UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
Instituto Universitario de Sanidad Animal
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Instituto Canario de Ciencias Marinas · ICCM ·

Grupo de Investigación en Acuicultura

Seahorses in Gran Canaria Island (Spain): Ecology and Aquaculture – Combined Tools for Marine Conservation Issues
Francisco Otero-Ferrer - PhD Thesis 2011

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Anexo I

Dª MARÍA SORAYA DÉNIZ SUÁREZ, SECRETARIA DEL INSTITUTO UNIVERSITARIO DE SANIDAD ANIMAL Y SEGURIDAD ALIMENTARIA DE LA UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA.

CERTIFICA

Que el Consejo de Doctores del Departamento en su sesión de fecha 9 de enero de 2012 tomó el acuerdo de dar el consentimiento para su tramitación, a la tesis doctoral europea titulada: **"Seahorses in Gran Canaria islands (Spain): Ecology and Aquaculture-Combined Tools for Marine Conservation Issues" ("Los caballitos de mar en Gran Canaria (España): La ecología y la Acuicultura como herramientas complementarias para la Conservación Marina"),** presentada por el doctorando Francisco Otero Ferrer, dirigida por la Dra. Lucía Molina y el Dr. Rogelio Herrera.

Y para que así conste, y a efectos de lo previsto en el Artº 73.2 del reglamento de Estudios de Doctorado de esta Universidad, firmo la presente en Las Palmas de Gran Canaria, a diez de enero de dos mil doce.

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Anexo II

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

Departamento: Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Programa de Doctorado: Acuicultura: producción controlada de animales acuáticos

Título de la Tesis

"Seahorse´s populations in Gran Canaria Island (Spain): Ecology and aquaculture - Combined Tools for Marine Conservation issues" ("Los caballitos de mar en Gran Canaria (España): La ecología y la Acuicultura como herramientas complementarias para la Conservación Marina").

Tesis Doctoral presentada por **D. Francisco José Otero Ferrer**

Dirigida por **la Dra. Dña. Lucía Molina y por el Dr. D. Rogelio Herrera**

El Director, **El Director,** **El Doctorando,**

Las Palmas de Gran Canaria, a 28 de Febrero de 2012

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA

IUSA Instituto Universitario de Sanidad Animal y Seguridad Alimentaria

Dña. Lucía Molina Domínguez, Doctora en Biología, y D. Rogelio Herrera Pérez, Doctor en Ciencias del Mar

CERTIFICAN

que la Tesis Doctoral titulada "*Seahorses in Canary Islands (Spain): Ecology and Aquaculture – Combined Tools for Marine Conservation Issues*" presentada por el licenciado Don Francisco José Otero Ferrer ha sido realizada bajo nuestra dirección, reúne las condiciones de calidad y rigor científico para poder ser presentada y defendida ante el tribunal nombrado al efecto para optar al grado de Doctor.

Y para que conste a los efectos oportunos firmamos la presente, en Las Palmas a 18 de febrero de 2012.

Fdo. Dra. Lucía Molina Domínguez

Fdo. Dr. Rogelio Herrera Pérez

universidade de aveiro dbio departamento de biologia

Aveiro, Portugal, 22nd of February 2012

To whom it may concern,

Report on the thesis entitled "Seahorse's Populations in Gran Canaria Island (Spain): Ecology and Aquaculture – Combined Tools for Marine Conservation Issues" presented by Mr. Francisco Otero-Ferrer to achieve the degree of Doctor of Philosophy (PhD) by the University of Las Palmas de Gran Canaria, Spain.

This is a well-structured thesis, with the first chapter providing an in depth and up to date review of seahorse's population biology studies, as well as breakthroughs and current challenges of seahorse aquaculture. The gap of knowledge on the taxonomical and ecological status of seahorse populations in the Canary Islands (at least prior to the present study) is well evidenced by the author, as well as the need to clarify the spatial and temporal distribution of these animals, in order to implement a suitable monitoring and management plan. The importance of establishing standardized culture protocols for the captive production of these organisms, with emphasis on live prey production and quality, is also highlighted by the author. The approach of combining ecological data and experimental results from captive culture trials as a way to predict the outcome of future restocking efforts being is well-thought and highly relevant for modern bio-remediation efforts.

The detailed description of all methodologies employed in *in situ* and *ex situ* studies, from the life-support system to stock and culture seahorses, to tagging methods, genetic studies, morphometrics and biochemical, osteological and statistical analysis make the second chapter of this thesis an excellent handbook for all those interested in studying seahorse using any of this techniques.

Chapters three to seven report original research work that is either submitted, in press or already published in peer-reviewed journals. A rage of very interesting findings are presented, from the identification of spatial and seasonal shifts in the distribution of *Hippocampus hippocampus* in Gran Canaria, to the potential hybridization of *Hippocampus* species (e.g. *H. hippocampus* and *H. algiricus*) due to habitat tropicalization promoted by global climate changes; the relevance of providing suitable live feeds for the husbandry and culture of *H. hippocampus* is highlighted and the possibility of modulating settlement of cultured seahorses by manipulating their culture conditions opens a new path for innovative experiments on stock enhancement.

In chapter eight, the relevance of the major findings of this thesis is summarized under the scope of sustainable management and conservation. The author highlights once again the need to merge ecological and aquaculture research, an approach which I also advocate, in order to advance our knowledge on endangered marine species biology. These endangered may soon need to be restocked if we aim to prevent their permanent loss from the wild.

I must refer that this is certainly the most graphically appealing thesis I have ever evaluated and unquestionably “the looks” are matched by the scientific content. Overall the thesis is

of a very high standard, which is reflected by part of the work presented already being published in well renowned international peer-reviewed journals.

I have no hesitation in stating that this thesis fulfils all the scientific standards required for a researcher to achieve the academic degree of Doctor of Philosophy (PhD) and that the thesis entitled "Seahorse's Populations in Gran Canaria Island (Spain): Ecology and Aquaculture – Combined Tools for Marine Conservation Issues", presented by Mr. Francisco Otero-Ferrer, should be approved by the University of Las Palmas de Gran Canaria.

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Università Politecnica delle Marche
Dipartimento Scienze della Vita e dell'Ambiente

Ancona, 27.02.2012

TO WHOM IT MAY CONCERN

I had the pleasure to read the thesis entitled "Seahorses in Gran Canaria Islans (Spain): Ecology and aquaculture-Combined tools for marine conservation issues" by Francisco Otero Ferrer.

The overall objective of the studies was to develop insights on the local populations of seahorses as well as to develop culture techniques for restocking purposes.
This work presents a well written and complete introduction that addresses very well the problematic about these fishes. The literature cites is appropriate and complete. Moreover, the materials and methods are appropriate and include both standard and modern/innovative techniques such as mitochondrial and PCR analysis. Statistics are appropriate for the different case studies and the results herein obtained are very promising and well supported by a deep and well organized discussion.
It is clear that the diet plays a key role in both reproduction and larval rearing of the selected species.
It would have been interesting testing alternative diets such as copepods during the experiment and evaluate their effects through the different techniques used, especially on larval survival and growth.

In summary the work is original and detailed, and I have no hesitation in considering it worthy of defence and approval by the University authorities.

Please fell free to contact me for further queries.

Regards,
Dr Ike OLIVOTTO

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Thesis for the degree of *Doctor of Philosophy*
University of Las Palmas de Gran Canaria
2011

Directors:
Ph.D. Lucía Molina and Ph.D. Rogelio Herrera

« ***Réussir sa vie, c'est réaliser à l'âge adulte ses rêves d'enfant*** »

- André Lefrançois

Été 2009. Henryville - Québec

CONTENTS

ABBREVIATIONS

A *Artemia* treatment
AF Anal fin
AH Artificial holdfast
ANCOVA Analysis of co-variance
ANOVA Analysis of variance
AOAC Association of Official Analytical Chemists
AR Hour after release
ARA Arachidonic acid (20:4n-6)
AS Artificial support
ASR Accumulative survival rate
Au Autumn
BI Bayesian inference
Br Brownish
BWd Body weight per day
CE Coracoid bone
CF Condition factor
CH Coronet height
CITES Convention on International Trade in Endangered Species of Wild Fauna and Flora
CNs *Cymodocea nodosa* seagrass
CPUE Capture per unit effort
CSIC Consejo Superior de Investigaciones Científicas
Cyt Cytochrome
DAB Day after birth
DD Data deficient
DF Dorsal fin
DHA Docosohexanoic acid (22:6n-3)
DL Dorsal fin length
DNA Deoxyribonucleic acid
EFA Essential fatty acids
EPA Eicosopentanoic acid (20:5n-3)
FA Fatty acid
FAO Food and Agriculture Organization
FID Frame ionization detector
GIA Grupo de Investigación en Acuicultura
GLC Gas liquid chromatography
GMAD Global marine aquarium database
H High level (density factor)
HD Head depth
HL Head length
HT Height
HUFA Highly unsaturated fatty acid
ICCM Instituto Canario de Ciencias Marinas
IUCN International Union for Conservation of Nature
IUSA Instituto Universitario de Sanidad Animal
K Condition factor
K Number of clusters
L Low level (density factor)
LSS Life-support system
M Mortality
MCMC Marko chain Monte Carlo
MNHN Museum National d'Histoire Naturelle
mtDNA maternal inherited DNA
NE North-East
NH Natural holdfast
NJ Neighbour-joining
NW North-West
OA Oleic acid
OD Orbital diameter
PCoA Principal coordinate analysis
PF Pectoral fin
PL Pectoral fin length
PO Post-orbital length
ppm part per million
PCR Polymerase chain reaction
PVC Polyvinyl chloride
Pw Pair-wise test
Q Ancestry of each individual
R Rays
RA Rotifer-*Artemia* treatment
RAD Distal radial rays

RAM Medial radial rays
RAP Proximal radial rays
RB Rocky bottoms
rDNA Ribosomal DNA
Re Reddish
SCUBA Self contained underwater breathing apparatus
SD Standard deviation
SGR Specific growth rate
SL Standard length
SnD Snout depth
Su Summer
Sp Spring
SPSS Statistical package for the social sciences
TCM Traditional Chinese medicine
TM Traditional medicine
TaR Number of tail rings
TD4 trunk depth between the 4^{th} and 5^{th} rings
TD9 trunk depth between the 9^{th} and 10^{th} rings
TL Tail length
TrL Trunk length
TrR Number of trunk rings
UNEP United nations environment programme
UV Ultra violet
UVC Underwater visual census
VFC Visual fast count
VIE Visible implant elastomer
VIFE Visible implant fluorescent elastomer
W Winter
WCMC World Conservation Monitoring Center
Wf Final weight
WG Weight gain
Wi Initial weight
Ww Wet weight
Y Yellowish

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Con la finalización de esta tesis doctoral, una etapa de mi vida se cierra. Hace ya unos años, cuando desembarque en Las Palmas de Gran Canaria (España), me sentía una persona afortunada, el destino me daba la oportunidad de invertir los próximos años de vida en trabajar en un proyecto interesante y que al mismo tiempo me permitiría culminar ese sueño ansiado de obtener mi doctorado.

Hoy, casi 5 años después, una parte del trabajo queda plasmada en este documento. Otra, forma parte del proceso de formación profesional y personal que ha constituido esta tesis. De la misma manera, muchas personas han colaborado en su realización. Algunas de modo directo y otras estando a mi lado para apoyarme cuando había que apretar los dientes y tirar hacia adelante. TODOS, en mayor o menor medida, HABÉIS puesto vuestro grano de arena para que este proyecto pudiera ver la luz. Por ello, pido perdón de antemano por si alguno se me pudiera olvidar en esta larga lista. En cualquier modo para vosotros y vosotras, GRACIAS, GRACIAS, GRACIAS…

En primer lugar, me gustaría darle las gracias a mis directores, la Dra. Lucía Molina y el Dr. Rogelio Herrera por haberme guiado hasta el final del camino. Gracias Lucía por confiar en mí y abrirme las puertas de la investigación hace ya muchos años; y a ti Rogelio por todo lo que me has enseñado, por animarme siempre y por nuestras discusiones siempre eternas , pero siempre constructivas. Gracias a los dos.

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natural para encontrar una solución donde parecía no haberla. También al Dr. Juan Manuel Afonso y a la Dra. María Jesús Zamorano, que me acogieron en su laboratorio de genética como uno más y siempre dispuestos a contestar mis innumerables preguntas. Al Dr. Daniel Montero, tu rigor científico ha sido un ejemplo para mi. Y como no, también gracias a la cabeza de todo ese grupo humano, a la Profesora Marisol Izquierdo. Marisol gracias por tu tiempo, tus aportaciones han impregnado de calidad este documento. Al lado de ellos y sin restarle méritos, a todos mis compañeros de doctorado, a los técnicos del IUSA y el ICCM: Alex, Silvia, Juan, Mohamed, Ada, Carmen, Yurena, Guacimara y todo el resto. Gracias por vuestra amistad y disponibilidad para ayudarme siempre.

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A Alex, Silvia, Juan y Moneiba; gracias por haber sido una piedra angular de nuestra vida personal en Canarias. Por todo lo que me habéis enseñado y compartido tanto profesional como personalmente. Por los consejos y ayudas para redactar este documento. Muchas gracias de verdad.

“A mis amigos más próximos, que desde lejos y cada uno a su manera, también habéis empujado para que este proyecto salga adelante. Gracias.

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A mi familia, a quién le debo todo. A mis padres, por el ejemplo que han sido siempre para mi, que siempre me han dado todo, y gracias a los cuáles nunca habría llegado hasta este punto. A mis hermanos, Marta, Pablo y José Luis, la única parte negativa de esta tesis ha sido el tiempo que he pasado lejos de vosotros. GRACIAS por estar ahí!

Et finalement à toi, ELODIE. Je te dédie cette thèse ; jamais je ne l’aurais réussie sans toi. MERCI pour tous tes encouragements, ton aide, ta compréhension, ton ENORME patience, ton support pendant les moments difficiles, et aussi, pour ton amour. Je t’aime.

Chapter 1

Introduction

The general subject of this thesis was the study of various aspects related to biology and ecology of seahorse species of Gran Canaria Island (Spain). The scarce information about this species as well as their protection status require a multidisciplinary approach combining ecology and aquaculture methods to achieve conservation goals.

A general overview of genus *Hippocampus* regarding their taxonomic status, biology (including aspects related to morphology, physiology, anatomy and reproduction) as well as distribution and habitat is presented along this first chapter. Moreover, the main methods employed for marine fish populations assessment focused on seahorse populations are described. Then a review of seahorse aquaculture (history and trade) is reported, regarding seahorse facilities, nutritional and diseases issues. Also a description of global seahorse conservation status, including their threats and management actions related to seahorse conservation, is mentioned.

Finally a brief description of seahorse species studied during this thesis is outlined including ecology and aquaculture background.

The main lines of this introduction also reflect the recent research effort made by several scientists to review different subjects related to seahorse ecology

(Foster and Vincent, 2004), aquaculture (Koldeway and Martin-Smith, 2010) and conservation (Vincent *et al.*, 2011), denoting the importance of these first two topics in the conservation of marine threatened species as seahorses, as the African environmentalist Baba Dioum said, "*In the end, we will only conserve what we love. We will only love what we understand. We will only understand what we are taught*."

1.1 THE SEAHORSE

1.1.1 SEAHORSE TAXONOMY

Seahorses are bony fish (Teleosts) belonging to *Syngnathidae* family (Lourie *et al*., 2004; Kuiter, 2009). The family name, which means "jaw-fused" in ancient Greek, groups also pipefish, pipehorses and seadragons. There are 56 genera described inside this family that includes more than 320 species. The most characteristic feature of this family is the unique reproductive strategy in where the female deposits the eggs in the incubating area placed on the ventral side of the male body. As it will be explained in detail later, he provides all post-fertilization parental care and fully developed, independent newborn seahorses are released from the pouch (Kornienko, 2001; Wilson *et al.*, 2001; Vincent and Giles, 2003; Foster and Vincent, 2004; Lourie *et al.*, 2004). Moreover, based on similar characteristics such as the location and development of the male brood pouch, head/body axis, development of the caudal fin, and prehensile ability of the tail, the *Syngnathidae* family is divided in four identifiable subfamilies (Herald, 1959; Wilson *et al.*, 2001; Lourie *et al.*, 2004; Kuiter, 2009) (Fig. 1.1):

a. Subfamily *Syngnathinae* (Pipefish)

This is the largest group, the actual pipefish, which are mostly stick-like with their head in line with the body. Usually they have a small caudal fin and the tail is not normally prehensile. The eggs are incubated by males using a pouch placed under the trunk or tail, which is formed by simple to overlapping or interlocking membranes (Kuiter, 2009).

b. Subfamily *Doryrhamphinae*

There is also a group of free-swimming pipefish characterized by mostly exposed broods and a large flag-like caudal fin. An unprotected patch of skin onto which the brood is attached composes incubation area in males.

c. Subfamily *Solegnathinae*

Seadragons and pipehorses comprise the subfamily *Solegnathinae*, in which the head is held at a slight angle to the body, the tail is more or less prehensile, the caudal fin is absent, and incubated eggs are exposed under the tail or trunk section.

d. Subfamily *Hippocampinae*

Together, seahorses and pygmy seahorses are inside the subfamily *Hippocampinae*, characterized by a fully enclosed brood pouch with a small opening for the incubation of eggs, an absent or vestigial caudal fin, and a prehensile tail.

Concerning seahorse taxonomy, species identification is problematic, since morphological differences between species are not always clear due to their capacity to change colour and develop skin filaments on their body in order to camouflage themselves (Lourie *et al.*, 2004). Numerous misidentifications, synonyms and even spelling mistakes have resulted in a taxonomic chaos (Leysen, 2011). As it will be explained later, this fact raises concern due to the global overexploitation of seahorses for traditional medicines, aquarium trade and curiosities in the last decades (Vincent, 1996). Therefore, this subfamily has been recently under revision to standardize their nomenclature making easier their sustainable management and conservation (Lourie *et al.*, 2004).

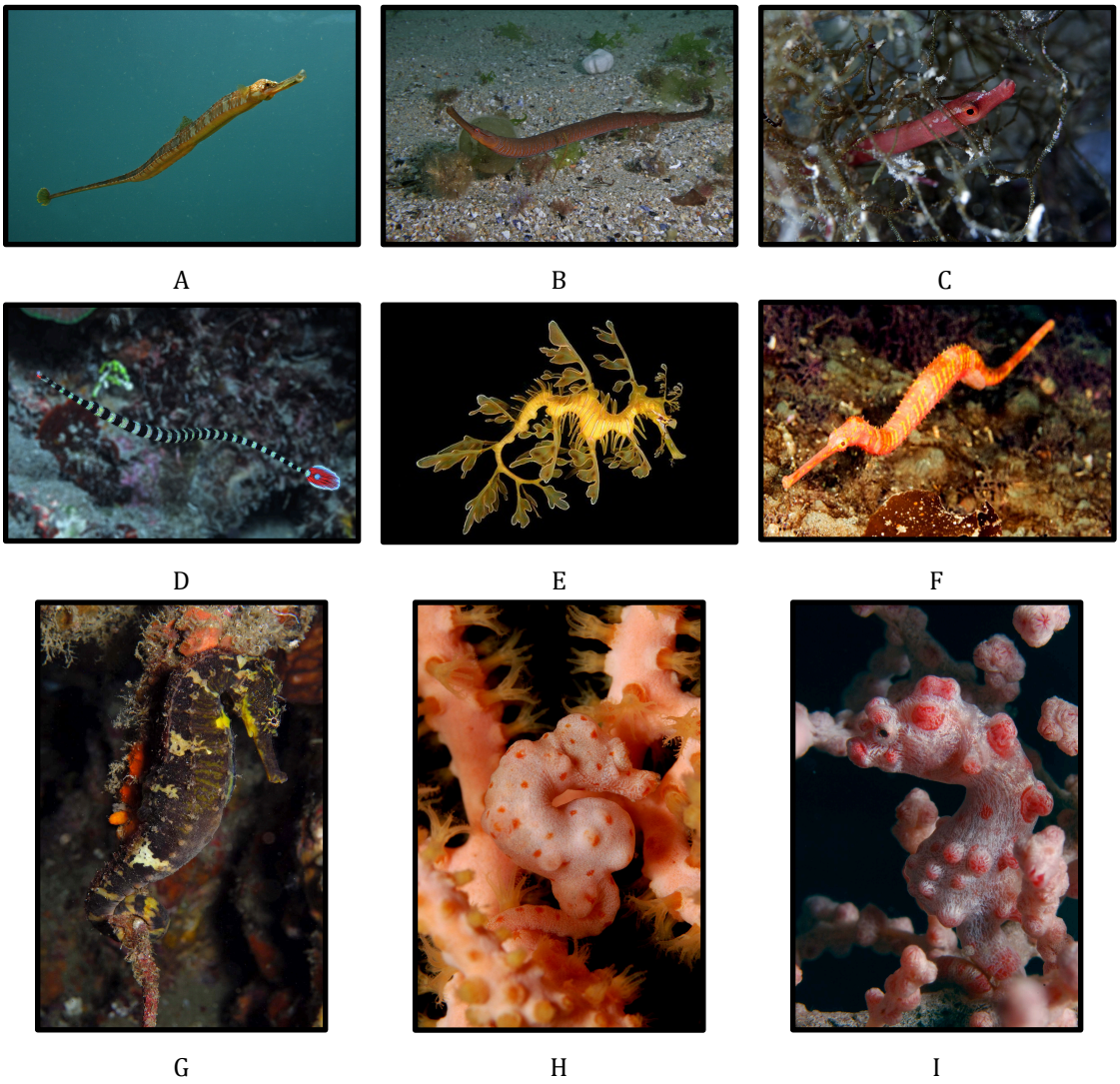

Figure 1.1 Examples of specimens included in different subfamilies of *Syngnathidae*: (A) *Syngnathinae* – *Syngnathus acus* (source: S. Jamme); (B) *Syngnathinae* – *Entelurus aequoreus* (source: S. Jamme); (C) *Syngnathinae* – *Nerophis maculatus* (source: S. Jamme); (D) Doryrhamphinae – *Dunckerocampus dactyliophorus* (source: T. Jimenez); (E) *Solegnathinae* – *Phycodorus eques* (source: www.daveharasti.com); (F) *Solegnathinae* – Solegnathus spinosissimus (source: Dr. P. Ryan); (G) *Hippocampinae* – *Hippocampus taenopterus* (source: E. Alemán), (H) *Hippocampinae* – *Hippocampus denise* (source: E. Alemán), (I) *Hippocampinae* – *Hippocampus barbiganti* (source: E. Alemán).

Thus, the latest studies using morphological, genetic and behavioural evidence suggest there are currently 48 species, once synonyms have been reconciled (Lourie, unpubl. data; Vincent *et al.*, 2011), all placed in the only genus *Hippocampus*. Research based on genetic analyses suggests that this genus was probably originated before the final closure of the Tethyan seaway (Fritzsche, 1980), in the Indo-Pacific region approximately 15 or 16 million of years (Myr) ago (Teske *et al.*, 2004; 2005; 2007; Wilson *et al.*, 2001; Casey, 1999; Casey *et al.*, 2004; Kuiter, 2009). This hypothesis has been confirmed by the fossils of only two

known extinct seahorse species found in the Middle Miocene (13 Myr ago) beds in Slovenia (Žalohar *et al.*, 2009). Thus, *H. slovenicus* and *H. sarmaticus* were "fully developed seahorse species" similar to extant pygmy seahorses (*H. bargibanti, H. denise, and H. colemani;* Žalohar *et al.*, 2009) for *H. slovenicus* and *H. trimaculatus* for *H. sarmaticus*. Even more, *H. sarmaticus* might represent the ancestor of the *H. trimaculatus* or at least a close relative of this ancestor (Žalohar *et al.*, 2009) (Fig. 1.2)

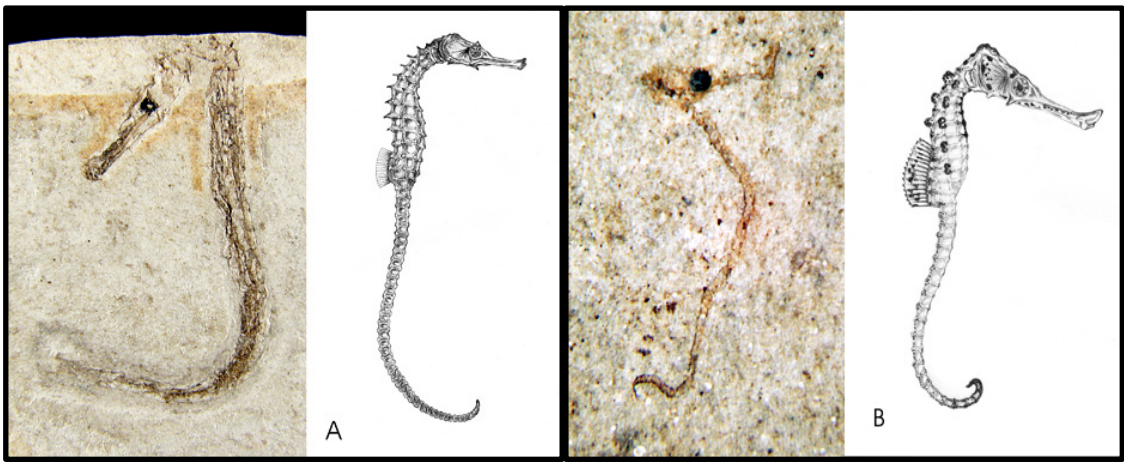

Figure 1.2 Seahorse fossils: (A) 5 cm adult female of *H. sarmanticus*; (B) 5 mm young seahorse of *H. slovenicus* (source: J. Žalohar).

1.1.2 SEAHORSE BIOLOGY

Unlike other fish, seahorses have unusual morphology without scales over their skin. Instead, their body is covered by bony plates arranged into a series of rings to protect them against predation (Lourie *et al.*, 2004; Kuiter, 2009; Leysen, 2011). The number of trunk and tail rings does not vary much among individuals of a species, but does differ among species (9-13 for trunk and 28-47 for tail rings; Lourie *et al.*, 2004). Where the bony plates intersect, the skin is raised into small tubercles or spines, whose degree of development varies with species and sometimes age of the seahorse (Woods, 2007).

The head of seahorses is held at a right angle to the body, provided by a tubular snout with a terminal, toothless mouth. Besides, their eyes can move independently, which helps them catch small crustaceans, their primary food source (Lourie *et al.*, 2004).

Seahorses have a prehensile tail to anchor themselves to various substrates or holdfast, but also employed during courtship and male competition (tail

wrestling) (Vincent, 1994; Hale, 1996; Lourie *et al.*, 2004; Bruner and Bartolino, 2008; Leysen, 2011). They lack pelvic and caudal fins therefore the rapid movements of the large dorsal fin provide the main propulsive force. Meanwhile, the smaller pectoral fins behind the gills provide steering control and a reduced anal fin has a minor role in ascending propulsion (Lourie *et al.*, 2004; Woods, 2007; Kuiter, 2009).

As the rest of syngnathids, seahorses exhibit great camouflage capacities, fading almost completely within the habitat in where they live. Thus they are able to change colour pattern or grow skin filaments or fronds that make them look like algae, seagrass or natural sticks (Lourie *et al.*, 2004; Kuiter, 2009). This behaviour allows them to remain unnoticed by their predators and also to approach their prey (Foster and Vincent, 2004). In general, the majority of their predators are considered to be opportunistic and/or generalist feeders, suggesting that seahorses were not specifically targeted as prey (Kleiber *et al.,* 2010) (Figure 1.3). As for seahorses, they are ambush predators which feeding mainly occurs during diurnal or crepuscular hours (Felício *et al.*, 2006; Woods, 2007; Kuiter, 2009). Prey is captured with a rapid movement of the seahorses' head and simultaneous buccal cavity expansion, which creates a strong inhalant current that may break larger preys (Bergert and Wainwright, 1997; Curtis and Vincent, 2005; Felício *et al.*, 2006; Woods, 2007). This process is astonishingly fast: adult seahorses capture prey in less than 5 ms after the start of the pivot movement (Wassenbergh *et al.*, 2009).

Data concerning seahorses feeding habits in the wild found that little epifaunal and planktonic crustaceans are their preferred dietary items (Texeira and Musick 2001; d'Entremont, 2002; Kendrick and Hyndes, 2005; Kitsos *et al.*, 2008; Storero and González, 2008). Although the diet can vary among seahorse species, availability and abundance of prey in the ecosystem (Woods, 2007), amphipods (*Gammaridae, Caprellidae* and *Hiperidae*) decapods and mysids are the most dominant prey category as registered in stomach contents of two Europen seahorses species (*H. hippocampus and H. guttulatus*) studied by Kitsos *et al.* (2008) (Fig. 1.3). Sometimes algae can be found inside seahorse guts due to their involuntary consumption to obtain animals adhered to them (Storero and González, 2008).

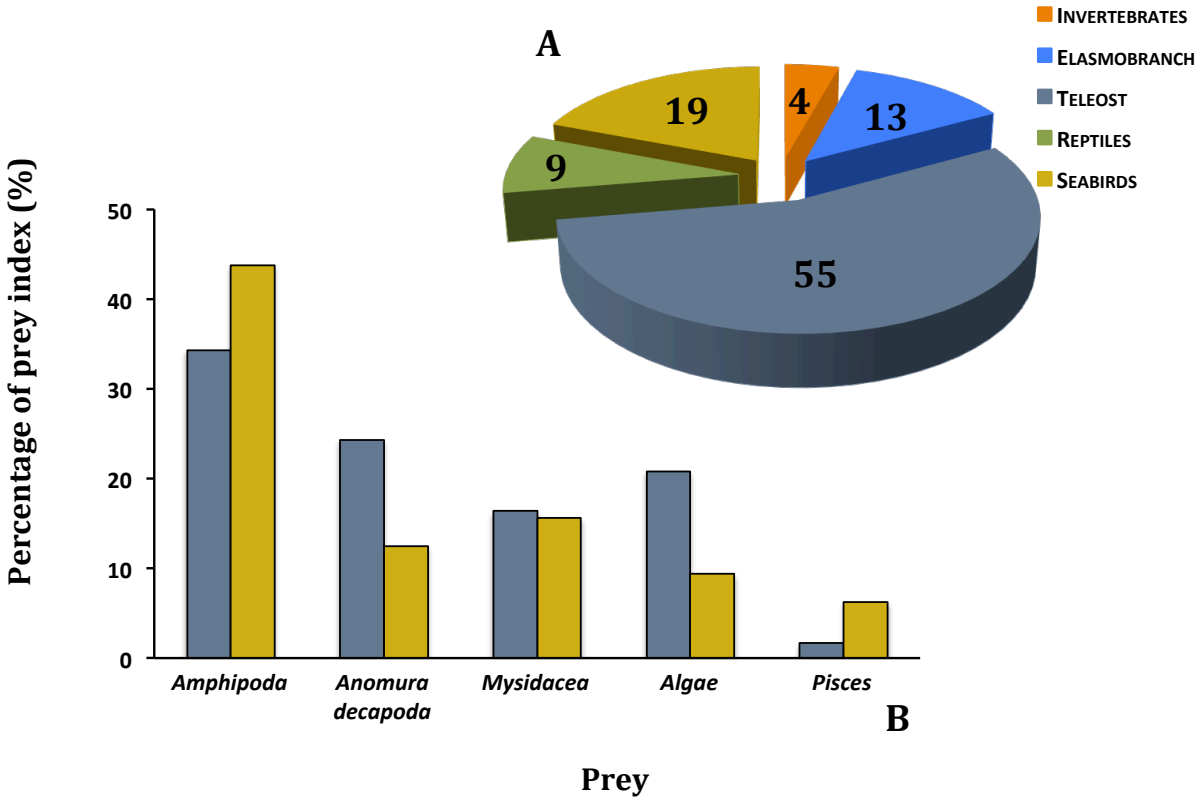

Figure 1.3 Percentages of (A) main seahorse's predators (Data from Kleiber *et al.*, 2010) and (B) main prey found in the gut of *Hippocampus guttulatus* (blue) and *Hippocampus hippocampus* (yellow) (Data from Kitsos *et al.*, 2008).

On the other hand, cannibalism on juveniles appears to be a common phenomenon of certain seahorse species such as *H. erectus* (Scarratt, 1995), *H. reidi* (Rosa *et al.*, 2005), *H. guttulatus* and *H. hippocampus* (included inside *Pisces* prey class; Kitsos *et al.*, 2008). Embryos and juveniles were identified in their guts but in a lesser extent than crustacean groups. This behaviour can be conditioned by limited alternative food (Storero and González, 2008).

Seahorse species range in sizes varying among species from 2 cm in *H. denise* (Fig. 1.1) to 35 cm in *H. abdominalis* (Lourie *et al.*, 2004), with males and females of most species reaching approximately the same size (Choo and Liew, 2003; Foster and Vincent, 2004). However, in the majority of species there is sexual dimorphism regarding body proportions. Normally, males have a relatively longer tail than females. In opposition, females show bigger trunks (Table II in Foster and Vincent, 2004). Longer tails may enable males to support a large caudal brood pouch whilst still grasping a holdfast. This pouch is the most important feature for sexual

differentiation in most species of seahorses. Normally it is placed below the last trunk ring, located in front of the tail. Although in a seahorses' group with a significant low size called "pygmy seahorses", its members lack this structure, brooding their eggs and young within the abdomen (Tackett and Tackett, 1997; Lourie and Randall, 2003; Lourie and Kuiter, 2008) (Fig. 1.1).

On the other hand, seahorses show the most specialized forms of mating and male parental care among teleost's species (Foster and Vincent, 2004). Mating starts with an elaborate courtship called "greetings" and ends with eggs transfer from female to male (Vincent and Sadler, 1995; Damerval *et al.*, 2003; Woods 2003c; Lin *et al.*, 2008). The greeting plays an important role in the maintenance of seahorse pair-bonds helping females synchronize their eggs hydration with male parturition (Vincent and Sadler, 1995). It also groups different behaviours or interactions between male and female. Thus the male, growing pale, is constantly active and pursuing the female, also changing her colour, swimming beside her and tangling her tail, or head-pointing her. The male also often repeatedly inflates his pouch with water ("pumping"). They swim every time higher and higher in the water column, while the male always opening his brood pouch. During an ultimate upward swim the female deposits her eggs in the opened pouch (Woods, 2000a; Damerval *et al.*, 2003) (Fig. 1.4).

Typical seahorse eggs are pear-shaped with a characteristic orange-yellow yolk, result of the carotenoids obtained from their crustacean-dominated diets (Kitsos *et al.*, 2008). The number of eggs per female can range from 5 (*H. zoosterae*, Vincent, 1990) to more than 1000 (*H. erectus*, Teixeira and Musick, 2001) depending on adult size and species (Foster and Vincent, 2004).

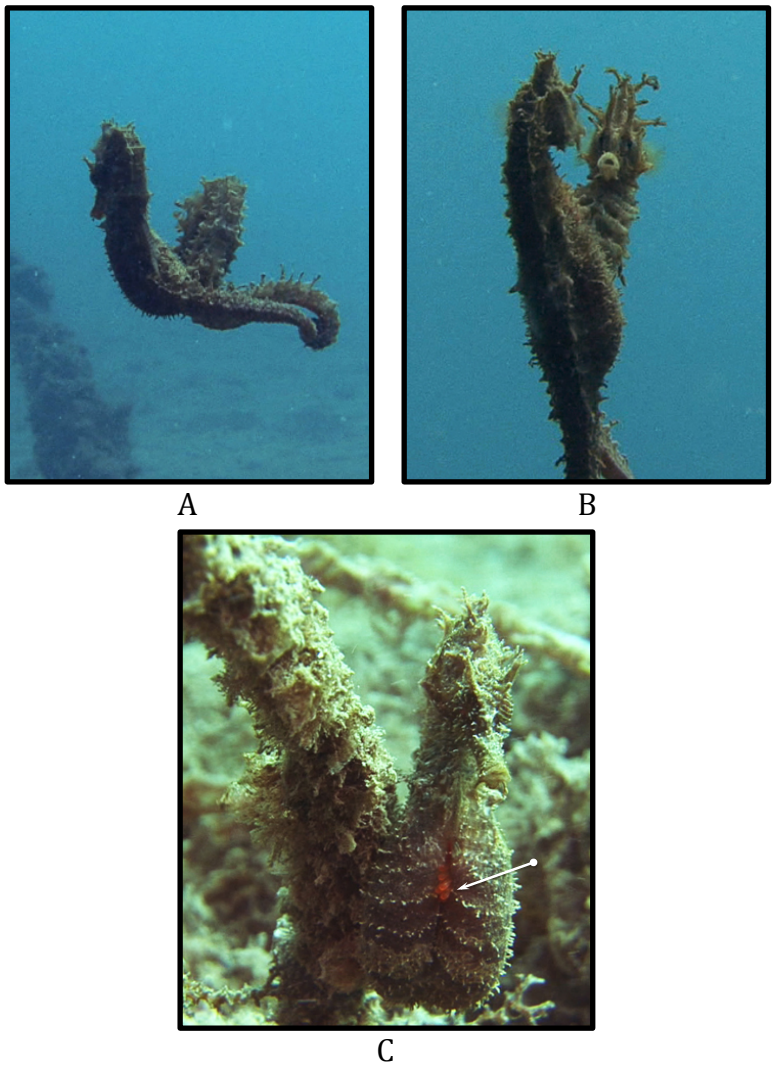

Figure 1.4 (A) Male and female swimming upwards; (B) female depositing her eggs in the male's brood pouch; (C) male's brood pouch just after the transfer. White arrow shows eggs (source: E. Turpin).

Regarding the mechanism of fertilization of eggs, it appears to occur just prior to their deposition into the male's pouch (Boisseau, 1967; Van Look *et al.*, 2007) as described in other syngnathids species (Monteiro *et al.*, 2002). Once the female's fertilized eggs are deposited in the brood pouch, it is sealed and the eggs are then isolated from the external environment. Several authors presume that sealed brood pouch works as a "pseudo-placenta", giving protection and providing oxygen and additional nutrients to embryos (Wilson and Vincent, 2001; Carcupino *et al.*, 2002; Foster and Vincent, 2004; Melamed *et al.*, 2005; Laksanawimol *et al.*, 2006). Thus, the developing embryos are kept by the male until they are ready for birth, being then released from the brood pouch. The duration of the male's

pregnancy ranges from approximately 9 (*H. comes*) to 45 (*H. capensis*) days, also depending on species and latitude (Table VIII in Foster and Vincent, 2004).

At the end of pregnancy the male gives birth (usually at night), during several hours, actively forcing the offspring out of his pouch (Vincent, 1990). The young seahorses look like miniature adult, with a fully functional prey-capture system and from that moment, they will not receive any further parental care (Wassenbergh *et al.*, 2009).

The number of newborn per brood ranges from 100 to 300 on average, with species that can produce more than 2000 (*H. ingens*) or only 5 (*H. zosterae*). Their size at birth is also variable ranging from 2 (*H. barbiganti*; Fig. 1.1) to 16 mm (*H. abdominalis*) in length (Woods, 2000b; Foster and Vincent, 2004; Lourie *et al.*, 2004). The newborn of some species are planktonic, entering the water column immediately after birth. The extent of juvenile dispersal by passive means is unknown, but may provide some gene flow among populations (Lourie *et al.*, 2004). Moreover, older animals can be carried out passively by ocean currents while grasping floating objects (Foster and Vincent, 2004). This phenomenon is quite common because the quantity of rafting material has increased dramatically with the spread of human population (Barnes, 2002).

The natural life span, mortality rates, growth rates, and disease prevalence for different seahorse species are largely unknown in their natural environment. Inferred life spans range from 1 year in the very small species (*H. zosterae*) to an average 3–5 years for larger species (*H. capensis*, *H. guttulatus* or *H. hippocampus*) (Foster and Vincent, 2004; Woods, 2007).

Most of seahorse species appear to be monogamous at least within a single breeding season, accepting eggs from only one female (Foster and Vincent, 2004; Kvarnemo *et al.*, 2000; 2007; Wilson and Martin-Smith, 2007; Woods, 2007; Koldeway and Martin-Smith, 2010). This behaviour increases reproduction efficiency (larger broods and less time spent on courtship) and shortens their inter-brood intervals, reducing the time necessary to locate and synchronize with their mate (Wilson and Martin-Smith, 2007), while, other specimens can also mate with several partners in the same season (polygamy) (*H. subelongatus*, Kvarnemo *et al.*, 2000).

Otherwise, seahorses have long been thought to have conventional sex roles during mating displays, with males competing for mating opportunities and females mate choice (Vincent and Sadler, 1995; Foster and Vincent, 2004). Recent studies of the potbellied seahorse (*H. abdominalis*) have shown that sex-role reversal occurs in high-density female-biased populations, indicating that male mating preferences may lead to sexual selection on females in this species. Thus, Wilson and Martin-Smith (2007) presume that sex roles in this species are "flexible" and that the strength of intra-sexual competition and mate choice is likely influenced by local conditions including sex ratio and population density.

Finally, the mate recognition system developed in seahorses appears to be size-assortative with breeding pairs comprising male and female with similar size (Jones *et al.*, 2003; Faleiro *et al.* 2008; Lin *et al.* 2008).

1.1.3 DISTRIBUTION AND HABITAT

Seahorses are widely distributed around the world, occupying both temperate and tropical coastal waters, with a distribution from about 48 degrees north to 43 degrees south (Foster and Vincent, 2004). Most species live in tropical and subtropical zones, but few can be found in the sub-temperate zones of Australia, New Zealand, Japan, South Africa, South America and Europe (Villares, 2006; Kuiter, 2009). Depending on species, seahorse distribution can range mainly from estuaries and lagoons to shallow coastal areas of tropical, as well as temperate seawaters living in many habitats. Sometimes estuaries and lagoons can aggregate high seahorse abundances related to their capacity to provide them large quantities of food (Choo and Liew, 2003) and protection against strong tidal currents (Curtis and Vincent, 2005). This is the case of Ria Formosa (Portugal) and Thau Lagoon (France). However, most of seahorses in the wild are generally found in low densities (Foster and Vincent, 2004) and patchy distribution (Foster and Vincent, 2004; Murugan *et al.*, 2011; Vincent *et al.*, 2011).

Concerning habitat, most seahorses are usually found among corals, macro algae, mangrove roots and seagrasses, but also on open sandy or muddy bottoms, over shell fragments, live holdfasts (sea urchins, sponges, bryozoans, ascidians or spiral tube worms) or even associated with anthropogenic structures such as ropes, nets or moorings (Lourie *et al.*, 2004; Curtis *et al.*, 2004). Usually seahorse

habitat depth remains between 5 to 30 m depth, depending on species or area. Nonetheless certain species can be found deeper as the recently *H. paradoxus* described in the South-eastern coast of Australia 102 m depth living on soft-bottom substrates colonized by sponges and bryozoans (Foster and Gomon, 2010).

Otherwise most seahorse species studied exhibit high site-fidelity and small home range sizes, at least during the breeding season without territorial defence (Vincent and Sadler, 1995; Perante *et al.*, 2002; Foster and Vincent, 2004; Curtis and Vincent, 2006; Woods, 2007). Sex differences in home ranges have been also observed for some species such as *H. whitei*, *H. breviceps*, *H. reidi*; *H. guttulatus* and *H. hippocampus*, where females have larger home ranges (Vincent and Sadler, 1995; Foster and Vincent, 2004). Moreover, Kvarnemo *et al.*, (2007) reported that pregnant males of *H. subelongatus* maintained relatively small home ranges whereas both mated and unmated females and unmated males moved over significantly larger areas. It has been postulated that sex differences in home range, if they exist, may arise due to physical and energetic constraints associated with male pregnancy (Vincent *et al.*, 2005).

1.2 FISH POPULATION ASSESSMENTS

1.2.1 GENERAL OVERVIEW

The sustainable management of seahorses as biodiversity resource requires the biological and ecological understanding of their wild populations (Foster and Vincent, 2004). Thus, different population assessments must be carried out to learn more about their abundance (Meffe and Carroll, 1997), size-frequency distribution (Perante *et al.,* 2002; Bell *et al.*, 2003), habitat use (Morgan and Vincent, 2007; Teske *et al.*, 2007) and life-history (Curtis and Vincent, 2006). Regular population monitoring can provide reliable data (e.g. trends in population abundance) on the current status of a population (English *et al.*, 1994; Curtis *et al.*, 2004) and their hypothetical extinction risk. In this way, this basic biological information will be very useful for marine resource managers to evaluate the impact of environmental disturbance events (natural or anthropogenic) that could possibly affect the seahorse communities studied, and determine the management actions required for their conservation (King, 1995; Bell *et al.*, 2003).

In general, *in situ* population assessment studies can be divided into two main groups. The first includes methodologies that involve destructive sampling and the second, observations that are non-destructive (Bortone *et al.*, 2000; Güll, and Lök, 2008). The selection of one or both techniques will be conditioned by the species surveyed (Herrera, 1998; Lockyear *et al.*, 2006; Teske *et al.*, 2007) and habitat type in where the study is conducted (English *et al.*, 1994; Kingsford and Battershill, 1998).

1.2.1.1 Destructive techniques

These methods are associated with animal capture using different gears, which can also damage the habitat. The most destructive technique is trawling, although in marine sampling it is commonly used (Güll and Lök, 2008). The information recovered may vary depending on whether animals were released after their capture or sacrificed. Thus, in the first option, data associated with species identification, feeding habits, growth or sex could be recorded. Additionally, sacrificed individuals can yield additional information about certain life-history parameters not obtainable from living specimens (Moring *et al.*, 1989, Bortone and Kimmel, 1991; Güll and Lök, 2008) such as growth and age (otolith and bones), feeding habits (stomach contents), and fecundity and sex-ratio (gonads) among others.

These techniques consider each specimen as a sample and data are recorded in Capture per Unit Effort (CPUE). Although these values can be mentioned in several studies as abundance estimation (Meeuwig and Samoilys, 2003), their precision along temporal and space scale is still under debate (Connell *et al.*, 1998). Ideally CPUE data might be supplemented with non-destructive techniques such as underwater visual census (Meeuwig and Samoilys, 2003).

1.2.1.2 Non-destructive techniques

The non-destructive assessment methods are in general more selective than the destructive ones, being able to assess a high number of species. Because, they can be adapted to different habitat types, they are generally preferred for sampling benthic communities. Moreover, they can be easily repeated on every habitat

(establishment of replicas), during short intervals and with a low impact on the habitat (Herrera, 1998; Güll and Lök, 2008).

Non-destructive methods are divided into two main groups that range from underwater visual census to remote sensing methods such as hydro acoustic technology or video recording (Bortone *et al.*, 2000; Güll and Lök, 2008).

1.2.1.2.1 Underwater Visual Census (UVC)

Visual assessments of fish are the most frequently used techniques used to obtain data related to population abundance, distribution and habitat use and other life-history parameters on these organisms (Bortone *et al.*, 1994; Samoilys, 1997; Herrera, 1998; Samoilys and Carlos, 2000; Curtis *et al.*, 2004; Samoilys *et al.*, 2007). UVC methods are flexible and can be adapted to a variety of diving conditions and substrates, providing reliable and repeatable relative estimates of population abundance (Samoilys and Carlos, 2000; Curtis *et al.*, 2004). Nonetheless these methods are subject to observer bias (species identification skills or size-estimation experience; Curtis *et al.*, 2004), underestimation of abundance due to habitat heterogeneity or cryptic species (Harmelin-Vivien *et al.*, 1985; Buckley and Hueckel, 1989; Samoilys and Carlos, 2000; Curtis *et al.*, 2004; Samoylis *et al.*, 2007) and environmental factors (depth or visibility conditions; Bortone *et al.*, 1989; DeMartini *et al.*, 1989; Bortone and Kimmel, 1991; English *et al.*, 1994).

UVC methods can be grouped in several categories depending if observer moves in predetermined (line or strip transect count) or random direction (species-time random count), or remains stationary (stationary point count) (Bohnsack and Bannerot, 1996; Herrera, 1998). Here below, the methods most widely used in fish assessments (Bortone *et al.*, 2000).

a. Line transect count

It is the most frequently employed method for fish surveys. Divers generally swim along line transects, recording species, abundance and size in a three dimensional corridor that can vary in width and height. Transects are randomly placed and their length can vary according to habitat homogeneity (Güll and Lök, 2008). Line transects use the perpendicular distances to individual sightings to model a detection function, which quantifies the probability of observing an object

given its distance from the track line. The resulting perpendicular sighting distributions are then used to estimate the surface area effectively searched during surveys (Hyrenbach *et al.*, 2007).

b. Strip transect count

Also called belt transect, this method has been usually used in benthic fish population studies (Samoilys and Carlos, 2000) overall in homogeneous habitats (Herrera, 1998). It is also currently employed for seahorse surveys (Curtis *et al.*, 2004). During strip transect census, divers move along specified length and width transects, randomly placed and recording relative abundance and size of species sighted (Hyrenbach *et al.*, 2007; Güll and Lök, 2008). Observation features should be standardized (length, width, height, duration direction) in order to compare survey results (Bortone *et al.*, 2000). These parameters can vary according to target species (Harmelin-Vivien *et al.*, 1985), habitat, or environmental conditions (e.g. depth or visibility; Hollacher and Roberts, 1985; Herrera, 1998).

c. Species-time random count

This method is adequate to estimate biodiversity of low-density or cryptic species (Bortone *et al.*, 1986; 1989; Herrera, 1998). The divers swim haphazardly over the global survey area, recording species observed within a set time period (Bortone *et al.*, 2000). Thus, the number of specimens of each species can be estimated as relative abundance values based on the time interval (Herrera, 1998; Güll and Lök, 2008). This difficulty to compare these abundance data with other UVC methods, which values are expressed by surface or volume units (e.g. strip or line transect counts), is one the most important limitations of this method. Kimmel (1985) tried to improve this technique, developing the "Visual Fast Count (VFC)", in where divers also move randomly during a defined time interval, recording the number of individuals of one species, but in a previously selected habitat.

d. Stationary point count

This method has been usually employed for heterogeneous benthic substrates (Herrera, 1998). To make stationary point counts, observation area should be designed as a geometric shape (usually circular) with specific

dimensions (height and width). Then diver occupies the centre of the observation area and slowly turns for a prescribed period of time (Bortone *et al.*, 2000; Güll and Lök, 2008) while simultaneously recording the specimens observed inside this water volume (Herrera, 1998; Güll and Lök, 2008). The size of the observation area will be determined by the visibility conditions.

1.2.1.2.2 Remote sensing methods

Within non-destructive methods, hydro-acoustic is the most advanced technology employed for fish and macro-invertebrate assessments (Thorne *et al.*, 1989; Güll and Lök, 2008; Bograd *et al.,* 2010). This technology allows to obtain long-term, time series data on assemblage abundance when it is combined with computerized data recorders (Güll and Lök, 2008), and also to study the movements and habitat use of fish (acoustic tags; Connolly *et al.*, 2002). A first experience in seahorses using acoustic tags has been recently reported (Caldwell *et al.*, 2011). The difficulty to identify marine species from their sonar signal and the unknown interference of these techniques with their behaviour in the wild remains one of the main limitations on their use (Bortone *et al.*, 2000; Güll and Lök, 2008; Caldwell *et al.*, 2011).

Video cameras can be useful to record transect surveys when linked to SCUBA divers or mounted in remote operated devices. The use of this technology decreases the time to collect data such as abundance and species size (Güll and Lök, 2008). However environmental conditions such as water visibility or strong currents may limit their employment.

1.2.2 SEAHORSE POPULATION ASSESSMENTS

The main method employed to assess seahorse populations in the wild is the UVCs (Perante *et al.*, 2002; Bell *et al.*, 2003; Curtis *et al.*, 2004; Foster and Vincent, 2004; Moreau and Vincent, 2004; Curtis and Vincent, 2005; Vincent *et al.*, 2005; Curtis and Vincent, 2006; Lockyear *et al.*, 2006; Villares, 2006; Morgan and Vincent, 2007; Teske *et al.*, 2007; Woodall, 2009). As mentioned before, UVC surveys are a non-destructive, fishery-independent method that can be used to estimate abundance, distribution and population structure of seahorse communities (Curtis *et al.*, 2004). UVC is subject to observer bias, underestimation

of density due to seahorse camouflage, and depth constraints (English *et al.*, 1994). Thus, seahorse experienced divers are strongly recommended to perform these studies (Bell *et al.*, 2003).

Generally, strip transect count is the UVC method usually employed to assess seahorse populations (Curtis *et al.*, 2004) (Table 1.1). The belt transects' length and width can range between 20-50 m and 2-5 m, respectively (Bell *et al.*, 2003; Curtis *et al.*, 2004; Lockyear *et al.*, 2006, Woodall, 2009). Also line transects (50 – 100 m) have been used to survey *H. comes* populations in Philippines (Perante *et al.*, 2002; Morgan and Vincent, 2007). Finally, some authors focus UVCs inside grids deliberately selected, where seahorse presence was previously described (Perante *et al.*, 2002). Thus, seahorse locations can be recorded using 1x1 m sections of these grids (Moreau and Vincent, 2004). Hence seahorse density estimated using focal study grid could be biased (Foster and Vincent, 2004).

Sometimes, UVCs can be complemented with data provided by direct fish capture (Boisseau, 1967; Lourie *et al.*, 1999; Vincent, 2001; Vincent and Giles, 2003; Kendrick and Hyndes, 2005) or by incidental capture of animals using non-selective gears such as seahorse bycatches derived from trawl-fisheries (Lourie *et al.*, 1999; Vincent, 2001; Foster and Vincent, 2004) (Table 1.2). Thus, UVCs have been combined with capture pushnet method to sample areas characterized by dense vegetation (approximately 60% vegetation cover or more; Lockyear *et al.*, 2006; Teske *et al.*, 2007), in where seahorse visual searching was impractical for the divers. On the other hand, morphometric information as well as meristic data (trunk and tail rings, fin rays, and cheek spines among others; Lourie *et al.*, 2004) obtained from sacrificed specimens can be employed as additional criteria with genetic analysis in taxonomic issues (Lourie *et al.*, 2004; Woodall, 2009).

The information recovered from capture methods and UVCs has allowed to increase data regarding life history and ecology of several seahorse species (Foster and Vincent, 2004). However basic biological information about seahorse distribution, habitat use, natural densities, population structure, behaviour and life histories is lacking for most species (Curtis *et al.*, 2004). Thus, over the 33 seahorse species registered on the Red List of Threatened Species of the International Union for Conservation of nature (IUCN, 2010), 23 are listed as "Data Deficient" (DD) and even several are heavily exploited nowadays (Foster and Vincent, 2004).

Table1.1 Recorded seahorse densities (Individuals·m^{-2}) using non-destructive methods (Modified from Foster and Vincent, 2004; Masonjones, 2011).

SPECIES	DENSITY	HABITAT	LOCALITY	STUDY TYPE	REFERENCE
H. abdominalis	0.007	Silt with anthropogenic debris, shells and rocky reef	Tasmania, Australia	Strip transect	Martin-Smith and Vincent 2005
	0.06-0.2	Not described	Tasmania, Australia	Not described	Wilson and Martin-Smith, 2007
H. breviceps	Site A: 0.17 Site B: 0.31	Site A: Algal patches over bare sand Site B: Seaweed beds divided by sandy strip	Port Philip Bay, Australia	Focal grid	Moreau and Vincent, 2004
H. capensis	0.0089	Seagrass and seaweeds	Knysna estuary, South Africa	Strip transect	Bell *et al.*, 2003
	0.22	Seagrass and seaweeds	Knysna estuary, South Africa	Strip transect	Bell *et al.*, 2003
H. comes	0.001	Coral reef, wild and farmed macroalgal and seagrass beds	Bohol, Philippines	Line transect	Morgan and Vincent, 2007
	0.02	Coral reef, sponge, *Sargassum*	Bohol, Philippines	Line transect	Perante *et al.*, 2002
H. guttulatus	0.073	Seagrass, macroalgae and sand flat	Ria Formosa Lagoon, Portugal	Strip transect	Curtis and Vincent, 2005
	Grid A: 0.33 Grid B: 1.52	Seagrass	Ria Formosa Lagoon, Portugal	Focal grid	Curtis and Vincent, 2006
H. hippocampus	0.007	Seagrass, macroalgae and sand flat	Ria Formosa Lagoon, Portugal	Strip transect	Curtis and Vincent, 2005
H. reidi	0.51	Pier colonized by different biological holdfast such as macroalgae, sponges cnidarians and tunicates	Rio Grande do Norte, Brasil	Visual census	Dias and Rosa, 2003
	0.006	Mangroves	Rio Grande do Norte, Brasil	Visual census	Dias and Rosa, 2003
	0.18	Rocky shores with macroalgae	Rio de Janeiro, Brazil	Focal grid	Freret-Meurer and Andreata, 2008
H. whitei	North: 0.215 South: 0.088	Seagrass (*Posidonia*)	Sydney, Australia	Focal grid	Vincent *et al.*, 2005

Table 1.2 Recorded seahorse densities (Individuals·m^{-2}) using destructive methods (modified from Foster and Vincent, 2004; Masonjones, 2011).

SPECIES	DENSITY	HABITAT	LOCALITY	STUDY TYPE	REFERENCE
H. erectus	CPUE data	Seagrass beds	Florida, USA	Bycatch from fishing gears	Baum *et al.*, 2003
H. fuscus	CPUE data	Dead coral reef, seagrass and seaweeds	Gulf of Mannar, India	Bycatch from fishing gears	Murugan *et al.*, 2011
H. guttulatus	Catch data		Ria Formosa Lagoon, Portugal	Capture: Experimental seines and trawls	Curtis and Vincent, 2006
H. kelloggi	Catch data	Gorgonians	Peninsular Malaysia	Capture: Pushnets	Choo and Liew, 2003
H. kuda	Catch data	Macroalgae, eelgrass and mangrove roots	Peninsular Malaysia	Capture: Pushnets	Choo and Liew, 2003
	CPUE data	Dead coral reef, seagrass and seaweeds	Gulf of Mannar, India	Bycatch from fishing gears	Murugan *et al.*, 2011
H. spinosissimus	Catch data	Octocorals, macroalgae, sponges and seagrass	Peninsular Malaysia	Capture: Pushnets	Choo and Liew, 2003
	CPUE data	Dead coral reef, seagrass and seaweeds	Gulf of Mannar, India	Bycatch from fishing gears	Murugan *et al.*, 2011
H. trimaculatus	Catch data	Octocorals, macroalgae, sponges and seagrass	Peninsular Malaysia	Capture: Pushnets	Choo and Liew, 2003
	CPUE data	Dead coral reef, seagrass and seaweeds	Gulf of Mannar, India	Bycatch from fishing gears	Murugan *et al.*, 2011
H. zosterae	0.084	Seagrass beds	Tampa bay, USA	Capture: Long pushnet transect	Masonjones *et al.*, 2010

1.3 SEAHORSE AQUACULTURE

1.3.1 GENERAL OVERVIEW

1.3.1.1 History

In general, the world's aquaculture industry has positively increased in the last half-past century. According to Food and Agriculture Organization (FAO) (2008), the production of fish for food has reached the 50 million tonnes per year. This global fact also has a direct impact on world ornamental trade. It is estimated that more than 2 billion live ornamental fish are moved annually worldwide (Monticini, 2010). Thus, aquarium hobbyists have become a rapidly growing sector of the industry (Tlusty, 2002). Nowadays, almost 2 million people keep marine aquariums (Wabnitz *et al.*, 2003), mainly in industrialized countries (Koldeway and Martin-Smith, 2010). Nevertheless, only 2% of marine ornamentals come from aquaculture sources (Moe, 2001). For this reason, the development of sustainable ornamental aquaculture industry could have a positive effect on the conservation of wild stocks. Furthermore ornamental fisheries related to aquaculture activities must be implemented in a sustainable way (Molina and Segade, 2011).

In this sense, seahorse populations have suffered a progressive decline in the last decades due to their overexploitation and habitat loss (Vincent, 1996; Martin-Smith *et al.*, 2004). Dried seahorses are extensively exploited in traditional medicine, particularly traditional Chinese medicine (TCM), and, to a lesser extent, as curios, while live seahorses are traded as aquarium fish (Vincent, 1996; Koldeway and Martin-Smith, 2010). Thus, aquaculture could be an answer to the global demand for these animals in traditional medicine and ornamental trade (Vincent, 1996; Payne and Rippingale, 2000), reducing animal collection in the wild (Woods, 2000a).

The first references related to seahorse aquaculture are relatively recent. Although some enthusiasts had already breed *H. trimaculatus* in captivity in 1957 (China; Fan, 2005), the first serious efforts to breed seahorses were carried out in early 1980s in different Asian countries (China and Japan) and Australia (Koldeway and Martin-Smith, 2010). However, these facilities had to deal with the scarce knowledge about seahorse life-history, nutritional requirements, behaviour and diseases, affecting their success. The recent research effort in seahorse husbandry and breeding techniques performed in the last 15 years, have allowed

to project larger-scale seahorse farms in Australia, USA and New Zealand (Forteath, 1997; Koldeway and Martin-Smith, 2010) (Fig. 1.5) in the early 2000s. Nowadays, there were reported at least 28 seahorse operation infrastructures in 15 different countries where many of the common and larger species of seahorses such as *H. abdominalis*, *H. barbouri*, *H. kuda*, *H. reidi* and *H. erectus* are kept and bred in captivity mainly for aquarium purposes (Kuiter, 2009; Koldeway and Martin-Smith, 2010) and also in low proportion for dried seahorse market (Vincent *et al.*, 2011).

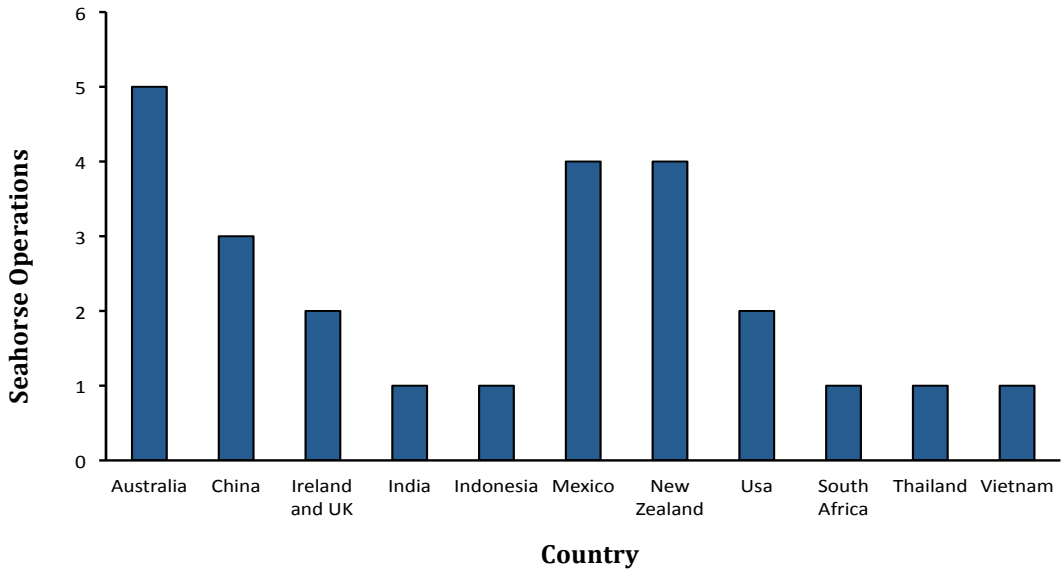

Figure 1.5 Seahorse aquaculture operations in the early 2000s (Data from Koldeway and Martin-Smith, 2010).

However, despite global seahorse demand for trade, the expansion of large-scale aquaculture facilities in developed countries may be limited by the high production cost compared to the retail price. On the contrary, in developing countries, limitations may be related to technical problems associated to rearing activities and diseases (Koldeway and Martin-Smith, 2010).

1.3.1.2 Seahorse trade

As it is well known, seahorses (*Hippocampus spp*.) are traded extensively for use in traditional medicine (TM), as aquarium fish, and as curiosities (Vincent, 1996). However, ninety-five percent of seahorses in trade are sold dried for use in

TM and particularly in traditional Chinese medicine (TCM; Tang, 1987; Vincent *et al.*, 2011). While the aquarium trade, hobby market and public aquariums, use fewer seahorses than the dried trade, it places heavy pressure on particular populations or species (Vincent, 1996). Otherwise, little is known about the curio trade for seahorses but, globally, large numbers are involved. Accordingly, large numbers of dried seahorses have been sold as curios in markets of ornamental shops (Vincent, 1996; Vincent *et al.*, 2011) all over the world (Fig. 1.6).

Figure 1.6 Dried seahorse retail in Joal Fadiouth (Senegal) (A, B) and Marseille harbour (France) (C) (source: J.F. André and F. Otero-Ferrer).

In response to concerns about potential impacts of trade upon wild seahorse populations and to increase their culture sustainability, all seahorses and their relatives have been included in Appendix II of CITES (Convention for the International Trade in Endangered Species of Wild and Flora) in 2002, which was implemented in May 2004. Normally CITES regulates seahorse trade, favouring their culture in a sustainable way without negative interference with wild stocks (Koldeway and Martin-Smith, 2010). However, trade records from CITES should be

viewed with caution due to their recent application, species misidentification or export/import information gaps (Vincent *et al.*, 2011). Another source of information on live seahorses in the aquarium trade is the Global Marine Aquarium Database (GMAD). GMAD includes data from representative wholesale exporters and importers of marine aquarium species (also seahorses) but only limited from 1988 to 2003 (Koldeway and Martin-Smith, 2010).

Despite these limitations, these data are the only available way to examine temporal trends and changes in seahorse trade (live or dried) due to aquaculture or wild-caught animals supply (Koldeway and Martin-Smith, 2010). Recently, additional global trade surveys were conducted over the period 1993–1995 and 1998–2001 (McPherson and Vincent, 2004; Baum and Vincent, 2005; Giles *et al.*, 2006; Martin-Smith and Vincent, 2006; Perry *et al.*, 2010; Vincent *et al.*, 2011), being useful to compare with data of international authorities.

1.3.1.2.1 Live seahorse trade:

According to Vincent *et al.* (2011) the live seahorse trade involves tens to hundreds of thousands of animals annually. Generally, Southeast Asian countries are the major suppliers of live specimens, principally exported to both North America and European Union (Koldeway and Martin-Smith, 2010; Evanson *et al.*, 2011; Vincent *et al.*, 2011). The lower numbers reported in the CITES compared to data reported by *in situ* surveys (Table II in Vincent *et al.*, 2011) may be related to illegal trade still observed in many countries (Evanson *et al.*, 2011). Thus, CITES trade records showed that tank-raised or cultured animals comprised 80% (2008) of seahorse live trade (Koldewey and Martin-Smith, 2010), in contrast to recent reports in where live trade derived almost entirely from wild populations (Vincent *et al.*, 2011).

Independently of quantities, seahorses live trade derived from a captive breed origin has experienced an increasing trend in the last decade probably related to technical implementation in seahorse aquaculture facilities. Nowadays, live seahorse trade provided by aquaculture includes almost 50% of seahorse species involved in live trade (Koldeway and Martin-Smith, 2010) (Fig. 1.7). This trend is also reported in GMDA data during overlapped periods with CITES. These data include 32 different species in where 99% of captive reared seahorses

grouped only 7 species *(H. abdominalis, H. breviceps, H. barbouri, H. comes, H. ingens, H. kuda* y *H. reidi)*. Other species appeared as good opportunities to diversify seahorse captive reared live trade (*H. coronatus, H. erectus, H. hixtrix, H. zoosterae, H. guttulatus* y *H. hippocampus*) (Fig. 1.7).

1.3.1.2.2 Dried seahorse trade

Asian countries remain the main source and destination for dried seahorses. According to Vincent *et al.*, (2011), most specimens are provided from trawl by-catch (mainly Malaysia and Thailand; Koldeway and Martin-Smith, 2010), although target species fisheries from Philippines and India are also important.

The majority of the dried trade targets the traditional medicine market. Thus, data exports of dried seahorses reported by CITES are quite variable, ranging between 1 to more than 10 million per yr^{-1}. This value is lower compared to data registered in other surveys, involving over 20 million of seahorses per yr^{-1} (Vincent, 1996; McPherson and Vincent, 2004; Baum and Vincent, 2005; Giles *et al.*, 2006; Vincent *et al.*, 2011). Thus only the estimated annual trawl catches of seahorses in Malaysia and Thailand overcome the 3 million specimens (1998-1999; Perry *et al.*, 2010), and nearly all were purchased as dried seahorses. Hence, as in live trade, the difference is likely to be a result of under-reporting trade data from CITES sources (Koldeway and Martin-Smith, 2010). Meanwhile, insignificant proportion of captive breed seahorses are included as dried in the international trade (<0.02% overall from Australia and New Zealand; Koldeway and Martin-Smith, 2010; Vincent *et al.*, 2011) due to the higher cost production of reared animals compared to wild caught, in particular with those provided by bycatch fisheries (Baum and Vincent, 2005; Giles *et al.*, 2006; Meeuwing *et al.*, 2006; Koldeway and Martin-Smith, 2010; Perry *et al.*, 2010; Vincent *et al.*, 2011).

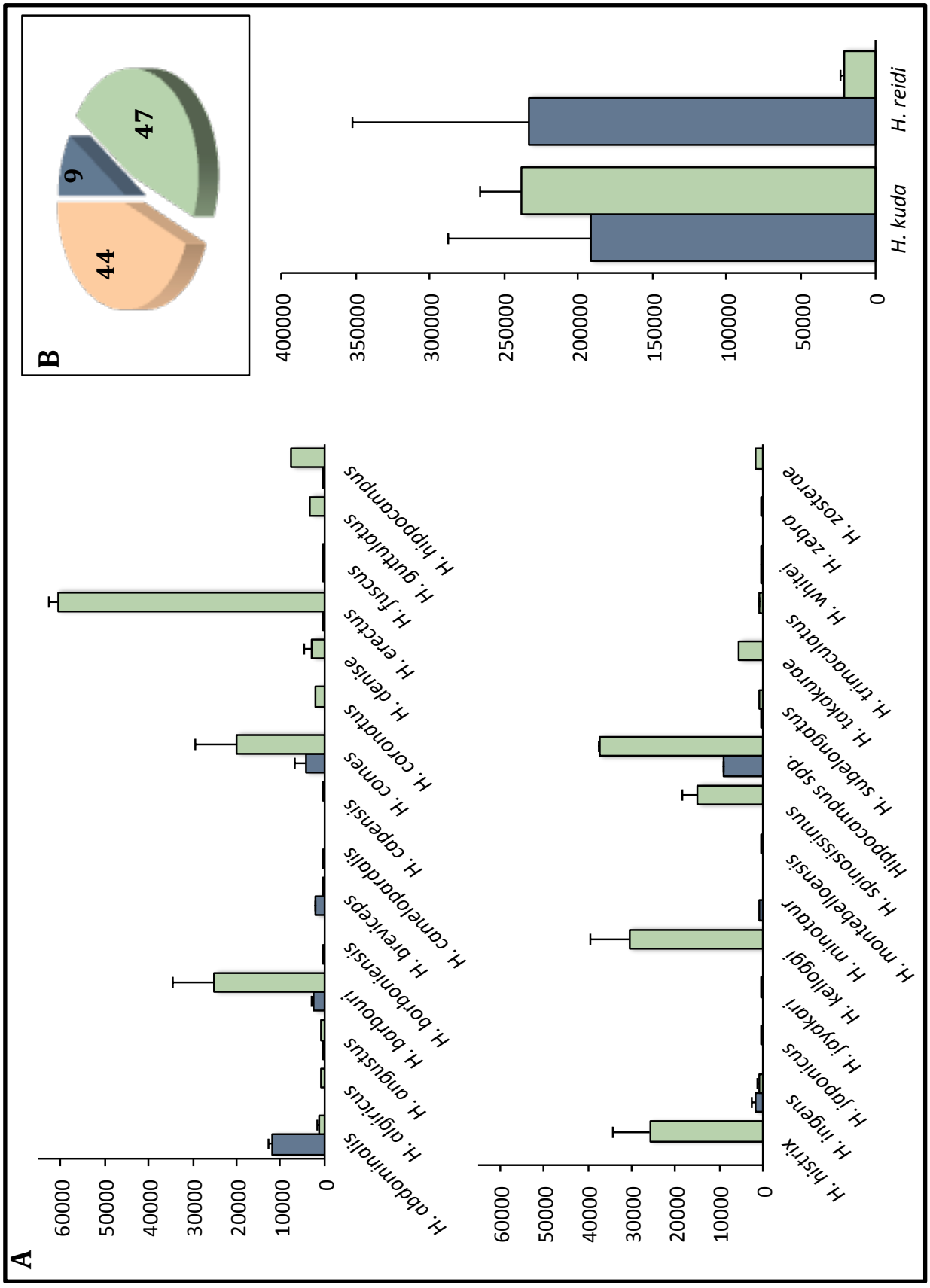

Figure 1.7 **(A)** Number of live seahorses species traded internationally as recorded in the CITES trade database by declared species and source between 1997-2008. **(B)** Percentage of Live seahorse trade species separated by source. Blue colour represents captive-reared animals, green shows wild-caught animals and orange includes both sources (data modified from Koldeway and Martin-Smith, 2010).

1.3.1.3 Seahorse culture

As described before, the global demand for seahorses has produced an enormous development of their culture in the last decades. This feature can be associated overall with improvements in technical (facilities and husbandry techniques) as well as nutritional issues, related to all seahorse life-history stages. However this performance has also been correlated with the increase of seahorse densities on captivity that made them more vulnerable to different pathologies. Here below, a review of these topics.

1.3.1.3.1 Facilities and husbandry

Different materials are employed to maintain and rear seahorses in captivity. Thus, glass or acrylic (PVC or polyethylene) aquariums or even large concrete ponds are frequently used to keep newborn, juveniles or adults (Woods and Martin-Smith, 2004; Ortega-Salas and Reyes-Bustamante, 2006; Martinez-Cardenas and Purser, 2007; Palma *et al.*, 2008; Planas *et al.*, 2008). Also *in situ* structures such as bamboo sea cages have been investigated as a possible alternative to grow out seahorses, providing a sustainable option to local fishermen (García and Hilomen-García, 2009).

On the other hand, seahorse facilities do not have any special features regarding their shape or volume, although in broodstock aquariums, tank height is an important feature to aquarium design. Thus, seahorse pairs need minimal water column to enable them to perform successful courtship and egg transfer during mating events (Vincent and Sadler, 1995; Koldeway, 2005; Kuiter, 2009). Also, several artificial holdfasts such as plastic plants, nets or PVC pipes must be placed inside tanks to provide seahorse attachments and then reduce stress in captivity (Kuiter, 2009). Otherwise, newborn seahorses are usually removed from broodstock tank after birth, and then placed into rearing tanks with different sizes and shapes (Koldeway and Martin-Smith, 2010).

Concerning seahorse husbandry parameters, the data reported in peer-reviewed publications reveals that these animals might be able to tolerate big variations in temperature and salinity, and also high ammonia levels (Kuiter, 2009; Koldeway and Martin-Smith, 2010). Concerning photoperiod conditions, some authors employed natural light cycle for most of the year (Villares, 2006; Kuiter,

2009). Under artificial light conditions, these values can change along species and life-history stage although they usually range among 10-16h light and 8-14h dark (Table 5; Koldeway and Martin-Smith, 2010). For larval stages, recent studies have shown that extended photoperiod (no more than 16h) can have a positive effect on growth (Kuiter, 2009).

Otherwise, even if seahorses are described as robust animals, high stocking densities have been shown to have a negative effect on growth and survival of adults Woods, 2003c; Kuiter, 2009; Lin *et al.*, 2009b; Gomez-Jurado, pers. comm.). Nonetheless, juveniles less than 2 months old can be stocked quite densely without effect on survival (Kuiter, 2009). Data concerning stocking densities employed in seahorse experiments are summarised in Tables 1.3 and 1.4. Moreover, a moderate water-flow is always desired, enough for ample water turnover and essential to increase food distribution in the tank overall in early larval stages (Kuiter, 2009). Hence, when strict control is applied to water quality, cleaning protocols and feeding regimes (Planas *et al.*, 2008), seahorses respond best to the aquaculture environment (Wilson and Vincent, 1998).

1.3.1.3.2 Feeding

Feeding seahorses in captivity has provided some of the initial challenges for seahorse aquaculture. The provision of suitable food sources for all seahorse life-history stages (overall offspring and broodstock), or the impossibility to be weaned to inert food, have been identified as some of the main constraints for seahorse aquaculture large-scale development (Adams *et al.*, 2001; Chang and Southgate, 2001; Lin *et al.*, 2007; Kuiter, 2009). No feeding protocols have been defined for most seahorse species; therefore one of the main topics in seahorse research in the last years concerned nutritional issues (Table 5 in Koldeway and Martin-Smith, 2010). A review of the principal advances in feeding protocols divided by life-history-stage is presented below in Tables 1.3 and 1.4. In order to avoid mistakes with literature cited, all offspring with less than 1 day old were registered with the term “newborn”, and the others were recorded as “juveniles”, although the term “seahorse larvae” has been recently described as a previous stage of seahorse juveniles (Novelli *et al.*, 2010).

a. Offspring and juveniles

The relatively large size of offspring (compared to most other marine fish larvae) should make it easier to rear them (Koldeway and Martin-Smith, 2010). Even if some experiments have been conducted with artificial food (Woods, 2003b; Wilson *et al.*, 2006), seahorses need live food to survive in the first few weeks after their release from the male pouch. Thus, live copepods or other zooplanktonic animals caught in the wild have been used in seahorse culture with high survival rates (Gardner, 2004; Bentivegna, 2006; Jones and Lin, 2007). However, copepods are not often used for rearing seahorse, as reliable large-scale production methods are usually difficult to attain (Payne and Rippingale, 2000). By contrast, rotifers and *Artemia* are commonly used as live prey food organisms in aquaculture, and have been also employed in seahorse breeding with different degrees of success, depending upon animal size and species (Damerval *et al.*, 2003). Thus rotifers have been usually used in seahorse breeding, combined with other live prey such as *Artemia*, copepods or both. This is due to their adequate size and better digestibility for the first 5 days of culture, which is believed to be the critical bottleneck in seahorse rearing (Damerval *et al.*, 2003; Jones and Lin 2007). Other authors used exclusively *Artemia*, copepods, or both, with other seahorse species born in aquaria with varied results (Reyes-Bustamante and Ortega-Salas, 1999; Wilson and Vincent, 1998; Payne and Rippingale, 2000; Woods, 2000a, b; Choo and Liew, 2006; Gonzalez *et al.*, 2006; Jones and Lin, 2007; Martinez-Cardenas and Purser, 2007; Sheng *et al.*, 2007; Truong and Hoang, 2007) (Table 1.3). Moreover, high survival rates in breeding experiments have been related to the enrichment of the live prey before feeding, sometimes not enough to cover seahorse nutritional requirements and also to the use of proper acclimation procedures using overlapping feeding protocols among different kinds of foods.

b. Adults and broodstock

In comparison with juveniles, few studies have been conducted on feeding habits and nutritional requirements of different seahorse species (Table 1.4). Little crustaceans such as amphipods, decapods or mysids have been reported as their main food in their natural environment (Kitsos *et al.,* 2008). Thus, feeding regimes based on these preys have been successfully employed for seahorse grow out and

reproduction. Nevertheless in commercial seahorse aquaculture, the collection of wild live food such as mysids is unreliable, not only being restricted by legislation, but also probably having a negative impact on the ecosystems (Woods and Valentino, 2003). Meanwhile *Artemia* brine shrimps have been already employed in adult seahorse husbandry (Olivotto *et al.* 2008; Planas *et al.*, 2008). *Artemia* are easily cultured and commercially available (Wong and Benzie 2003; Woods and Valentino 2003; Planas *et al.* 2008), although frequently they must be adequately enriched or combined with wild prey to increase their nutritional profile (Table 1.4). Overall for broodstock, the importance of good feeding during reproduction periods has been recognised as one of the main factors on spawning quality of teleost fish (Luquet and Watanabe 1986; Fernández-Palacios *et al.*, 2011) and therefore also in seahorses (Wong and Benzie 2003; Lin *et al.*, 2006).

Accordingly, adult seahorse aquaculture would require new methods that allow the culture of these little crustaceans or provide improvements on enrichment protocols as already done with seahorse rearing techniques (Ortega-Salas and Reyes-Bustamante, 2006; Olivotto *et al.*, 2008).

Table 1.3 (3 following pages) Feeding parameters for juvenile seahorse aquaculture research published in peer-reviewed journals arranged by species (Modified from Koldeway and Martin-Smith, 2010). For meaning of abbreviations please see footnotes to table.

SPECIES	AGE	DENSITY (sh/L)	FOOD ITEMS	FOOD RATIO	FREQUENCY (times/day)	REFERENCE
abdominalis	>42dab	1/3	(L)Enriched *Artemia*	100/3L	1	**Martinez-Cardenas and Purser, 2007**
	>7dab	1/3	(L)Enriched *Artemia*	100/3L	1	**Martinez-Cardenas and Purser, 2007**
	>3dab	15/3	(L)Enriched *Artemia*	7.5%BWW	2	**Martinez-Cardenas and Purser, 2007**
	Nb	NR	(L)Enriched *Artemia*	NR	2	**Martinez-Cardenas *et al.*, 2008**
	>30dab	4/25	(L)Enriched *Artemia*	2.5%BWW	2	**Shapawi and Purser, 2003**
	Nb	45/25	(L)Enriched *Artemia*, (L)Mysids,(AF)Pellets, (AF)Crumble food	2.5%BWW	2	**Wilson *et al.*, 2006 (5 treatments)**
	Nb	NR	(L) Enriched Artemia	1000/Nb/day	NR	**Woods, 2000a**
	>7,30dab	15/2	(L) *Artemia*	50/2L	1	**Woods, 2000b (2 treatmens)**
	>180dab	9/9	(L)Enriched *Artemia*	*Ad libitum*	NR	**Woods, 2003a**
	Nb	10/9	(L) Enriched *Artemia*+(AF) golden perls	AF:33%BWW Art:1/mL	3	**Woods, 2003b (5 treatments)**
	>30dab	10/9	(L) Enriched *Artemia*+(AF) golden perls	AF:33%BWW Art:1/mL	3	**Woods, 2003b (5 treatments)**
	>60dab	7/9	(L) Enriched *Artemia*+(AF) golden perls	AF:33%BWW Art:1/mL	3	**Woods, 2003b (5 treatments)**
	>30dab	10/9	(L) Enriched *Artemia* and (F) copepods	Cop:33%BWW Art:1/mL	3	**Woods, 2003b (5 treatments)**
	>60dab	7/9	(L) Enriched *Artemia* and (F) copepods	Cop:33%BWW Art:1/mL	3	**Woods, 2003b (5 treatments)**
	>180dab	1,2,5/L	(L)Enriched *Artemia* + (L)amphipods	*Ad libitum*	3	**Woods, 2003c (3 treatments)**
	>300dab	5/9	(L)Enriched *Artemia*, (F)mysids	25%BWW	NR	**Woods and Valentino, 2003 (3 treatments)**
barbouri	Nb	33/35	(L)Enriched *Artemia*	1-5/mL	Not below 2/mL	**Wilson and Vincent, 1998**
	>7dab	NR	(L) Copepods	1-10/mL	Not below 2/mL	**Wilson and Vincent, 1998**
	>29dab	NR	(L)Mysids	*Ad libitum*	2	**Wilson and Vincent, 1998**
	>65dab	NR	(F)Mysids	*Ad libitum*	2	**Wilson and Vincent, 1998**

Species	Age	Density (sh/L)	Food items	Food Ratio	Frequency (times/day)	Reference
comes	Nb	430/3500	(L) Wild Zooplankton	*Ad libitum*	NR	**Job *et al.*, 2006**
	>7dab	NR	(L) Enriched *Artemia*	1-2/mL	2	**Job *et al.*, 2006**
	>28dab	NR	(L) Enriched *Artemia*	0.1-0.2/mL	2	**Job *et al.*, 2006**
erectus	Nb	100/38	(L) Artemia, (L) Wild zooplankton	NR	Not below 0.5/mL	**Gardner, 2004 (6 treatment)**
	Nb	15/18	(L) Enriched *Artemia*	*Ad libitum*	2	**González *et al.*, 2006**
	>2dab	15/38	(L) *Artemia*	4-6/mL	4	**Lin *et al.*, 2008**
	>16dab	15/38	(L) Enriched *Artemia*	4-6/mL	4	**Lin *et al.*, 2008**
	>41dab	15/38	(L) Enriched *Artemia* + (F) mysids	Art:3-6/J/d My: NR	NR	**Lin *et al.*, 2008**
	>70dab	6/38	(F) mysids	5%BWW	3	**Lin *et al.*, 2009a**
	>84dab	0.25,0,5,1,1.5/ L	(F) mysids	5%BWW	3	**Lin *et al.*, 2009a (4 treatments)**
	>78dab	4/38	(F) mysids	20,10,7,5%BW W/day	1,2,3,4	**Lin *et al.*, 2009a (4 treatments)**
	>90dab	4/38	(F) mysids	5%BWW	3	**Lin *et al.*, 2009a**
	>42,49,56, 63,70 dab	8/38	(F) mysids, (L) *Artemia*	3.75%BWW	4	**Lin *et al.*, 2009b (4 treatments)**
	>112dab	8/38	(F) mysids	3.75% BWW	4	**Lin *et al.*, 2009b**
fuscus	Nb	33/35	(L)Enriched *Artemia*	1-5/mL	Not below 2/mL	**Wilson and Vincent, 1998**
	>7dab	NR	(L) Copepods	1-10/mL	Not below 2/mL	**Wilson and Vincent, 1998**
	>29dab	NR	(L)Mysids	*Ad libitum*	2	**Wilson and Vincent, 1998**
	>65dab	NR	(F)Mysids	*Ad libitum*	2	**Wilson and Vincent, 1998**
guttulatus	Nb	NR	(L) Enriched rotifers	*Ad libitum*	NR	**Damerval *et al.*, 2003**
	>3dab	NR	(L) Enriched *Artemia*	*Ad libitum*	NR	**Damerval *et al.*, 2003**
	>60dab	NR	(L) Enriched *Artemia* + (L)mysids	*Ad libitum*	NR	**Damerval *et al.*, 2003**
hippocampus	Nb	NR	(L) Enriched rotifers	*Ad libitum*	NR	**Damerval *et al.*, 2003**
	>3dab	NR	(L) Enriched *Artemia*	*Ad libitum*	NR	**Damerval *et al.*, 2003**
	>60dab	NR	(L) Enriched *Artemia* + (L)mysids	*Ad libitum*	NR	**Damerval *et al.*, 2003**
ingens	Nb	12/60	(L)Enriched rotifers+ (L)Enriched *Artemia*	Rot:75/mL Art: 1-2/mL	NR	**Ortega-Salas and Reyes-Bustamante, 2006**
	>20dab	50/1000	(L) Enriched *Artemia*	2/mL	NR	**Ortega-Salas and Reyes-Bustamante, 2006**

Species	Age	Density (sh/L)	Food items	Food Ratio	Frequency (times/day)	Reference
kuda	Nb	90/60	(L)Rotifers, (L) Copepods	Rot: 15/mL Cop: 0.05/L	Rot: Not below 5/mL Cop: Not below 0.05/mL	**Celino *et al.*, 2011**
	Nb		(L)Enriched *Artemia*	NR	4	**Dzyuba *et al.*, 2006**
	0-42dab	300/3500	(L)Wild zooplankton, (L)*Artemia*, (L)mysids	1-4/mL	2	**Job *et al.*, 2002**
	>42dab	5/18	(L)Enriched *Artemia*	NR	2	**Job *et al.*, 2002**
	Nb	50/48	(L)Copepods	NR	NR	**Lin *et al.*, 2006**
	Nb	20/4	(L) Copepods	5/mL	NR	**Sheng *et al.*, 2007**
	Nb	150/10	(L) Copepods	5/mL	NR	**Sheng *et al.*, 2007**
	Nb	NR	(L)*Artemia*, mysids	*Ad libitum*	NR	**Thangaraj *et al.*, 2006**
	Nb		(L)Enriched *Artemia*	1-5/mL	Not below 2/mL	**Wilson and Vincent, 1998**
	>7dab	33/35	(L) copepods	1-10/mL	Not below 2/mL	**Wilson and Vincent, 1998**
	>29dab	NR	(L)Mysids	*Ad libitum*	2	**Wilson and Vincent, 1998**
	>65dab	NR	(F)Mysids	*Ad libitum*	2	**Wilson and Vincent, 1998**
reidi	Nb	205/50	(L)Wild zooplankton,(L)Enriched *Artemia*	5/mL	5	**Hora and Joyeaux, 2009**
	>3dab	205/50	(L)*Artemia*	5/mL	5	**Hora and Joyeaux, 2009**
	>22dab	205/50	(L+F)mysids	*Ad libitum*	5	**Hora and Joyeaux, 2009**
	Nb	33/20	(L)Enriched rotifers, (L)enriched copepods	10/mL	4	**Olivotto *et al.*, 2008 (2 treatments)**
	>6dab	33/20	(L)enriched *Artemia*, (L)enriched copepods	10/mL	4	**Olivotto *et al.*, 2008 (2 treatments)**
subelongatus	Nb	2/1	(L)Enriched copepods, (L)Enriched *Artemia*	Cop:0.5-1.2/mL Art: 0.2-0.8/mL	2	**Payne and Rippingale, 2000 (2 treatments)**
	>5dab	5/1	(L)Enriched copepods+ (L)Enriched *Artemia*	5/mL	2	**Payne and Rippingale, 2000**
trimaculatus	Nb	50/80	(L) copepods+(L) rotifers	NR	1	**Murugan *et al.*, 2009**
	>7	2/L	(L) copepods+ (L) *Artemia*	NR	1	**Murugan *et al.*, 2009**
	125dab	15/500	(L) amphipods+(L) shrimps	*Ad libitum*	2	**Murugan *et al.*, 2009**
	Nb	20/4	(L) Copepods	5/mL	NR	**Sheng *et al.*, 2007**
whitei	Nb	0.5, 1, 2/L	*Artemia*	1.5-1.7/mL	2	**Wong and Benzie, 2003 (3 treatments)**
Unidentified	>3dab	20/10	(L)Enriched *Artemia*	0.5-3/mL	2	**Chang and Southgate, 2001**

Nb: Newborn seahorse; NR: Not Reported; dab: day after birth; BWW: Body Wet Weight; L: Live; F: Frozen; AF: Artificial Food.

Table 1.4 Feeding parameters for adult seahorse aquaculture research published in peer-reviewed journals arranged by species (Modified from Koldeway and Martin-Smith, 2010). For meaning of abbreviations please see footnotes to table.

SPECIES	ORIGIN	DENSITY (sh/L)	FOOD ITEMS	FOOD RATIO	FREQUENCY (times/d)	REFERENCE
abdominalis	CR	NR	(F) mysids	NR	2	**Martinez-Cardenas *et al.*, 2008**
	WS	2/75(1:1)	(L)Enriched *Artemia*+when available: (L)Amphipods, shrimps, mosquito larvae and white worms	Art: 573±81/A/day	NR	**Woods, 2000a**
	CR	15/75	(L)Enriched *Artemia*+when available: (L) shrimps and amphipods	Art: 300/A/day S&Amp:5/A/day	NR	**Woods, 2000b**
	CR	6/23(3:3)	(F) Mysids	2.5,5,7.5,10% BWW	2	**Woods, 2005 (4 treatments)**
capensis	WS	2/50 (1:1)	(L)Enriched *Artemia* + (L)shrimp	*Ad libitum*	2	**Lockyear *et al.*, 1997**
comes	WS	12/500	(L+F) shrimp	NR	NR	**Job *et al.*, 2006**
erectus	WS	NR	(F) shrimp+(F)mysids	*Ad libitum*	3	**Gardner, 2004**
	CR	20/430	(L) *Artemia*+(F) mysids	*Ad libitum*	3	**Lin *et al.*, 2008**
guttulatus	WS	1/65	(L)Mysids+(L)*Artemia*	*Ad libitum*	NR	**Damerval *et al.*, 2003**
	WS	12/180 (6:6)*	(L+F)Enriched *Artemia*+(L+F)mysids	*Ad libitum*	2	**Faleiro *et al.*, 2008**
	WS	6/90 (3:3)*	(L+F)Enriched *Artemia*+ (L+F)mysids+(L+F)shrimp	5%BWW	2	**Palma *et al.*, 2008**
	WS	8/320 (4:4)*	(L) Enriched *Artemia*	*Ad libitum*	2	**Planas *et al.*, 2008**
	WS	4:160 (3:1)*	(L) Enriched *Artemia*	*Ad libitum*	2	**Planas *et al.*, 2008**
	WS	4:160 (1:3)*	(L) Enriched *Artemia*	*Ad libitum*	2	**Planas *et al.*, 2008**
hippocampus	WS	1/65	(L)Mysids+(L)*Artemia*	*Ad libitum*	NR	**Damerval *et al.*, 2003**
kuda	CR	NR	(L+F)Enriched *Artemia*+(L+F)mysids	NR	4	**Dzyuba *et al.*, 2006**
	CR	NR	(L)Mysids	2-5/mL	NR	**Lin *et al.*, 2006**
reidi	CR	NR	(L+F)mysids	*Ad libitum*	5	**Hora and Joyeaux, 2009**
	CR	2/200 (1:1)*	(F)*Artemia*+Mysids	*Ad libitum*	2	**Olivotto *et al.*, 2008**
subelongatus	WS	2/30 (1:1)*	NR	NR	NR	**Payne and Rippingale, 2000**
trimaculatus	CR	14/2000	(L) Shrimps, (L) amphipods	*Ad libitum*	2	**Murugan *et al.*, 2009**
whitei	WS	10/300(5:5)*	(F) mysids	*Ad libitum*	2	**Wong and Benzie, 2003**

NR: Not Reported; WS: Wild Stock; CR: Captive Reared; BWW: Body Wet Weight; L: Live; F: Frozen; AF: Artificial Food. * Inside ( ) the ratio males:females is showed

1.3.1.3.3 Diseases

The recent increase in seahorse aquaculture operations can be correlated with increases in animal densities and a possible substandard water quality conditions within facilities. As for other culture fish species, this fact makes seahorses more vulnerable to both opportunistic and/or virulent bacterial infections.

As reported by Koldeway and Martin-Smith (2010), laboratories and aquaculture facilities have reported a variety of diseases affecting different seahorse species such as *H. kuda (Vibrio harveyi;* Tendencia, 2004), *H. ingens* (flagellates; Berzins, 2005) or *H. trimaculatus* (trematodes; Shen, 1982) among others. In particular, vibriosis and mycobacteriosis are frequent diseases related to these fish (Berzins, 2005; Koldeway, 2005). With vibrio, the most frequent clinical signs include external haemorrhages and haemorrhagic liver and ascitic fluid accumulation in the intestinal cavity (Alcaide *et al.*, 2001; Tendencia, 2004). It can be treated with antibiotics mixed with food or injectable. However the treatment effectiveness is according to bacterial strain (Koldeway and Martin-Smith, 2010). Concerning mycobacteriosis, it can be presented as a chronic infection that may involve skin, sub-cutis and/or underlying skeletal muscle but also organs and organ systems, which often include the spleen, liver and kidney. Successful treatment is associated with long-term administration of antibiotics but with high toxicity for fish and also moderate efficacy (Berzins, 2005).

Finally, the major health issue in culture seahorses is associated with the bubble-gas problem. This disease has been traditionally related to gas accumulation in the brood pouch (exclusively encountered in males), subcutaneous emphysema of the tail segment and/or over-inflation of the swim bladder (Berzins, 2005). Affected fish have postural and buoyancy problems. In caudal emphysema, the gas bubbles can also be easily observed. Even if histological findings denoted no infectious agents or association with gas super-saturation of the water column, causative agents remain unclear (Berzins, 2005). Treatment attempts are most successful in cases that are caught early. Normally gas bubbles can be aspirated with a needle accompanied with a later antibiotic disinfection (Koldewey, 2005).

Accordingly, there is a lack of information about seahorse diseases and therefore, prophylaxis remains the best option to avoid disease outbreaks. Improvements in good water quality monitoring, quarantine protocols and adequate nutritional regimes can mean the difference between survival and death of sick animals and the seahorse aquaculture feasibility (Berzins, 2005; Koldeway and Martin-Smith, 2010).

1.4 SEAHORSE CONSERVATION

In recent years, several researchers have referenced the importance to establish guidelines for conservation of marine biodiversity. Some of these initiatives already implemented focused on specific ecosystems or "charismatic" species such as coral reefs, mammal's, seagrasses and seahorses (Lourie and Vincent, 2004). The selection of these "flagship-species" can be conditioned by different socio-economic and cultural factors, or their threatened status. In the seahorses' specific case, these fish are concerned by several issues related to marine conservation (Vincent *et al.*, 2011). Therefore, the efforts conducted to protect seahorse populations will produce indirect benefits for the habitats in where they live as well as for other non-target species, usually less studied.

As described before, seahorses have life histories (obligate parental care) and behaviours (site fidelity and small home ranges) that might make them particularly vulnerable to important threats that affect marine fish populations such as overexploitation and habitat loss (Jackson *et al.*, 2001; Foster and Vincent, 2004; Vincent *et al.*, 2011). Accordingly, seahorse populations might be threatened as a result of overfishing, non-selective fishing methods (bycatch), and habitat degradation (McPherson and Vincent, 2004; Baum and Vincent, 2005; Giles *et al.*, 2006). The direct or indirect fishing can affect seahorse individuals, populations and species in a variety of ways such as injuring or killing specimens, disrupting social structure by selectively capturing females, reducing reproduction by disrupting pair bonds in monogamous species, affecting cohorts differentially (sizes-selective) and damaging habitat by removing seagrasses or potentially other seahorses (Baum *et al.*, 2003; Vincent *et al.*, 2011).

Moreover, even if global demand for seahorses decreases and direct fishing activity is controlled, the fishing pressure on the wild stocks would not necessarily reduce, given the considerable quantities caught incidentally each year by non-selective fishing gears (Perry *et al.*, 2010). Thus bycatch from trawlers appears to be the largest source of seahorses in international trade (Fig. 1.8). Moreover, the trawl gear also damages many of key habitats in where seahorses are distributed (seagrass beds, coral reefs, mangroves, and estuaries; Hodgson, 1999; Kaiser *et al.*, 2002) (Perry *et al.*, 2010).

Meanwhile, habitat loss and pollution related to anthropogenic activities may also reduce seahorse holdfast availability as well as their food sources, also affecting seahorse breeding cycles and becoming more vulnerable to their predators. However, changes in habitat configuration resulting from their destruction may sometimes favour increases in certain seahorse populations. For example, habitat damage caused by seine fishing in the Ria Formosa lagoon system (Portugal) benefited *Hippocampus hippocampus* (Linnaeus 1758) while creating problems for the sympatric *H. guttulatus*. *H. hippocampus* prefers the sparsely vegetated habitats that emerge from fishing while *H. guttulatus* occurs on the more complex habitats as seagrass beds, macroalgae and colonial invertebrates (Curtis and Vincent, 2005; Curtis *et al.*, 2007).

Accordingly, a combination of direct and indirect fishing pressures and/or environmental impacts led to the inclusion of all seahorses in the IUCN Red List as Endangered, Vulnerable, or Data Deficient (IUCN, 2010). One seahorse *spp* (*H. capensis*) is on IUCN Red list as Endangered because of its restricted and fragmented distribution and habitat decline (Knysna estuary; Bell *et al.*, 2003). Seven others (all found in seahorse trade) are listed as Vulnerable, based on observed, estimated, inferred or suspected population declines (30% over a 10-year period). Finally, most seahorse species are listed as Data Deficient (DD), because there is inadequate information to make an assessment of their risk of extinction based on their distribution and population status (Dulvy *et al.*, 2003; Vincent *et al.*, 2011).

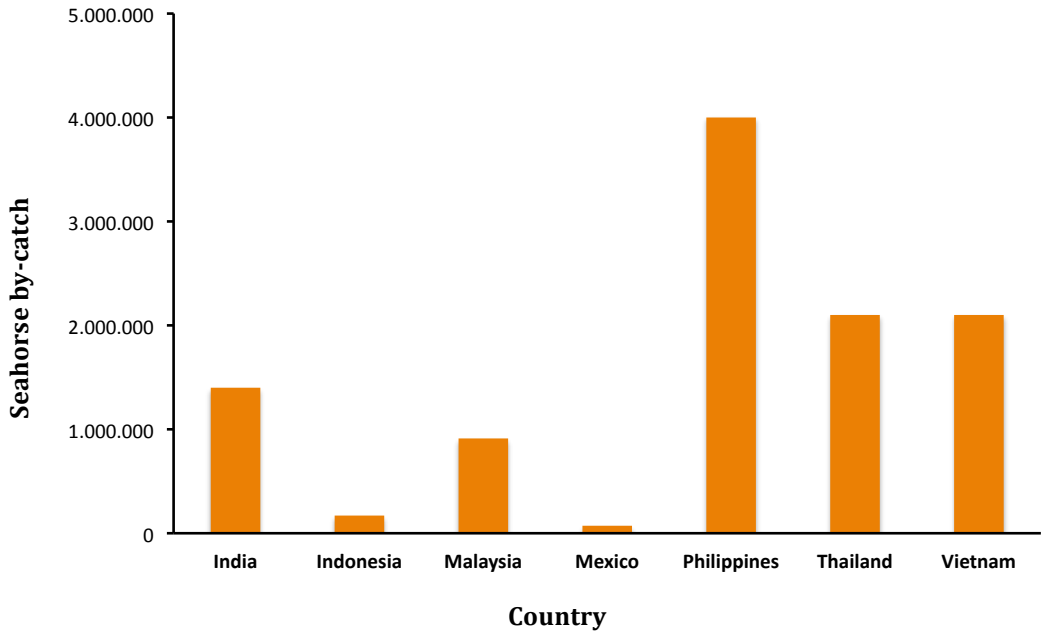

Figure 1.8 Estimates of annual seahorse by-catch by country. All estimates are from data up to 1999, except India (2001), Mexico (2000) and the Philippines (2001) (Data from Vincent *et al.*, 2011).

Meanwhile, declines in seahorse landings were reported by the majority of fishers in all regions surveyed in a series of trade surveys conducted around the world (Vincent 1996; Salin and Yohannan, 2005; Perry *et al.*, 2010; Vincent *et al.*, 2011). Also *in situ* assessments conducted in the wild reported a decrease trend in seahorse population abundances (Murugan *et al.*, 2011).

In this context, conservation and management of seahorses depends on interdisciplinary approaches such as seahorse populations, ecosystem protection, fisheries management, captive breeding, trade management and CITES and social development (Vincent *et al.*, 2011). Even if biological responses will simply not be enough, more accurate information concerning ecological variables could help to redefine species status for IUCN, establish better sustainable exploitation of seahorse fisheries (e.g. recommendation quotas, delimit trawls zones, etc.) and trade (e.g. seahorse minimum size limits), improve culture techniques and create no-take marine protected areas (Martin-Smith *et al.*, 2004), although, their beneficial effects on seahorse populations is still unclear (Samoylis *et al.*, 2007; Vincent *et al.*, 2011).

On the other hand, aquaculture operations will have conservation benefits if they reduce pressures of exploitation on wild populations (Murugan *et al.*, 2011).

This approach is particularly important on small seahorse fisheries communities, in where marine ornamental fish do present an opportunity for community-based, conservation-focused aquaculture initiatives in developing countries (Job, 2005) and providing an alternative livelihood to local people. Furthermore, setting up hatcheries in source countries would also reduce the risk associated with escapes and, by extension, the risk of introducing exotic species (Vincent and Koldewey, 2006; Wabnitz *et al.*, 2003).

Besides, captive rearing can be used to provide animals for re-stocking activities. This topic often emerges in discussion because captive-reared animals could damage wild populations by carrying disease, altering genetics and/or disrupting social and spatial behaviour (Bell *et al.*, 2003; Naylor *et al.*, 2000).

Stock enhancement could be a possible way for highly threatened seahorse populations, being considered if all other conservation approaches fail (IUCN, 1998). However, prior to mass-scale release, environmental studies might be carried on to identify the pressures that led to seahorse population decline and their effects in the wild stocks (Vincent and Koldewey, 2006; Vincent *et al.*, 2011). Additionally pilot-small-scale extremely controlled release (out of local seahorse population distribution) using genetically characterized, tagged and healthy animals can be integrated with previous environmental data to plan stock enhancement strategy employed as an ultimate resort when previous conservations actions failed.

Hence, aquaculture appears to be the solution to replace wild-caught animals or to reduce the fishing pressure over wild stocks (Woods, 2000a), however some authors denoted the importance to study ecological aspects related to population characteristics and life-history of these species (Curtis and Vincent, 2006). The combination of both variables will avoid the natural resources depletion and it will make the difference in their conservation status (Koldeway and Martin-smith, 2010).

1.5 SEAHORSE IN CANARY ISLANDS

Biological and ecological information about seahorses in Canaries Archipelago remains scarce. References about seahorse wild populations are coming from general reports (not always published) conducted in the last 30 years on general fish communities all over the Archipelago. Thus, different surveys were performed in rocky bottoms and seagrass meadows (*Cymodocea nodosa*) using UVCs and destructive methods (Table 1.5). All reports denoted the low seahorse presence in all sites studied, being also cited as an occasional species by Brito *et al.*, 2002.

Table 1.5 References about seahorse's populations in Canaries archipelago.

SPECIES	HABITAT	LOCALITY	STUDY TYPE	REFERENCE
H. hippocampus	Sandy bottoms	F,G, GC,H,L,LP,T	Dredging	Haroun and Herrera, 1991 (UP)
H. ramulosus[1]	Rocky shores	F, GC, L,T	Stationary point count	Falcón *et al.*, 1993a,b; 1996
H. hippocampus	Seagrass beds	T	Line transect/ dredging	Mena *et al.*, 1993
H. hippocampus	Rocky shores	LP,G,H	Line transect	Espino and Herrera, 2002; Espino *et al.*, 2008 (UP)
H. hippocampus	Rocky shores and seagrass beds	GC	Line transect/ seine nets	Herrera *et al.*, 2003 (UP)
H. hippocampus	Rocky shores and seagrass beds	GC	Strip transect/ Species-time count	Villares, 2006

*In where; F: Fuerteventura; G: Gomera; GC: Gran Canaria; H: El Hierro; L: Lanzarote; LP: La Palma and T: Tenerife. UP: Unpublished Data. [1] Later identified as *H. hippocampus* (Brito *et al.*, 2002)

Recently, preliminary seahorse studies were performed in 11 sites along Gran Canaria Island coast (Villares, 2006). After 63 dives, only 13 seahorses were recorded using strip transects, and species-time random counts technique. All animals, morphologically identified as *H. hippocampus* (Lourie *et al.*, 2004), were mostly associated with shallow inshore waters among rocks, artificial holdfast and seagrass meadows patches and also mainly concentrated in two zones, Melenara and Sardina del Norte bays (Villares, 2006). However, no data concerning animal abundance, population structure and appearance along spatial and seasonal scale, were published, and comparisons with values obtained during this study were not feasible and therefore their reliability could not be quantitatively assessed.

On the other hand, other references related to seahorse populations in Canary Islands, reported the presence of the two European seahorses spp., *Hippocampus guttulatus* (Cuvier 1829) and *H. hippocampus* (Linnaeus 1758) (Brito, 1991). However, these records were in controversy since many years (Brito *et al.*, 2002). Although *H. guttulatus* or other seahorse species presence cannot be discarded in other islands (López *et al.*, 2010), recent surveys (Villares, 2006) and molecular marker studies conducted in Gran Canaria Island denoted only the presence of *H. hippocampus* specimens (López *et al.*, 2010).

According to IUCN (2010) and Seahorse's taxonomic guides (Lourie *et al.*, 2004), *H. hippocampus* is catalogued as "Data Deficient" (DD) species, which means that there is currently insufficient information available on this species to make a direct, or indirect, assessment on its risk of extinction based on its distribution and/or population status (Planas *et al.*, 2008).

In general, the short-snouted seahorse, *H. hippocampus*, has an extremely broad latitudinal range, ranging on Atlantic coasts from Wadden Sea southward to Portugal, the Mediterranean and Black Seas, including Sea of Azov. Elsewhere, the Canaries and the African coast southwards to the Gulf of Guinea (Whitehead, 1986; Lourie *et al.,* 2004; Woodall, 2010).

1.6 OBJECTIVES

As described before, the seahorse populations have suffered a worldwide progressive decline trend in the last decades (Vincent *et al.*, 2011). Meanwhile, the information concerning seahorse wild stocks in Canary Islands is insufficient to make an assessment of their risk of extinction based on their distribution and population status (IUCN, 2010). Therefore their conservation and management need an interdisciplinary approach based on Ecology and Aquaculture as main research tools.

Temperate waters seahorse species as reported in the Canarian Archipelago (Brito *et al.*, 2002) have been in general scarcely studied. Thus, population assessment was conducted to estimate different ecological data related to wild seahorse stocks such as abundance, population structure or species diversity. Accordingly, specific conservation actions based on their spatial and temporal

distribution could be established, leading to their sustainable management. This assessment has been conducted in Gran Canaria Island, one of the seven islands of the Archipelago, where seahorses are regularly observed.

Concerning breeding in captivity, few peer-review journals have described parameters or protocols related to husbandry and culture (Boisseau, 1967; Damerval *et al*., 2003) of seahorse species observed in these latitudes. Moreover, no data regarding first feeding regimes in offspring or spawning quality in broodstock have been published. This is why aquaculture methods were also applied on seahorse species observed in the wild, to close their life-cycle in captivity. Hence, the optimization of seahorse culture techniques could allow decreasing pressure over wild stocks, enlarging the knowledge about biological topics that cannot be studied in the wild (Curtis, 2007), and also producing easily animals for an eventual stock enhancement if required when all other conservations measures failed (IUCN, 1998).

Finally we have tried to combine some data previously obtained with ecology and aquaculture tools, to design and carry out a pilot scale reared-seahorse release in the wild. This study could provide useful cues about acclimatization of captive-reared animals to wild conditions.

Data obtained through this thesis could be employed as a reference for future studies developed in the same areas or even as a guideline to study other seahorse populations inside Canaries.

According to this interdisciplinary approach several specific objectives were addressed as follows (Fig. 1.9):

1. To improve the knowledge of *Hippocampus hippocampus* wild populations on Gran Canaria Island, along spatial and seasonal scale (**Study I** – **Chapter 3**).

2. To identify seahorse species on Gran Canaria Island following morphometric as well as genetic criteria.

   Not previously expected, this objective was derived from population assessment study (Chapter 3). However due to the importance of the results obtained, it was addressed in a separate chapter (**Study II** – **Chapter 4**).

3. To evaluate the effect of first feeding regimes on survival and growth of newborn short-snouted seahorses *H. hippocampus* (**Study III - Chapter 5**).

4. To compare the effect of live prey on the spawning quality of *H. hippocampus* broodstock (**Study IV - Chapter 6**).

5. To evaluate the effect of some seahorse life-history parameters on settlement of captive-bred *H. hippocampus* released in the wild (**Study V - Chapter 7**).

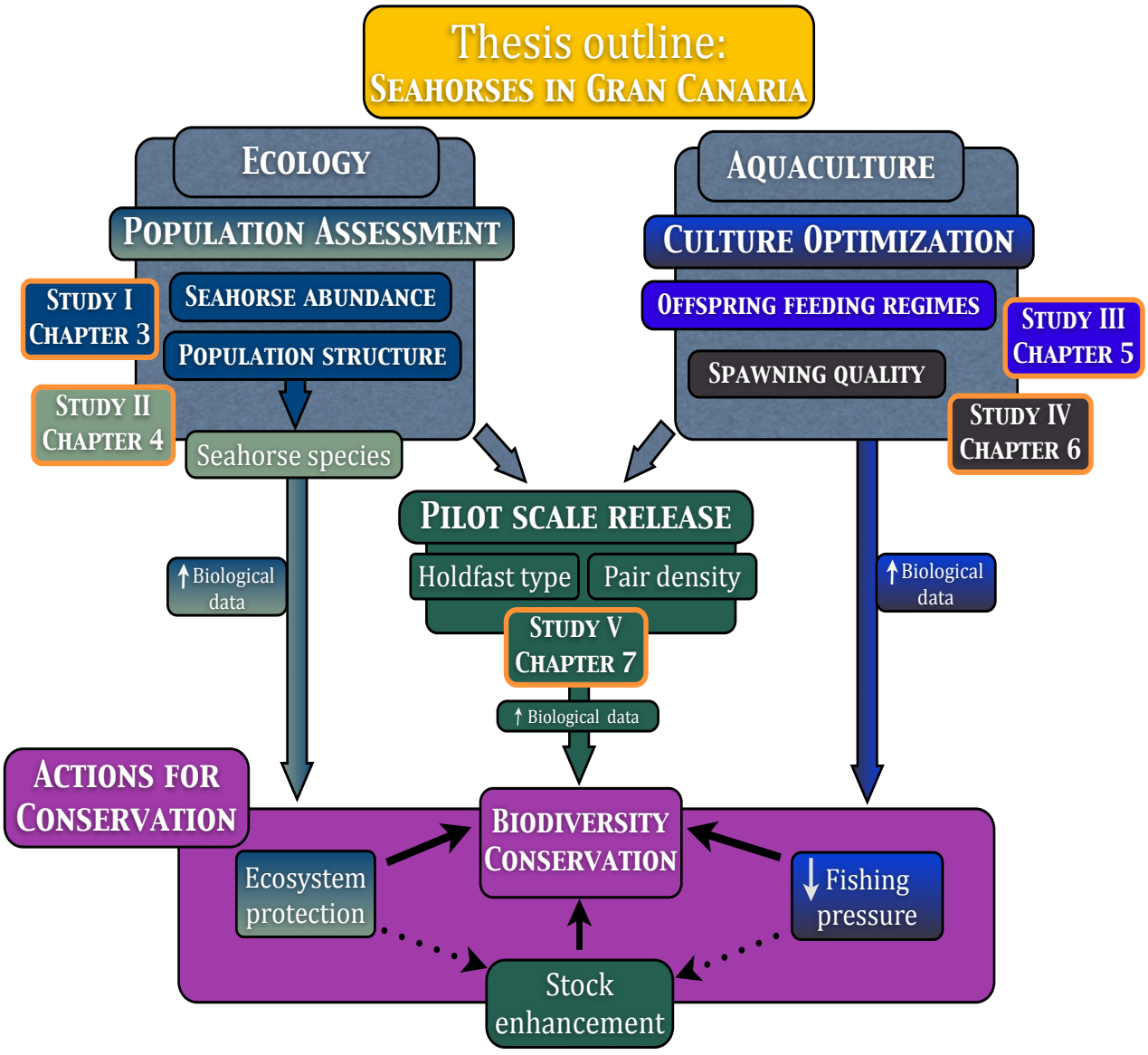

Figure 1.9 Schematic diagram of thesis including the various studies performed and their relation with conservation actions. Arrows with dots note the option employed only when other conservation actions failed.

Chapter 2

Material and Methods

Along this thesis, the material and methods employed can be divided in two main sections regarding *in situ* or *ex situ* studies. *In situ* studies are referred to seahorse population assessments (**study I – Chapter 3**) and stock enhancement research (**study V - Chapter 7**). On the other hand, *ex situ* experiments concerned rearing (**study III - Chapter 5**) and spawning quality (**study IV - Chapter 6**) studies in relation to optimization of seahorse reproduction in captivity. At the same time, the experiments conducted to identify a seahorse species sighted during **study I** (**study II - Chapter 4**) are also included in this section. A general description of material and methods is represented in Figure 2.1.

All seahorses handling performed along this thesis, including capture for rearing experiments and release of captive-bred animals, have been carried out with the official permissions of Gobierno de Canarias (Resolución nº, 2007/22, 2007/302 and 2010/0753).

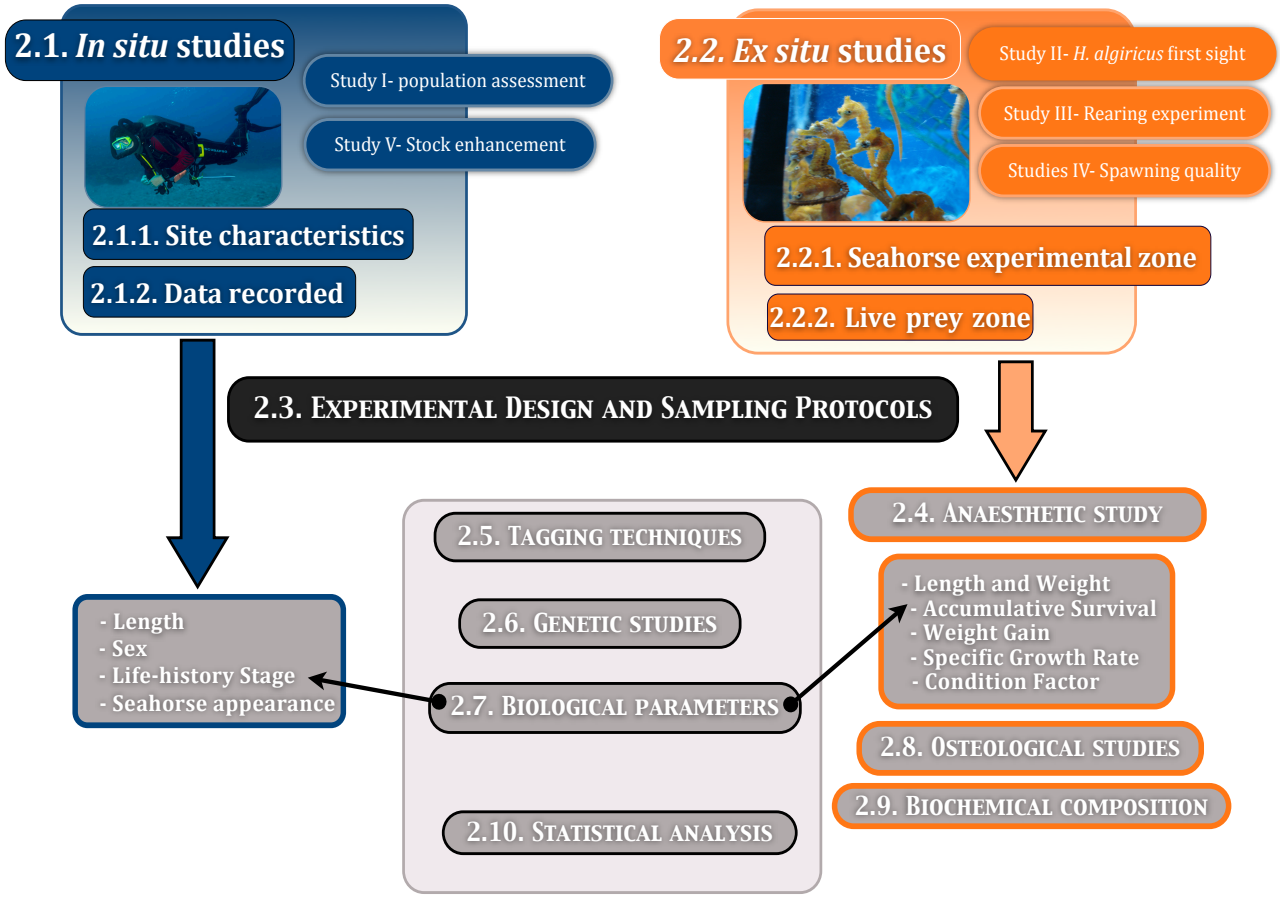

Figure 2.1 General description of material and methods.

2.1 *IN SITU* STUDIES

2.1.1 SITE CHARACTERISTICS

Before starting seahorse population assessment, it is important to define the characteristics of the study area and the parameters that will be recorded along the surveys. Thus, all *in situ* studies carried out along this thesis were conducted in the shallow coastal waters of Gran Canaria Island (North-Eastern Atlantic Ocean), one of the seven major islands included in the Canarian Archipelago (Fig. 2.2).

Seahorse population assessment (**study I**) was conducted simultaneously in two little harbour-bays called Sardina del Norte and Melenara, respectively located in the Northern (28° 9' N, 15° 42' W) and Eastern (27° 59' N, 15° 22' W) parts of the island (Fig. 2.2). Meanwhile, Melenara bay was also chosen to perform the stock enhancement experiment (**study V**).

Little underwater cliffs colonized by macroalgal beds characterize both sites. In Melenara, seaweed coverage is mainly dominated by Phaeophytes (e.g. *Cystoseira abies-marina*, *Sargassum sp* and *Stypocaulon scoparium*in) (Herrera *et*

al., 1993). On the contrary, algal coverage in Sardina del Norte is dominated by calcareous red algae (*Corallinacea*) accompanied by groups of Phaeophytes (*Dictyota sp.*, *Saragassum sp.*, *Padina pavonica* and *Colpomenia sinuosa*), and green algae (*Derbesia marina*) in lesser percentage (Hernández Zerpa *et al.*, 2010).

Just below rocky areas, sandy bottoms with dispersed naturalized-anthropogenic substrates like ropes, hoop-nets combined with rocky patches are also sparsely found in both sites (Villares, 2006). Also, in the northern part of Melenara bay there are some patches of *Cymodocea nodosa* seagrass (Espino *et al.*, 2008).

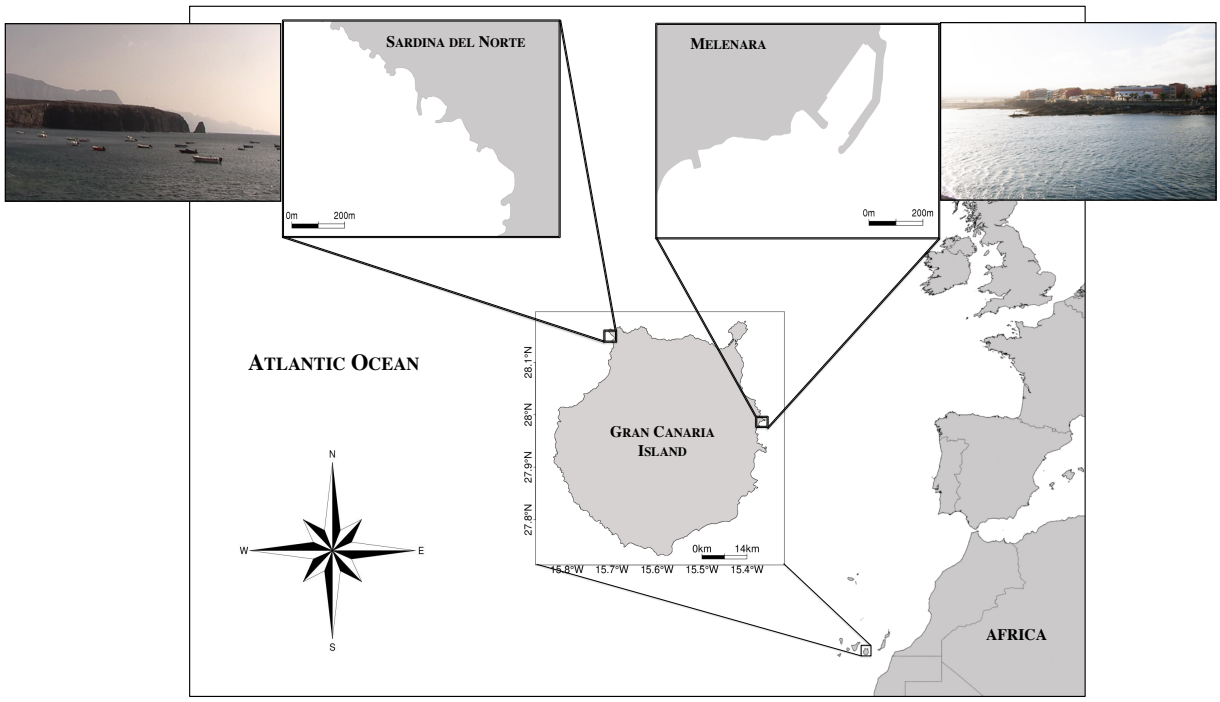

Figure 2.2 Map of Gran Canaria Island showing the studied sites, Melenara and Sardina del Norte bay (source: J.L. Otero, F. Otero-Ferrer and E. Turpin).

2.1.2 DATA RECORDED

All *in situ* studies were conducted using SCUBA and data was recorded on underwater PVC data sheet (Fig 2.3). Thus, information such as time, date, time spent underwater, depth, sea temperature, habitat sampled, seahorse sights and some biological parameters associated to these animals (described in detail in section 2.7) was also noted during each dive. Dive parameters were recorded using a dive computer (Aladin prime, Scubapro-Uwatec, Oberwilerstrasse, Switzerland).

Each seahorse sight was registered using an alphanumeric code: KT for Melenara and KS for Sardina, adding also a correlative identification number. When environmental conditions were adequate, animals were also photographed and tagged *in situ*, using visible implant fluorescent elastomer (VIFE; Northwest Marine Technology, Inc., Shaw Island, Washington) following the techniques described by Morgan and Martin-Smith (2004). These tags have already been employed in other seahorse mark-recapture studies without effect on mortality (Curtis, 2006a).

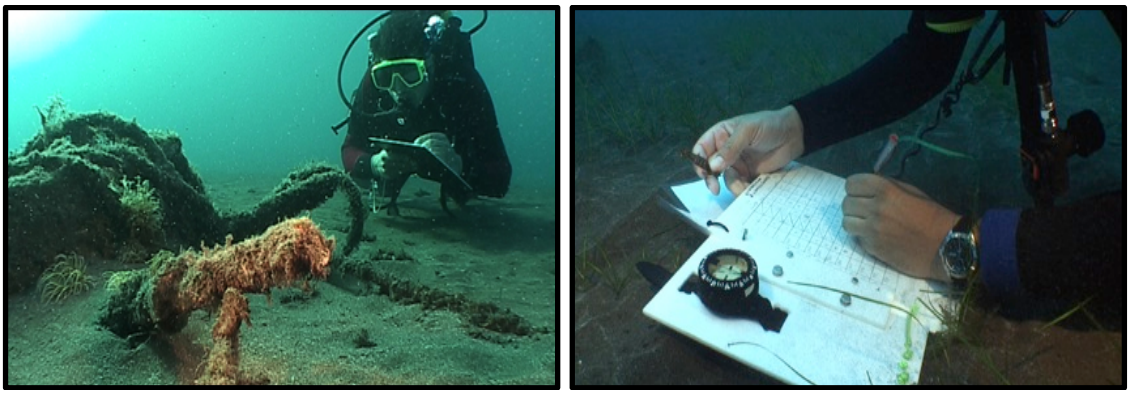

Figure 2.3 Diver recording data along underwater census conducted along this thesis (source: E. Turpin).

2.2 *EX SITU* STUDIES

In order to develop *ex situ* experiments, new facilities were built in the Instituto Canario de Ciencias Marinas (ICCM – Gobierno de Canarias) in Telde (Gran Canaria Island, Spain). These facilities were divided in two separate sections. The "**Seahorse experimental zone**", designed to perform breading and broodstock studies and the "**Live prey zone**", reserved to culture seahorse food, mainly rotifers and *Artemia*.

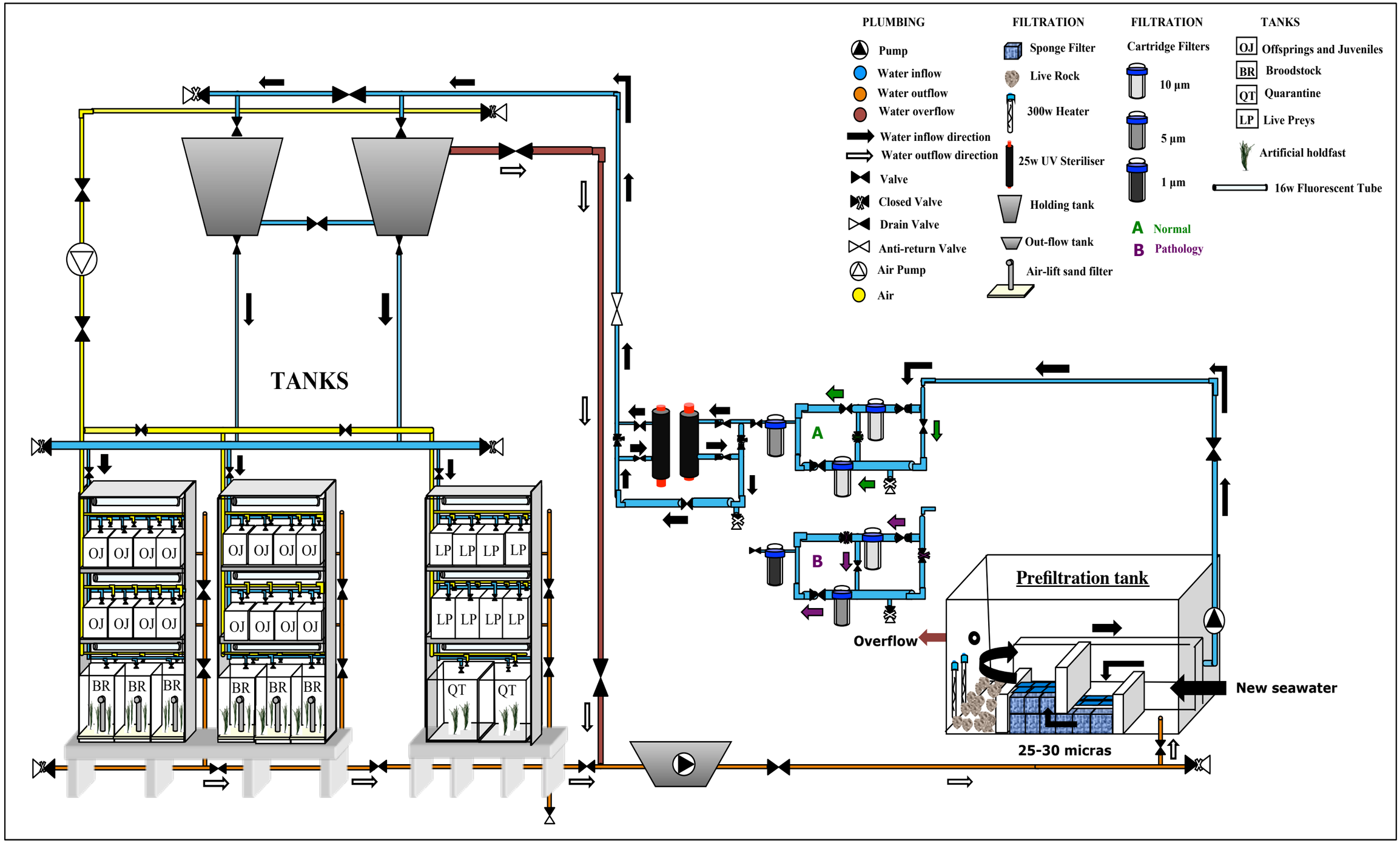

Figure 2.4. Schematic diagram of seahorse experimental zone, including Life-Support System (LSS) section, and quarantine and culture tanks.

2.2.1 SEAHORSE EXPERIMENTAL ZONE

This area has its own life-support system (LSS), able to work in open or semi-closed water circulation conditions. This characteristic allowed good water quality and also high feasibility, easy animal handling, avoiding daily variations of water quality parameters. Accordingly, this first section was also divided in three main zones: LSS, acclimatizing and quarantine zone and culture aquariums. The total volume of this area was approximately 4000 L. A schematic diagram of seahorse experimental zone is presented in Figure 2.4.

2.2.1.1 Life Support System (LSS)

New flow-through ambient seawater was directly pumped from harbour facilities of ICCM and stocked in "decantation tank" prior to be sent by gravity to seahorse LSS. The first LSS element was a 1500 L fiberglass pre-filtration tank. It was subdivided in three compartments to increase filtration efficiency. These compartments were filled with different filtration media such as sponge filters (mechanical and biological filtration) and live rock (biofilter) (Fig 2.5a). Moreover, two 300w heaters (Jäguer, 300W, Wuestenrot, Germany) were also integrated in the tank to maintain seawater temperature. Thus, the temperature values remained between 20-24°C, following natural seasonal variations. Submerged pump (Pedrollo Top 2, Moore Road, South Africa) sent pre-filtered seawater through plastic polyethylene pipes towards the next filtration step, cartridge filters. Two alternative circuits were designed in order to improve system feasibility. As shown in Figure 2.4, cartridge filters could work in parallel (configuration A), filtering 5 µm size-particles, or in serial (configuration B) until 1 µm size-particles. Normally, this last configuration was only employed to reduce filtration particle-size during pathology episodes. Two 25w ultraviolet lights (Philips, 25W/G25T8UV-C, Amsterdam, Holland) were established as the last LSS filtration-barrier (Fig. 2.5c).

Afterwards, filtered seawater was sent to two 500 L holding-tanks where it was stocked prior to be sent by gravity to the different aquarium racks. Thus, if submerged pump failures occurred at night water supply for aquariums was always insured. Moreover, a re-circulation system towards pre-filtration tank was installed to prevent holding-tanks overflow.

Aeration was provided by an air pump located outside the seahorse experimental zone. Then, air was transferred towards aquarium racks through polyethylene pipes net. Each water volume was aerated with air diffusers to maintain oxygen levels ranged from 6.5 to 7 mg/L.

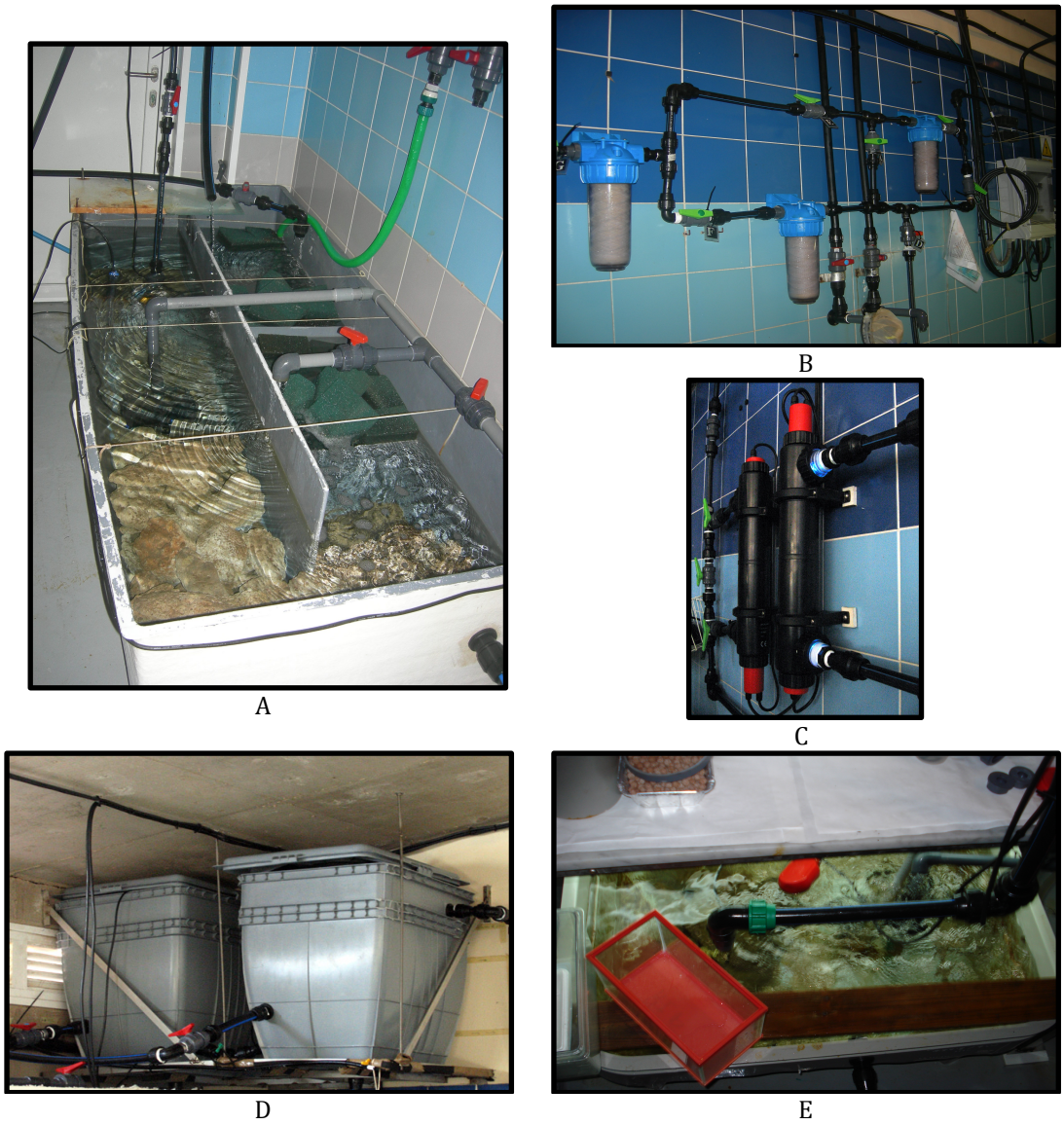

Figure 2.5 Elements of life support system (LSS): (A) Pre-filtration tank, (B) cartridge filters, (C) U.V. sterilisers, (D) holding tanks and (E) out-flow tank (source: F. Otero-Ferrer).

2.2.1.2 Acclimatizing and quarantine zone

The acclimatizing and quarantine zone was composed by a three-shelf stainless-steel-rack, which held eight 35 L glass aquariums mounted on the middle and upper levels, and two 120 L glass aquariums on the lower shelf. Each shelf was illuminated by one broad-spectrum fluorescent tube (Sera blue daylight, 36W,

12000 °K, Heinsberg, Germany). Photoperiod was adjusted depending on animal conditions. Generally, small volumes were used to maintain live prey collected directly from the wild or to isolate unhealthy animals for treatments during pathology episodes while the bigger ones were employed to acclimatize new seahorses to captivity conditions. All aquaria were supplied with flow-through ambient seawater from holding-tanks, previously purified by LSS. Each one was separated from the others and equipped with two valves to regulate air and water flow-rate. Artificial holdfasts such as plastic plants were also placed to help seahorse along acclimatizing processes (Fig. 2.4). Water outflow was recovered by a PVC pipe and then transferred directly to the drain.

2.2.1.3 Culture aquaria

Two racks were installed for seahorse culture experiments. Each was a three-shelf stainless-steel-rack, holding eight 35 L glass aquaria (breeding study) mounted on the middle and upper levels, and three 90 L glass aquaria (broodstock study) on the lower shelf. Each shelf was illuminated by one broad-spectrum fluorescent tube (Sera blue daylight, 36W, 12000 ºK, Heinsberg, Germany). During the different studies, all aquaria were illuminated 10h per day (Woods, 2000a) and light intensity just above the water surface ranged between 60 to 100 lx in 35 L aquaria, and 800 to 1000 lx (HT170N Digital Light Meter, HT instruments, Barcelona, Spain) in 90 L aquaria. As in acclimatizing zone, each aquarium was independent from the others and equipped with two valves to regulate air and water flow-rate. Water outflow of all aquaria was recovered by a PVC pipe and then transferred directly to a 300 L out-flow tank, equipped with a submersible pump (Resun King 6, Guandong, China) used to recirculate seawater towards prefiltration tank.

2.2.1.3.1 Breeding aquaria

Thirty-five litres aquaria were employed to rear newborn seahorses and also to maintain juveniles out of experimental periods. Each aquarium was equipped with a recirculation pump (Universal mini pump, Tunze Aquarientechnik GmbH, Penzberg, Germany). This pump was separated from the animals by a blue painted-glass wall placed in the aquarium backside, leaving 28 L as real breeding

volume. Two 500 µm nylon meshes connected both compartments, preventing newborn seahorses from running away (Fig. 2.6).

During the rearing study, the stocking density within tanks was 2.3 seahorses/L. Moreover, each aquarium was supplied with flow-through ambient seawater previously treated by LSS at a flow-rate of 2 L/min. This high flow-rate was established to assure that untaken living food (Rotifers and *Artemia*) was removed after each feeding period. PVC-plastic mesh (12 cm x 5 cm) was added in each aquarium to provide holdfasts and habitat for the animals when it was necessary (Villares, 2006) (Fig. 2.6).

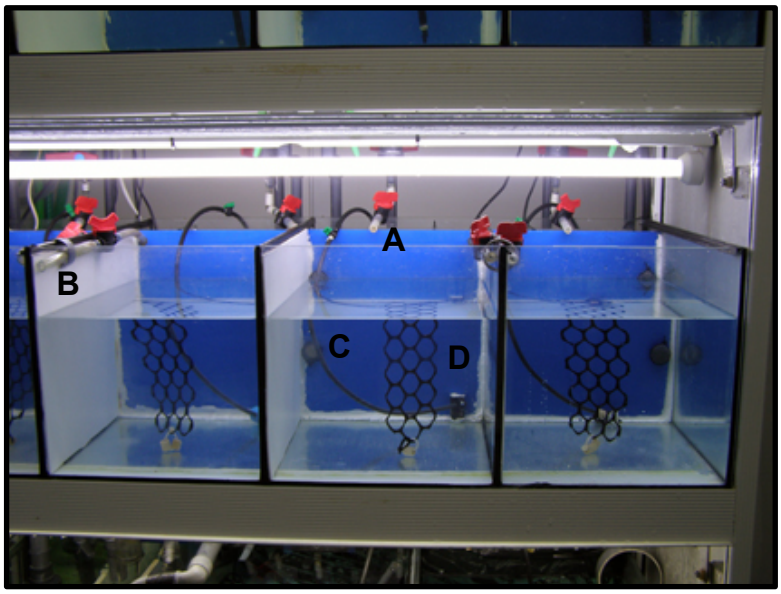

Figure 2.6 Breeding aquaria employed to rear newborn seahorses equipped with (A) filtered seawater valve, (B) recirculation seawater valve, (C) 500 µm nylon-mesh and (D) plastic net holdfast (source: F. Otero-Ferrer).

2.2.1.3.2 Broodstock aquaria

Ninety litres tanks supplied with purified flow-through ambient seawater (flow-rate 0.5 L/min) were employed to study spawning quality in broodstock seahorses. At the bottom of every aquarium an under-gravel filter equipped with an airlift was installed in order to increase water quality (Fig. 2.7). Lateral black PVC walls placed between aquaria, avoided visual interactions among animals, and also increased prey contrast during feeding episodes.

During the study, the stocking density within tanks was 1 seahorse pair per aquarium. Different plastic supports or artificial seaweeds were added to provide holdfasts for each pair.

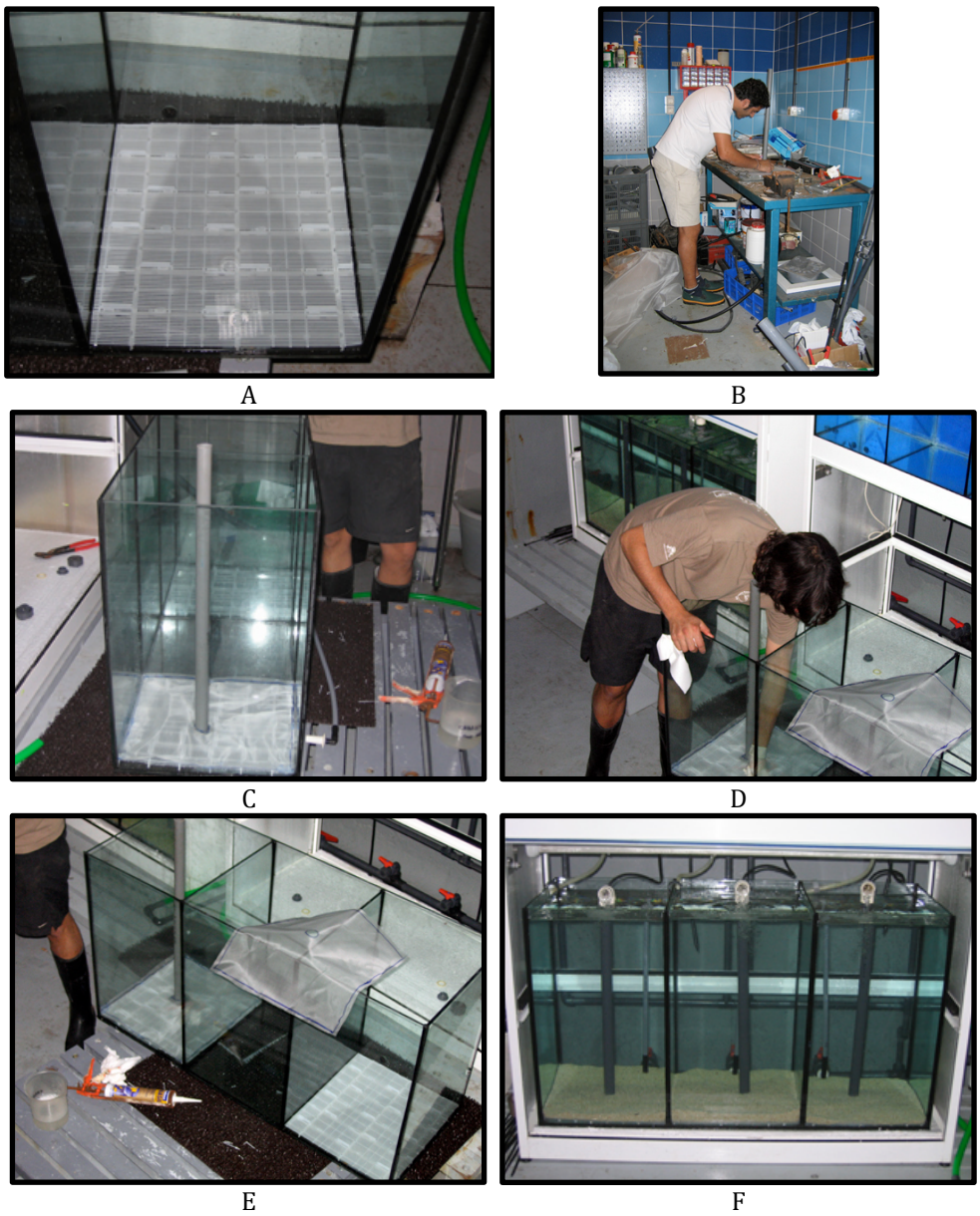

Figure 2.7 (A-F) Different steps in construction of air-lift sand filter included in broodstock aquaria (source: F. Otero-Ferrer).

2.2.1.4 Water quality

Different protocols were established to control water quality of the seahorse experimental zone. Thus, cartridge filters were changed every week and a daily cleaning of aquaria was carried out to remove uneaten food and animal faeces before feeding episodes. Also, measurements of physical parameters such as

temperature (°C) and oxygen level (mg/L) were carried out twice a day using hand-held probe (Handy Polaris, Oxyguard International ®, Birkerød, Denmark). Nitrogen compounds that can be lethal for fish, including ammonia and nitrites (Otero-Ferrer, 2001), were measured twice a week (Red Sea Europe, Marine and Freshwater Test Lab, Verneuil Sur Avre, France). Values were always below detectable levels for ammonia (NH_4 < 0.2 mg/L) and nitrites (NO_2 < 0.02 mg/L).

2.2.2 LIVE PREY ZONE

Depending on life-history stage of seahorses (offspring, juveniles and adults), different preys were employed as live food along experiments conducted in captivity.

2.2.2.1 Offspring

Newborn seahorses were fed with rotifers (*Brachionus plicatilis*, L-strain, 240 mm) and/or *Artemia* Instar II (EG type 850 mm, INVE Aquaculture) according to experimental design. Rotifers had been grown out in a batch culture system fed with commercial yeast (*Saccharomyces cerevisiae*) and enriched with DHA protein-Selco (INVE Aquaculture, Dendermonde, Belgium) following the standard protocols of ICCM (Roo *et al.*, 2009). *Artemia* was hatched in 2-L plastic container during 24 h. Then they were transferred to 8-L plastic tanks, taking another 24h to reach the next stage of development called Instar II. At this point, brine shrimps were also enriched during 24 h with EASY DHA-Selco (INVE Aquaculture, Dendermonde, Belgium) prior to be employed as live food.

2.2.2.2 Juveniles and adults

Juvenile and adult seahorses needed bigger sizes of prey. Accordingly, *Artemia* were grown out in a batch culture system using 50 L conical tanks and fed with commercial ORI-culture (SKRETTING®, Vervins, France) (Fig. 2.8). Twenty-four hours before feeding episodes, *Artemia* were enriched following the standard protocols of the ICCM (Roo *et al.*, 2010). Moreover, wild benthic mysids (*Leptomysis sp*) were captured with a 500-µm hand net in the area of Risco Verde (27º 51' N, 15º 23' W), Gran Canaria (Spain). Mysids were then transported at ICCM facilities and held in captivity in 40-L quarantine square glass aquaria.

Mysids were fed twice a day with *Artemia* Instar II (1/mL) nauplii, previously enriched with Easy DHA-Selco, (INVE Aquaculture, Dendermonde, Belgium), following the Instituto Canario de Ciencias Marinas (ICCM) standard protocols (Roo *et al.*, 2010).

Figure 2.8 Live prey zone with conical tanks employed on *Artemia* grow out (source: F. Otero-Ferrer).

2.3 EXPERIMENTAL DESIGN AND SAMPLING PROTOCOLS

A general overview of experimental designs and samplings protocols are included in this section. Detailed methodologies are described in the chapters corresponding to each study. Accordingly, only a summarized schematic representation for each individual study has been included in this Materials and Methods section (Fig. 2.9, 2.10, 2.11, 2.12 and 2.13 corresponding to **studies I**, **II**, **III**, **IV** and **V**, respectively).

Figure 2.9 Schematic representation of experimental design and sampling followed along **study I**.

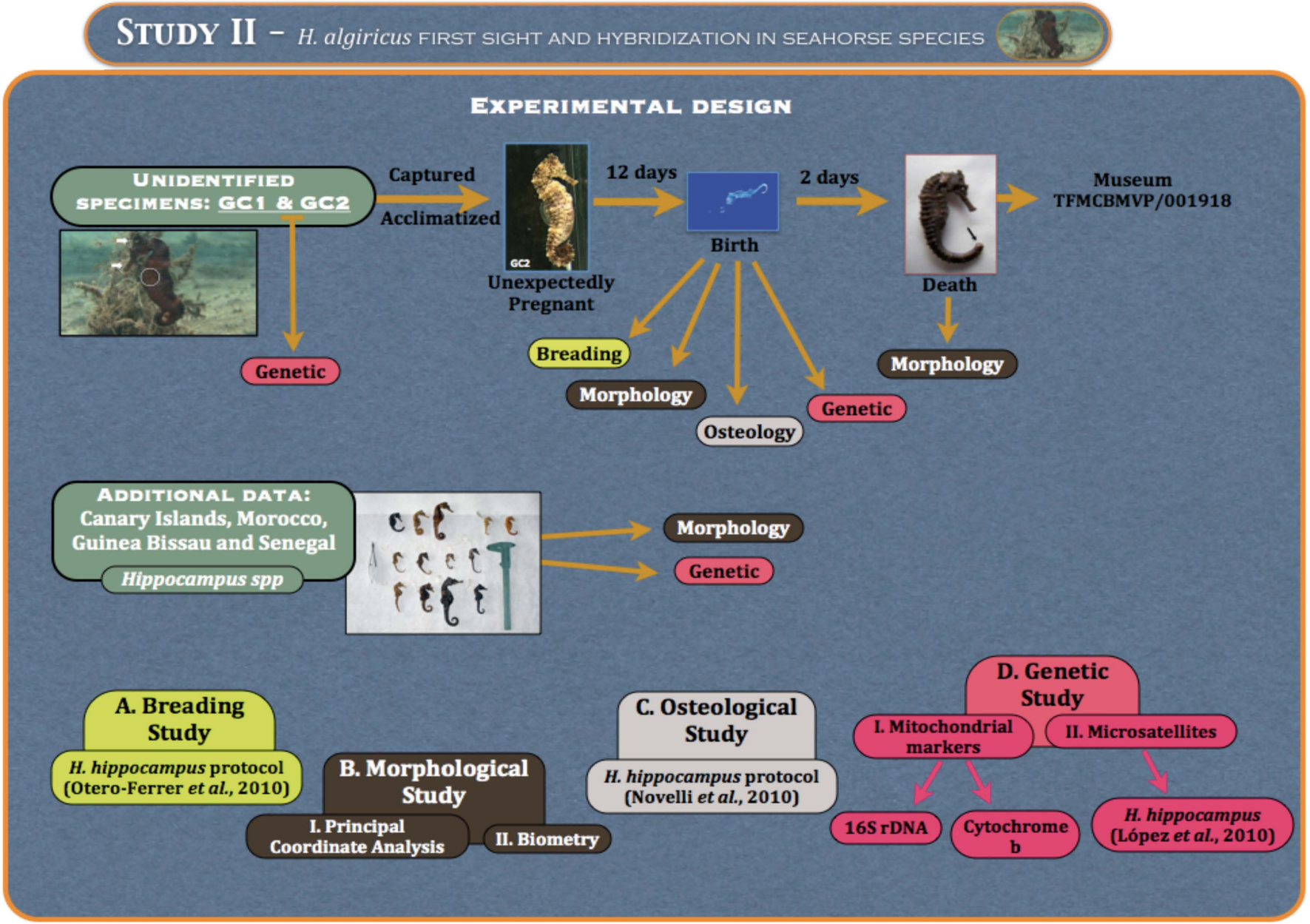

Figure 2.10 Schematic representation of experimental design and sampling followed along **study II.**

Figure 2.11 Schematic representation of experimental design and sampling followed along **study III**.

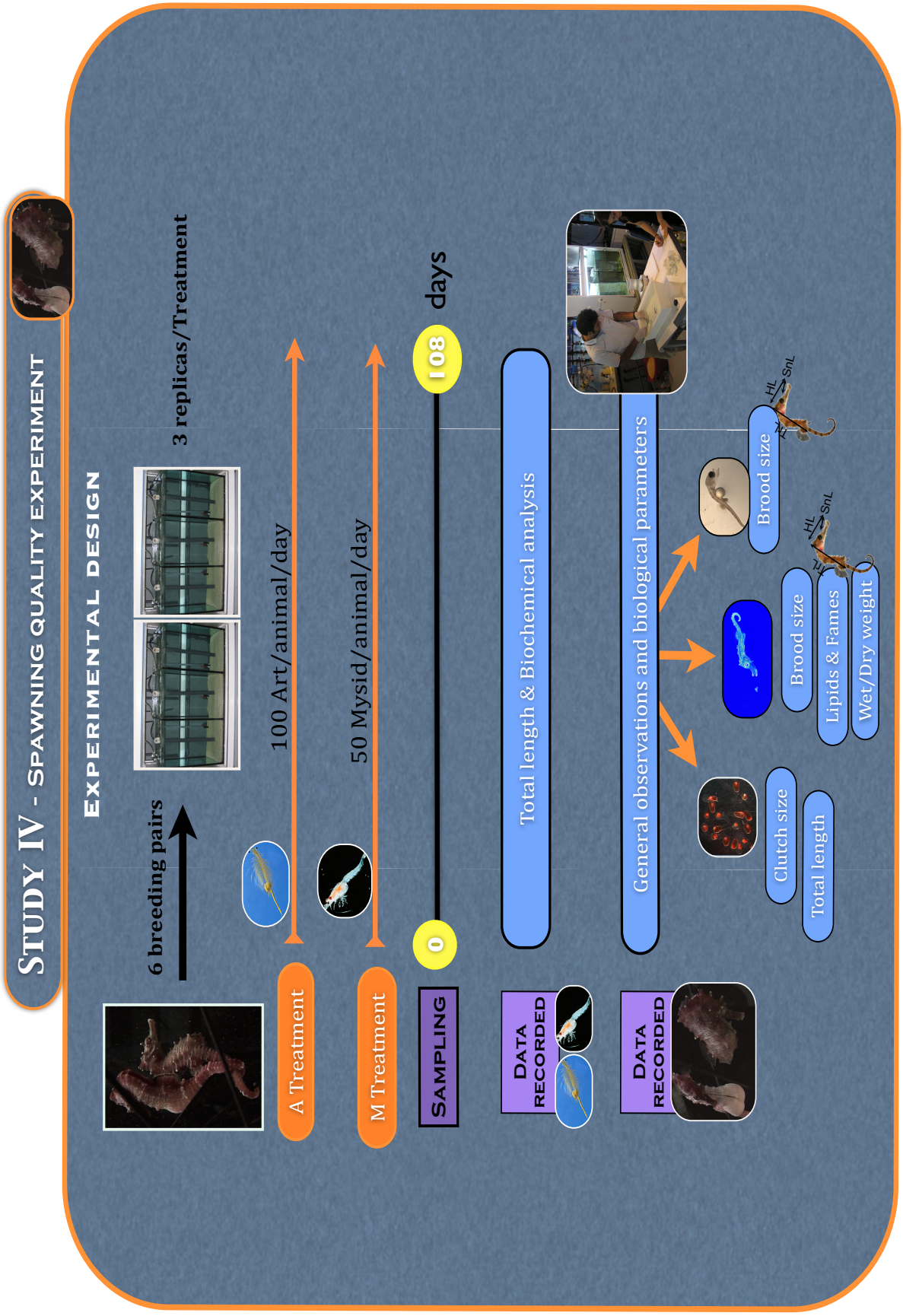

Figure 2.12 Schematic representation of experimental design and sampling followed along **study IV**.

Figure 2.13 Schematic representation of experimental design and sampling followed along **study V**.

2.4 ANAESTHETIC STUDY

In order to avoid animal sacrifice during seahorse handling in captivity, an anaesthetic study was carried out using natural clove (*Syzygium aromaticum*; Guinamas, Valencia, Spain) essential oil, containing 87% eugenol anaesthetic. This product has several advantages for routine tasks in marine fish cultured (Munday and Wilson, 1997; Waterstrat, 1999; Woody *et al.*, 2002), such as a high efficacy at low doses, cost-efficiency and non-toxicity among others (García-Gómez *et al.*, 2002). This protocol was employed to reduce stress during seahorses' measurements (length and weight) of offspring, juveniles and adults. Moreover, it was also used to help animal handling during tagging.

Offspring were anaesthetized in 100mL plastic beakers using a 25 ppm clove oil solution. Before returning to culture tanks, animals were transferred to recovery tanks (2 L beakers) to make sure that they did not suffer any side effects.

2.5 TAGGING TECHNIQUES

Seahorses were tagged along different trials in order to identify animals during their population assessment (**study I**), to study spawning quality in seahorse broodstocks (**study IV**) and also to follow holdfast preference in captive-bred *H. hippocampus* released in the wild (**study V**).

Seahorses were tagged using visible implant fluorescent elastomer (VIFE; Northwest Marine Technology, Inc., Shaw Island, Washington), applying the methodology described by Morgan and Martin-Smith (2004) and adapted by the author for *in-situ* (**study I**) and *ex-situ* experiments (**study IV** and **study V**). These tags have already been employed in other seahorse mark-recapture studies without effect on mortality (Curtis, 2006a). VIFE is an injected plastic polymer that when mixed with a curing agent, hardens under the fish skin to leave a permanent, flexible, fluorescent and biocompatible mark (Morgan and Martin-Smith, 2004). The four fluorescent colours selected to tag animals were green, orange, red and pink; avoiding combinations such as red-orange or red-pink when 2 colours were employed. Normally, at least three marks were injected in lateral body segments to reduce the probability of tagging loss during the trials. Also coding systems based

on 8 body locations (Appendix 2 in Morgan and Martin-Smith, 2004) were employed for animals tracking.

During *in-situ* studies, small amount of VIFE was loaded in two injecting syringes in which the curing agent was added just before diving to prevent VIFE polymerization along the surveys. In captivity, seahorses were placed in 10 L plastic tank during tagging episodes (Fig. 2.14). In all cases, morphometric measurements were performed before tagging to avoid animal manipulation after injection.

Figure 2.14 *In-situ* and *ex-situ* setup for tagging *Hippocampus hippocampus* along the different studies (source: E. Turpin, F. Otero-Ferrer, S. Jamme and R. Herrera).

2.6 GENETIC STUDIES

2.6.1 SAMPLES

Three different sampling protocols were established depending on seahorse life-history stage and if animals were dried or alive. Non-invasive technique described by Lourie (2003a) has been used *in situ* or *ex situ*, to collect a small piece of dorsal fin (2-3 mm) on live specimens found in Melenara bay (**Study II**) or during characterization of animals reared in captivity (**study V**). In newborn seahorses, the entire specimen was sacrificed using anaesthetic overdose. Finally, in dried seahorses (**study II**), a little piece of tail (1 cm) was clipped to complete genetic sample collection. All samples were stored in microcentrifuge 2.0 mL tubes (Eppendorf ibérica, Madrid, Spain) and conserved in 100% ethanol PA (Panreac, Barcelona, Spain) to further analyses.

2.6.2 DNA EXTRACTION

According to quality and quantity of DNA samples, different protocols were conducted in order to proceed to DNA extraction. Thus, DNA was extracted from the piece of dorsal fin (**studies II and V**), following the phenol-chloroform method described by Sambrook *et al.* (1989). Chelex® resin was used for extracting DNA from newborn seahorses (Estoup *et al.*, 1996) and for dried *H. hippocampus* and *H. algiricus* specimens, a little piece of tail was hydrated and then DNA was purified using NucleoSpin® Tissue columns (Macherey-Nagel), following Sanders *et al.* (2008). For very small tissue samples yielding low DNA concentration, further genomic amplification was carried out using GenomiPhi V2 kit (GE Healthcare) (López *et al.*, 2010) or NucleoSpin® Tissue XS kit (Macherey-Nagel). When it was necessary, quality and quantity of DNA was evaluated using a Nanodrop 1000 spectrophotometer (Termo scientific, Wilmington, USA).

2.6.3 GENETIC ANALYSIS

Genetic analyses were performed using different DNA molecular markers (mitochondrial and microsatellites) to study some aspects related to seahorse phylogeny, parental relationships or species identification (Teske *et al.*, 2004; López *et al.*, 2010).

2.6.3.1 Mitochondrial analysis

All mitochondrial analyses were carried out by the research group "Acuigen" of the University of Santiago de Compostela (Spain), and therefore only a brief description of the methodology employed is included in this chapter. Acuigen was a partner involved in the project that partially funded this study.

Thus, DNA samples were sequenced for two mitochondrial markers, 16S rDNA and cytochrome *b* (cyt *b*), to study phylogenetic relationships among different seahorse specimens (**study II**). The first marker was employed to discriminate between *H. hippocampus* and *H. guttulatus* (López *et al.*, 2010) species, while cyt *b* was used to differentiate similar species within the group of seahorse species from Indo-Pacific (*kuda* complex: *H. algiricus*, *H. reidi*, *H. capensis*, *H. kuda;* Casey *et al.*, 2004; Teske *et al.,* 2004; 2007).

All analyses were performed following the methodology employed by López *et al.* (2010). Phylogenetic relationships among 16S rDNA and cyt *b* haplotypes were established using the neighbour-joining and Bayesian inference analyses as previously reported by López *et al.* (2010).

2.6.3.2 Microsatellites

Nine microsatellite loci previously used in *H. hippocampus* (López *et al.*, 2010) were employed in study II to discriminate seahorse specimens and also to investigate parental relationships among offspring and adults. The analyses were conducted at the University of Santiago de Compostela by researchers of Acuigen (Spain), and therefore only a brief description of the methodology employed is included in this thesis.

Accordingly, Hgu-USC2, Hgu-USC4, Hgu-USC5, Hgu-USC9, Hgu-USC13, Habd3, Habd9, Hcaμ27 and Hcaμ33 were amplified in unidentified seahorse specimens (GC1 and GC2), and also in offspring obtained from the male GC2. PCR were carried out following López *et al.* (2010) conditions. Genotyping was performed on an ABI 3730xl DNA Analyzer using GENEMAPPER v3.7 (Applied Biosystems) for comparison with allelic categories observed in *H. hippocampus* from Melenara by López *et al.* (2010).

On the other hand, microsatellites were also employed to characterize 10 *H. hippocampus* batches of reared seahorses, held in captive at the ICCM laboratory

research facilities. Thus, thirty-two seahorse pairs were selected using microsatellite-based criteria (maximum allelic differentiation among groups) without mixing animals from the same batch. Analyses were conducted at the Instituto Universitario de Sanidad Animal (IUSA - Arucas, Canary Islands, Spain) in our facilities.

Four microsatellites were selected from those reported by López *et al.*, (2010) considering more polymorphic criteria. Thus, Hcaμ27, Hcaμ33 and Habd9 were firstly described by Galbusera *et al.* (2007), (Wilson and Martin-Smith, 2007), and Hgut USC4 by Pardo *et al.*, (2007) (Table 2.1).

Table 2.1 Characterization of 4 microsatellite primer sets used in **study V**, including locus name, fluorochrome label (F), primer sequences, specific annealing temperature (°C), buffer conditions (mM), primers concentrations (mM) and references of microsatellites described authors.

Locus	F	Primers sequences (5'-3')	T_a	$MgCl_2$	Primer (F+R)	References
Hcaμ33	5' 6-FAM	F:TTGTGGCAGCTGAGTACACC R:CCTGCTTGGCATTTTCTTCT	54	1.5	0.4	Galbusera *et al.*, 2007
Hcaμ27	5' VIC	F:GGACAGGCATGCTTTTTGTC R:GCTCAGAGGAAGGTGAGTGC	54	1.5	0.4	Galbusera *et al.*, 2007
Hgu-USC4	5' NED	F:CCGACAGGAAGTAGCTGGAA R:GTGGCAGTTGCACAGAGGTA	60	1	0.4	Pardo *et al.*, 2007
Habd9	5' PET	F:GCTAATTGCGGATACCCAGA R: TAGTCCCTCACCTCCCAAAA	54	2.5	0.4	Wilson and Martin-Smith, 2007

2.6.3.3 Polymerase Chain Reaction (PCR) conditions

Initially, six samples were tested for original described PCR conditions for each microsatellite (Table 2.1). Thus, Hcaμ27, Hcaμ33 and Hgut USC4 amplified correctly with the original conditions. However, Habd9 did not amplify. Therefore, temperature and magnesium chloride ($MgCl_2$) gradient were tested to optimize the PCR conditions for this microsatellite.

PCR conditions consisted of an initial denaturalization at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s, 54-60 °C for 30 s (depending of annealing temperature of each primer) and 72°C for 30 s, with a final extension of 72°C for 10 min. Reactions were carried out in a final volume of 10 μl with the following component concentrations: 1X PCR Buffer (100 mM Tris–HCl pH 8.3, 500 Mm KCl) (Primer®), 1-2.5 mM MgCl2, 0.25 mM of each dNTP, 0.04 mM AmpliTaq DNA polymerase (Primer®), 40 ng of DNA template, and 0.4 mM of each primer.

After obtaining the optimal concentrations of microsatellite markers, all samples were amplified with the studied microsatellites in order to confirm allelic segregation of each locus, to test genetic variability of all markers.

Before running PCR reactions in the automatic sequencer, an aliquot of amplified products was checked on 2% agarose gel for 30 min (8 v cm−1) to assess the correct amplification of amplicons. Later, for each sample, 0.25 µl from the amplified product of each microsatellite was mixed. Then total 1 µl solution was mixed with 9.75 µl of Hi-Di formamide and 0.25 µl of GeneScan 500-250 LIZ (Applied Biosystem®) size standard, and run on an ABI Prism-3130-XL Genetic Analyzer (Applied Biosystem®) with 36 cm capillary arrays and POP-4 polymer (Applied Biosystem®) (60°C, 3000v, 1500s). Electropherograms and genotypes were evaluated with GeneScan (v3.7) and Genotyper (v3.7) (Applied Biosystems, Inc.) software.

2.7 BIOLOGICAL PARAMETERS

2.7.1 LENGTH

All measurements reported in this thesis are standard lengths, except when stated otherwise. They were performed *in* or *ex* situ for juveniles and adults using a plastic calliper, or through a profile projector (Mitutoyo PJ-A3000, Kawassaki, Japan) when offspring were measured in captivity (Fig. 2.15). Thus, standard length (SL) was defined as the sum of head (HL, cm), trunk (TrL) and tail (TL) length (Fig. 4b, Lourie, 2003b) However, different criteria were employed if measurements were carried out on live or dried specimens, always following the methodology described by Lourie (2003b).

Thus, on live specimens, lengths were calculated as straight line to the nearest cm between the appropriate reference points, with the head positioned at a right angle to the body (Lourie, 2003b) to reduce handling time, and keeping the animal in the water (*ex situ* studies). Additional measurement commonly employed in seahorse taxonomy was height (HT). This measurement is defined as the vertical distance from the tip of the coronet, to the tip of the outstretched tail, with the head held at right angles to the body (Lourie, 2003b).

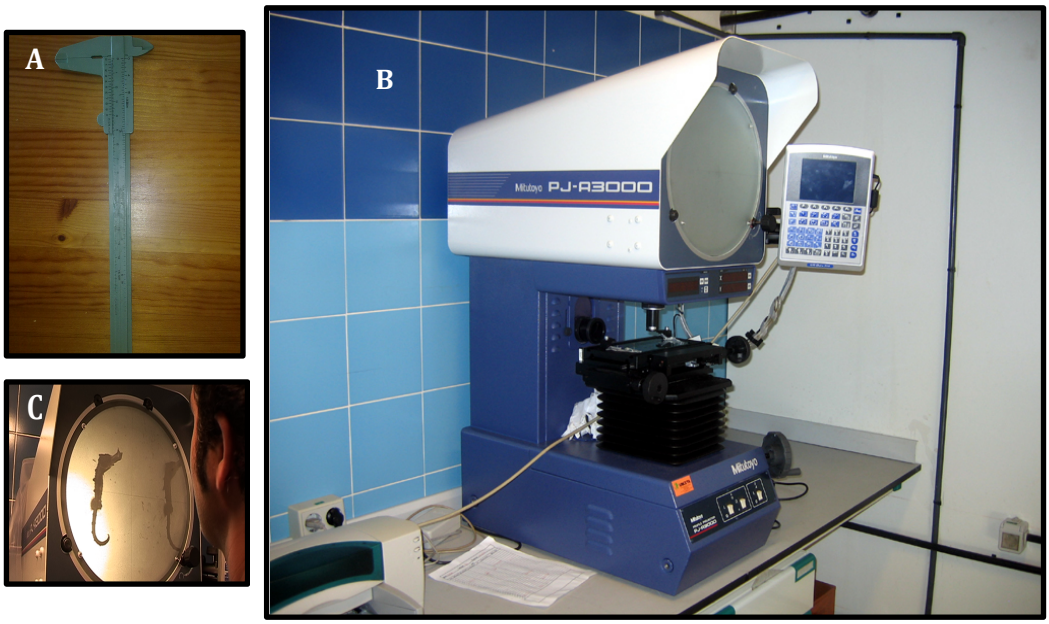

Figure 2.15 Tools used for seahorse measurements: (A) Plastic calliper employed in both *in situ*, *ex situ* studies and (B-C) profile projector used to measure young seahorses along breeding experiments (source: F. Otero-Ferrer and E. Turpin).

For preserved seahorses lengths were calculated following the mid-lateral line using a piece of fine wire that was straightened out and measured with calliper. Also other morphometric and meristic features could be recorded in these specimens to accurate species identification. All measurements and counts were made under a binocular microscope (Leica M-125, Wetzlar, Germany), and were repeated to ensure accuracy. Thus, head (HD) depth, orbital diameter (OD), post-orbital length (PO) and coronet height (CH) were reported as proportions of HL. Other morphological variables also employed were the length of pectoral (PL) and dorsal (DL) fin base, the trunk depth between the 4th and 5th (TD4) and between the 9th and 10th (TD9) trunk rings (from the superior to the inferior trunk ridge), and Snout depth (SnD) (Fig.4, Lourie, 2003b). Regarding meristic variables, the counts made during the study were, the number of trunk (TrR) and tail (TaR) rings; the dorsal (DF), pectoral (PF) and anal (AF) fin rays; and finally, the number of cheek and eye spines (Fig.4, Lourie, 2003b).

2.7.2 WEIGHT

As well as in length measures, methodology changed with wet or dry weight. For wet weight, once taken out from the tank, seahorses were blotted briefly on

filter paper and then weighted with a precision balance (Mettler Toledo, AG204, Greifensee, Switzerland) (Job *et al.*, 2002). On the contrary, for dry weight animals were sacrificed with an anaesthetic overdose (clove oil) and then placed in an oven (Jouan, EU 280 EL TS SN Inox, Saint-Herblain, France) at 105°C until the weight was not reducing any further (AOAC 2010). The measurements were taken with a precision balance.

2.7.3 SEX

Male seahorses were differentiated visually from females by the presence of a brood pouch in the lower part of the trunk (Lourie, 2003b), which was recorded in individuals having a standard length between 3-4 cm or longer (unpubl. data). Hence, individuals that lacked a brood pouch were thus recorded as females — if their standard length was 4cm or longer — or as juveniles if it was less than 4cm.

2.7.4 LIFE-HISTORY STAGE

Specimens were classified in adults or sub-adults according to their standard length. Thus, the status "sub-adult" was assigned to individuals with SL lesser than 8.7 cm, following the criteria established by Curtis (2004) along her *H. hippocampus* population assessment in Ria Formosa (Portugal).

2.7.5 SEAHORSE APPEARANCE

Seahorse appearance included two characteristics such as the presence or absence of skin filaments and the seahorse body colouration. Skin filaments are threadlike structures of skin that extend from the spines of many seahorse species (Curtis, 2006c).

On the other hand, seahorse body colouration varied within specimens observed during the trial. Under artificial light, different shades of yellow to green, and brown to red (also rust) were noted as seahorse colour background. To compare data, they were pooled in three colour groups: mainly yellowish (Y), brownish (Br) and reddish (Re). Additionally to colour pattern, some trunks showed white spots or stripes. The number and position of them were registered during 2011 surveys (**study I**). The same experimented diver carried out all the observations.

2.7.6 ACCUMULATIVE SURVIVAL RATE (ASR)

In order to evaluate the efficiency of different prey in breeding experiment, accumulative survival for each treatment's replicate was determined as follow:

$$\textbf{ASR}\ (\%) = ((N_i - M_{ij})/ N_i) \times 100$$

N_i was the initial number of seahorses put in each aquarium at Day 1.

M_{ij} was the total seahorses dead since day 1 until the day J.

2.7.7 WEIGHT GAIN (WG)

This parameter was employed to evaluate the effect of different prey regimes in broodstock growth. It was defined as the relation between the increases in biomass (g) compared to initial weight (g). The WG could be expressed in absolute values as well in percentage and it is corrected in relation to the individual fish weight through the following equation:

$$\textbf{WG}\ (\%) = (\textbf{Final weight} - \textbf{Initial weight})/\textbf{Initial weight} \times 100$$

2.7.8 SPECIFIC GROWTH RATE (SGR)

This parameter indicates the increase in weight gain (g) in relation to the number of days of feeding period and it is expressed in percentage values. These data were used to calculate the following parameters:

$$\textbf{SGR}\ (\%) = ((\ln \textbf{Final weight} - \ln \textbf{Initial weight})/\textbf{days}) \times 100$$

2.7.9 CONDITION FACTOR (K)

The Standard Length (cm) and wet weight of broodstock were measured at the beginning and at the end of the trial. Condition factor is an expression of the relationship between fish length and weight. The equation is defined as follows:

$$\textbf{K} = (\textbf{Wet weight}/(\textbf{Standard length})^3) \times 100$$

2.8 BIOCHEMICAL ANALYSIS

Along the **studies III** and **IV**, live preys (rotifers, mysids and *Artemia*) and seahorse proximate composition was conducted at the Instituto Universitario de Sanidad Animal (IUSA - Arucas, Canary Islands, Spain) laboratories. Live prey samples were collected with an adequate-sized nylon mesh, measured with a precision balance (Mettler Toledo, AG204, Greifensee, Switzerland) and washed twice with distilled water and freeze-dried (-80°C) for subsequent analysis.

2.8.1 TOTAL CRUDE LIPID CONTENT

Crude lipids from weighted samples were extracted by a chloroform:methanol (2:1) mixture as described by Folch *et al.* (1957). Extracted lipids were diluted in chloroform and stored at -80°C under nitrogen atmosphere to avoid oxidation.

2.8.2 FATTY ACID METHYL ESTERS PREPARATION AND QUANTIFICATION

Fatty acid methyl esters were obtained by transmethylation with 1% sulphuric acid in methanol (Christie, 1982). The reaction was carried out in dark conditions under nitrogen atmosphere for 16 h at 50°C. Then, fatty acid methyl esters were extracted with hexane: diethyl ether (1:1 v/v) and purified by adsorption chromatography on NH_2 Sep-Pack cartridges (Waters S.A., Massachusetts, USA). Fatty acid methyl esters were separated by GLC (GC-14A, Shimadzu, Japan) in a Supercolovax-10-fused silica capillary column (Length: 30 m; internal diameter: 0.32 mm; Supelco, Bellefonte, USA) using helium as a carrier gas. Column temperature was 180°C for the first 10 min, increasing to 215°C at a rate of 2.5°C/min and then held at 215°C for 10 min, following the conditions described by Izquierdo *et al.* (1992). Fatty acid methyl esters were quantified by frame ionization detector (FID) and identified by comparison with external standards and well-characterized fish oils (EPA 28, Nippai, Ltd Tokyo, Japan) (Torrecillas, 2011).

2.8.3 CRUDE PROTEIN

Crude protein analyses were carried out according to the Kjeldahl Method (AOAC, 2010). Briefly, samples were digested with 37% sulphuric acid in presence

of a cupper catalyst converting all the N_2 present in the sample to $(NH_4)_2SO_4$. After that, NH_3 was released from the digested sample by the addition of NaOH in excess. This ammoniac acid was distilled in boric acid and finally the ammoniac acid released was quantified by titration with chloridic acid 0.1 N. The total nitrogen in the sample was converted to total crude protein value by multiplying by the empirical factor 6.25.

2.8.4 MOISTURE CONTENT

Dry matter content was determined by thermal drying to constant weight in an oven at 110°C, with a first 24 h period followed by 1h periods until weight became constant (AOAC, 2010). Sample weight was recorded before drying and after each drying period, previous to a cooling period in desiccators until achieving ambient temperature. Moisture was expressed as a percentage of sample weight according to Official Methods of Analysis (AOAC, 2010).

2.8.5 ASH CONTENT

Ash content was determined by combustion in a muffle furnace at 600 °C for 12 hours (AOAC, 2010). Sample weight was recorded before combustion and after combustion period, previous to a cooling period in desiccators until achieving ambient temperature. Ash content was expressed as a percentage of sample weight according to Official Methods of Analysis (AOAC, 2010).

2.9 OSTEOLOGICAL STUDIES

Osteological studies were conducted to describe and identify cartilages and bones in offspring of the unidentified seahorse specimen found in Melenara bay (Gran Canaria – Spain) (**study II**).

Different body regions were included in these studies; skull, including branchiocranial and neurocranial parts (Helfman *et al.,* 2009), trunk, tail and also, all fins (pectoral, anal, dorsal and caudal). Seahorses were processed according to protocols established for Taylor and Van Dyke (1985) and Digerkus and Uhler (1977) for the alcian blue-alizarin red double staining technique and adapted to *H. hippocampus* by Novelli *et al.*, (2010) as next described.

Samples were fixed in 10% neutral-buffered formalin (10% Formalin, Panreac T-143091, Barcelona, Spain) for at least 24 hours. Prior to analysis, they were washed with 1%KOH (85% KOH, Panreac T-201515, Barcelona, Spain) and 3%H_2O_2 (3% H2O2, Panreac T-622772, Barcelona, Spain) bleaching solution (for 100 ml: 9.09 ml H_2O_2, 1 g KOH and distilled water) until pigments disappear from seahorse skin surface (30-60 min). Then samples were transferred into an alcyan blue (Alcyan blue 8GX, SIGMA T-5268, Steinheim, Germany) solution (for 100ml: 10mg alcian blue, 80 ml 95% ethanol and 20ml acetic acid) to cartilage staining. The incubation time to achieve staining saturation was 2 hours. In the following step, newborn seahorses were hydrated in decreasing ethanol (96% ethanol, Panreac T-141085, Barcelona, Spain) series (95, 95, 95, 75, 40, 15%), one hour each, and remained in distilled water until animals sank. Then a trypsin solution (SIGMA T-4799, Steinheim, Germany) (for 100 ml: 90 mg trypsin, 70 ml distilled water and 30 ml sodium borate saturated solution) was employed during one hour to tissue digestion. In the next step, the bone staining, samples were incubated (90 min) in alizarin red solution (Alizarin Red S, SIGMA T-4799, Steinheim, Germany) (1g/l alizarin red in 0.5% KOH). Finally newborn were incubated in the following increasing series of glycerol (Gly): 0.5%KOH solution (for each step at least 12 hours, maximun 24 hours):

- **Step 1** (for 100 ml: 75 ml KOH 0.5% and 25 ml Gly) – 1:3
- **Step 2** (for 100 ml: 50 ml KOH 0.5% and 50 ml Gly) – 1:1
- **Step 3** (for 100 ml: 25 ml KOH 0.5% and 75 ml Gly) – 3:1

To preserve samples, stained seahorses were stocked in 100% Gly solution (Glycerin, SIGMA G-2289, Steinheim, Germany), adding also some grains of thymol.

2.10 STATISTICAL ANALYSIS

All data obtained along this thesis were analysed and ordinated using various statistical tools. The premises of normality and homogeneity of the variables were previously performed using Kolmogorov-Smirnov and Levene's test ($P < 0.05$), respectively (Zar, 2009). When it was necessary, data were transformed to obtain

variance homogeneity. Means and standard deviations (SD) were calculated for each parameter measured.

Various statistical analyses were carried out depending on various studies. (Zar, 2009):

a. **Study I**: Two-way permutations ANOVAs were used to detect variations in the *H. hippocampus* abundances during surveys. Pair-wise test comparisons were also performed for factors showing significant differences in the test. The variation of sex-ratio (males:females), body colouration, and the presence or absence of skin filaments were studied taking in account different factors (season, habitat, sex and size). In all cases, significant deviations from the expected 1:1 ratio were analysed using a chi-square test. Moreover, comparative studies regarding mean size of seahorses were analysed using Student's *t*-test. Finally length-frequency distributions of seahorses were tested statistically using Kolmogorov-Smirnov test and the strength of relation among life-history stage and habitats was calculated through crosstabs tables, using Eta as statistic to measure the association level.

b. **Study II**: Multivariate methods such as principal coordinate analysis, PCoA, (Gower, 1966), were applied to ordinate different preserved seahorse specimens using various morphological characteristics. Also, proportional measurements and meristic counts performed on seahorses were compared among species and statistically tested using Student's *t*-test.

c. **Study III**: All comparative studies regarding *H. hippocampus* offspring (growth, biochemical composition and mortality observed during anaesthetic experiment) and preys (biochemical composition) were analysed using Student's *t*-test. Furthermore, their survivals were statistically tested with the loglineal model.

e. **Study IV**: All comparative studies regarding spawning quality and biochemical composition of prey and offspring were analysed using Student's *t*-test. Additional ANOVA tests were performed for differences in broodstocks between treatments and sexes as well as seasonal variations among mysids samples.

f. **Study V**: Two-way permutation-based analysis of covariance (ANCOVA) was used to detect differences in the seahorse abundances (male, female and

total). Fish and invertebrates observed during surveys were included as covariables in experimental design. Pair-wise test comparisons were also performed for factors showing significant differences in the test.

Significant differences were considered for $P < 0.05$-0.001 depending on the parameters studied. All results were processed and analysed with ©PRIMER 6.0 statistical packages for Windows (Clarke and Warwick, 2001) and SPSS Statistical Software System ver. 17.0. (SPSS Chicago, Illinois, 1999).