al principio, (Aristóteles, Plinio) se pensaba que el ejemplar que tenía la bolsa incubadora fuera la hembra, y el que carecía de ella, el macho. Esta particular forma de reproducción conlleva la eclosión dentro de la bolsa incubadora y tras un periodo de gestación, la expulsión de recién nacidos, que hasta la actualidad se consideraban juveniles por su alto grado de desarrollo.

Balon (1999) divide el desarrollo de los peces en periodos, o intervalos (huevo, embrionario, larvario, juvenil, adulto y senescente), definiendo el periodo larvario como el periodo en el que la larva pasa progresivamente desde la nutrición endógena a una nutrición exógena (y que generalmente dura hasta la formación/osificación del esqueleto axial), y el periodo juvenil como el periodo en el que comienza mostrarse un fenotipo definitivo en el que la mayoría de estructuras embrionarias y larvarias se degeneran y la mayoría de órganos o estructuras permanentes de adultos se han formado. Prácticamente el periodo larvario es aquel periodo que empieza con la eclosión, que en caballitos de mar ocurre dentro de la bolsa incubadora, y dura hasta que el ejemplar muestra el mismo fenotipo del adulto (sin la capacidad de reproducirse).

Una primera duda sobre el estadío de los recién nacidos, considerados como juveniles, procede de un estudio de morfología,

en el cual los autores (Choo y Liew 2006) afirman que estos animales cuando salen de la bolsa incubadora paterna están completamente metamorfoseados, pero al mismo tiempo describen, en *Hippocampus kuda*, la aparición progresiva de la coronilla, que está completamente formada solo después de 9 días de vida, y la desaparición de la aleta caudal presente solo en este primer periodo de vida. El desarrollo de estructuras ausentes al ser expulsados y los cambios morfológicos han sido descritos también en recién nacidos de *H. reidi* (Silveira 2000) y *Hippocampus spinosissimus* (Kang et al. 2005).

Lo que se ha observado en la presente Tesis Doctoral durante el estudio del desarrollo embrionario, es que *H. reidi* muestra un desarrollo indirecto peculiar, durante el cual después de la eclosión no aparece una aleta primordial, y la formación de las aletas ocurre antes de la reabsorción del saco vitelino, lo que no es común entre los teleósteos (Kendall et al., 1984). Las larvas de teleósteos generalmente pasan por dos estadíos principales, en la fase inicial poseen una aleta primordial continua que se origina dorsalmente, y se extiende alrededor del cuerpo hasta el borde posterior del saco vitelino (larva con aleta primordial), mientras que en la fase siguiente muestran aletas formadas (larva con aletas formadas). Estas características han sido descritas también por otros autores en otras especies de caballitos de mar (*H. guttulatus,* Brunelli 1922; *Hippocampus zosterae*, Wetzel and Worums 2004), mientras que, en peces pipa (pertenecientes a la misma familia) se producen larvas transparentes que poseen la aleta primordial (*Nerophis lumbriciformis*, *Nerophis ophidion* and *Entelurus aequoreus*, Russell 1976; Monteiro et al. 2003). Este contraste entre miembros de la

misma familia puede ser debido a las importantes diferencias entre caballitos de mar y peces pipa relativas a la propulsión natatoria. Mientras que los caballitos de mar mantienen una posición vertical y la propulsión se realiza a través de la aleta dorsal, los peces pipa, como la mayoría de los teleósteos, siguen usando la aleta caudal, y este profundo cambio evolutivo puede reflejarse en la pérdida de la aleta primordial en larvas de caballitos de mar.

Aunque los recién nacidos poseen aletas formadas al salir de la bolsa incubadora, y no se puede observar la típica forma redondeada del saco vitelino tanto en *H. reidi* como en otras especies de caballito de mar (Choo y Liew 2006), se describió un fenotipo muy distinto del fenotipo del adulto, con elementos morfológicos ausentes, que resultan distintivos para cada especie (como la coronilla), e imprescindibles para asumir el fenotipo del adulto (como los anillos y placas óseas).

Una vez asumido que podía haber diferencias morfológicas importantes entre adulto y recién nacidos, se estudiaron en detalle los primeros 30 días de vida del caballito de mar desde el punto de vista histológico y osteológico.

A pesar de la elevada capacidad de cazar presas vivas de los recién nacidos, éste es el periodo más delicado, y el verdadero cuello de botella en el cultivo masivo de caballitos de mar en el que se observan las mayores tasas de mortalidad.

El estudio histológico en *H. reidi* y el estudio osteológico en *H. hippocampus* demuestran que los recién nacidos de estas especies están inmersos en una metamorfosis que afecta tanto la morfología

exterior como la interior requiriendo cambios profundos para alcanzar el estadío juvenil.

Desde el punto de vista histológico, la estructura interna de los recién nacidos es todavía muy diferente a la estructura interna del adulto, observándose cambios morfológicos durante la organogénesis que se pueden asemejar con los cambios morfológicos que ocurren en larvas de edad avanzada de otras especies de peces marinos. Como se ha descrito en otros estudios de desarrollo larvario de otras especies de peces marinos, también en *H. reidi* se observan entre los resultados más significativos, reminiscencias de saco vitelino al salir de la bolsa incubadora, los cambios morfológicos de enterocitos y hepatocitos, el cambio de absorción desde la pinocitosis hasta la digestión extracelular, la formación de curvas intestinales, el aumento de la cantidad de papilas gustativas y folículos tiroideos, y la formación de los islotes de Langerhans (Sarasquete et al. 1995; Tanaka et al. 1995; Ribeiro et al. 1999; García Hernández et al. 2001; Santamaría et al. 2004; Hachero-Cruzado et al. 2009; Ortiz-Delgado et al. 2011; Papadakis et al. 2013). Este estudio demuestra que los caballitos de mar recién nacidos como otras larvas de peces marinos de edad avanzada también necesitan un periodo de desarrollo para transformarse en juveniles, durante el cual es necesario cubrir requerimientos específicos que le permitan completar la organogénesis.

Se ha descrito en la literatura como los caballitos de mar recién nacidos son depredadores visuales excelentes (Hilomen-García et al. 2003), y se ha confirmado con el estudio histológico el alto grado de desarrollo del sistema visual. Sin embargo, también en un sistema tan

desarrollado se han encontrado diferencias entre adultos y recién nacidos, que carecen de fóvea, estructura asociada a una visión aguda.

El incremento de la capacidad digestiva se refleja en el desarrollo del sistema digestivo descrito en la presente Tesis Doctoral. Inicialmente los caballitos de mar poseen un intestino recto, como observado en otras especies de caballitos de mar (Wardley 2006; Palma et al. 2014) que no es completamente funcional y que aumenta su capacidad de asimilación con la formación de curvas intestinales durante el desarrollo. El desarrollo del intestino observado es análogo al desarrollo del intestino de otras larvas de peces de teleósteos marinos de edad avanzada (Santamaría et al. 2004; Sanchez-Amaya et al. 2007). Efectivamente, cuando se alimenta a los recién nacidos con presas vivas de alta calidad como zooplancton o nauplios de copépodos silvestres (probablemente más fácilmente digeribles), se alcanzan tasas de mortalidad muchos más bajas (Job et al. 2006; Hora and Joyeux 2009; Payne and Rippingale 2000; Zhang et al. 2015). Sin embargo, suministrando presas vivas como rotífero y *Artemia* (y aún enriquecidas), comúnmente utilizadas en el cultivo larvario de las especies acuícolas, los caballitos de mar muestran una mortalidad muy elevada (Payne y Rippingale 2000; Olivotto et al. 2008; Otero-Ferrer et al. 2010; Willadino et al. 2012; Sales et al., 2016). Sin embargo, zooplancton o nauplios de copépodos silvestres no se utilizan en el cultivo a gran escala debido a su escasa disponibilidad de mercado y a su alto coste. Como en otras especies de larvas de teleósteos marinos, se tendría que considerar la alimentación de los caballitos de mar recién nacidos como "alimentación larvaria" y ampliar la investigación sobre sus requerimientos específicos.

Desde el punto de vista osteológico, se confirman las diferencias entre el fenotipo de los recién nacidos y el fenotipo de los adultos en *H. hippocampus*. Se realizó este estudio en otra especie de caballitos de mar, debido al hecho que en *H. reidi* el desarrollo osteológico ya había sido descrito (Silveira 2000). En el estudio de Silveira (2000) en *H. reidi*, al igual que en la descripción de Choo and Liew (2006) en *H. kuda*, se observan diferencias morfológicas importantes entre recién nacidos y adultos, no obstante, ninguno de estos autores propuso una fase de desarrollo larvario para las especies descritas.

Recién nacidos de *H. kuda* al nacer presentan anillos y placas óseas (Choo y Liew 2006), a diferencia de otras especies, en las que los estudios osteológicos describen ocho días para su osificación, como en *H. subelongatus* (Franz-Odendaal and Adriaens 2014) y en *H. zosterae* (Azzarello 1990). En la presente tesis, *para H. hippocampus* se ha observado que se necesitaban 20 días para la osificación completa, mientras que en *H. reidi* 25 días después de la expulsión de la bolsa incubadora no fueron suficientes para que estuvieran completamente osificados (Silveira 2000, Franz-Odendaal and Adriaens 2014). Esto puede indicar que cada especie, según su talla o según la temperatura ambiental (o ambos), sigue un modelo de desarrollo específico para formar estructuras óseas características del fenotipo de adulto y por lo tanto cada especie podía tener una fase larvaria de con duración distinta.

En recién nacidos de *H. hippocampus* se puede observar la falta de osificación del esqueleto axial cuando salen de la bolsa incubadora, como en *H. reidi* se puede observar le presencia de la notocorda. Como en las larvas de otros teleósteos, el desarrollo osteológico

empieza en la fase lavaria a partir de la notocorda y lleva a la formación de características morfológicas internas y externas típicas de los adultos, como la presencia de un sistema vertebral (Koumoundouros et al. 2001; Sfakianakis et al. 2004; Socorro 2006; Park et al. 2016).

En la presente tesis, no solo ha sido observada la ausencia de estructuras como la coronilla, las espinas, las placas óseas y los anillos óseos en los recién nacidos de *H. hippocampus*, sino también la presencia de una pequeña aleta caudal ausente en adultos, al igual que sucede con *H. kuda* (Choo and Liew 2006). La presencia de una aleta caudal, en la presente Tesis Doctoral, ha sido relacionada con el cambio de hábitat (planctónico a bentónico), y con la osificación progresiva de la cola que le permite adquirir unas de las habilidades más importantes para cambiar de hábitat, como su carácter prensil, que permite vivir a los adultos anclados y mimetizados al sustrato. Efectivamente la mayoría de los recién nacidos de *Hippocampus spp.* poseen una fase planctónica inicial desde los pocos días hasta las 8 semanas según la especie (*H abdominalis*, Woods 2000; *H. Fuscus, H. kuda, y H. barbouri,* Wilson and Vincent 2000; *H Subelongatus* y *H. Ingens*, Koldewey 2005; *H. comes* Job et al. 2006; *H. Erectus*, Alexandre 2009; *H. reidi*, Hora and Joyeux 2009; *H. trimaculatus*, Murugan et al. 2009; *H. hippocampus* Otero-Ferrer et al. 2010; *H. kuda*. Pawar et al., 2011; *H. guttulatus*, Blanco 2014). La capacidad prensil de la cola en *H. hippocampus* se adquiere al tiempo que sucede la osificación del esqueleto axial y la progresiva desaparición de la aleta caudal. La presencia de aleta caudal descrita en varias especies de caballitos de mar recién nacidos y que desaparece durante

el desarrollo (Choo and Liew 2006; Odendaal and Adriaens 2014), probablemente es una adaptación que permite mejorar la natación en la primera fase planctónica. En un estudio de Bishop et al. (2006) sobre la metamorfosis, 14 autores explican y describen este periodo de cambios, tomando en consideración entre varios conceptos, incluyendo también el cambio de hábitat presente entre larva y juvenil, que es uno de los factores distintivos en la metamorfosis de peces planos (McMenamin y Parichy 2013). Más del 70% de los autores considera este cambio como directamente relacionado con la presencia de metamorfosis en larvas de peces marinos. De acuerdo con este alto porcentaje de autores, en este trabajo se considera que el cambio de hábitat planctónico a bentónico esté también directamente relacionado con la existencia de metamorfosis en caballitos de mar. La existencia de metamorfosis durante el desarrollo excluye una fase juvenil inmediatamente después de la expulsión de la bolsa incubadora paterna de los recién nacidos en las especies estudiadas.

En el último estudio, se analizó la bioquímica digestiva de los primeros 20 días de vida de *H. reidi* y se observó un incremento progresivo de las actividades enzimáticas durante el desarrollo (menos que en la actividad de la fosfatasa ácida), como se ha descrito en otras larvas de peces marinos (Moyano et al. 1995; Cara et al. 2003) incluidas otras especies de caballitos de mar (Blanco et al. 2015). Este estudio enzimático demuestra el incremento de las capacidades digestivas durante el desarrollo, respaldando las observaciones histológicas en las que constataron que desde el día del nacimiento hasta las tres semanas de vida los caballitos de mar no tienen un sistema digestivo maduro a diferencia de lo que sucede en

el periodo juvenil. Se demuestra como en los primeros días después de la expulsión, los caballitos de mar pasan de procesos de pinocitosis y digestión intracelular a mecanismos de transporte relacionados con la digestión extracelular, con los consecuentes cambios en la actividad de la fosfatasa ácida que decrece y la actividad de la fosfatasa alcalina que se incrementa.

Los valores más bajos de fosfatasas alcalina y ácida, implicadas en los procesos de digestión "parietal" coinciden con el pico de mortalidad alrededor de los 4–7 días de vida observado en esta especie (Hora and Joyeux, 2009; Olivotto et al., 2008). Elevados porcentajes de mortalidad han sido descritos también en otras especies de caballitos de mar (6 días en H. *subelongatus*, Payne y Rippingale 2000; 3 días en *H. trimaculatus*, Murugan et al. 2009, 5 días en *H. hippocampus*, Otero-Ferrer et al. 2010). Esto quiere decir que mientras que el caballito de mar está consumiendo las últimas reservas vitelinas, si no se suministra un alimento adecuado para su sistema digestivo poco desarrollado durante esta fase, el animal no es capaz de digerirlo provocando una elevada mortalidad. Como en otras larvas de peces marinos la capacidad digestiva depende de la maquinaria enzimática con la que cuentan. El estudio de la ontogenia de las enzimas digestiva demuestra que los caballitos de mar no presentan un equipo enzimático completo como deberían si se tratara de juveniles y no tienen la capacidad de digerir eficientemente el alimento en el momento del nacimiento. Dicha capacidad aumenta progresivamente desde el nacimiento con el desarrollo del sistema digestivo, como se ha descrito en todas las larvas de peces marinos (Civera-Cercedo et al. 2004).

Todos los eventos descritos y observados en esta tesis durante el desarrollo de los recién nacidos fuera de la bolsa incubadora siempre han resultado comparables con los descritos para larvas de otras especies de peces marinos de edad avanzada. La presente tesis tras el seguimiento detallado de las primeras fases de desarrollo en caballitos de mar confirma la hipótesis de la existencia de una fase larvaria y demuestra su presencia en las especies *H. hippocampus* e *H. reidi*.

Se propone ampliar esta hipótesis a las demás especies de caballitos de mar abriendo las puertas al estudio de la nutrición larvaria en caballitos de mar recién nacidos, considerando sus requerimientos nutricionales como requerimientos nutricionales larvarios. Se propone revisar el termino juvenil actualmente usado para definir los recién nacidos de caballitos de mar, sustituyéndolo por el termino de larva, que, como ha sido demostrado en los distintos estudios llevados a cabo en esta Tesis Doctoral, es el término que corresponde.

# Conclusiones

# Conclusiones

1- Los huevos fecundados de *H. reidi* fuera de la bolsa incubadora, en agua de mar, muestran un progresivo incremento de volumen hasta su ruptura, por el contrario, los huevos no fecundados resultan menos permeables y, en consecuencia, más estables.

2- *H. reidi* después de la eclosión, que ocurre dentro de la bolsa incubadora, muestra un desarrollo indirecto peculiar al carecer de aleta primordial y desarrollar las aletas pectorales, dorsal anal y caudal antes de la reabsorción del saco vitelino.

3- Los recién nacidos de *H. reidi* carecen de las estructuras morfológicas que confieren la característica forma del adulto como son coronilla, espinas, placas y anillos óseos. Además, carecen de la capacidad prensil de la cola, que muestra una pequeña aleta caudal, que no presentan los adultos.

4- Tras la salida de la bosa incubadora paterna *H. reidi* presenta un alto grado de desarrollo en los sistemas locomotor, visual, inmunológico, excretorio y sensorial, mientras que el sistema digestivo sufre importantes modificaciones que mejoran progresivamente su funcionalidad durante los treinta primeros días de vida libre.

5- Los recién nacidos de *H. reidi* poseen notocorda y restos de saco vitelino el primer día de vida libre, con un intestino posterior capaz de asimilar glúcidos mediante una digestión por pinocitosis. La asimilación lipídica en el intestino medio no tiene lugar hasta los cuatro días de vida libre

6- A la salida de la bolsa incubadora *H reidi* muestra una funcionalidad digestiva incompleta, con una actividad enzimática mayor de tripsina y lipasa frente a heptanoato, que se incrementa durante los primeros 20 días de vida libre en relación al desarrollo del intestino.

7- Una fase crítica durante el desarrollo de *H. reidi* transcurre entre los cuatro a seis días de vida, periodo en el que las actividades de fosfatasas ácida y alcalina son muy bajas reflejando el paso desde una digestión parcialmente endógena, con la reabsorción de los últimos restos del saco vitelino, a una digestión totalmente exógena.

8- En *H. reidi* el tubo digestivo durante los 30 días de estudio aumenta progresivamente, como la capacidad de asimilación de nutrientes. Entre el día 11 y el día 13, los caballitos de mar sufren la primera torsión del intestino medio, que se repite posteriormente, con un máximo de tres durante el primer mes de vida.

9- En *H. reidi*, se destacan algunas peculiaridades como la ausencia de glomérulos en el riñón y la precocidad en el desarrollo del aparato reproductor femenino que aparece a los 10 días de vida, y alcanza el tercer estadío previtelogénico a los 30 días.

10- A la salida de la bolsa incubadora *H. hippocampus* carece totalmente de columna vertebral, que muestra una osificación progresiva durante todo el periodo de estudio.

11- A la salida de la bolsa incubadora *H. hippocampus* posee solo algunas estructuras cartilaginosas, y la osificación es un proceso que evoluciona durante todos los 30 días con el desarrollo de estructuras que confieren la característica forma del adulto como coronilla, espinas, placas y anillos óseos.

12- A la salida de la bolsa incubadora *H. hippocampus* posee una aleta caudal que desaparece progresivamente durante el proceso de osificación. Los primeros 30 días de vida fuera de la bolsa incubadora paterna no son suficientes para la desaparición completa de la estructura.

13- El carácter prensil de la cola en *H. hippocampus* es un proceso que se adquiere paulatinamente en relación a su osificación permitiendo el cambio de hábitat, de planctónico a bentónico, durante los primeros 15-18 días.

14- En *H. reidi* las actividades de las fosfatasas acida y alcalina, la ratio de tripsina/esterasa y la evaluación combinada de actividades enzimáticas fueron consistentes con los resultados de crecimiento y pueden ser considerados como buenos indicadores del estado nutricional de los individuos.

15- *H. hippocampus* e *H. reidi* presentan desarrollo larvario que se pone de manifiesto por las profundas diferencias entre los recién nacidos y los adultos. La particular estrategia de reproducción en ambas especies divide el periodo larvario (de embrión a juvenil) en dos fases, la primera ocurre dentro de la bolsa incubadora mientras que la segunda fase ocurre fuera de ella. El proceso de metamorfosis que se ha observado indica que los recién nacidos de ambas especies tienen que ser considerados larvas e incluir, por lo tanto, este estadío de desarrollo en el ciclo de vida de *H. reidi* y *H. hippocampus*.

# Conclusions

# Conclusions

1- Fertilised eggs of *H. reidi* out of the male brood pouch, in sea water, showed a progressive swelling until the rupture, contrary to the non-fertilized eggs resulting less permeable and therefore more stable.

2- *H. reidi* after hatching, inside the male brood pouch, displays a peculiar indirect development during this phase lacks primordial fin and develops the dorsal, anal, pectorals, and caudal fins before the reabsorption of the yolk sac.

3- Newborns of *H. reidi* lack of morphological structures confer the characteristic adult shape as the coronet, spines, bony plates and bony rings. In addition, lack of tail prehensile capacity, that shows a small caudal fin absent in adults.

4- After the release, *H reidi* shows a high degree of development in the locomotor, visual, immune, excreting and sensory systems, while the digestive system suffers important changes during thirty days of free life, promoting the functionality in relation to the development.

5- Newborns of *H. reidi* show notocorda, reminiscence of yolk sac, and the hindgut able to assimilate nutrients through pynocitic digestion., during the first day of life. The lipids assimilation in the midgut does not take place until the 4 days of life.

6- At birth, *H reidi* shows an incomplete digestive tool, with a higher enzymatic activity of trypsin and lipase against heptanoate, which increases during the first 20 days of free life in relation to the development of the intestine

7- Around the 4th-6th day after birth there is critical point during the development of *H. reidi* due to the very low activities of acid and alkaline phosphatases, which reflect the step from a partially

endogenous digestion with the yolk sac reabsorption to a complete exogenous digestion.

8- In *H. reidi* the elongation of the digestive tube increase progressively as well as the capacity to assimilate the nutrients. Between the 11th and the 13th day after birth seahorses suffer the first torsion of the anterior intestine. Subsequently a maximum of three loops, until the 30th days after birth, were present.

9- In *H. reidi*, some peculiarities are observed as the absence of glomeruli in the kidney, or the precocity to develops female reproductive system that appears at the 10th day after birth and reaching the third previtellogenic stage to 30th day after birth.

10- When released from the male brood pouch, *H. hippocampus* lacks totally of vertebral system, which shows a progressive ossification throughout the study period.

11- When released from the male brood pouch, *H. hippocampus* shows some cartilaginous structures, and the ossification evolves progressively during the development, forming structures that confer the characteristic adult shape, as the coronet, spines, bony plates, and bony rings.

12- When released from the male brood pouch *H. hippocampus* shows the caudal fin, that disappears progressively during the ossification process. The first 30 days of life out of the male brood pouch are not sufficient for the complete disappearance of the structure.

13- The prehensility in *H. hippocampus*, is a progressive process acquired in relation to the ossification of the tail, that make it possible the habitat change, from planktonic to benthic, during the first 15-18 days.

14- In *H. reidi*, the activities of acid and alkaline phosphatases, the ratio trypsin/esterase, and the combined evaluation of enzyme

activities were consistent with the results of growth and may be considered as good indicators of nutritional status in this species.

15- *H. hippocampus* and *H. reidi* presented larval development evidenced by the marked differences between newborns and adults. The peculiar reproduction strategy in both species divides the larval period (from embryo to juvenile) in two phases, the first happens inside the male brood pouch, while the second phase occurs outside. The process of metamorphosis indicates that newborn seahorses of both species would be considered as larvae, and this new stage of development in the life cycle of *H. reidi* and *H. hippocampus* would be included.

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