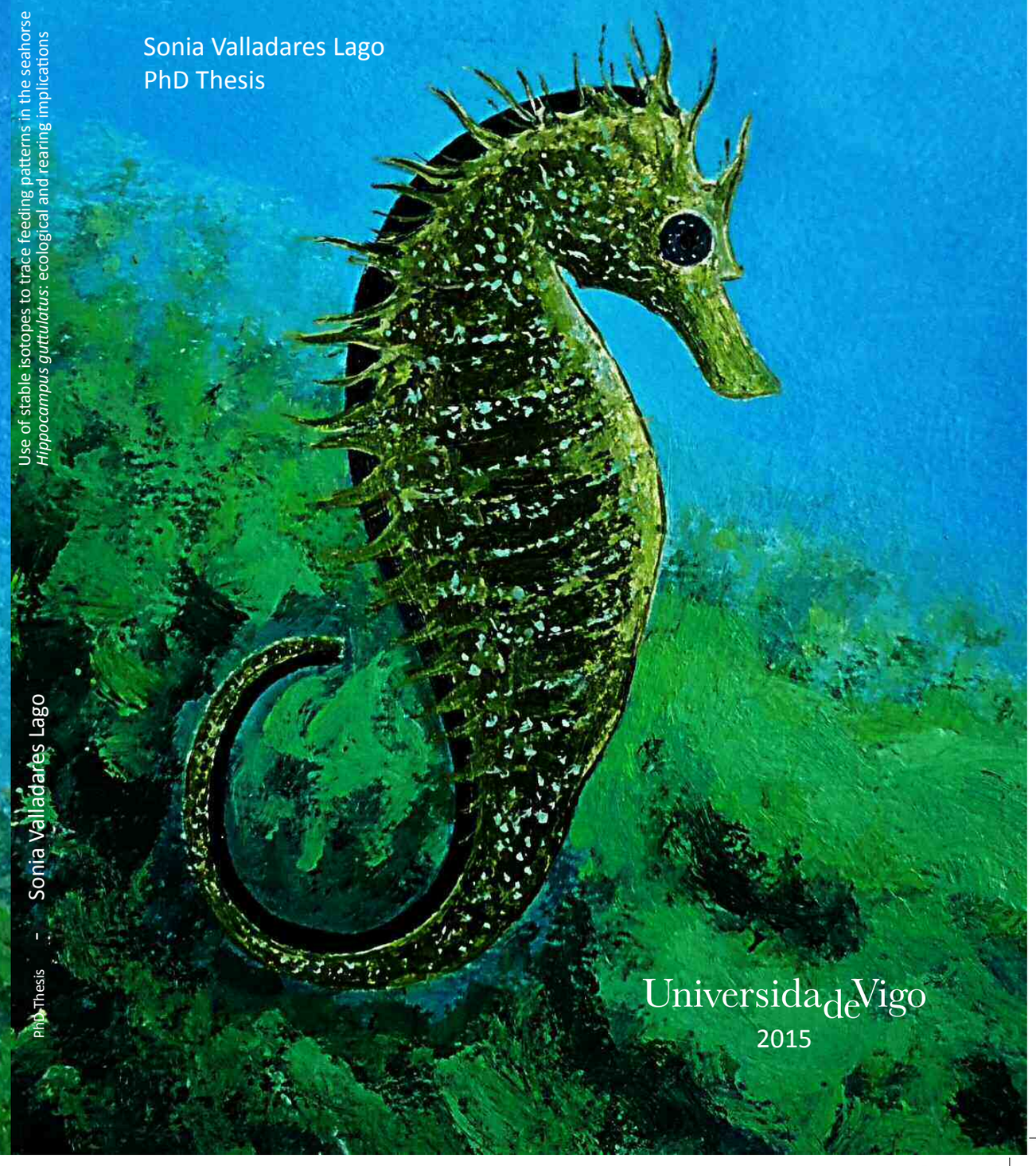
Universidade Vigo

Use of stable isotopes to trace feeding patterns in the seahorse *Hippocampus guttulatus*: ecological and rearing implications

Sonia Valladares Lago

PhD Thesis



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Use of stable isotopes to trace feeding patterns in the seahorse *Hippocampus guttulatus*: ecological and rearing implications

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Certifies:

The present Thesis, entitled **"Use of stable isotopes to trace feeding patterns in the seahorse *Hippocampus guttulatus*: ecological and rearing implications"** and presented to obtain the PhD degree from University of Vigo, has been fully written by Sonia Valladares Lago and carried out under my advisement at the Department of Marine Ecology and Resources at the Institute of Marine Research (IIM – CSIC).

The University tutor, Dra. Celia Olabarria Uzquiano allows its defence to the Department of Ecology and Animal Biology, responsible of the doctorate programme of Biodiversity and Ecosystems.

Vigo, May 27 2015

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‘Ondiñas veñen e van…’

Table of contents

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Preface

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SEAHORSES, one of the most fascinating creatures of the sea. Legend, fantasy and myths have surrounded them since ancient times. The HIPPOCAMPUS or HIPPOKAMPOI was a mythological creature described by Phoenician and Greek mythology as an aquatic beast with the head and forelegs of a horse and the serpentine tail of a fish. Nereid nymphs and sea gods were depicted in ancient art riding on the back of dolphins, Hippokampoi and other sea creatures. In Homeric poems Hippokampoi was considered the symbol of Poseidon (god of the sea and horses), whose chariot was drawn by two or four of these sea beasts. Likewise, depictions of Neptune in Roman mosaics represent the god in his seahorse-drawn chariot. Hippokampoi appears in Etruscan civilization as a theme in tomb wall-paintings and reliefs, and were sometimes provided with wings. In the Middle Ages, the mythic Hippocampus came to be regarded as real, but it was considered a sea monster (e.g. Olaus Magnus and Abraham Ortelius) or a sea dragon (e.g.Vincent de Beauvis and Albertus Magnus). During the Renaissance, descriptions of seahorses were often a combination of fact and fiction, as, for example, in John Josselyn's description: 'the most strange fish is a small one, so like the picture of Saint George his dragon, as possible can be, except his legges and wings' (New England's Rarities Discovered, 1672). Some naturalists classified them wrongly as insects or shellfish, as in early natural history texts. Even Linnaeus, listed them under Amphibia (amphibians) in one edition of the Systema Naturae (1758). In 1881, Ryder describes a seahorse specimen found as probably no teleostean fish due to the profoundly modified structure compared with the ordinary bony fish type. The term Hippocampus is now the scientific name to refer to the real animal commonly known as the seahorse. Although is still believed by many to be just a creature of myth, rather than a real animal.

Summary

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The worldwide decrease of wild seahorse populations is attributed to habitat degradation, incidental captures (by-catch) and overfishing for traditional medicines, curio and aquarium trades. A global conservation concern on the state of wild seahorse populations resulted in the inclusion of all seahorse species in the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species in 1996. Additionally, seahorses were also listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in November 2002. Conservation and management efforts are required to ensure seahorse population persistence in the wild, including captive breeding programmes as an alternative to the capture of wild individuals and research on seahorse life history traits for the improvement of the effectiveness of conservation programmes.

This PhD Thesis aimed to investigate the feeding patterns of one of the European seahorse species inhabiting in the Galician coast (NW Iberian Peninsula), the long-snouted seahorse *Hippocampus guttulatus*, whose wild populations have declined or disappeared in many areas in the last decades. The research was based on rearings in captivity and ecology approaches, specifically evaluating food assimilation in the early development of juvenile seahorses and assessing the dietary composition of wild seahorses. Both approaches were exclusively based on the application of the stable isotope analysis, which represent an important tool in ecological and physiological research. Stable isotopes are natural tracers widely used to determine nutrient assimilation and to reconstruct animal diets, among other applications. The method is based on the fact that stable isotopes are transferred from prey to predators in a predictable manner, that is, consumers incorporate the isotopic composition of the resources that they use. This Thesis represents the first attempt to investigate seahorse feeding issues using the stable isotopes approach. Increasing our knowledge on feeding aspects of this seahorse species would help to redefine their status and to (i) reduce the number of Data Deficient species on the IUCN Red List, (ii) optimise culture techniques, (iii) monitor population trends and (iv) guide future management and conservation actions.

The main critical bottleneck in seahorse rearing is the low survival of early juveniles, which is essentially related to feeding and nutritional requirements. A breeding program and a rearing methodology of *H. guttulatus* were firstly initiated by 'Proyecto Hippocampus' in 2006. The limited information available on the rearing of juveniles of this species resulted in initial low survivals and massive mortality events, which needed to be solved to achieve a successful rearing procedure for this species. In spite of significant recent improvements regarding survival rates, the feeding and nutritional requirements and their effects on initial mortalities in juveniles were scarcely studied. In this framework, the research performed in the first part of this Thesis were addressed to estimated diet assimilation for understanding

nutrition processes occurring in the early development of *H. guttulatus* juveniles, which may help in the interpretation of their growth and mortality rates.

Temperature is one of the most important environmental factors affecting seahorse growth and survival. Temperature has also a direct effect on carbon and nitrogen stable isotope values. Accordingly, the influence of seawater temperature on carbon and nitrogen stable isotope values (δ^{13}C and δ^{15}N) was investigated to assess food assimilation in early life stages of the seahorse *H. guttulatus* reared at different temperatures (15, 18 and 21 °C) and submitted to feeding and starvation conditions. Under feeding conditions, both δ^{13}C and δ^{15}N values in juveniles shifted progressively towards those of the dietary source (copepods and *Artemia*) especially under the most active feeding conditions (18 and 21 °C), which suggests a more efficient diet assimilation in seahorses maintained at warmer temperatures. The highest growth rate was observed at 21 °C, with a weight gain up to 11.80 ± 9.96 mg and a size gain of 23.73 ± 11.31 mm at the end of the experiment period (day 30). Conversely, the highest survival at day 30 was achieved at 18 °C (75.5 ± 14.2 %), which was significantly higher than at 15 °C (18.5 ± 7.2 %) and 21 °C (61.8 ± 27.1 %) (Kruskal-Wallis test, $p < 0.05$). The C:N ratios of juveniles were also higher at 18 °C suggesting a better physiological condition compared to those reared at 15 and 21 °C. This suggestion is also supported by the highest Fulton's condition factor achieved at 18 °C. On the other hand, the effect of food deprivation did not have a significant effect on δ^{13}C and δ^{15}N values. Starving seahorses maintained at 21 °C showed the lowest condition index (K_F = 1.01 ± 0.04), indicating that at that temperature juveniles would consume faster their own reserves. The proposed optimum seawater temperature for the development of *H. guttulatus* juveniles would be of 18 °C or slightly higher. At that temperature, higher survivals, best physiological conditions and more efficient food assimilation would be met.

In the rearing of seahorses, juveniles are generally fed on rotifers and *Artemia*, depending on the species and hence mouth size at birth. Feeding juveniles exclusively on *Artemia* has resulted in poor survivals, probably due to their poor digestibility. In this regards, copepods provided alone or supplemented with *Artemia* would be an alternative diet enhancing survival and growth rates. The assimilation of two life preys (*Artemia* and copepods) was traced in early juveniles of *H. guttulatus* using carbon stable isotopes to establish the most adequate diet for a successful rearing in the early life stages. For that, two experiments were performed: initial feeding on *Artemia vs.* copepods (Experiment 1), and shifting from copepods to *Artemia* (Experiment 2). Differences in growth between feeding regimes in both experiments were clearly noticed. At the end of Experiment 1 (day 20), the growth of juveniles fed on copepods (6.20 ± 1.07 mg; 30.8 ± 1.9 mm) was significantly higher than in those fed on *Artemia* (4.42 ± 1.36 mg; 27.96 ± 2.3 mm) (*t*-test = 2.97, p = 0.011; *t*-test = 2.84, p = 0.014, respectively). Similarly, a dietary shift from copepods to *Artemia* at day 20 resulted in a lower, but not significant (*t*-test = 1.74, p = 0.112), mean dry weight in 30-d old juveniles (7.46 ± 3.27 mg) in relation to those fed on a copepods feeding regime (10.80 ± 3.34 mg). However, not significant differences were noticed in mean standard lengths at day 30 between both feeding regimes (35.29 ± 3.4 and 35.08 ± 2.2 mm, respectively) (*t*-test = -0.85, p = 0.41). Regarding the survival, juveniles fed on *Artemia* in Experiment 1 exhibited significantly very low survivals (7.87 ± 1.12 %) compared to those from the copepods diet (98.66 ± 2.36 %) (*t*-test = 48.91, $p < 0.001$). After the dietary shift from copepods to *Artemia* in experiment 2, the average survival from days 20 to 30 decreased from 98.3 to 91.2 %, whereas no mortalities occurred in juveniles maintained on the copepods feeding regime. Since active foraging and ingestion was

observed from the onset of the Experiment 1, it seems that copepods are more efficiently assimilated than *Artemia* by *H. guttulatus* juveniles as growth and survival were clearly improved when copepods were offered, especially in early development. The progressive changes in $\delta^{13}C$ values toward those of the corresponding diet (copepods or *Artemia*) exhibited by juvenile seahorses in both experiments would indicate a successful assimilation of the food offered. Although the assimilation of *Artemia* may occur, its low nutritional quality would limit its contribution to tissue growth in juveniles, promoting higher mortalities, as clearly noticed in Experiment 1. Conversely, it is feasible that a better digestibility of ingested copepods would enhance their assimilation and hence that of essential nutrients with higher nutritional quality, promoting higher survival and growth rates (both experiments). Considering also the fact that the diet of juvenile seahorses in their natural environment is primarily based on copepods, it is reasonable to consider copepods as a more adequate diet than *Artemia* for the early rearing of juvenile seahorses.

The second part of this Thesis regards the assessment of the dietary composition of wild seahorses. Unlike more traditional dietary methods (e.g. gut content analysis, field and laboratory observations), stable isotope analysis provides information of assimilated food sources over longer time periods, ranging from days to months depending on the tissue analysed. Sampling of fish tissues (e.g. muscle, liver) for stable isotope analysis has traditionally acquired by the use of lethal techniques. Considering the conservation concern in seahorses, traditional methods are inappropriate and non-lethal methodologies are a must. Hence, the suitability of a fin tissue sampling (fin-clipping) methodology to provide a non-lethal tool for stable isotope analysis in adult seahorses was assayed. For that study, tissue samples were obtained entirely from naturally dead *H. guttulatus* seahorses. Firstly, comparisons of stable isotope values between common tissues (liver and muscle) and non-lethal tissues (dorsal fin) were performed to assess differences since isotope values can show variability among tissues. The similarity between $\delta^{15}N$ and $\delta^{13}C$ values in *H. guttulatus* dorsal fin and muscle tissue suggests that both tissues are adequate for stable isotope analysis to provide dietary information in a relatively long term. Secondly, the effects of tissue lipid extraction on carbon and nitrogen stable isotope values in dorsal fin tissue was evaluated, as it is known that the lipid content in tissues can potentially affect stable isotope values. Due to the very low lipid content in dorsal fin tissue (2.6 % lipids; C:N = 3.3), lipid removal had no effect on stable isotope values. Consequently, lipid removal in dorsal fin tissue of seahorses would not be necessary to perform stable isotope analysis. Finally, the limited availability of dorsal fin tissue obtained from fin-clipping in seahorses made necessary a previous assessment of sample size to evaluate its specific use in stable isotope analysis. The smaller section of the dorsal fin analysed (19.99 ± 9.10 mm^2 surface, 0.21 mg minimum dry weight) fully satisfied the minimum amount of carbon and nitrogen required (20 and 50 µg, respectively) for stable isotopes analysis with the analytical equipment used. The isotope values were found to be independent of the size of fin analysed (Linear regression, $F_{1,17} = 2.22$, $p = 0.15$, $F_{1,17} = 0.009$, $p = 0.92$, for nitrogen and carbon respectively). Therefore, fin-clipping is a non-lethal sampling procedure ensuring accurate and reproducible stable isotopes analysis in adult *H. guttulatus* seahorses. This sampling procedure could also be advisable for stable isotope analysis in other seahorse species. Within the conservation framework, the fin-cliping procedure allows to determine feeding habits in wild seahorses reducing the impact on the population under study.

Despite the increasing worldwide concern over the conservation status of seahorses there is a current lack of detailed information about their feeding ecology, which restricts

the effectiveness of management strategies. As all seahorse species, adult *H. guttulatus* are ambush predators feeding primarily on live crustaceans, especially amphipods, mysidaceans and decapods, but knowledge on its specific dietary composition is considerable scarce. Given that lack of information, the dietary composition of *H. guttulatus* seahorses inhabiting coastal waters of Galician was assessed using Bayesian stable isotope mixing models. Seasonal and spatial variations in food sources were analysed considering seahorse breeding period and three wild populations (Site 1: Toralla, Site 2: Bueu; Site 3: Ribeira). The three sites differed in their habitat characteristics, Toralla and Bueu were dominated by macroalgae and seagrass beds, and Ribeira was mainly characterised by accumulations of *Ulva* sp. and anthropogenic debris. Along a two years survey, 132 dorsal fin samples of seahorses were collected as well as main potential preys (benthic invertebrates) at several time periods. Potential preys were grouped in five taxonomic categories: Gammaridea, Caprellidea, Caridea, Mysidae and Annelida. The results of stable isotopes analysis in the samples showed that the breeding period did not have a significant effect on the isotopic composition of seahorses (MANOVA, Wilks, $F_{1, 128} = 0.36$, $p = 0.70$). However, significant differences were caused by sex and site (MANOVA, Wilks, $F_{1, 128} = 7.83$, $p < 0.001$; $F_{2, 128} = 9.34$, $p < 0.001$, respectively). The Bayesian stable isotope mixing model (MixSIAR) estimated the relative contributions of the prey items to the diet of *H. guttulatus* and revealed that Caprellidea would be the primary food source for *H. guttulatus* in the three sites; meanwhile, Mysidae and Annelida represented the less dominant preys. Diet proportion by site showed differential contributions of Gammaridea and Caridea to the seahorse diet, which could be attributable to the different habitat characteristics of each site. The second dominant prey group in Site 3 was Caridea, whereas Gammaridea was in Sites 1 and 2. The large proportion of Caprellidea, Gammaridea and Caridea suggest that these prey are consistent in the diet of *H. guttulatus*, being consumed regularly. In contrast, the low proportion of Mysidae and Annelida indicates that they are not essential dietary components for seahorses, and might be occasionally consumed. Prey selectivity of seahorses can be explained by their foraging behaviour. The relatively reduced swimming ability and low motion of seahorses would limit their success in capturing fast swimming preys such as mysids (Mysidae), and would benefit their efficiency on hunting benthic and less mobile crustaceans such as Caprellidea, Gammaridea and Caridea. Among other factors, feeding habits would explain the preference of *H. guttulatus* in occupying vegetated habitats where they can forage on these benthic crustaceans, instead of open areas where foraging on more scarce benthic crustaceans or more mobile mysids results more difficult. The spatial differences in diet could be the result of the diverse habitat characteristics among the three sites and the possible different habitat use by seahorses within each site, as each type of habitat would have specific distinct preys associated to.

The results presented in this Thesis provide relevant data regarding the feeding patterns of the seahorse *H. guttulatus* to support conservation actions of this endangered species.

Resumen

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Las poblaciones de caballitos de mar (*Hippocampus* spp.) se encuentran en regresión a nivel mundial como consecuencia de la degradación del hábitat, capturas accidentales (*by-catch*) y sobreexplotación y comercio indiscriminado con fines medicinales (medicina tradicional china principalmente), acuariofilia y decorativos (*souvenirs*). La capacidad de recuperación de estas especies en zonas donde ha desaparecido es muy lenta debido a su baja densidad poblacional, su distribución dispersa y su movilidad limitada. Debido al estado de las poblaciones de caballito de mar y la preocupación a nivel mundial sobre su conservación, en 1996 todas las especies de caballitos de mar se incluyeron en la Lista Roja de la Unión Internacional para la Conservación de la Naturaleza (IUCN) como especies vulnerables. Además, la Convención Internacional sobre el Comercio Internacional de Especies Amenazadas de Flora y Fauna Silvestres (CITES) incluyó todas las especies de caballitos de mar en su Apéndice II de especies amenazadas en noviembre de 2002. Una adecuada gestión y conservación de estas especies amenazadas es altamente necesario para una recuperación progresiva y asegurar la permanencia de sus poblaciones salvajes. El desarrollo de técnicas de reproducción y cría en cautividad, como alternativa a la captura de ejemplares salvajes, junto con la adquisición de un mayor conocimiento de los parámetros de la historia de vida de la especie considerada, permitirá mejorar la efectividad de futuros programas de conservación.

Esta Tesis doctoral se centra en investigar los hábitos alimentarios de una de las especies europeas de caballito de mar que habita en las costas gallegas (NO de la Península Ibérica), el caballito de mar narizón *Hippocampus guttulatus* Cuvier 1829. Las poblaciones de dicha especie en esta región han sufrido una disminución considerable o incluso desaparición en muchas áreas durante las últimas décadas. Desde 2003 la especie está incluida en el estado 'Deficiente en información' de la Lista Roja de la IUCN debido a la escasa información disponible para determinar el nivel de regresión de las poblaciones naturales y elaborar una correcta evaluación de su riesgo de extinción. La investigación que se presenta incluye dos áreas de estudio, la acuicultura y la ecología, con los objetivos de evaluar la asimilación del alimento en las primeras fases del desarrollo de crías de caballito de mar y examinar la composición de la dieta de los caballitos de mar salvajes. Ambas metodologías se basan exclusivamente en la aplicación del análisis de los isótopos estables, que representa una herramienta importante en estudios de ecología y fisiología animal. Los isótopos estables son trazadores naturales ampliamente empleados en el estudio de asimilación de nutrientes, así como en la reconstrucción de la dieta natural de animales, entre otras aplicaciones. La técnica se basa en la premisa de que la composición isotópica es transferida desde las presas a sus consumidores de manera predecible, esto es, los consumidores incorporan en sus tejidos la composición isotópica de los recursos que utilizan. La investigación de esta Tesis representa un innovador estudio de los aspectos alimentarios de los caballitos de mar mediante la aplicación de la técnica de los isótopos estables. El aumento de nuestro conocimiento sobre los aspectos alimentarios de esta

especie de caballito de mar puede ayudar a redefinir su estado de protección y reducir el número de especies de caballito de mar catalogadas como 'Deficientes en información' en la Lista Roja de la IUCN, optimizar las técnicas de cultivo, monitorizar las tendencias de sus poblaciones salvajes y guiar las futuras acciones de gestión y conservación.

El principal cuello de botella en el cultivo de los caballitos de mar es la baja supervivencia en las primeras fases de desarrollo de los juveniles, lo cual está fundamentalmente relacionado con requerimientos alimentarios y nutricionales. El desarrollo de técnicas de reproducción y cultivo en cautividad del caballito de mar *H. guttulatus* se inició por primera vez en 2006 con el 'Proyecto Hippocampus'. En la fase inicial del proyecto, la limitada información sobre la cría en cautividad de esta especie de caballito de mar resultó en tasas de supervivencia muy bajas y masivos eventos de mortalidad. De manera que una de las prioridades del proyecto se centró en el desarrollo de una técnica eficaz de cultivo para solventar dichas tasas de supervivencia de los juveniles. A pesar de importantes mejoras conseguidas en la producción de juveniles, los aspectos relacionados con los requerimientos alimentarios y nutricionales y su efecto sobre las mortalidades iniciales de los juveniles no fueron estudiados. En este marco, la investigación correspondiente a la primera parte de esta Tesis está dirigida a evaluar la asimilación del alimento para comprender los procesos nutricionales que ocurren en las primeras fases del desarrollo de los juveniles del caballito de mar *H. guttulatus*, y que a su vez ayudará a interpretar las tasas de crecimiento y mortalidad.

La temperatura es considerada uno de los factores físicos ambientales potencialmente más relevantes con efecto sobre la supervivencia y crecimiento de los caballitos de mar, además también tiene un efecto directo en los valores de isótopos estables de carbono y nitrógeno. Por consiguiente, se investigó la influencia de tres temperaturas del agua de mar (15, 18 and 21 °C) en los valores de isótopos estables de carbono y nitrógeno (δ^{13}C and δ^{15}N) para evaluar la asimilación del alimento en las primeras fases del desarrollo de los juveniles del caballito de mar *H. guttulatus* cultivados en estas tres condiciones de temperaturas. También se comparó la evolución de los valores de isótopos estables de juveniles mantenidos bajo condiciones de alimentación con juveniles sometidos a inanición. En condiciones de alimentación, se observó que los valores de δ^{13}C and δ^{15}N de los juveniles cambiaron progresivamente a lo largo del experimento hacía los valores de δ^{13}C y δ^{15}N de la dieta correspondiente (copépodos y *Artemia*). Estos cambios fueron especialmente notables en el caso de las condiciones de alimentación más activas (18 y 21 °C), lo cual sugiere una asimilación del alimento más eficiente en juveniles mantenidos a temperaturas más cálidas. La mayor tasa de crecimiento se observó a 21 °C, con un incremento de peso máximo de 11.80 ± 9.96 mg y un incremento de talla de 23.73 ± 11.31 mm al final del experimento (día 30). Por el contrario, la supervivencia más elevada al final del experimento se alcanzó a 18 °C (75.5 ± 14.2 %), que fue significativamente más elevada que a 15 °C (18.5 ± 7.2 %) y 21 °C (61.8 ± 27.1 %) (Kruskal-Wallis test, $p < 0.05$). La ratio C:N de los juveniles fue también más elevada a 18 °C lo que sugiere una mayor condición fisiológica para estos individuos comparada con los aquellos cultivados a 15 y 21 °C. Esta posible conclusión puede verse afirmada por el factor de condición de Fulton, el cual fue mayor a 18 °C. Por otro lado, el efecto de las condiciones de inanición no fue significativo ni en los valores de δ^{13}C ni δ^{15}N. Los juveniles sin alimentar y mantenidos a 21 °C manifestaron el índice de condición más bajo del experimento (K_F = 1.01 ± 0.04), por lo que se puede deducir que a esta temperatura los juveniles consumirían de una manera más rápida sus propias reservas. Según estos resultados, se propone que la temperatura del

agua de mar para obtener el desarrollo óptimo de los juveniles del caballito de mar *H. guttulatus* sea de 18 °C o algo más elevada. A esta temperatura, se obtendrá simultáneamente mayor supervivencia, mejor condiciones fisiológicas y una asimilación del alimento más eficiente.

En la cría de caballitos de mar, los juveniles han sido comúnmente alimentados con rotíferos y *Artemia*, según la especie de caballito de mar considerada y en función del tamaño inicial de la boca. Cuando la alimentación consistía únicamente de *Artemia* los resultados de supervivencia que se obtenían eran de valores muy bajos, probablemente debido a su mala digestibilidad. En este sentido, la introducción de copépodos como presa complementaria a la *Artemia* o subministrados como única presa inicial supuso una eficaz alternativa que mejoró las tasas de supervivencia y crecimiento de los juveniles de caballitos de mar. Con el fin de establecer la dieta más adecuada en las primeras fases del desarrollo del caballito de mar *H. guttulatus* para su cultivo exitoso, la asimilación de dos tipos de presas (*Artemia* y copépodos) en los juveniles se determinó mediante el análisis del isótopo estable de carbono (δ^{13}C). Se diseñaron dos experimentos: alimentación inicial *Artemia vs.* copépodos (Experimento 1), y cambio de alimentación de copépodos a *Artemia* (Experimento 2). Las diferencias de las tasas de crecimiento entre los dos tipos de alimentación fueron evidentes en ambos experimentos. Al final del Experimento 1 (día 20), el crecimiento, tanto en peso como en longitud total, de los juveniles alimentados con copépodos (6.20 ± 1.07 mg; 30.8 ± 1.9 mm) fue significativamente mayor que los juveniles alimentados con *Artemia* (4.42 ± 1.36 mg; 27.96 ± 2.3 mm) (*t*-test = 2.97, p = 0.011; *t*-test = 2.84, p = 0.014, respectivamente). De manera similar, los juveniles sometidos al cambio de dieta de copépodos a *Artemia* a día 20 mostraron un peso menor al alcanzar 30 días de edad (7.46 ± 3.27 mg), aunque no significativo (*t*-test = 1.74, p = 0.112), que los juveniles mantenidos hasta día 30 con la alimentación de copépodos (10.80 ± 3.34 mg). Sin embargo, no se detectaron diferencias significativas en relación a las longitudes totales de los juveniles de 30 días de edad entre los dos tipos de alimentación (35.29 ± 3.4 and 35.08 ± 2.2 mm, respectivamente) (*t*-test = -0.85, p = 0.41). Con respecto a la supervivencia, en el Experimento 1 los juveniles alimentados con *Artemia* manifestaron unas tasas de supervivencia (7.87 ± 1.12 %) significativamente menores comparado con los juveniles alimentados con copépodos (98.66 ± 2.36 %) (*t*-test = 48.91, p < 0.001). Después del cambio de dieta de copépodos a *Artemia* (Experimento 2), la supervivencia disminuyó de día 20 a día 30 desde un 98.3 a un 91.2 %, mientras que no se observaron mortalidades en los juveniles mantenidos con la alimentación de copépodos. Dado que desde el inicio del Experimento 1 se observó un comportamiento activo de captura e ingesta de los dos tipos de presa, se puede pensar que los juveniles de *H. guttulatus* asimilan de una manera más eficiente los copépodos que la *Artemia* ya que los experimentos demuestran una clara mejora en el crecimiento y la supervivencia cuando los copépodos están disponibles en la dieta de los juveniles. La evolución de los valores de δ^{13}C de los juveniles hacía los valores de δ^{13}C de la dieta correspondiente (copepods or *Artemia*) observada en los dos experimentos indica que los dos tipos de presas subministradas fueron asimiladas correctamente por los juveniles. Aunque estos cambios en los valores de δ^{13}C indican la asimilación de la *Artemia*, la baja calidad nutricional de dicha presa limitaría su contribución al crecimiento de los tejidos de los juveniles del caballito de mar, derivando en altas tasas de mortalidad, tal como se observó claramente en el Experimento 1 y en menor grado en el Experimento 2. Contrariamente, es posible que una mayor capacidad de digestión asociada a los copépodos ingeridos favorezca su asimilación y, por lo tanto, originando la disponibilidad de nutrientes

esenciales con elevada calidad nutricional, resultando en mejores tasas de supervivencia y crecimiento que se observaron en los dos experimentos. Considerando el hecho de que la dieta de los juveniles de caballitos de mar en su medio natural se basa principalmente en copépodos, es razonable considerar los copépodos como dieta adecuada y preferente para la cría en cautividad de juveniles de *H. guttulatus*, en lugar de la *Artemia*.

En la segunda parte de esta Tesis se aborda la determinación de la composición de la dieta de los caballitos de mar salvajes. A diferencia de los métodos tradicionales empleados en el estudio de la dieta de animales (como por ejemplo el análisis de contenidos estomacales, observaciones directas en el campo o laboratorio), el análisis de los isótopos estables proporciona información de las fuentes de alimentación que han sido ingeridas y asimiladas por el consumidor a lo largo de un períodos de tiempo extenso, que puede ser de días o meses dependiendo del tejido analizado. En peces, los tejidos que comúnmente se muestrean (como por ejemplo músculo, hígado) para el análisis de los isótopos estables se obtienen mediante técnicas letales para el animal. Considerando el estado de conservación de los caballitos de mar, descrito anteriormente, los métodos tradicionales de muestreo se consideraron inapropiados de manera que era prioritario establecer una metodología no letal para el muestreo de estas especies protegidas. En este contexto, se comprobó la utilidad del método de muestreo de las aletas (*fin-clipping*) como herramienta no letal para obtener las muestras de tejido necesarias para el análisis de los isótopos estables en caballitos de mar adultos. Para este estudio las muestras se obtuvieron únicamente de individuos de caballito de mar *H. guttulatus* que murieron de manera natural en las instalaciones del 'Proyecto Hippocampus'. En primer lugar, los valores de los isótopos estables de carbono y nitrógeno (δ^{13}C y δ^{15}N) se compararon entre dos tipos de tejido comúnmente usados (hígado y musculatura) y el tejido de aleta dorsal obtenido de manera no letal, para evaluar las posibles diferencias en los valores de los isótopos ya que puede existir variabilidad según el tejido considerado. La similitud entre los valores de δ^{13}C y δ^{15}N de la aleta dorsal y la musculatura sugiere que los dos tejidos por igual son adecuados para llevar a cabo análisis de los isótopos estables con el objetivo de obtener información de la dieta acerca de un período de tiempo relativamente largo. En segundo lugar, se estudiaron los efectos de la extracción lipídica sobre los valores de los isótopos estables de carbono y nitrógeno de la aleta dorsal, porque se conoce que el contenido lipídico de los tejidos puede potencialmente afectar a los valores de los isótopos estables. Debido a la baja cantidad de contenido lipídico de la aleta dorsal (2.6 % lipids; C:N = 3.3), la extracción lipídica no tuvo efecto en los valores de los isótopos estables. De este modo, la extracción lipídica de la aleta dorsal de los caballitos de mar previamente al análisis de los isótopos estables no sería necesaria. Por último, debido a la limitada cantidad de tejido de aleta dorsal que se puede obtener mediante el muestreo *fin-clipping* en caballitos de mar fue necesario una previa comprobación en relación al tamaño de muestra adecuada para su uso específico en el análisis de isótopos estables. La porción de aleta dorsal más pequeña analizada (19.99 ± 9.10 mm^2 de superficie, 0.21 mg de peso seco mínimo) contenía las cantidades mínimas de nitrógeno y carbono (20 and 50 µg, respectivamente) necesarias para el adecuado análisis de los isótopos estables en el equipo analítico utilizado. Además, los valores de isótopos estables fueron totalmente independientes del tamaño de la aleta analizada (Regresión Lineal, $F_{1,17}$ = 2.22, p = 0.15, $F_{1,17}$ = 0.009, p = 0.92, para nitrógeno y carbono respectivamente). Por lo tanto, se puede concluir que el método *fin-clipping* es un procedimiento de muestreo no letal que asegura el análisis de isótopos estables precisos y reproducibles en ejemplares adultos del caballito de mar *H. guttulatus*. Este procedimiento

de muestreo podría también ser aplicado adecuadamente en otras especies de caballitos de mar. En el contexto propio de conservación, el procedimiento descrito permite el estudio de los hábitos alimentarios de caballitos de mar salvaje reduciendo el impacto causado en la población objeto de estudio.

A pesar de la creciente preocupación a nivel mundial sobre el estado de conservación de los caballitos de mar, mencionada anteriormente, existe actualmente una gran falta de información detallada sobre los hábitos de alimentación en su medio natural lo cual limita la efectividad de los planes de gestión sobre estas especies. Como todas las especies de caballito de mar, los adultos *H. guttulatus* son depredadores visuales que se alimentan principalmente de crustáceos, especialmente de anfípodos, misidáceos y decápodos, sin embargo el conocimiento de su composición detallada de la dieta es muy escaso. Dado este vacío de información, se determinó la composición de la dieta del caballito de mar *H. guttulatus* de poblaciones localizadas en costa gallega mediante la aplicación de modelos Bayesianos de isótopos estables (*mixing models*). Las variaciones temporales y espaciales en las fuentes de alimento preferidas por los caballitos de mar fueron analizadas considerando período reproductor y tres poblaciones (Sitio 1: Toralla, Sitio 2: Bueu; Sitio 3: Ribeira). Estas tres poblaciones se caracterizan por presentar diferente tipo de hábitats, Toralla y Bueu están dominadas por parches de macroalgas y praderas de fanerógamas (*Zostera marina*), mientras que Ribeira está dominada principalmente por acumulaciones del alga *Ulva* sp. y de restos antropogénicos. Los muestreos se llevaron a cabo durante dos años distribuidos en diferentes períodos de tiempo. En total se muestrearon 132 muestras de aleta dorsal de los caballitos de mar, y también se recogieron muestras de las principales presas potenciales para el caballito de mar (invertebrados bentónicos). Las presas potenciales se agruparon en cinco categorías taxonómicas: Gammaridea, Caprellidea, Caridea, Mysidae y Annelida. Los resultados de la ANOVA multivariante indicaron que el período reproductor no tuvo efecto significativo en la composición isotópica de los caballitos de mar (MANOVA, Wilks, $F_{1, 128} = 0.36$, $p = 0.70$). Las diferencias significativas fueron causadas por los factores sexo y sitio (MANOVA, Wilks, $F_{1, 128} = 7.83$, $p < 0.001$; $F_{2, 128} = 9.34$, $p < 0.001$, respectivamente). El modelo Bayesiano de isótopos estables (MixSIAR) estimó las contribuciones relativas de cada una de las presas a la dieta del caballito de mar *H. guttulatus* revelando que Caprellidae representa la principal fuente de alimento para esta especie de caballito de mar en los tres sitios evaluados, seguido por Gammaridea y Caridea, mientras que Mysidae y Annelida serían las presas con menor representación en la dieta. Analizando la proporción de la dieta de tres sitios por separado, las diferencias en las contribuciones a la dieta se debieron únicamente a los grupos Gammaridea y Caridea, que puede ser debido a los diferentes hábitats que caracterizan cada sitio. En Ribeira, el segundo grupo dominante en la dieta fue Caridea, mientras que en Toralla y Bueu fue Gammaridea. La mayor proporción de los grupos Caprellidea, Gammaridea y Caridea sugiere que son presas consumidas regularmente por el caballito de mar. Por el contrario, la baja proporción de Mysidae y Annelida indica que no son componentes esenciales en la dieta del caballito de mar *H. guttulatus* y podrían ser consumidos ocasionalmente. La preferencia de presas por los caballitos de mar puede ser explicada por su comportamiento de alimentación, su reducida capacidad de natación y baja movilidad limitaría el éxito de captura de presas activamente nadadoras como los misidáceos (Mysidae), y beneficiaría su eficacia en la captura de crustáceos bentónicos menos móviles como los grupos Caprellidea, Gammaridea y Caridea. El conocimiento de los hábitos alimentarios ayuda a explicar la documentada preferencia del caballito de mar *H.*

guttulatus en ocupar hábitats con vegetación donde podría alimentarse de estas presas bentónicas, en lugar de permanecer en áreas sin vegetación donde tendría más dificultad en encontrar crustáceos bentónicos y/o capturar los misidáceos. Por otro lado, la variabilidad del tipo de hábitat entre los tres sitios estudiados y las posibles diferencias en la preferencia de hábitats entre los caballitos de cada sitio podría explicar las diferencias espaciales en su dieta, ya que distintos tipos de vegetación pueden tener asociadas ciertas presas reflejando así la diferente selección de presas por el caballito de mar.

Los resultados presentados en esta Tesis proporcionan información relevante con respecto a los patrones de alimentación del caballito de mar *H. guttulatus* que puede servir para apoyar futuras acciones de conservación de esta especie amenazada.

Chapter 1

General Introduction

General Introduction

§

1. Seahorses

Seahorses are an extraordinary and enigmatic group of teleosts marine fishes, extensively considered as the most unfish-like in existence. Their unique biological features and the traditional legends that surround them have attracted the interest of many, becoming significant worldwide fish for conservation, economic and medicinal reasons.

1.1. Seahorse taxonomy

The term syngnathids refers to all members of the family Syngnathidae, which include pipefishes, pipehorses, seadragons and seahorses. They are assigned within the order Gasterosteiformes or Syngnathiformes, a diverse group of fishes with the evolutionary relationships among the component families still confused (Pietsch, 1978; Fritzsche, 1983; Bowne, 1994; Nelson, 1994; Keivany and Nelson, 2006; Kawahara et al., 2008; Wilson and Orr, 2011) (Fig. 1.1.). The family name Syngnathidae means 'jaw fused', from the Greek words *syn*-fused and *gnathus*-jaws, a common trait represented in the family and used for the taxonomical grouping. There are around 55 genera and close to 300 species estimated inside this family (Nelson, 1994; Kuiter, 2000). Within the family Syngnathidae, the four subfamilies identified are based on the position in the body and development of the male brood pouch (specialised incubation area): eggs incubated in a fully enclosed brood pouch under its tail (Hippocampinae – pygmy seahorses and seahorses), eggs incubated in a well defined brood pouch under the trunk or tail formed by lateral membranes (Syngnathinae – pipefishes), exposed eggs in a spongy mass under the tail or trunk section (Solegnathinae – pipehorses and seadragons), exposed eggs without spongy mass located under the abdomen (Doryrhamphinae – flag-tail pipefishes) (Herald, 1959; Wilson et al., 2001; Stölting and Wilson, 2007) (Fig. 1.1.).

Seahorses comprise a single genus, *Hippocampus* (meaning horse-*hippos* and sea-monster-*campus* in Greek), which represents the largest species-rich genera in the family Syngnathidae. This monophyletic group exhibits a number of distinctive morphological traits, including the position of the head at right angle to the trunk, the absence of a caudal fin, a prehensile tail and a specialised brood pouch closed along the midline (Ginsburg, 1937; Fritzsche, 1980). The taxonomy of the genus has been studied for many years, initiated with the original description of the seahorse *Syngnathus hippocampus* in Linnaeus' *Systema Naturae* (1758). Several authors have since described new species of seahorses and reviewed their taxonomy, including Kaup (1856), Günther (1870), Duméril (1870), Ginsburg (1937), Fritzsche (1980), Vari (1982) and Dawson (1985). More than 100 names for seahorses have been recorded in that early literature, leading to a great deal of discrepancy

in the real and valid seahorse species mainly due to misidentifications, synonyms, incorrect museum labels or spelling errors (Lourie et al., 1999; Kuiter, 2001). Since the first seahorse identification guide (Lourie et al., 1999), which recognised 32 species, further revisions based on morphological and genetic evidences still show a disagreement on the number of recognised *Hippocampus* species: 72 species (ITIS, 2004), 54 species (Fishbase, 2014), 48 species (Vincent et al., 2011), 38 species (IUCN, 2014).

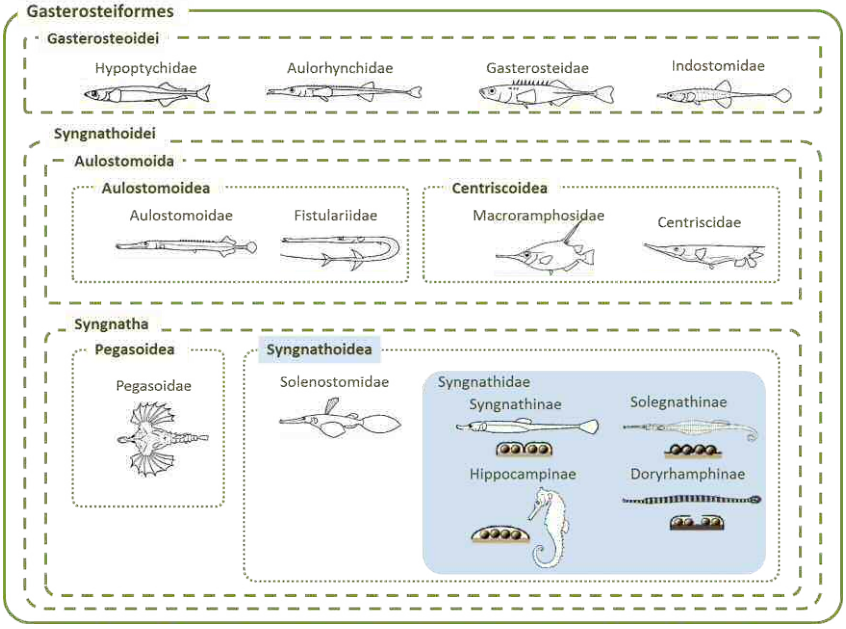


Figure 1.1. Schematic classification of the order Gasterosteiformes. Based on Kawahara et al., 2008. Drawings from Nelson, 1994. Pouch morphology of Syngnathidae from Stölting and Wilson, 2007.

Because seahorse species in existence are spread worldwide, it was estimated that the genus *Hippocampus* evolved before the final closure of the Tethys seaway (Fritzsche, 1980), which connected the Indo-Pacific with the Atlantic until the Middle Miocene. Genetic analyses suggest that this genus originated approximately 15 or 16 million of years ago (Teske et al., 2004; Žalohar et al., 2009; Wilson and Orr, 2011). The oldest known fossil records of seahorses found in the Middle Miocene (Lower Sarmatian) beds in Slovenia proved the presence of seahorses in the Central Paratethys Sea at that time (Žalohar et al., 2009). The two seahorse species identified, *Hippocampus sarmaticus* and *H. slovenicus*, represent 'fully developed seahorse species' (Fig. 1.2.).

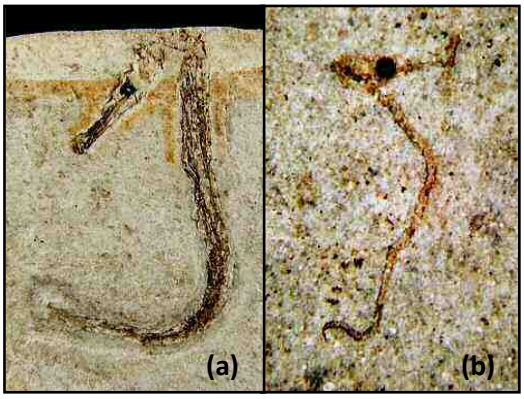


Figure 1.2. *Hippocampus sarmaticus* (a) and *Hippocampus slovenicus* (b) seahorse fossils from the Middle Miocene (lower Sarmatian) in Slovenia (Žalohar et al., 2009).

1.2. Seahorse biology

All seahorses share the same unusual morphological characteristics and extraordinary adaptations (Vincent, 1996; Lourie et al., 1999; Kuiter, 2000) (Fig. 1.3.). They have a horse-like head positioned at a right angle to the body and acquire an upright posture. The evolution of their vertical posture would have favoured seahorses by improving their camouflage when seagrass habitat expansion occurred in the Indo-West Pacific during the Late Oligocene. Contrarily, pygmy seahorses (with horizontal posture they are an evolutionary link between seahorses and other syngnatids) would not have benefited from the seagrass radiation, and remained restricted to the macroalgal reefs in which they still occur today (Teske and Beheregaray, 2009). Furthermore, the unique head and trunk position of seahorses potentially improve their prey-capture performance thereby enhancing their fitness and survival (Van Wassenbergh et al., 2011). The head terminates in an elongated and narrow snout with an un-teethed jaw adapted for specialised suction feeding (Ryder, 1881; Muller, 1987; Leysen et al., 2010). Their eyes move independently of each other like a chameleon, allowing them to improve their predation success and observe its environment for predator avoidance (Lourie et al., 1999; Foster and Vincent, 2004; Choo and Liew, 2006). Instead of scales over the skin, like other fishes, the seahorse's body is covered by robust internal bony plates arranged into a series of articulating rings visible along the length of the fish (Ryder, 1881; Ginsburg, 1937) (Fig. 1.4.). This subdermal armour provides seahorses with body support, as well as helping them to protect against predation (Lourie et al., 1999; Porter et al., 2013).

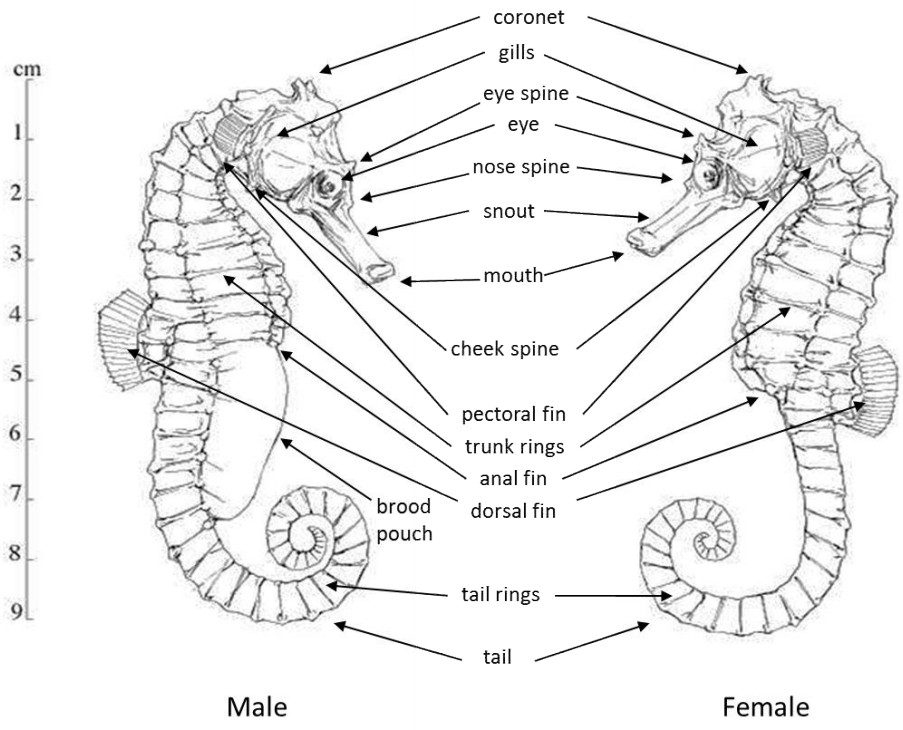


Figure 1.3. General external morphology of male and female seahorses. Drawings from Lourie et al., 2004.

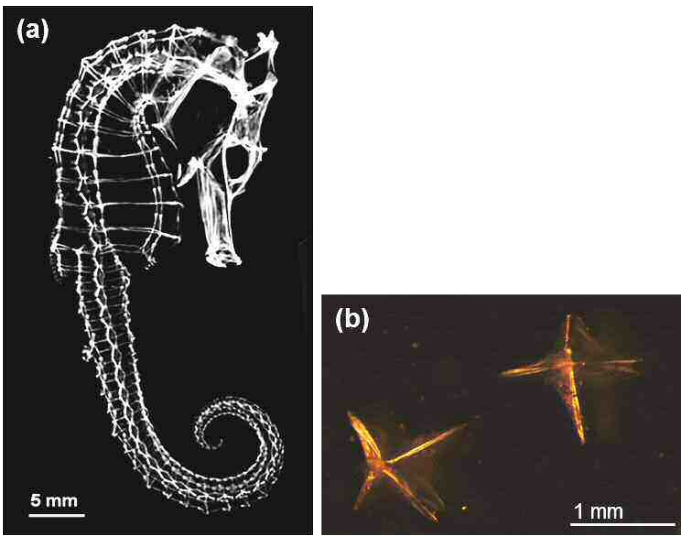


Figure 1.4. (a) Scan of a juvenile seahorse skeleton (*Hippocampus kuda*) from Porter et al., 2013; (b) subdermal bony plates of a juvenile seahorse (*Hippocampus guttulatus*) reared in captivity at the IIM-CSIC facilities, picture taken under dissecting microscope.

Seahorses are considered sedentary fishes with poor swimming abilities, lacking both the form and the propelling mechanism for speed. With the lack of pelvic and caudal fins, a certain independent locomotion is produced exclusively by rapid undulations of the small dorsal fin (on their back) for a slow propulsion in a vertical position, while the two small pectoral fins (below the gills opening) serve as steering control and the reduced anal fin could aid in the ascending propulsion (Ryder, 1881; Breder and Edgerton, 1942; Consi et al., 2001). The tail is an extension of the vertebral column covered by a chain of articulating rigid bony plates and a complex system of muscles and tendons, a combination that provides sufficient flexibility to function as a prehensile organ (Hale, 1996; Bruner and Bartolino, 2008; Van Cauter et al., 2010; Praet et al., 2012). The ability of bending the tail is a unique trait among fishes and yet a common trait among seahorses and pipehorses and represents an important role in numerous aspects of their biology. With their prehensile tail they tend to grasp the vegetation or holdfasts (any structure used as attachment sites). Also the tail has an important function in the social behaviour of these fishes: males strongly hang on to other with the tail when competing for females and it is used gently by the couple during mating (Vincent, 1994; Vincent and Sadler, 1995). There is not a large intraspecific variation in the number of trunk and tail rings, but the existing difference among species is used for seahorse species identification (Lourie et al., 1999).

Adult seahorse size range from less than 2 cm of the tiny *Hippocampus denise* up to 35 cm of the large *Hippocampus abdominalis* (Foster and Vincent, 2004). Beyond the distinct sexual difference of the brood pouch on males, sexual dimorphism in body proportions is consistently found in seahorses; usually males have longer tails and females longer trunks. A longer tail may enable a male to support a large caudal brood pouch while still grasping a holdfast, or may give them an advantage in the tail-wrestling exhibited during mating competition (Foster and Vincent, 2004). Seahorses remain attached to various substrates by the tail for much of the time and are generally more active during daylight than at night (Foster and Vincent, 2004). They are highly specialised in camouflage, being cryptic with the surrounding. Hence it is commonly very difficult to spot them in the wild. Their flexibility to blend in with the habitat where they live is achieved by changing colours and sometimes growing extra skin filaments (Kuiter, 2000; Foster and Vincent, 2004). Colour modification is involved in social interaction among seahorses, as a method to communicate interest,

recognition, and perhaps health and vitality (Vincent, 1994; Vincent and Sadler, 1995; Lourie et al., 1999). Overall, seahorses rely more on camouflage to avoid detection by predators than on speed for escape. When this strategy is ineffective, adult seahorses may be refused by predators due to their unpalatable bony plates, their low digestibility and caloric value of these fishes (Lourie et al., 1999; Harris et al., 2007). However, they can be opportunely taken by cephalopods, crabs, large pelagic fishes, turtles, marine birds and marine mammals (Kleiber et al., 2011). In the case of the planktonic juveniles, larger predation may occur by many fish and invertebrates (Lourie et al., 2004). Unfortunately, mortality rates for all life history stages are generally unknown. The inferred life spans of seahorses, generally from laboratory observation, range from about one year in the very small species to about three to five years in the larger species (Foster and Vincent, 2004; Curtis and Vincent, 2006).

The camouflage also helps seahorses to act as visually and ambush predators by reducing their visibility to prey. It has been observed that feeding behaviour occurs mostly during diurnal or crepuscular time (James and Heck, 1994; Felício et al., 2006). Because seahorses have no teeth and a digestive tract without a differentiated stomach (Foster and Vincent, 2004) (Fig. 1.5.), prey items must be generally swallowed whole and pass rapidly through the digestive system, although it has been observed they can break large prey into two pieces before ingestion (Woods, 2002). During the feeding process, they usually grasp on holdfast and wait until prey approaches the mouth, while the body remains reasonably stationary which is more energy efficient and more advantageous than swimming towards the prey (Muller, 1987; James and Heck, 1994). This particular feeding strategy requires the combination movement of the different components of the feeding system (neurocranium, hyoid, lower jaw and snout) to generate a successful prey capture. The prey is captured with a rapid movement of the head towards the prey (first phase) followed by a coordinated expansion of the buccal cavity (second phase) in order to create a strong flow of water towards and into the mouth opening (Muller, 1987; Bergert and Wainwright, 1997; Roos et al., 2009a, b; Leysen et al., 2010; Van Wassenbergh et al., 2013). The entire process occurs incredibly fast, in less than 5 ms adult seahorses are able to capture prey. Unlike other fishes, the complex integrated musculoskeletal system involved in the feeding process appears to be fully functional in seahorses at birth, which provide juvenile seahorses with an effective and rapid means of capturing prey (Van Wassenbergh et al., 2009; Leysen et al., 2010). This underlines the essential role of the brood pouch in male seahorses, allowing a later developmental stage and the maturation of the feeding system, and may help to overcome critical periods during early ontogeny (Kornienko, 2001; Van Wassenbergh et al., 2009; Leysen, 2011). Nevertheless, feeding success in juveniles has been reported slightly low compared with adults (Van Wassenbergh et al., 2009; Roos et al., 2010); such a result could be explained by some developmental limitations.

The morphology of the snout undergoes significant changes throughout ontogeny, getting larger while the seahorse grows, and this could affect the kinematics of the feeding process (Choo and Liew, 2006; Leysen et al., 2010; Roos et al., 2010; Roos et al., 2011; Blanco et al., 2014), as well as the prey selection in terms of size. Preference for larger prey items with increasing seahorse sizes have been reported in *Hippocampus zosterae* (Tipton and Bell, 1988), *H. mohnikei* (Kanou and Kohno, 2001), *H. erectus* (Teixeira and Musick, 2001) and *H. abdominalis* (Woods, 2002). An increase in prey size is presumably more energy efficient and directly benefits the growth of a seahorse.

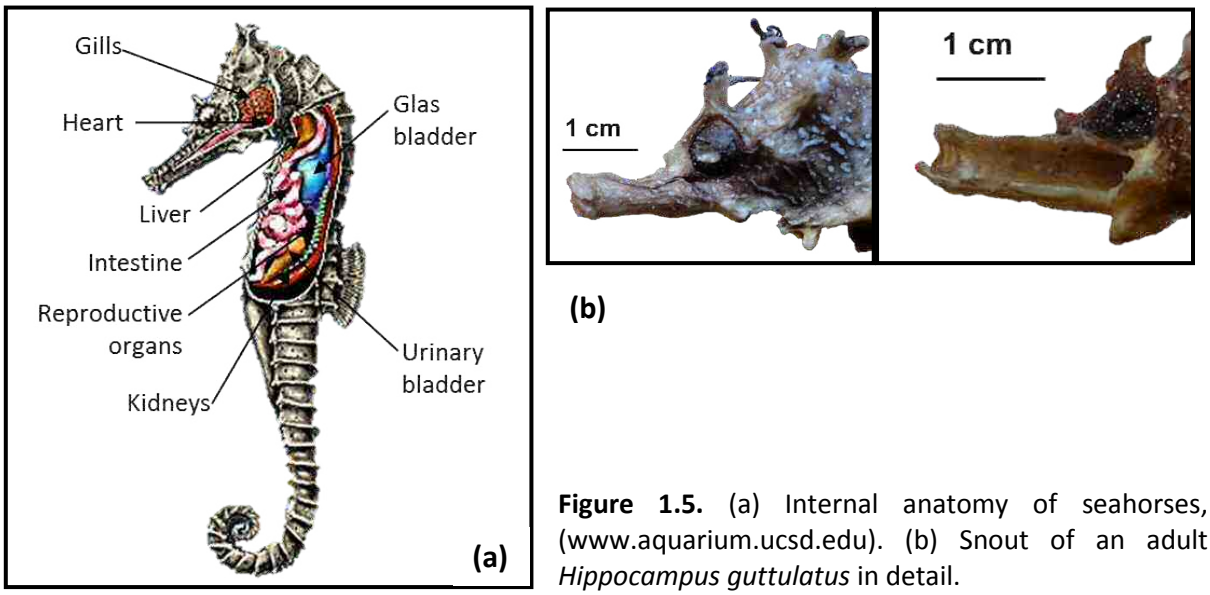


Figure 1.5. (a) Internal anatomy of seahorses, (www.aquarium.ucsd.edu). (b) Snout of an adult *Hippocampus guttulatus* in detail.

Other factors like habitat change or feeding capabilities development can be involved in the variation of the diet in growing seahorses (Tipton and Bell, 1988). Very few studies have reported the natural diet in juvenile stages, being copepods undoubtedly a prevalent dietary item (Tipton and Bell, 1988; Castro et al., 2008) or of secondary importance (Teixeira and Musick, 2001). In the case of adult seahorses, studies in the wild have indicated that they feed primarily on live crustaceans, small enough to fit into their mouths. Few studies have dealt with the identification of the specific prey items ingested, mostly determined by examination of gut contents. Harpacticoid and Calanoid copepods comprised the major prey items eaten by *H. zosterae* in Tampa Bay, Florida (Tipton and Bell, 1988). *H. erectus* from Virginia fed almost exclusively on amphipods (Teixeira and Musick, 2001). For *H. abdominalis* from New Zealand amphipods, mysids, and caridean shrimp represented the main dietary components (Woods, 2002). Gammarids amphipods were the most important prey for *H. breviceps* in south-western Australia (Kendrick and Hyndes, 2005). Nematods and Copepods were described as the main preys consumed by *H. reidi* in Brazil (Castro et al., 2008). The diet of *H. patagonicus* from Argentina consisted mainly of decapods and amphipods (Storero and González, 2008). Diet of *H. guttulatus* from south Portugal consisted mainly of amphipods, decapods and isopods (d'Entremont, 2002). In the Aegean Sea, the main prey groups in the diet of *H. hippocampus* and *H. guttulatus* were Amphipoda, Mysidacea and Anomura Decapoda (Kitsos et al., 2008; Gurkan et al., 2011). The presence of sand and vegetation fragments observed uncommonly in the gut contents indicates that an accidental ingestion of both particles may occur during the suction of prey on sandy seabeds and when eating small preys adhered to the vegetation (d'Entremont, 2002; Woods, 2002; Kendrick and Hyndes, 2005). Cannibalism of juveniles appears to be a common phenomenon described in various seahorse species, *H. reidi* (Rosa et al., 2005), *H. patagonicus* (Storero and González, 2008), *H. guttulatus* and *H. hippocampus* (Kitsos et al., 2008) and *H. abdominalis* (pers. obs.).

The diet specialisation among adult seahorse species can be explained by their snout morphology, individual size, method of predation, availability and abundance of prey in the ecosystem (Tipton and Bell, 1988; Kendrick and Hyndes, 2005; Woods, 2002). Considering the large variation in snout dimensions among adult seahorses, species with relative long

snouts exhibit a faster prey capture (de Lussanet and Muller, 2007; Roos et al., 2010) and a greater success attacking fast swimming (planktonic) preys such as mysids, while short snouts are more advantageous to capture less mobile preys and epifaunal preys in complex habitats (Kuiter, 2001; Kendrick and Hyndes, 2005). Different prey captured has been related to the foraging behavior of seahorses; more sedentary species would hunt preferentially on epibenthic rather than planktonic preys (Kendrick and Hyndes, 2005; Castro et al., 2008). Seahorses are able to occupy a wide range of habitats and, hence, the type of prey they consume could be related to the habitats in which they forage, influenced by the likelihood of encountering a particular type of prey (James and Heck, 1994; Kendrick and Hyndes, 2005). Besides the differences in diet attributed to the seahorse behavior, other factors are involved in such changes. Seasonal differences in feeding habits may be caused by temporal variations in the relative abundance of preys (Woods, 2002). According to sex, differences in food preference between female, non-pregnant and pregnant male were evident depending on the species considered (d'Entremont, 2002; Woods, 2002; Castro et al., 2008).

One of the most extraordinary features exhibited by seahorses is their unique reproductive biology. Pregnancy of male seahorses provides the most extreme example of paternal care known. They have a specialised brood pouch or marsupium (incubating area) where eggs are deposited, fertilised and incubated during the entire gestation period (Ryder, 1881; Ginsburg, 1937; Herald, 1959; Fritzsche, 1983). Sexual maturity of males is easily recognised and inferred from the fully developed of the brood pouch (Wilson and Vincent, 1998; Baum et al., 2003; Curtis and Vincent, 2006). The time at first maturity has been frequently determined by the seahorse age, and ranged from 3 months in case of smaller seahorse species (*H. zosterae*, Strawn, 1958) to 5-12 months for many other species (Wilson and Vincent, 1998; Whitfield, 1995; Woods, 2000a; Curtis and Vincent, 2006). However, the size would serve as a better predictor of first maturity (Foster and Vincent, 2004).

Seahorses have a long and elaborated courtship rather similar among species in which males and females start "dancing" up and down repeatedly in the water column by flexing tails and bending their bodies, showing coloration variation, and culminate with eggs transfer from female to male via her ovipositor. The male fertilises the eggs once are deposited in the brood pouch, then embryos develop into the pouch, protected and provided with oxygen and additional nutrients, although most nutrition comes from the egg yolk (Linton and Soloff, 1964; Hardy, 1978; Vincent et al., 1992; Wilson et al., 2001; Carcupino et al., 2002; Foster and Vincent, 2004; Monteiro et al., 2005; Stölting and Wilson 2007). The complex interactions before copulation play an important role in the reproductive synchrony between sexes (Vincent, 1995). The eggs are hydrated by the female before copulation but, as the storage appears not to last for long, they are commonly dumped if she has not found a mate within about 24 hours of hydrating eggs. Seahorse eggs are pear-shaped or oval, semi-transparent, and with a characteristic orange colour as a result of the carotenoids from their crustacean-dominated diets (Ryder, 1881; Hardy, 1978). The number of eggs per female can range from 9 (*H. zosterae*, Masonjones and Lewis, 1996) to more than 1000 (*H.erectus*, Teixeira and Musick, 2001) depending on adult size and species.

Period pregnancy ranges from 9 (*H. comes*) to 45 (*H. capensis*) days, depending on species and is positively correlated with latitude and water temperature (Foster and Vincent, 2004). Breeding seasons vary from all year around or restricted to the summer

season depending on the species, and could be influenced by environmental factors such as light, temperature and food availability (Foster and Vincent, 2004). At birth, which generally occurs at night over several hours, the newborns are forced out of the brood pouch by means of continual contractions by the male (Vincent and Sadler, 1995; Faleiro et al., 2008; pers. obs.). Males of most seahorse species release 100–300 newborns on average per pregnancy, with a maximum of 2000 (*H. ingens*) and a minimum of only five (*H. zosterae*). Released newborns resemble miniature adult seahorses, fully developed, with sizes ranging from 2 mm (*H. bargibanti*) to 20 mm (*H. abdominalis*) which have been correlated with latitude (Foster and Vincent, 2004). The newborns are initially pelagic, receive no further parental care and, in contrast to the adult, are very vulnerable as prey. This pelagic phase can last for a few hours, days or weeks (Foster and Vincent, 2004).

Most seahorse species are considered socially monogamous mating exclusively with one partner in a single breeding season. Monogamy probably acts to increase the reproductive success in low population densities, resulting in larger broods and reducing the time spent on courtship (Kvarnemo et al., 2000; Vincent, 1995; Vincent and Sadler, 1995; Carcupino et al., 2002). Contrary, there are some pieces of evidence that suggest seahorses can also mate with several partners in the same breeding season (polygamy), a breeding behaviour that may have derived from a combined effect of greater population density and higher rate of movement increasing chances of extra-pair encounters (Wilson et al., 2003).

1.3. Distribution and habitat

Seahorses are found worldwide, from about 50° North to 50° South, with the highest diversity of species in the IndoPacific region. Latitude, in terms of photoperiod and temperature, has been linked to many life-history variables as it is known to affect physiological functions (Wong and Benzie, 2003; Lin et al., 2006; Planas et al., 2013).

They are exclusively marine, generally inhabit coastal regions above 30 m deep in shallow tropical and temperate waters, although many have been found between 40 and 100 m deep (Lourie et al., 1999; Foster and Vincent, 2004). Some estuarine species appear to tolerate fluctuating salinities, although they experience high rates of mortality during freshwater flooding (Russell, 1994; Bell et al., 2003). Temperate species predominantly occur in seagrass and macroalgae beds, while tropical species are primarily found among coral reefs, but also among mangroves forests and sponge gardens. Some seahorse species have been shown to have a preference for using particular species of plant and animal as holdfasts (Lourie et al., 2004; Vincent et al., 2005). Each species displays habitat preferences in order to obtain the optimum opportunity for food and the best predator avoidance. Although seahorse species are generally encountered inhabiting vegetated habitats, they are also found in open sandy or muddy seabeds (Bell et al., 2003; Garrick-Maidment, 2004; Lourie and Vincent, 2004; Curtis and Vincent, 2005). Seahorses are also often found associated with artificial structures including wooden piers, cages, ropes and nets (Lourie et al., 2004; Curtis et al., 2004).

Density of seahorse populations tend to be low and are found in a pattern of patchy distribution (Foster and Vincent, 2004; Murugan et al., 2011; Vincent et al., 2011). Nevertheless, sometimes estuaries and lagoons can aggregate high seahorse density related to their capacity to provide them with large quantities of food and protection against strong tidal currents (Curtis and Vincent, 2005). Most seahorse species maintain small home

ranges, commonly less than one individual per m^2, and high levels of site fidelity, 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). Small home ranges may have enabled the seahorses to adopt camouflage appropriate for their environment, and to maintain a stable social structure (Vincent et al., 2005; 2011). Sex differences in home range size have been documented for some species; where females had significantly larger home ranges than males (Foster and Vincent, 2004; Moreau and Vincent, 2004; Vincent and Sadler, 2005), probably as a consequence of the physical limitations associated with male pregnancy (Vincent et al., 2005).

Variation in habitat preferences between species seems to influence the dispersal capabilities and hence distribution of species. Some seahorses have revealed an ontogenetic shift in habitat and depth use (Perante et al., 2002; Curtis and Vincent, 2006). While most species have limited daily movements, adults of some species may undergo seasonal migrations to deeper waters in the winter months (Foster and Vincent, 2004; Garrick-Maidment, 2007). These shifts in habitat use has implications for seahorse conservation since the full range of habitats utilised by particular seahorse species throughout their lives will need to be protected to ensure their long term survival.

All seahorse species are restricted by their limited dispersal abilities. Adult dispersal over large distances appeared primarily to occur when adults were cast adrift by storms or carried away while grasping floating debris. Young seahorses during their planktonic phase are more likely to disperse than adults, but the degree to which they contribute to long-distance dispersal of seahorses remains unknown (Foster and Vincent, 2004).

1.4. Seahorse trade and conservation

The unique morphology and peculiarities of seahorses have made them highly valued and popular in traditional medicines, curio and aquarium trades. In order to fulfil the increasing demand of seahorses, wild populations have been heavily exploited in a global trade over the last decades (Vincent, 1996; Foster and Vincent, 2004; Koldewey and Martin-Smith, 2010). Dried seahorses have been used widely for over 600 years in traditional medicine, particularly traditional Chinese medicine, which represents the largest human consumption of seahorses, related to the belief of curative properties with regards to cholesterol, arthritis, impotence and kidney disorders among others (Vincent, 1996; Lourie et al., 1999). A great number of dried seahorses are also used for curios and ornamental purposes (e.g. key rings, jewellery, paperweights) (Vincent, 1996; Grey et al., 2005) (Fig. 1.6.). At least 20 million of dried seahorses, 56 tonnes, are traded globally each year, originating primarily from Asian countries (Vincent, 1996; Giles et al., 2006; Evanson et al., 2011). Within the aquarium trade the volume of live seahorses was estimated at about hundreds of thousands individuals annually around the world (Vincent, 1996). Reports into the seahorse trade indicated that the volume of seahorses traded and the number of countries involved has increased, and demonstrated the overexploitation of some species and populations (Vincent, 1996; Giles et al., 2006). In mid 1990s at least 32 countries traded seahorses (mainly Southeast Asian countries) and more than 45 tonnes of dried seahorses, but increased to 80 countries and 50 tonnes by 2000, with a particularly great expansion in Africa and Latin America (Vincent, 1996; McPherson and Vincent, 2004; Baum and Vincent, 2005). A total of 28 seahorse species involved in the international trade are reported by

CITES from 2004-2008, a slight increase from the 24 previously estimated from historic data (Evanson et al., 2011).

Seahorses for commercial trade are primarily obtained by direct exploitation or by-catch in non-selective fishing gears (Vincent, 1996; Grey et al., 2005). Such pressure on wild stocks has led to strong declines of seahorses in recent years, particularly in Southeast Asian waters (Vincent, 1996; Martin-Smith et al., 2004). The direct or indirect fishing can affect seahorses, both in an individual or population level, by injuring or killing specimens, disrupting social structure, reducing reproduction and damaging their key habitats (seagrass beds, coral reefs, mangroves and estuaries) (Foster and Vincent, 2004; Vincent et al., 2011). Moreover, due to their life history characteristics and association with shallow inshore habitats seahorses are particularly vulnerable to overfishing or other disruptions such as habitat degradation and environmental pollution related to anthropogenic activities (Vincent, 1996; Lourie et al., 1999; Foster and Vincent, 2004), main factors that contribute to the decrease of many wild seahorse populations. As a result of the state of seahorse populations, all seahorse species were listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in November 2002 (CITES, 2002). The legislation implies actions towards the sustainable exploitation of these fishes, by requiring all of the signatory nations to ensure that exports after 15 May 2004 did not threaten wild populations. Additionally, a worldwide concern over the conservation status of wild seahorse populations causes the inclusion of all seahorses in the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species in 1996. Initially, 88 % of seahorse species were listed as 'Vulnerable' and the rest as 'Data Deficient'. Currently, are cited 26 species as Data Deficient, 10 as Vulnerable, 1 as Endangered and 1 as Least Concern (IUCN, 2014).

Figure 1.6. Dried seahorses in Asian markets for medicinal, food and curios purposes.

Despite the increasing interest of seahorse fisheries and concern about their conservation status, there is a lack of detailed information about their biology, ecology, life history parameters and population structure (Curtis and Vincent, 2006), which restricts their suitable conservation assessment and management. Complete knowledge of the life history of species is essential to understand the impacts of the harvesting and trade of seahorses as well as for their management and conservation strategies to ensure their population persistence in the wild (Foster and Vincent, 2004). A broad individual and population characteristics (individual movement, geographic ranges, social behaviour, distribution, growth rates, dietary and breeding needs) have been correlated with vulnerability to population depletion in some marine fishes (Foster and Vincent, 2004; Vincent et al., 2011). It has been described that the study of the natural diet of fish species represents a very important tool towards a more sustainable management of their stocks and the development of conservation measures (Tipton and Bell, 1988; Rikardsen et al., 2000; Denny and Schiel, 2001; Gaskett et al., 2001; Watanabe et al., 2006; Storero and González, 2008).
Increasing biological and ecological knowledge could help to redefine species status and reduce the number of Data Deficient species on the IUCN Red List (Vincent et al., 2011), establish better sustainable exploitation of seahorse fisheries (e.g. recommendation quotas, delimit trawls zones, etc.) and trade (e.g. seahorse minimum size limits) (Foster and Vincent, 2005), improve culture techniques, monitor population trends, guide future management and conservation actions, and create marine protected areas (Martin-Smith et al., 2004). The charismatic and attractive nature of seahorses provides a powerful way of getting political and public support to conduct appropriate conservation programmes. Seahorses have already been used as flagship species to promote the protection not only of seahorses themselves, but also of their habitats (Lourie et al., 1999; Scales, 2010). Then, increasing the knowledge of their life history traits may help to improve the effectiveness of conservation programmes where seahorses act as a flagship species.

Overall, conservation and management of seahorses relays on interdisciplinary measures such as seahorse populations research, seahorse habitat protection, fisheries management, captive breeding efforts, trade management particularly under CITES, social and policy development (Lourie et al., 1999, Vincent et al., 2011).

1.5. Seahorse aquaculture

Concern over global declines in wild seahorse populations, and in recognition of their high economic value and marketability, have promote the aquaculture of many seahorse species to supply the increasing trade demand (Koldewey and Martin-Smith, 2010; Olivotto et al., 2011). Furthermore, the rearing of seahorses in captivity represents an additional tool for seahorse conservation as an alternative to the capture of wild individuals. Then, seahorse aquaculture integrates both conservation and sustainable development aims by reducing the fishing pressure on the wild seahorse stocks and providing alternative sources of income for seahorse fishers who, in the absence of alternatives to fishing, continue to exploit declining seahorse populations (Vincent, 1996; Wilson and Vincent, 1998; Job et al., 2002). Other benefit to consider when breeding seahorses is the generation of important life history information that can be used to support the conservation and management of exploited species.

Certainly seahorses represent an important part of the growing global trade in live marine animals, and their value per individual in the live trade is much higher than in the dried trade. According to CITES trade records seahorses supplied by aquaculture comprised less than 1 % in 2002, 25 % in 2004 and 84 % in 2008 of the total (Koldewey and Martin-Smith, 2010; Evanson et al., 2011) (Fig. 1.7.). Although these trade records may be overestimated mostly due to export and import information gaps and illegal trade actions (Vincent et al., 2011), the growing trend of live seahorse trade derived from captive breeding has been evident in the last decade. At present, seahorses provided by aquaculture facilities mainly go to the home aquarium market, tropical or subtropical species (*H. barbouri*, *H. kuda*, *H reidi* and *H. erectus*) are preferred in home aquarium but there is a potential new market for temperate species, such as *H. abdominalis* and *H. guttulatus*. In contrast, although they are also used for traditional medicine, tonic products (e.g., seahorse wine), curios and souvenirs, none of the many large-scale aquacultures developed to supply seahorses for these purposes (Koldewey and Martin-Smith, 2010).

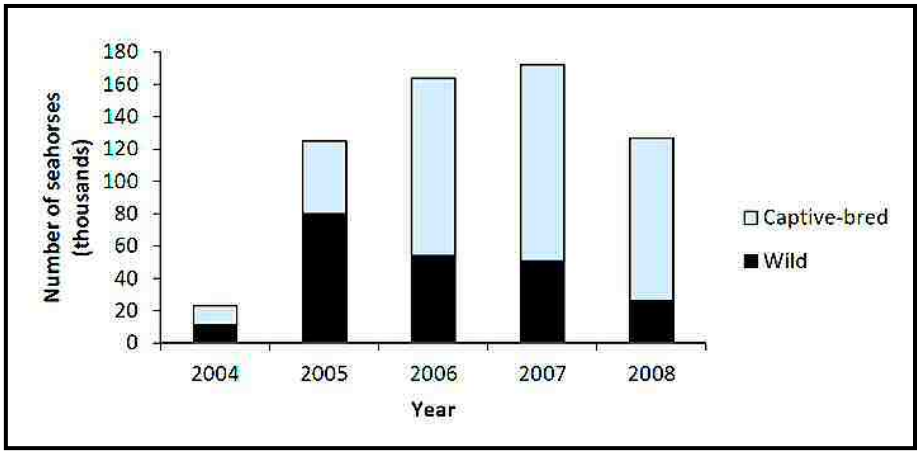


Figure 1.7. Total of life seahorses exported globally between 2004 and 2008, reported by CITES (extracted from Evanson et al., 2011).

The fact that production costs for seahorse aquaculture are considerably high in developed countries prompt the extremely prices of captive-bred seahorses and make hard to compete economically with the very cheap prices of wild-caught individuals while this is not considered an issue in a developing countries. Wilson and Vincent (1998) reported that live seahorses have originally a low value of about US $1.50 per animal but then they are sold for up to US $30-43 per individual in Canada and in the USA. The majority of those traded species usually come from developing countries and are sold in developed countries. Hence, such high values in developed countries may be balanced by consumers willing to pay a first-rate for a single individual, enhanced by the wide offer of sizes and attractive coloration patterns, and the higher adaptability to a home aquarium environment of cultured seahorses than wild-caught seahorses. However, as wild specimens are still being sold in developed countries at a cheaper price than captive-bred due to their low capture-cost, conservation aquaculture objectives are very difficult to achieve (Vincent and Koldewey, 2006). Therefore, conservation effort should also focus on meet commercial demand; otherwise, trade prices may increase and fishers would return to wild-caught practices (Tlusty, 2002). In view of this, there is an essential need to develop commercially

viable seahorse husbandry and rearing to totally eliminate the presence of wild individuals in the aquarium trade.

First attempts to breed seahorses were undertaken by the hobby aquarist community on a small scale in late 1950s and early 1960s, probably due to their interest in many aspects of seahorse husbandry (Koldewey and Martin-Smith, 2010). Commercial seahorse aquaculture started in Asia in the 1970s, particularly in China. However, disease and nutritional problems and the economic collapse resulted in the unsuccessful breeding and rearing of seahorses until the 1980s when a considerable expansion of the seahorse farms occurred (Vincent, 1996; Koldewey and Martin-Smith, 2010). At the same time, experimental breeding and rearing of seahorses was attempted in other countries (e.g. Australia, Japan, Venezuela), as a small-scale systems in research institutes and public aquariums. Prior to the 1990s, problems related to disease and feeding affected to the success of seahorse aquaculture. However, the considerable research efforts in late 1990s and early 2000s offered relevant information to improve the seahorse aquaculture, reflected in the increase of the number of aquaculture facilities and number of species cultured, particularly in Australia, New Zealand and the USA (Koldewey and Martin-Smith, 2010). The scale of seahorse culture facilities ranges from small aquariums to large pond systems and from research based to commercial operations (Vincent and Koldewey, 2006). Recently, interest in seahorse aquaculture projects has increased considerably around the world: Australia, New Zealand, Brazil, China, India, Indonesia, Philippines, Vietnam, Mexico, South Africa and the USA (Vincent and Koldewey, 2006).

Even the great interest in culturing and breeding these species and some commercial successful facilities, seahorse aquaculture is a relatively new industry that has to solve many technical challenges (Lourie et al., 1999; Foster and Vincent, 2004). Firstly, one of the critical bottlenecks in seahorse rearing is the low survival of early juveniles, affecting commercial economic return (Payne and Rippingale, 2000; Woods, 2003a; Olivotto et al., 2011). This may be related to many issues such as environmental factors (temperature, salinity, light intensity and photoperiod), food, feeding behaviour, as well as stocking density (Woods, 2000b; Sheng et al., 2006). Secondly, reproductive efficiency of the broodstock can be problematic to achieve a successful seahorse culture (Lin et al., 2006; Faleiro et al., 2008). In this framework, research works have mainly focused on the growth and survivorship of juveniles to establish appropriate rearing protocols. As a result, seahorse husbandry and reproduction in captivity have been achieved for several species: *Hippocampus abdominalis* (Woods, 2000b), *H. capensis* (Lockyear et al., 1997), *H. reidi* (Olivotto et al., 2008; Hora and Joyeux, 2009), *H. subelongatus* (Payne and Rippingale, 2000), *H. kuda* (Job et al., 2002; Lin et al., 2006), *H. erectus* (Lin et al., 2008), *H. comes* (Job et al., 2006), *H. whitei* (Wong and Benzie, 2003), *H. trimaculatus* (Sheng et al., 2006; Murugan et al., 2009), *H. hippocampus* (Otero et al., 2010), *H. guttulatus* (Palma et al., 2011; Planas et al., 2012; Blanco et al., 2014). In addition, the closure of life cycles for some species has also been achieved (Wilson and Vincent, 1998; Woods, 2000a; Blanco et al., 2014).

Besides the trade market goals, procedures for captive rearing of seahorses are investigated with the objective of providing specimens for ecological studies, public displays and restocking activities. The later, is a controversial initiative and release proposals to resolve conservation problems often emerge in discussions because may transmit diseases, alter genetics or disrupt social and spatial behaviour of wild populations (Naylor et al., 2000; Bell et al., 2003). The IUCN Re-introduction Specialist Group declares that the pressures leading to population declines must have been relieved and the effects on host populations

evaluated before considering any release. Subsequently, it is essential to study wild population characteristics, measure exploitation and threat status, identify a clear management goal and objectives, and execute the initiatives with great care (Vincent and Koldewey, 2006). Releases and further monitoring and assessment require long-term financial, political and local support in order to guarantee no biological risks on wild seahorse populations and marine ecosystems in general (Vincent, 1996; Tlusty, 2002).

The rearing of seahorses in captivity has contributed to increased understanding of seahorse biology and physiology, but there is still insufficient information on growth, feeding and nutritional requirements. Besides, the transfer of culture information among seahorse species is restricted because each seahorse species respond differently under same culture conditions due to interspecific differences in biological and physiological characteristics. Further research is required to improve and optimise culture protocols for seahorse culture, especially addressing the reproductive efficiency and survivorship of early juveniles, in order to progress on the economically viability of seahorse aquaculture (Vincent, 1996; Wilson and Vincent, 1998; Job et al., 2002, Vincent and Koldewey, 2006).

1.6. Species of study

The long-snouted seahorse (*Hippocampus guttulatus*) Cuvier 1829 (Fig. 1.8.) has been historically synonymised with *H. ramulosus* Leach 1814 (Lourie et al., 1999), which was the name designed to the fossil seahorses found in the Lower Pliocene in North-Eastern Italy (Teske et al., 2004). Other synonyms of *H. guttulatus* include *H. hippocampus microstephanus* Slastenenko 1937, *H. hippocampus microcoronatus* Slastenenko 1938, *H. guttulatus multiannularis* Ginsburg 1937 and *H. biscuspis* Kaup 1856, although *H. guttulatus* is the currently accepted name (Fishbase, 2014).

Figure 1.8. Adult specimen of *Hippocampus guttulatus* photographed off the coast of Galicia (Northwest Spain).

H. guttulatus has a similar distribution and habitat to its sympatric species the short-snouted seahorse *H. hippocampus*, both species occur in the Mediterranean Sea and the northeastern Atlantic Ocean and co-exist in some areas (Lourie et al., 1999; Garrick-Maidment, 2004; Curtis and Vincent, 2005). Some observations described variable abundance between *H. guttulatus* and *H. hippocampus* over regional spatial scale, *H. guttulatus* was the predominant species recorded in Ria Formosa lagoon (Southern Portugal) (Curtis and Vincent, 2005) and in Galicia (Northwest Spain) (Valladares et al., 2014). The confirmed distribution of *H.guttulatus* includes Netherlands, United Kingdom of Great Britain, Northern Ireland, France, Spain, Portugal, Morocco, Italy, Malta, Croatia, Greece and Cyprus (Lourie et al., 1999; Lourie et al., 2004) (Fig. 1.9.). In Galicia, scientific publications reported its occurrence (Bañón et al., 2010; Valladares et al., 2011), but scarce information is available on exact locations, population abundance and structure.

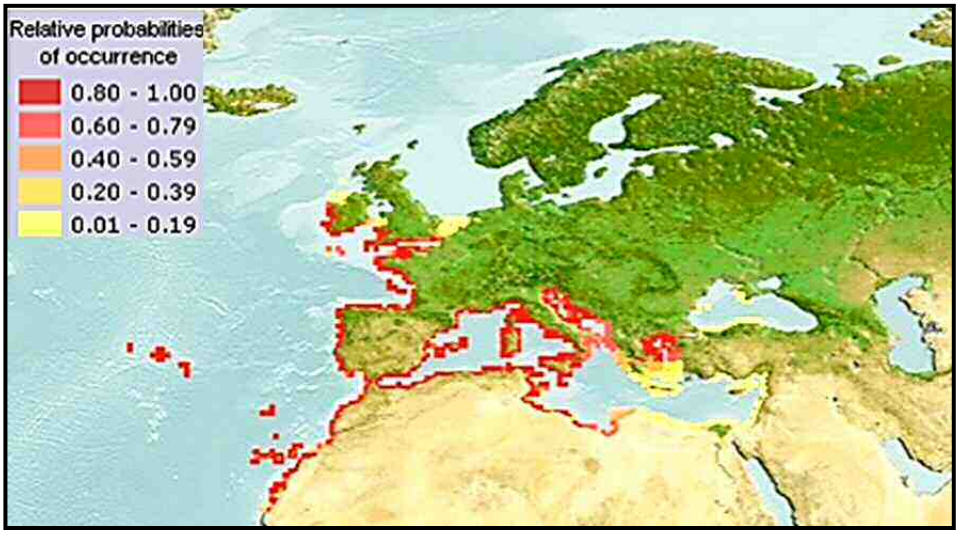


Figure 1.9. Reviewed native distribution map for *Hippocampus guttulatus* (modelled 2100 map based on IPCC A2 emissions scenario), www.aquamaps.org, version of August 2013. Accessed on February 2015.

Although the two European species are similar in appearance, there are clear morphological differences in the head, snout, trunk shape and patterns of white spots on the body that can be used to reliably identify these species (Fig. 1.10.). *H. guttulatus* is larger than *H. hippocampus*, growing up to 18 cm in height (22 cm, present PhD Thesis) and has a longer snout (Lourie et al., 1999). Adults are yellow to brown in coloration but sometimes olive brown to grey, with white spots on body (Lourie et al., 1999). *H. guttulatus* commonly bears skin filaments. However, the presence or absence of skin filaments is an unreliable character for taxonomic identification as both species can be found with or without skin filaments (Lourie et al., 1999; Curtis and Vincent, 2005; Curtis, 2006). Because the specific identification based on morphological traits can be uncertain, identifications are currently based on genetic studies (Pardo et al., 2007; López et al., 2010; López, 2011).

H. guttulatus is generally associated with seagrass beds (Zoostera or Posidonia) and macroalgae in shallow inshore waters, usually up to 12 m deep (Lourie et al., 1999; Curtis and Vincent, 2005). Within these habitat types, this species uses a variety of microhabitats as holdfasts including seagrass shoots, ascidians, tube-dwelling polychaetes, broken shells and open sand (Curtis and Vincent, 2005; pers. obs.). Adults exhibit high site fidelity during multiple years with small home ranges for an average of 20 m^2 (Curtis and Vincent, 2006). During the winter they disappear from their shallower breeding areas where seahorses are exposed to the severe weather conditions (low temperature, storms, strong tides and currents), and are believed to spend time in deeper waters (Lourie et al., 1999). Contrarily, in protected areas (e.g. harbours, estuaries and lagoons) seahorses appear to be resident all year around (Garrick-Maidment, 2007).

The breeding season of the species is considered from March, when water temperature starts to be warmer and daylight is becoming larger, until November, when water temperature starts to decrease and lightening is shortening (Planas et al., 2013). Annual spawning frequency of females varies among *H. guttulatus* populations, once per year was described in the Arcachon Basin (France) in contrast to 4 times per year reported in the Ria Formosa (Portugal), with a mean clutch size of 214 ± 111 eggs per breeding season

(Curtis, 2007). Female gonad development and hatching is affected by temperature, enhanced at 18–20 °C (Planas et al., 2010, 2013). The eggs are particularly large (0.9 to 2.0 mm in diameter) compared to other seahorse species and have a high weight per volume unit (237 µg/µl) (Planas et al., 2010). It has been suggested that nutrients in *H. guttulatus* eggs are highly concentrated and rich in protein and lipids since their yolk reserves represent about a 60 % of the total egg volume, but the relative yolk content would decrease at temperatures under 18 °C (Planas et al., 2010, 2013).

The gestation period is usually 28 days, but this varies with water temperature (Lourie et al., 1999; Foster and Vincent, 2004; Planas et al., 2008a). Brood sizes reported in the wild ranged from 180 to 567 juveniles, and newborn juveniles average 12 mm in length (Curtis and Vincent, 2006; pers. obs.). Immediately after birth juveniles are planktonic for at last 8 weeks and settle into benthic habitats at approximately 3 months of age, when their length is 65 - 215 mm (Curtis and Vincent, 2006). Length at maturity in wild individuals has been estimated when they reach 109 mm, and they begin reproducing at approximately 1 year of age (125 - 129 mm in length) (Curtis and Vincent, 2006). *H. guttulatus* is characterised by short lifespan, 4 - 6 years (Lourie et al., 1999; Curtis, 2004; Foster and Vincent, 2004; Curtis and Vincent, 2006).

As all seahorse species, adult *H. guttulatus* in the wild feed primarily on live crustaceans, but its specific dietary composition has been only documented by three studies by means of gut contents examination. The most dominant prey categories found were Amphipoda, Anomura Decapoda and Mysidacea in the Greece coasts of the Aegean Sea (Kitsos et al., 2008); Mysidacea and Decapoda in the Turkish coasts of the Aegean Sea (Gurkan et al., 2011); Amphipoda, Decapoda (Euphasids and Mysidaceans) and Isopoda in the Ria Formosa lagoon (Portugal) (d'Entremont, 2002).

Figure 1.10. Adult female specimens of *Hippocampus guttulatus* (long-snouted seahorse) (left) and *Hippocampus hippocampus* (short-snouted seahorse) (right) from Galician population (Northwest Spain).

The main threats on wild *H. guttulatus* populations are either intentional or incidental (by-catch) captures, occurring in most Mediterranean countries and Portugal. They are traded mainly as curiosities (dried) and are collected occasionally for local public aquariums or hobbyist use (life) (Lourie et al., 2004). In Galicia, whilst it is thought that *H. guttulatus* is not subject to high fishing pressure for international trade, habitat loss or degradation and by-catch fisheries are mainly considered its potential threats.

According to the IUCN (International Union for Conservation of Nature), *H. guttulatus* is currently listed as 'Data Deficient' species since 2003 (IUCN, 2014), modified from their previous 'Vulnerable' status in 1996 due to the insufficient information available on this species to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status. Additionally, the species was included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in November 2002 with implementation of the listing in 2004 (CITES, 2002), as well as it is listed in the Appendix II of the convention on the Conservation of European Wild life and Natural Habitats (the Bern Convention). *H. guttulatus* is also included in OSPAR list of threatened habitats and species in 2004 (OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic). Regionally, the species is listed in National Red Data Books in Bulgaria, France and Portugal. It is protected in Slovenia under the 1993 Protection of Threatened Animal Species Act, which prohibits trade in and bans the keeping of the animal in captivity. In the United Kingdom, it is protected by the Wildlife and Countryside Act of 2008 (DEFRA, 2008) and considered a UK Biodiversity Action Plan priority species (JNCC, 2010). In Spain, the species has been recently included in the Spanish List of Wild Species with Special Protection Regime and in the Spanish Catalogue of Endangered Species (BOE, 2011). However, in Galicia (the study area of the present PhD Thesis), there are no regional regulations by the Consellería de Medio Ambiente Territorio e Infraestructuras (Ministry of Environment, Territory and Infrastructures) nor the Dirección Xeral de Conservación da Natureza (Directorate for Nature Conservation) to protect wild populations of seahorse species inhabiting in the region. Besides this, none of these species is listed in the Galician Catalogue of Endangered Species (CGEA, 2007).

1.7. Stable isotopes

Stable isotopes have been commonly employed by scientists from different disciplines. In the late 1970s and 1980s, this technique became an important tool in ecological and physiological research as stable isotopes provide a natural tracer to directly follow details of element cycling or routing (Fry, 2006; Martínez del Rio et al., 2009). In particular, the application of stable isotopes in fish ecology and nutritional physiology has increased considerably in recent years (Schroeder, 1983; Hobson and Welch, 1992; Cabana and Rasmussen, 1996; Jennings et al., 1997; Pinnegar and Polunin, 1999; Frediksen, 2003; Schlechtriem et al., 2004; Jomori et al., 2008; Gamboa-Delgado et al., 2008; Vizzini and Mazzola, 2009).

Isotope chemistry and terminology

Isotopes are different forms of the same chemical element that differ in the number of neutrons in the nucleus. The term "isotope" means that forms of an element all occupy the same (*iso*) place (*topos*) in the periodic table of the elements. Stable isotopes are naturally occurring isotopes that do not spontaneously disintegrate or decay over time (non-radioactive). Each isotope of an element has a specific mass because of the different number of neutrons, for example an extra neutron in the ^{13}C isotope makes the nucleus more massive or heavier than the ^{12}C isotope. The tiny mass differences between isotopic forms of an element causes the isotopes behave differently in chemical reactions; the lighter isotope (e.g. ^{12}C or ^{14}N) tends to form weaker bonds and to react faster than the heavier isotope (e.g. ^{13}C or ^{15}N) (DeNiro and Epstein, 1978, 1981). As a consequence, the proportions or ratios (*R*) (e.g. $^{13}C/^{12}C$ for carbon, and $^{15}N/^{14}N$ for nitrogen) of isotopes of an element will vary among the substrates and products of a chemical reaction. These ratio differences produce enrichment or depletion of the heavy isotope relative to the light isotope. Enrichment happens when the heavier stable isotope is accumulated in the product (relative to the substrate), while the lighter isotope is preferably eliminated. On the other hand, depletion occurs when the lighter isotope is favoured. The terms "enriched" and "depleted" are relative comparisons of the heavier isotope among samples, referring to samples that have higher or lower proportion of heavy isotope, respectively (Bond and Hobson, 2012).

An isotopic value is the ratio of the isotopes in a sample expressed in conventional delta notation (δ) as parts per thousand (‰) relative to international standards according to the following equation:

$$\delta X = [\ (R_{Sample}/R_{Reference}) - 1\ ]$$

where X is the heavy stable isotope of an element (e.g. ^{13}C or ^{15}N) and R is the corresponding ratio (e.g. $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively)*.

The change in isotopic abundance between a substrate and product due to chemical processes is called fractionation and results in different isotopic ratios in different compounds. Fractionation is commonly denoted by the Greek symbol Δ and calculated by the equation:

$$\Delta = \delta_{\text{Substrate}} - \delta_{\text{Product}}$$

* Isotope ratios (*R*) are the simple ratio of the number of atoms of two isotopes in a material, and the δ value is a mathematical manipulation of isotope ratios.

There are a large number of terms employed to refer this process: fractionation, fractionation factor, enrichment, trophic enrichment, trophic fractionation, discrimination, trophic discrimination, discrimination factor, tissue-diet discrimination factor and isotopic discrimination factor (Martínez del Río et al., 2009). The term "discrimination factor" is considered more appropriate when referring to the difference in the isotopic composition between an animal's tissue and its diet (i.e. $\Delta\ \delta = \delta_{\text{tissue}} - \delta_{\text{diet}}$) (Martínez del Río and Wolf, 2005; Bond and Hobson, 2012), thus is the term used in this Thesis.

1.7.1. Animal ecology

Ecologists have used stable isotopes in a variety of applications, including analysis of food web structure, identification of sources supporting a food web, description of an animal's trophic level, reconstruction of animals diet, determination of habitat use and examination of animal migration and movement (Peterson and Fry, 1987; Hobson and Welch, 1992; Cabana and Rasmussen, 1996; Hobson, 1999; Pinnegar and Polunin, 1999; Vander Zanden and Rasmussen, 2001; Post, 2002; Fry, 2006; Bode et al., 2007). Stable isotopes have attracted a major interest on these research areas mainly because the advantages that offer over traditional methods that may be unethical (destructive sampling of endangered species), impractical (quantifying complex food webs over large temporal and spatial scales), prohibitively expensive (observational studies of ocean-going and deep-

sea organisms), or impossible (reconstructing diets of long-extinct species) (Boecklen et al., 2011). The two elements most commonly employed in ecological studies are carbon (C) and nitrogen (N). Ratios of nitrogen ($^{15}N/^{14}N$) are expressed as $\delta^{15}N$, and ratios of carbon ($^{13}C/^{12}C$) are expressed as $\delta^{13}C$.

The method is based on the fact that stable isotopes are transferred from prey to predators in a predictable manner, that is, consumers incorporate the isotopic composition of the resources that they use. As energy transfer happens throughout ecosystems isotopic discrimination takes place from one trophic level to the next, resulting in alterations of the consumer's stable isotope ratios relative to its diet because of a slight selective retention of the heavy isotope and excretion of the lighter isotope. As a result, tissues of consumers tend to become enriched in heavy isotopes (^{13}C and ^{15}N) as compared with their food (DeNiro and Epstein, 1978, 1981).

This enrichment, discrimination factor (Δ), is assumed to be about 3-4 ‰ for $\delta^{15}N$ and 0-1 ‰ for $\delta^{13}C$ between trophic levels (DeNiro and Epstein, 1978; Peterson and Fry, 1987; Vander Zanden and Rasmussen, 2001; Post, 2002; Sweeting et al., 2007a, 2007b) (Fig. 1.11.). Consequently, $\delta^{15}N$ is a powerful tool for estimating trophic position of organisms (Hobson and Welch, 1992) and $\delta^{13}C$ is mainly used to determine primary sources in a food web. In the marine environment, $\delta^{13}C$ indicates inshore vs. offshore, or pelagic vs. benthic, contribution to food intake (Hobson et al., 1994). However, discrimination factor can vary among tissues and species, as well as may be affected by environmental (e.g. temperature), physiological and nutritional conditions (e.g. stress, starvation) (Hobson and Clark, 1992a; Hobson et al., 1993; Herzka and Holt, 2000; Bosley et al., 2002; Barnes et al., 2007). Therefore, reliable interpretation of stable isotope data requires knowledge of the discrimination factor between diet and consumer, and its variation because small changes in its value affect estimates of trophic level and the contribution of different sources to a consumer (McCutchan et al., 2003). Discrimination factor estimates are usually derived from captive feeding experiments for application in wild studies, but an accurate estimation is complicated due to the potential sources that contribute to its variation. Otherwise appropriate values for similar taxa and tissues from the literature must be used. Measurements of discrimination factors in fish tissues are limited (Caut et al., 2009; Willis et al., 2013), and in particular for seahorses are lacking.

Diet studies

Stable isotopes have been widely used as dietary tracers to reconstruct animal diets of birds, mammals and fish (Tieszen et al., 1983; Gannes et al., 1997; Riera et al., 1999; Kharlamenko et al., 2001; Hobson et al., 2002; Moreno et al., 2010; França et al., 2011; Galván et al., 2012; Chiaradia et al., 2014). Conversely, diet studies of seahorses based on stable isotopes are lacking in the literature.

Its utility relies on the premise that "You are what you eat" (DeNiro and Epstein, 1978), that is, the isotopic composition of a consumer reflects the assimilated contributions of different prey sources, so that a consumer's diet can be inferred. Dietary calculations are based on two assumptions: sampled tissues are in equilibrium with the sampled diet, and discrimination factors between the consumed food and the assimilated tissue are known (Post, 2002). The isotopic equilibrium state depends on an animal's isotopic turnover rate (see Animal physiology section) and its dietary consistency. Hence, appropiate measures of discrimination factors play an important role for a reliable estimation of an animal's diet.

Carbon and nitrogen isotopic values of consumers and food sources are usually plot on a graph of $\delta^{15}N$ vs. $\delta^{13}C$ or *vice versa*, named "bi-plot", after applying appropriate discrimination factor corrections. In order to achieve solutions for diet composition from these food sources, the consumer isotopic values must fall within the range of the food source isotopic values (Fig. 1.12.).

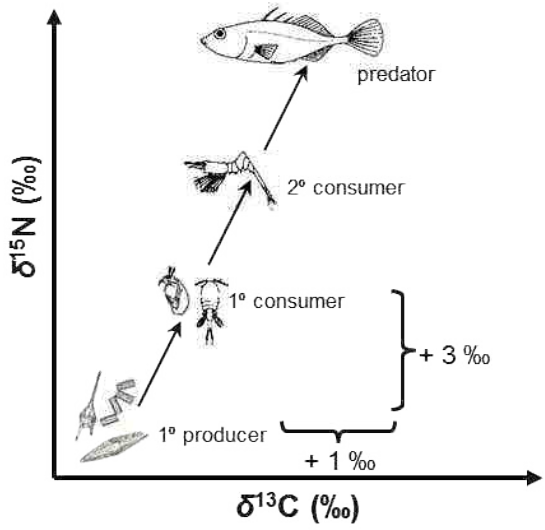


Figure 1.11. $\delta^{15}N$ and $\delta^{13}C$ variation throughout a food web.

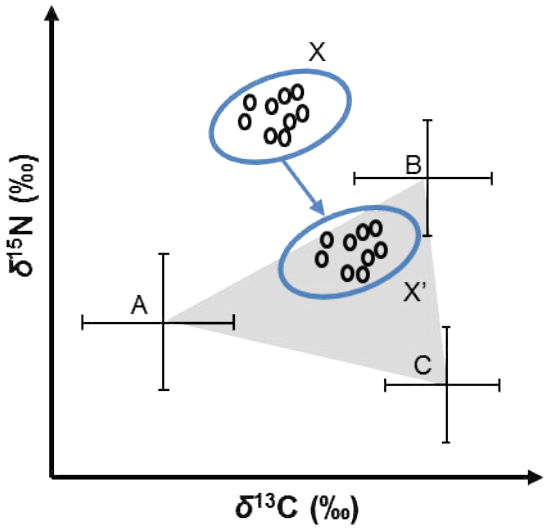


Figure 1.12. $\delta^{15}N$ vs. $\delta^{13}C$ bi-plot of food sources (A, B, C) and a consumer corrected (X') or not (X) for discrimination factor.

In spite of these critical assumptions, dietary reconstructions using stable isotopes have a number of advantages over conventional methods. Unlike conventional dietary analysis (e.g. behavioural observations, stomach content analysis), which provide information on recently ingested food sources, stable isotope analysis provides information of assimilated food sources over longer time periods, ranging from days to months depending on the tissue analysed (Tieszen et al., 1983; Fry, 1988, Hobson et al., 1995). For growing animals, the isotopic composition of newly added biomass will reflect that of the current food source (Fry and Arnold, 1982). The fact that different tissues integrate source use over different time scales can be explained by their different turnover rates (see Animal physiology section). For example, muscle tissue provides diet information over longer time periods due to its slow turnover rate, when compared to tissues with fast isotopic turnover rate, such as liver (Hobson and Welch, 1992).

Sampling of fish tissues (e.g. muscle, liver) for stable isotope analysis has traditionally acquired by the use of lethal techniques (Jennings et al., 1997; Frediksen, 2003; Vizzini and Mazzola, 2009). When dietary studies involve threatened species, such as seahorses, traditional methods are inappropriate and non-lethal methodologies are a must (e.g. fin, scales) (Kelly et al., 2006; Sanderson et al., 2009; Jardine et al., 2011; Valladares and Planas, 2012).

Mixing models

The relative contributions of various food sources to a consumer's diet can be calculated numerically from isotopic data using basically two mathematical approaches: Euclidean distance methods and mixing mass-balance models (Phillips, 2001). Regarding the latter, several mathematical methods and programs have been developed during recent years to improve these mixing models: lineal mixing models (Phillips and Gregg, 2001), multi-source mixing models (IsoSource, Phillips and Gregg, 2003) and Bayesian approaches (MixSIR, Moore and Semmens, 2008; and SIAR, Parnell et al., 2010; MixSIAR, Stock and Semmens, 2013). Generally, carbon and nitrogen values of consumer species and their potential food sources are used in mixing models, with appropiate discrimination factor correction, to estimate the feasible contributions of foods to the diet of consumer species. Limitations related to multiple sources and variability of parameters when applying the initial mixing models were solved thought Bayesian approaches. Bayesian mixing models have the potential to provide more robust results when it comes to quantifying feeding preferences in a complex system by estimating probability distributions for the proportional contribution of each source (prey items) to a mixture (consumer) while accounting for uncertainty associated with multiple sources, discrimination factors and isotope values (Moore and Semmens, 2008; Parnell et al., 2010). The most recent Bayesian mixing model developed, MixSIAR, has been employed in this Thesis to estimate the contributions of prey items to the consumer *H. guttulatus* seahorses.

Data for a mixing model

- Isotopic values of consumers
- Isotopic values of sources (preys) with isotopically distinct values
- Values for discrimination factors, Δ to correct isotopic values of consumers from experiments or literature
  big WEAKNESSES of mixing models
- Prior information
  gut contents, observations, previous studies

1.7.2. Animal physiology

In an extension of ecological studies, stable isotopes can be used in nutritional research to determine the allocation of assimilated nutrients, identify dietary components contributing to growth, determine ingestion and assimilation rates, and to assess the physiological condition of an animal (Hobson et al., 1993; Schlechtriem et al., 2004; Sears et al., 2009).

Nutrient assimilation in fish is frequently determined by indirect methods (faeces collection, gut contents or growth rates). The use of stable isotopes provides an alternative to directly trace nutrient incorporation from different dietary items into fish tissues (Schlechtriem et al., 2004; Jomori et al., 2008; Gamboa-Delgado et al., 2008).

As explained above, the use of this approach relies on the assumptions that isotope values in tissues of consuming organisms reflect those of their diet and that tissue isotope value may change over time due to dietary variations (Fry, 2006). The rate of change relies on the tissue considered, with the more metabolically active tissues turning over faster (e.g. liver) than less metabolically active tissues (e.g. muscle) (Tieszen et al., 1983; Hobson and Clark, 1992b). Isotopic turnover rate refers to how quickly the variation in isotopic value in a tissue occurs due to growth and metabolic tissue replacement. Hence, the isotope turnover rate defines the delay necessary to the isotope composition of the tissue of the consumer to

reach equilibrium with that of the food source. Isotope turnover rates have been determined through experimental studies, following an isotopic switch in their diet (Fry and Arnold, 1982; Hobson and Clark, 1992b; Martínez del Rio and Wolf, 2005), but little is known about the isotope turnover process in fish (Pinnegar and Polunin, 1999; Herzka and Holt, 2000; Bosley et al., 2002; Perga and Gerdeaux, 2005).

Growth and nutritional status are likely to affect $\delta^{15}N$ and $\delta^{13}C$ because of the differences in protein catabolism and nitrogen excretion processes, allowing predictions of body animal condition (Hobson and Clark, 1992a; Hobson et al., 1993). During starvation, endogenous nutrient stores may be mobilised as sources of energy and as substrates for tissue formation when exogenous nutrients are limiting. This process of mobilisation and deposition of endogenous sources may result in additional isotopic fractionation favouring the heavier isotope. Therefore, the animal becomes progressively more ^{15}N enriched over the course of starvation because the excreted lighter nitrogen is not replaced by dietary protein. Similarly, enrichment in ^{13}C is also observed as a result of the consumption of endogenous lipid reserves (DeNiro and Epstein, 1978, Hobson et al., 1993).

Carbon-to-nitrogen elemental ratios (C:N) reflect the amount of lipid content into a tissue and have been demonstrated useful for studies of lipid storage, and thus to assess the physiological condition of an animal (Post et al., 2007). The ratio increases due to the assimilation of exogenous nutrients and indicates increase of lipid reserves, so that C:N values are higher in animals with better physiological conditions. Conversely, samples with low C:N ratios indicate low lipid content because of the consumption of lipid reserves under starvation or limited resources.

1.8. Background to the Thesis

As stated in above, available biological and ecological information on the species *Hippocampus guttulatus* is considerable scarce, not only from Galicia, but from Europe in general. On a local scale, specific knowledge of seahorse wild populations in the Galician coast came exclusively from observations of recreational divers, fishers, marine naturalists and other citizens, who played a key role by reporting sightings of *H. guttulatus* and indicated a progressive population decline or disappearance in many areas in the last decades, although there is a lack of scientific research to quantify this statement. In this context, the 'Proyecto Hippocampus' (in which this PhD Thesis was framed) was initiated in 2006 at the Institute of Marine Research-CSIC (Vigo, Northwest Spain) as the first Spanish project focussed on the seahorse *H. guttulatus* research, with the main objective of the recovery of wild *H. guttulatus* populations in Galicia. The actions included in the Project were based on ecology and aquaculture approaches: studying the wild populations (genetic and biologically) in some areas of the Galician coast, developing a feasible and reliable *ex-situ* breeding programme in captivity and conducting the assessment of a genetically-controlled repopulation program in selected natural areas. Therefore the Project, as well as this PhD Thesis, has a clear significant value for assessment, management and conservation programmes of this seahorse species.

Concerning breeding in captivity, in accordance with the IUCN Captive Breeding Policy, the breeding programme in 'Proyecto Hippocampus' was in advance of a potential and irreversible fall of populations below a too low census size. In this sense, it was important that the breeders used in the husbandry and breeding programme were a high

representative fraction of the genetic diversity of the source populations including genetic analyses for broodstock management to retain maximum genetic diversity and minimise inbreeding (Planas et al., 2008a). Genetics on wild population showed moderate levels of genetic diversity, historical reductions in population size and a subsequent demographic expansion, which may aware of maintenance of genetic variability and a risk of short- and long-term adaptive potential for the species (López, 2011). In the context of limited information on captive *H. guttulatus* seahorse and specifically the absent research on the rearing of juveniles, 'Proyecto Hippocampus' established a breeding protocol for broodstock maintenance and reproduction of adult seahorses and the base of knowledge for the successful rearing of the juveniles of this species. In spite of recent improvements in the rearing procedures and successful progress in survival rates in reared *H. guttulatus* seahorses (Planas et al., 2012), feeding and nutritional requirements and their effects on initial mortalities in juveniles were not studied. Feeding issues are considered one of the most decisive factors in the initial survival of juvenile seahorses, especially regarding nutritional requirements, feeding efficiency and digestive capabilities (Payne and Rippingale, 2000, Olivotto et al., 2008, Planas et al., 2009). In this regards, one of the aims of the present PhD Thesis was to estimate the assimilation efficiency for understanding nutrition processes occurring in the early development of juvenile *H. guttulatus* seahorses, which may help the interpretation of their growth and mortality rates. The estimation of food assimilation was assessed by stable isotopes, which represent natural tracers that allow the direct determination of ingestion and assimilation rates in organisms and have been successfully applied in fish larval nutrition research (Schroeder, 1983; Schlechtriem et al., 2004; Jomori et al., 2008; Gamboa-Delgado et al., 2008).

Given the lack of information on feeding habits of *H. guttulatus* seahorses in the wild, this PhD Thesis also focused on assessing the diet composition of this European seahorse inhabiting coastal waters of Galician applying stable isotopes analysis. The study of the trophic ecology in fishes by means of stable isotope analysis has been extensively used over the last two decades (Hobson and Welch, 1992; Cabana and Rasmussen, 1996; Jennings et al., 1997; Frediksen, 2003; Vizzini and Mazzola, 2009), but it has never been applied to seahorses. Understanding how seahorses use food resources in the natural environment is essential for identifying factors that affect their distribution, abundance and choice of habitat, which are relevant parameters to guide conservation management of these endangered species.

1.9. Objectives and structure of the Thesis

The aim of this PhD Thesis was to improve the knowledge on the feeding ecology of the long-snouted seahorse *Hippocampus guttulatus* focusing on (1) food assimilation in the early development of juveniles, and (2) dietary composition of wild seahorse population. The research was based exclusively on the application of the stable isotope analysis. The results achieved would contribute to optimize seahorse culture techniques and to understand biological and ecological characteristics of wild seahorse populations, which in turn are relevant features to guide conservation management plans in seahorses.

The specific objectives of this study were:

1. To investigate the effects of temperature on carbon and nitrogen stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) and to determine the optimal seawater temperature for the early development of *H. guttulatus* juveniles (Chapter 3).

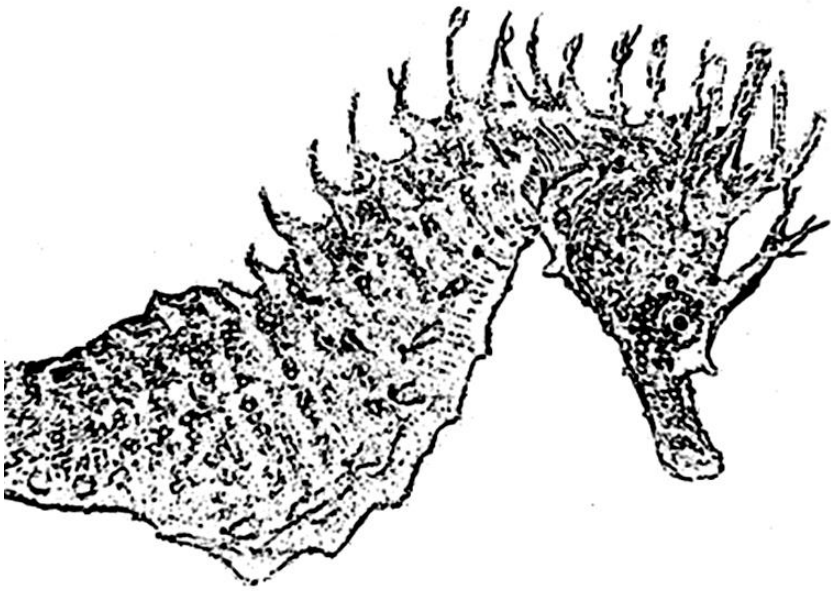
2. To determine the assimilation efficiency of two live preys (*Artemia* and copepods) and to establish the most adequate diet in early life stages of *H. guttulatus* (Chapter 4).

3. To assess the suitability of the fin-clipping sampling method as a non-lethal tool for stable isotope analysis in adult seahorses (Chapter 5).

4. To assess the dietary composition of wild *H. guttulatus* seahorses using Bayesian stable isotope mixing models (Chapter 6).

Apart from the four main sections presenting my own original work, an introductory chapter (Chapter 1) provides a general review about seahorse species and a brief description of the species of study, including biology, ecology and aquaculture aspects. In addition, the general material and methods employed in the Thesis are presented in Chapter 2. Finally, the general conclusions are presented in Chapter 7.

Versions of the main chapters of this Thesis have been published or submitted in peer-reviewed international journals, or are in preparation for publication. Some concepts may be repeated in these chapters, due to the fact that chapters have been considered as individual studies for publishing. I am the lead author of the manuscripts, conducting the experimental and field work, data analysis and writing. Co-authors have made significant contributions to the studies and improved the manuscripts considerably.



Chapter 2

General Material and Methods

General Material and Methods

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A general overview of the material and methods employed in both wild and reared seahorse studies is presented in this chapter. Also, a brief introduction to stable isotopes approach has been included in this section.

2.1. The study area

The Galician coast, located in the Northwest of the Iberian Peninsula, has a length of 1,195 km and represents about 35 % of the Spanish coastline (Bañón et al., 2010). Several coastal embayments named 'Rías' are present along the coast (Fig. 2.1.). The 'Rías' enter into the Galician coast tapering progressively from their mouth towards the innermost part with their axes almost perpendicular to the main coastline; their surface areas range from 12 km^2 to 230 km^2 (Prego et al., 1999). These 'Rías' have been traditionally classified into two groups: 'Rías Altas' and 'Rías Baixas', with the Cape Finisterre representing the boundary (Fig. 2.1.). The former, located to the North, exhibit a variety of geographic orientations and have higher values of primary production only during spring blooms. In contrast, the latter, located to the South, have approximately the same transverse geographical orientation in the NE-SW direction and are more productive during spring and summer upwelling events (Bode and Varela, 1998; Prego et al., 1999).

The Galician coast constitutes the Northern limit of the Eastern North Atlantic Upwelling System, which introduces the cold and nutrient-rich Eastern North Atlantic Central Water into the 'Rías' and increases the primary production (Fraga, 1981). Upwelling events along this coast are characterised by favourable northerly winds, frequent during spring and summer (from March–April to September–October) and generally predominant in the 'Rías Baixas' while they are less common in the 'Rías Altas' (Álvarez-Salgado et al., 1993, 2000).

In the 'Rías', the tidal regime is mesotidal and semidiurnal, with a mean amplitude between 2 and 4 m, and is considered the main force affecting the water circulation (Alvarez et al., 2006).

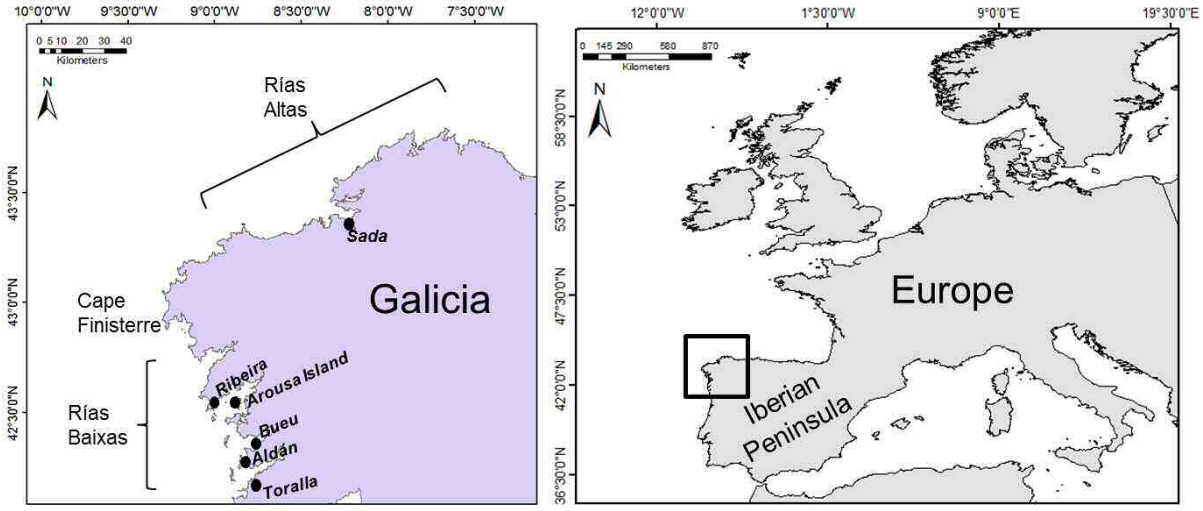


Figure 2.1. Map of the study area and sampling sites.

2.2. Seahorse collection

Wild seahorses *Hippocampus guttulatus* were collected from 2006 to 2012 in several locations along the South Galician coast ('Rías Baixas') for wild and captive studies (Fig. 2.1.). Different locations were considered to minimise the impact of wild captures and ensure an adequate genetic pool in the brood stocking. Toralla, Bueu, Aldán and Arousa Island localities are characterised by sandy or rocky bottom with patches of macroalgae (*Sargassum muticum*, *Dictyota* sp., *Chondrus crispus* and *Ulva* sp.) or seagrass beds (*Zostera marina*). In these sites, seahorses were mainly observed attached to different macroalgae or seagrass. On the contrary, Ribeira and Sada harbours were mainly dominated by muddy substrate and anthropogenic debris with accumulations of *Ulva* sp. and *Chlamiys varia*, respectively, where seahorses were effectively camouflaged. Generally seahorses were found in vegetated areas and stationary or holdfasted.

Surveys were conducted employing the underwater visual census method, which is the main technique used to obtain data of wild seahorse populations (Perante et al., 2002; Bell et al., 2003; Curtis et al., 2004; Foster and Vincent, 2004; Curtis and Vincent, 2005) (Fig. 2.2.). Seahorses were hand-caught by Scuba diving in shallow waters (<10 meters in depth and characterised by frequent poor visibility), placed in 5 L plastic bags with surrounding water and taken them out of the water for biological parameters measurements and sampling. Then, seahorses were immediately released to their origin, when they were not needed to supply the broodstock. For each seahorse found GPS position, depth of capture, habitat characteristics, sex, sexual maturity, reproductive status (males) and weight were recorded. Individuals were also photographed for standard length (SL) measurements.

External tagging with VIFE (Northwest Marine Technology Inc., USA), a visible implant fluorescent elastomer injected beneath the skin (Fig. 2.3.), was used for labelling and identification of individual seahorses marking different body segments and using a variety of colours. VIFE tags have been the most utilised tagging techniques in studies of syngnathid biology (Woods and Martin-Smith, 2004; Curtis and Vincent, 2006; Caldwell et al., 2011).

In order to study population genetics and to conduct an adequate individual identification of the seahorses, samples from skin filaments (cirrus) were collected for genetic analyses following specific protocols using microsatellites loci (Pardo et al., 2007). In

addition, a small sample of the dorsal fin was also collected as described by Valladares and Planas (2012) for stable isotope analyses (Chapter 5).

Figure 2.2. Underwater visual census conducted along the Galician coast to obtain data of wild *Hippocampus guttulatus* seahorses.

Figure 2.3. Wild seahorse *Hippocampus guttulatus* tagged with VIFE tags (left), tagging procedure (right).

Wild seahorses selected for the broodstock were individually placed into 5 L plastic bags containing 1/3 seawater from the capture site and 2/3 air and transported to the Institute of Marine Research (IIM-CSIC) facilities in isothermal conditions. On arrival, each seahorse was tagged using nylon collars (VI Alpha TAG collars, NMT Inc., USA) with a unique alphanumerical code for individual identification (Planas et al., 2008b) (Fig. 2.4.). Then, seahorses were gradually acclimatised to the captive conditions.

2.3. Broodstock maintenance

Seahorses collected in the wild were acclimatised to captive conditions by the gradual addition of seawater from the husbandry aquaria into the holding bag for a few hours. No therapeutic treatments or quarantine were applied. Seahorses were maintained in the husbandry aquarium units (630 L) as described by Planas et al. (2008b) (Fig. 2.4.). Each aquarium unit functioned as an autonomous closed systems. A water treatment system was located at the bottom of each aquarium, and consisted of a filtering system, a cooling unit with electronic thermostat control and pumps. Pumped seawater was filtered (5 µm) and UV treated, with a 10-15 % daily water exchange. Water quality was checked periodically for NO_2, NO_3 and NH_4/NH_3 content (0 mg l^{-1}) by using Sera Test Kits. Salinity and pH levels were 38 ± 1 ppt and 8.1 ± 0.1, respectively. The water temperature was kept under an annual temperature regime ranging from 15 °C in winter to 19 °C in summer and controlled by a cooling unit and a thermostat (± 0.5 °C). Each aquarium had an illumination intensity of about 850–1050 lx provided by a 20 W fluorescent tube (4000 K) located 25 cm above the

water surface. A natural-like photoperiod regime was applied (16L:8D in June-July; 10L:14D in December-January), the daily light regime was controlled by a timer and weekly adjusted. The aquaria were covered with black curtains for the dark phase. Soft plastic plants and plastic ropes were anchored to small stones and placed on the bottom of the aquaria as holdfasts for the seahorses. Wastes and uneaten food were removed daily by siphoning the bottom of the aquaria early in the morning.

Adult seahorses were predominantly fed on live adult enriched *Artemia* of about 15–25 days old (Fig. 2.6.). *Artemia* was offered *ad libitum* to seahorses twice a day. Food levels were adjusted daily (60–150 Artemia per seahorse) according to the season/temperature and to visual observations of *Artemia* remaining in the aquaria from the previous day. Supplemental food consisted of a single dose of wild-captured Mysidacea (*Leptomysis* sp. and *Siriella* sp., 15-20 per seahorse) or cultivated shrimp *Palaemonetes varians* (15-20 per seahorse), when available (Fig. 2.6.).

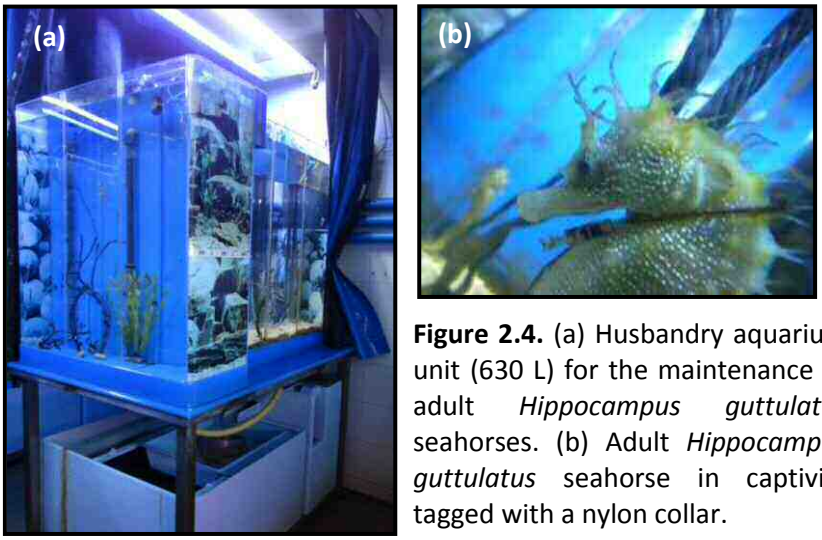


Figure 2.4. (a) Husbandry aquarium unit (630 L) for the maintenance of adult *Hippocampus guttulatus* seahorses. (b) Adult *Hippocampus guttulatus* seahorse in captivity tagged with a nylon collar.

2.4. Rearing of juveniles

By the end of pregnancy, pregnant seahorses were transferred from the broodstock aquaria to 30 L pseudoKreisel-type aquaria and maintained isolated for a few days until newborn release. After birth, total batch size was recorded and healthy live juveniles from each batch were randomly distributed (5 juveniles L^{-1}) into 30 L pseudoKreisel-type aquaria for feeding experiments or into 10 L rectangular aquaria (33×22×19 cm) for starvation experiments (Fig. 2.5.).

Pumped seawater was filtered by a series of filter-cartridges (20, 10, 5 and 1 µm) and UV treated (76 W; 16 L min^{-1}). The rearing aquaria operated in a semi-opened recirculation system including a degasifying column and two 50 L biofilters with mechanical (up to 20 µm) and biological filters, aerators and skimmers. From the biofilter units, the seawater was pumped to secondary UV units (36 W) and then to a 50 L reservoir aquarium, being finally routed by gravity towards the rearing aquaria.

The rearing system was submitted to a constant 16L:8D photoperiod regime supplied by 20 W fluorescent lamps (Power Glo), a continuous inflow flux of 700 ml min^{-1} and gentle aeration provided by a stand-pipe placed in the upper part of the water column. Initial

water temperature in the rearing aquaria (18 °C) was progressively modified by means of an inlet heating system to reach the desired experimental temperature. Detailed rearing conditions (water temperature and feeding) for the experiments undertaken in this Thesis are further described in the corresponding chapters.

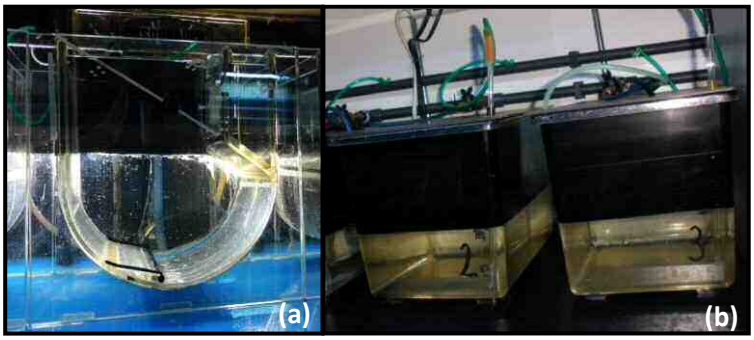


Figure 2.5. Rearing aquaria for juveniles *Hippocampus guttulatus* in experimental conditions, 30 L pseudoKreisel aquaria (a) and 10 L rectangular aquarium (b).

2.5. Food production

2.5.1. Microalgae

Microalgae strains (*Phaeodactylum tricornutum*, *Rhodomonas lens* and *Isochrysis galbana*) were kept under sterilised conditions in 250 ml Erlenmeyer flasks containing 100 ml of 0.22 µm filtered and autoclaved (Sartorius 11407-047-ACN) seawater supplemented with 200 µl F2P (100 g L^{-1}) media (VarAqua). Additional 200 µl of silicates (40 g L^{-1}) were added into the *P. tricornutum* flask, and 200 µl F2P media (100 g L^{-1}) into the *R. lens* flask. Culture flasks were maintained in a 22 ± 1 °C room under a continuous day-like white fluorescent light cycle. No aeration was supplied and manual mixing was applied to avoid cell sedimentation. After one week of culture, the microalgae reached their stationary growth-stage with approximately 10^7 cells ml^{-1} (*P. tricornutum*), $6x10^6$ cells ml^{-1} (*I. galbana*) and $4x10^6$ cells ml^{-1} (*R. lens*). Then, 5 ml of the culture were used to inoculate new Erlenmeyer flasks whereas the remaining volume was inoculated in 5 L glass flasks containing filtered (1 µm) and autoclaved seawater supplemented with 2 ml L^{-1} F2P media (100 g L^{-1}). Additional 2 ml L^{-1} F2P media (100 g L^{-1}) was added after 4 days into the *R. lens* 5 L flask culture, and *P. tricornutum* flasks were supplemented with 2 ml L^{-1} of silicates (40 g L^{-1}). When the microalgae cell density reached the stationary growth-phase, a 5 L flask was used to inoculate 80 L plastic bags, which were maintained at 22 ± 1 °C into an acclimatised room. Strong bubbling was supplied through a sterilised stand pipe. Continuous lightening was applied with day-like fluorescent lights. Seawater was sterilised adding bleach (0.5 ml L^{-1}) for 2h, then neutralised with sodium thiosulfate pentahydrated (0.5 ml L^{-1}) (Merck) and checked after 30 minutes with orthotolidine (Panreac).

2.5.2. *Artemia*

Artemia cysts (EG, Iberfrost or Inve, Spain), were incubated at 28 °C for 20 h in 15 L incubators. The cyst decapsulation was carried out by adding bleach (10 ml L^{-1}) for 30 min and further neutralising with sodium thiosulfate pentahydrated (10 ml L^{-1}). Then, chlorine free water was checked with orthotolidine (Panreac). Hatched *Artemia nauplii* were rinsed with tap-water for 30 minutes prior to be transferred to 90 L tanks for adult *Artemia*

production, or to 5 L buckets for *Artemia metanauplii* production, with initial densities of 100 *Artemia* ml^{-1} (Fig. 2.6.). Adult *Artemia* was cultured at 26–28 °C with gentle aeration and constant light, and enriched on a mixture of microalgae (*P. tricornutum* and *I. galbana*), Red Pepper (Bernaqua, Belgium) and Spirulina (Iberfrost, Spain). Extra doses of Red Pepper were supplied for at least 3 days prior to seahorse feeding (Quintas et al., 2007). *Artemia metanauplii* was grown up to different ages at 26 °C with gentle aeration and constant light. *Artemia metanuplii* was filtered through a 125 µm mesh and rinsed with tap water for 30 minutes prior to the daily enrichment. The enrichment consisted of a mixture of live microalgae *I. galbana* (10^7 cells ml^{-1}) and *P. tricornutum* ($1.6 \cdot 10^7$ cells ml^{-1}), and commercial products, Red Pepper (0.3 g L^{-1}) and dried Spirulina (0.8 g L^{-1}).

2.5.3. Copepods

Copepods (*Acartia tonsa* and *Tisbe* sp.) (Fig. 2.6.) were cultivated with an initial density of 1 adult copepod ml^{-1}, in 250 and 500 L tanks at 26–27 °C and 38 ppt salinity, and daily fed on a 10 L mixture (1:1 volume) of the microalgae *I. galbana* (10^7 cells ml^{-1}) and *R. lens* (16^7 cells ml^{-1}). Siphoning and water exchange (20 % of the total volume) was done every other day. Each tank was partially harvested when necessary for seahorse feeding. Once a month, the tanks were completely siphoned, bleached, rinsed and refilled with filtered seawater, previous retention of microalgae and copepods.

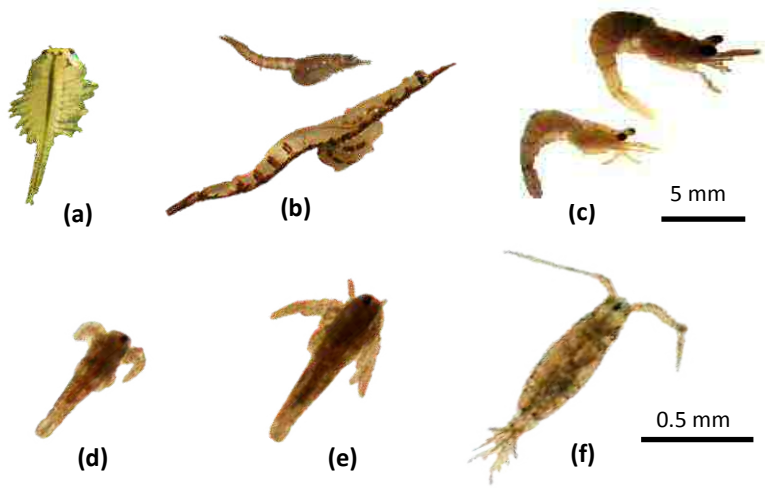


Figure 2.6. Live preys employed to feed adult and juvenile *Hippocampus guttulatus* seahorses in captivity. Adults: (a) Adult *Artemia*, (b) Mysidacea, (c) *Palaemonetes varians*; juveniles (d) *Artemia nauplii*, (e) *Artemia metanauplii*, (f) copepods.

2.6. Biological parameters

2.6.1. Length and weight

All standard lengths (SL) reported in this Thesis were measured as head length (HL) + trunk length (TrL) + tail length (TaL) (curved measurement), following the measuring protocol described by Lourie et al. (1999) (Fig. 2.7a.). Morphometric measurements were made on digital images using the image processing software NIS Elements (Nikon).

For sampling juvenile specimens, an overdose of MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma Aldrich, 0.1 g L^{-1}) was supplied to euthanise the juvenile seahorses. Then, individuals were transferred to Petri dishes over a grid paper and photographed for standard length (SL) measurements (Fig. 2.7b.). Subsequently, seahorses were blotted on filter paper to remove the excess of water and each seahorse was individually weighted (wet weight, W) on a Sartorius microbalance MC210P (± 0.01 mg). Finally, seahorses were rinsed with distilled water and maintained frozen at -80 °C or -20 °C, as further analysis requirements. Freeze-dried specimens were weighted for dry weight (DW).

In case of adult seahorses, weight and length were recorded at the time of capture. Individuals were photographed alive by placing them carefully over a gridded plate for standard length (SL) measurements (Fig. 2.7b.). Then, specimens were individually weighted (wet weight, W) on a Scaltec balance (± 0.01 g). Handling time was kept at a minimum to minimise their stress. No anaesthetic was applied.

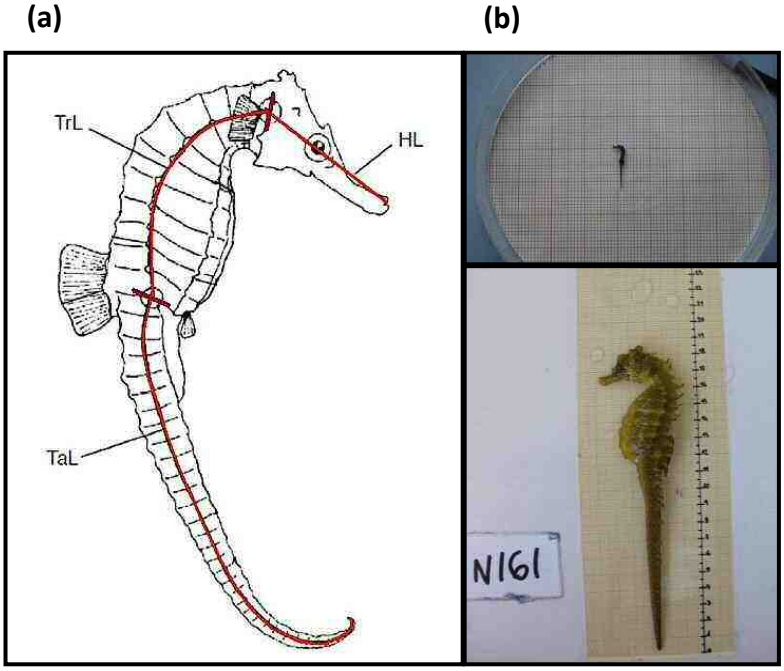


Figure 2.7. (a) Standard seahorse morphometric measurements from Lourie et al. (1999). HL: Head length; TrL: Trunk length; TaL: Tail length. Standard length (SL) = HL + TrL + TaL. (b) Photograph methodology for standard length measurements of juvenile (top) and adult (bottom) seahorses.

2.6.2. Size and weight gain

Size and weight gain (SG and WG, respectively) are determined as the difference between the final length (mm) or biomass (mg) and initial length (mm) or weight (mg), respectively.

2.6.3. Condition factor

Condition factor is a simple indicator of a fish condition, commonly referred as Fulton's condition factor (K_F). This index was calculated as the relationship between standard length (SL) and wet weight (W) of juvenile seahorses. The equation is defined as follows:

$$K_F = (W / SL^3) * 1000$$

2.6.4. Age

Wild seahorses were categorised into two age classes, adult and sub-adults, according to their standard length. Therefore, sub-adult age class (sexually immature) corresponded to individuals smaller than 109 mm, whereas larger individuals were included in the adult age class (mature) (Curtis, 2004).

2.6.5. Sex

In case of adult and sub-adult age classes, sexual differences were easily distinguished and inferred from the presence of the brood pouch developed in males. Hence, sexual differentiation in juvenile seahorses was not possible to estimate.

2.7. Analytical methods

2.7.1. Lipid content

Samples were submitted to lipid extraction following a modification of the procedure described by Bligh and Dyer (1959) (Fernández-Reiríz et al., 1989). Lipids were first extracted by a mixture of chloroform/methanol (1:2), centrifuged (3,246 x g) and extracted again with chloroform/methanol (2:1). Then, both supernatants were washed by a mixture of chloroform/methanol/water (8:4:3) (Folch et al., 1957). Total lipid content was quantified gravimetrically according to Herbes and Allen (1983).

2.7.2. Stable isotopes

Whole body (juvenile seahorses) or small portion of the dorsal fin (adult seahorses) was used for stable isotope analysis. Lipid extraction was not considered when lipid content in samples is below around 5 % lipids (C:N < 3.5) (Post et al., 2007).

Prior to stable isotope analyses, all samples were washed in distilled water, freeze-dried (Ilshin Lab Co., Ltd.) and homogenised, and then sub-samples of about 1 mg were weighted into tin capsules. Carbon and nitrogen isotopic analyses were conducted at Centro de Apoio Científico e Tecnolóxico á Investigación (CACTI), Universidade de Vigo (Spain), and Servizos de Apoio á Investigación (SAI), University of A Coruña (Spain). Samples were measured by means of a continuous-flow isotope ratio mass spectrometry (IR-MS).

Stable isotope abundances are expressed in conventional delta notation (δ) as parts per thousand (‰) relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air,

according to the following equation: $\delta X = [(R_{Sample}/R_{Reference}) - 1]$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively.

As part of an analytical batch run, a set of international reference materials for $\delta^{15}N$ (IAEA-N-1, IAEA-N-2, IAEA-NO-3) and $\delta^{13}C$ (NBS 22, IAEA-CH-6, USGS24) were used for system calibration. The analytic precision (standard deviation) of the laboratory for $\delta^{13}C$ and $\delta^{15}N$ are specified in the corresponding chapters.

2.8. Animal bioethics

Animal maintenance and handling practices were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government ('*Real decreto 1201/2005, de 10 de octubre*') and approved by the Bioethics Committee of CSIC.

Chapter 3

Influence of temperature and feeding status on isotopic composition in early developing seahorses

Influence of temperature and feeding status on isotopic composition in early developing seahorses

§

3.1. Introduction

The estimation of food intake, digestibility and assimilation patterns provides valuable information for the interpretation of growth and mortality rates of a consumer (Govoni et al., 1986; Conceição et al., 2007). The majority of techniques used to determine nutrient assimilation in fish are indirect (e.g. faeces collection, gut contents or growth rates) and often difficult to apply, particularly at the early life stages mainly due to size limitation, complexity of sample collection and quantification of food intake (Rønnestad et al., 2001; Conceição et al., 2007). Direct measurements are suitable for evading the difficulties associated with larval nutrition studies. Measurement of stable isotope values in larvae represents a direct measure of nutrient uptake, useful for understanding how consumers incorporate the nutrients they consume. This approach is based on the fact that stable isotope values of a consumer tissue reflect those of the food incorporated, with a small variation named trophic discrimination that occurs during food assimilation (DeNiro and Epstein, 1978). $^{15}N/^{14}N$ and $^{13}C/^{12}C$ ratios of a consumer are usually higher than those in its diet, mainly because the lighter isotope (^{14}N and ^{12}C) is preferred in metabolic processes.

Stable isotopes and particularly natural isotope ratios of carbon and nitrogen ($^{15}N/^{14}N$ and $^{13}C/^{12}C$, respectively measured and reported in delta notation as $\delta^{15}N$ and $\delta^{13}C$) have been successfully applied as nutritional tracers to support research on fish larval nutrition (Schroeder, 1983; Schlechtriem et al., 2004; Jomori et al., 2008; Gamboa-Delgado et al., 2008). Numerous factors such as environmental conditions (e.g. temperature), feeding rates, physiological and nutritional status of the consumer (e.g. stress, starvation) might cause modifications on food assimilation reflecting differences on the isotopic composition of the consumer (Hobson et al., 1993; Herzka and Holt, 2000; Gaye-Siessegger et al., 2004; Barnes et al., 2007). Experimental studies have been developed to aid in the interpretation of stable isotopes changes when comparing diets and consumers. It has been reported that the amount of food consumed may influence the isotopic ratios of animal tissues but the results obtained for fish are different. Focken (2001) determined higher $\delta^{15}N$ and $\delta^{13}C$ values at higher feeding rates in Nile tilapia (*Oreochromis niloticus*). Contrarily, Gaye-Siessegger et al., (2004) observed a decrease of $\delta^{15}N$ and $\delta^{13}C$ values in common carp (*Cyprinus carpio*) with increasing feeding rate. In the same way, energy reserves depletion during starvation cause an important effect on the isotopic composition, usually reflected in a clear $\delta^{15}N$ increase (Hobson et al., 1993; Gaye-Siessegger et al., 2007) but a small $\delta^{13}C$ increase (Schlechtriem et al., 2004; Jomori et al., 2008). Also, it has been pointed out that

water temperature may influence the effect of starvation on the isotopic composition of fish (Gaye-Siessegger et al., 2007). Studies on the effects of environmental conditions on stable isotope incorporation may be relevant for larval nutrition, recognition of the environmental preferences of larvae, interpretation of ecological studies and improvement of rearing techniques. Temperature is among the environmental factors that affect fish development, and thus larval rearing success, with a strong influence on juvenile growth rates and metabolism (Hidalgo et al., 1987; Houde, 1989; Blaxter, 1992; Hart et al., 1996; Planas et al., 2012). In particular, the direct effect of temperature on carbon and nitrogen stable isotopes of fish has been reported by a few studies (Herzka and Holt, 2000; Bosley et al., 2002; Witting et al., 2004; Barnes et al., 2007).

Breeding seahorses in captivity, as an alternative to reduce the fishing pressure on wild stocks (Vincent, 1996; Lourie et al., 1999), is a relatively recent activity that confronts many culture difficulties, particularly the low survival in the early life stages (Wilson and Vincent, 1998; Foster and Vincent, 2004; Koldewey and Martin-Smith, 2010; Olivotto et al., 2011). The current knowledge gap about factors affecting the low survival occurrence needs to be analysed and solved. Growth and mortality rates can be linked with nutrient assimilation. Thereby, it is a priority to estimate the assimilation efficiency for understanding nutrition processes in first feeding seahorses, improving feeding strategies in their rearing system and understanding feeding dynamics of seahorses in the natural environment.

The long-snouted seahorse *Hippocampus guttulatus* Cuvier 1829 is a threatened species, mainly due to habitat loss/degradation and incidental captures, which cause a negative impact on wild populations. Recent research studies have focused much effort on studying factors (e.g. prey type, food enrichment, temperature and photoperiod) affecting survival and growth of early juvenile *H. guttulatus* seahorse (Faleiro et al., 2008; Palma et al., 2008; Blanco et al., 2011; Olivotto et al., 2011; Valladares and Planas, 2011; Planas et al., 2012; Blanco et al., 2014). In spite of improvements achieved in rearing procedures and successful progress in survival rates on *H. guttulatus* culture, food assimilation in first feeding juveniles and factors influencing its variation still remain unknown. Regarding temperature, its effect on seahorse growth and survival has been reported in the past (Wong and Benzie, 2003; Lin et al., 2006; Sheng et al., 2007; Planas et al., 2012) but the relationship between temperature and food assimilation efficiency has not been previously studied. Estimates of the optimal water temperature level may represent a key parameter for understanding the processing of nutrients and achievement of more efficient rearings. Fish reared at warmer temperatures would increase growth rate, with faster tissue isotopic turnover and higher nutrient assimilation reflecting changes in the isotopic composition.

The present study investigates the influence of seawater temperature on carbon and nitrogen stable isotope values to evaluate the food assimilation in early life stages of the seahorse *H. guttulatus*. The influence of starving conditions on the isotopic composition was also evaluated. This study represents the first attempt to investigate environmental parameters affecting food assimilation in early life stages of a seahorse species using stable isotopes. The results reported can contribute to understand how biological and ecological features of wild populations (e.g., geographical distribution and breeding season length) may vary under temperature fluctuations.

3.2. Material and methods

Adult seahorses *Hippocampus guttulatus* collected off the Galician coast (NW Spain) were maintained in *ad hoc* aquaria (Planas et al., 2008b) at the Instituto de Investigaciones Marinas (IIM-CSIC) in Vigo (Spain). The water temperature was kept under an annual temperature regime ranging from 15 °C in winter to 19 °C in summer (± 0.5 °C). A natural-like photoperiod regime was also applied (16L:8D in June-July; 10L:14D in December-January). Pumped seawater was filtered (5 µm) and UV treated, with a 10-15 % daily water exchange. Water quality was checked periodically for NO_2, NO_3 and NH_4/NH_3 content (0 mg L^{-1}) by using Sera Test Kits. Salinity and pH levels were 38 ± 1 ppt and 8.1 ± 0.1, respectively. Captive seahorses were fed *ad libitum* twice daily on a diet comprising enriched adult *Artemia* (EG, Inve, Spain) and supplemented with captured Mysidacea (*Leptomysis* sp. and *Siriella* sp.).

Juvenile seahorses from two batches were obtained from a male hold in captivity for 19 months. Immediately after birth, juveniles from each batch were randomly transferred (5 juveniles L^{-1}) into three 30 L Kreisel-type aquaria (15, 18 and 21 °C) for feeding experiments; and into three 10 L rectangular aquaria (33×21×17 cm) for starvation experiment (15, 18 and 21 °C). The rearing system was submitted to a 16L:8D photoperiod regime and lightening was supplied by 20 W fluorescent lamps (Power Glo). The initial water temperature in the rearing system was 15 °C. During the following two days, the temperature was progressively adjusted to reach the desired experimental temperatures of 15, 18 and 21 °C (± 0.5 °C), which were maintained constant until the end of the experiments (day 30). The total volume of seawater in the rearing system was renovated twice per hour by means of an external inflow (24 L h^{-1}) of 20 µm filtered and UV-treated seawater.

In the feeding experiment, seahorses were initially fed on a single daily dose of cultivated copepods (*Acartia tonsa* and *Tisbe* sp.) (0.6 copepods ml^{-1}) until day 5. A daily dose of copepods (0.3 copepods ml^{-1}) and Great Salt Lake *Artemia nauplii* (1 *Artemia* ml^{-1}) were added from day 6 to 10. *Artemia nauplii* and 24 h enriched *Artemia metanauplii* (1 *Artemia* ml^{-1} $dose^{-1}$) was offered, three daily doses on weekdays and two daily doses with a single dose of copepods (0.3 copepods ml^{-1}) on weekends, from day 11 until the end of the experiment at day 30. The feeding scheme applied in this study was based on the optimal feeding schedule proposed by Blanco (2014).

Copepods were cultivated in 250–500 L tanks at 26–27 °C and 38 ppt salinity and fed on mixtures of the microalgae *Isochrysis galbana* and *Rhodomonas lens*. Only copepods retained by a 125 µm mesh were used to feed seahorses. The enrichment of *Artemia* was performed in 5 L buckets at 26 °C and with initial densities of 100 *Artemia* ml^{-1}, and consisted of a mixture of the microalgae *Isochrysis galbana* (10^7 cells ml^{-1}), *Phaeodactylum tricornutum* ($1.6 \cdot 10^7$ cells ml^{-1}) and *Rhodomonas lens* (16^7 cells ml^{-1}). Each aquarium was kept under gentle aeration in the upper part of the water column and a continuous flow rate of 700 ml min^{-1}. At night, water outlets were screened with 250 µm mesh to avoid prey and seahorses from leaving the aquaria and allowed water to circulate. Twice daily, wastes and faeces were siphoned out, and dead seahorses removed and counted.

An experiment was also carried out with newborns deprived of food to obtain information about potential changes on isotopic levels in seahorses under starvation conditions and comparison with seahorses under normal feeding conditions. The rearing

system was set up with a constant water flow rate of 300 ml min^{-1} and moderate aeration. Mortalities were recorded daily throughout the experimental period.

At the onset of the experiments, individuals were sampled to determine initial carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values, weight and length. Samples of *Artemia* and copepods were also collected, rinsed with distilled water and kept frozen at -20 °C for further isotopic determination. Copepods, *Artemia nauplii* and *Artemia metanauplii* had $\delta^{13}C$ values of -19.81, -21.99 and -19.87 ‰, $\delta^{15}N$ values of -1.07, 11.60 and 9.08 ‰, and C:N ratios of 3.96, 5.00 and 4.18, respectively. Fed seahorses were randomly collected before feeding time from each aquarium (at ages of 5, 15 and 30 days) for determination of stable isotope ratios, weight and length. Starved seahorses were sampled only at day 5, prior to 100 % mortality at days 7-9. For standard length (SL) measurements, seahorses were anesthetized with MS222 (0.1 g L^{-1}), transferred individually to Petri dishes and photographed. Then, the excess of water was removed and the seahorses weighted on a Sartorius microbalance (± 0.01 mg). Then, seahorses were rinsed with distilled water and frozen at -20 °C. Standard lengths were measured as head + trunk + tail length (curved measurement), following Lourie et al. (1999). Measurements were made on digital images using image processing software (NIS, Nikon).

Whole juvenile seahorses were lyophilized, homogenized and sub-samples of 1 mg were weighted into tin capsules for stable isotope analysis. Carbon and nitrogen isotopic ratios and elemental composition were analysed at Servizos de Apoio á Investigación (SAI) of the University of A Coruña (Spain). Samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyser (ThermoFinnigan, Italy) coupled to a Deltaplus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface. Carbon and nitrogen stable isotope abundance was expressed as parts per thousand (‰) relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air, according to the following equation: $\delta X = [(R_{Sample}/R_{Reference}) - 1]$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. As part of an analytical batch run, a set of international reference materials for δ^{15} N (IAEA-N-1, IAEA-N-2, IAEA-NO-3) and $\delta^{13}C$ (NBS 22, IAEA-CH-6, USGS24) were analysed. The precision (standard deviation) for the analysis of $\delta^{13}C$ and $\delta^{15}N$ of the laboratory standard (acetanilide) was ± 0.15 ‰ (1-sigma, n=10).

Carbon and nitrogen isotopic changes over time were determined applying two different time indices: chronological (days) and effective day degrees ($D°_{eff}$). Effective day-degrees ($D°_{eff}$) is an index of developmental progress based on a species-specific threshold temperature (T_o) at which development is theoretically arrested (Weltzien et al., 1999). $D°_{eff}$ was calculated from the following equation:

$$D°_{eff} = \Delta t \cdot T_{eff} = \Delta t \cdot (T - T_o)$$

where T_{eff} is the biologically effective temperature ($T_{eff} = T - T_o$) and T_o the threshold temperature for the species (13.1 ± 0.9 °C) (Planas et al., 2012).

In starving larvae, mortality data (M; %) were fit to the sigmoidal model:

$$M = a + ((b-a) / (1 + e^{(-(t-c)/d)}))$$

with a, b and d constants, t time (days or $D°_{eff}$) and c time of 50% mortality (M_{50}).

Fulton's condition factor (K_F) was calculated as:

$$K_F = (W / SL^3) * 1000$$

with W wet weight and SL standard length.

Variables were checked for normality using the Shapiro–Wilk test. Analysis of variance (ANOVA or Kruskal-Wallis, based on assumptions fulfilled or not) was applied to estimate effects of temperature on survival, growth parameters and isotopic values at different times. When significant differences were found, Tukey's HSD post-hoc test was applied. SPSS v.15.0 software was used to perform the statistical analyses at a significant level of $p < 0.05$.

Animal maintenance and manipulation practices were conducted in compliance with all bioethics standards of the Spanish Government and approved by the Bioethics Committee of CSIC.

3.3. Results

Larval growth parameters and C:N ratios through the experimental period are summarized in Table 3.1. Growth rates in the feeding experiment differed between temperature conditions. The highest growth rate was observed in warmer conditions (21 °C), with a weight gain up to 11.80 ± 9.96 mg and a size gain of 23.73 ± 11.31 mm at day 30, although the difference between temperatures was not statistically significant ($F_{2,5} = 1.28$, $p = 0.39$ and $F_{2,6} = 1.66$, $p = 0.33$, respectively). In spite of the highest growth rate corresponded to the 21 °C, the highest survival at the end of the experiment (day 30) was achieved at 18 °C (75.5 ± 14.2 %), which was significantly higher than at 15 °C (18.5 ± 7.2 %) and 21 °C (61.8 ± 27.1 %) (Kruskal-Wallis test, $p < 0.05$). Mortalities at 15 °C started at day 4, while at 18 and 21 °C initial mortalities were delayed until day 6 (Fig. 3.1a.). Starving juveniles showed a weight loss at all temperatures; however, it was observed a small size gain (Table 3.1.). Unfed seahorses survived longer at 15 °C than at warmer temperatures and full mortalities occurred at day 9 at 15 °C, day 8 at 18 °C and day 7 at 21 °C (Fig. 3.1b.). M_{50} was estimated to occur at days 5.6, 6.3 and 6.7 for 21, 18 and 15 °C, respectively.

Fulton's K_F index in fed juveniles decreased at day 5 (1.18 ± 0.08 at 15 °C, 1.19 ± 0.08 at 18 °C, 1.17 ± 0.03 at 21 °C), compared to the initial values (1.46 ± 0.04). K_F values were similar at day 15 (1.19 ± 0.01 at 15 °C, 1.16 ± 0.01 at 18 °C, 1.05 ± 0.12 at 21 °C) and increased at day 30 (1.20 ± 0.04 at 15 °C, 1.38 ± 0.18 at 18 °C, 1.23 ± 0.14 at 21 °C). Starved seahorses at day 5 showed Fulton's K_F values lower than the initial values (1.46 ± 0.04) and inversely related to temperature level (1.16 ± 0.07 at 15 °C, 1.07 ± 0.02 at 18 °C, 1.01 ± 0.04 at 21 °C).

Table 3.1. Larval growth parameters and C:N ratios for seahorses *Hippocampus guttulatus* in two experimental regimes (feeding and starvation) under three water temperature conditions (15, 18 and 21 °C). Mean and SD of two batches. SL = Standard length.

			Dry Weight (mg)		Weight gain (mg)		SL (mm)		Size gain (mm)		C:N	
Experiment	Temp (°C)	Day	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Onset	15	0	1.00	0.24	n/a	n/a	15.30	0.69	n/a	n/a	3.46	0.06
Feeding	15	5	0.86	0.11	-0.14	0.13	17.22	0.86	1.92	0.16	3.60	0.13
	15	15	1.11	0.39	0.11	0.14	18.08	1.70	2.78	1.01	3.52	0.02
	15	30	1.53	0.39	0.53	0.14	21.32	2.98	6.02	2.28	3.49	0.01
	18	5	0.81	0.42	-0.02	0.06	17.84	0.42	2.53	0.28	3.60	0.07
	18	15	2.65	1.63	1.65	1.39	23.92	5.46	8.62	4.76	3.57	0.04
	18	30	7.57	7.28	6.57	7.04	30.49	12.98	15.19	12.29	3.60	0.09
	21	5	1.32	0.86	0.32	0.62	19.58	4.05	4.28	3.36	3.56	0.14
	21	15	4.49	2.45	3.49	2.21	29.37	4.53	14.07	3.83	3.53	0.01
	21	30	12.80	10.20	11.80	9.96	39.13	12.15	23.73	11.31	3.48	0.12
Starvation	15	5	0.65	0.07	-0.35	0.17	16.34	0.37	1.03	0.33	3.55	0.02
	18	5	0.75	0.14	-0.42	0.14	16.35	0.70	1.05	0.00	3.63	0.01
	21	5	0.56	0.09	-0.45	0.15	16.16	0.70	0.86	0.01	3.47	0.09

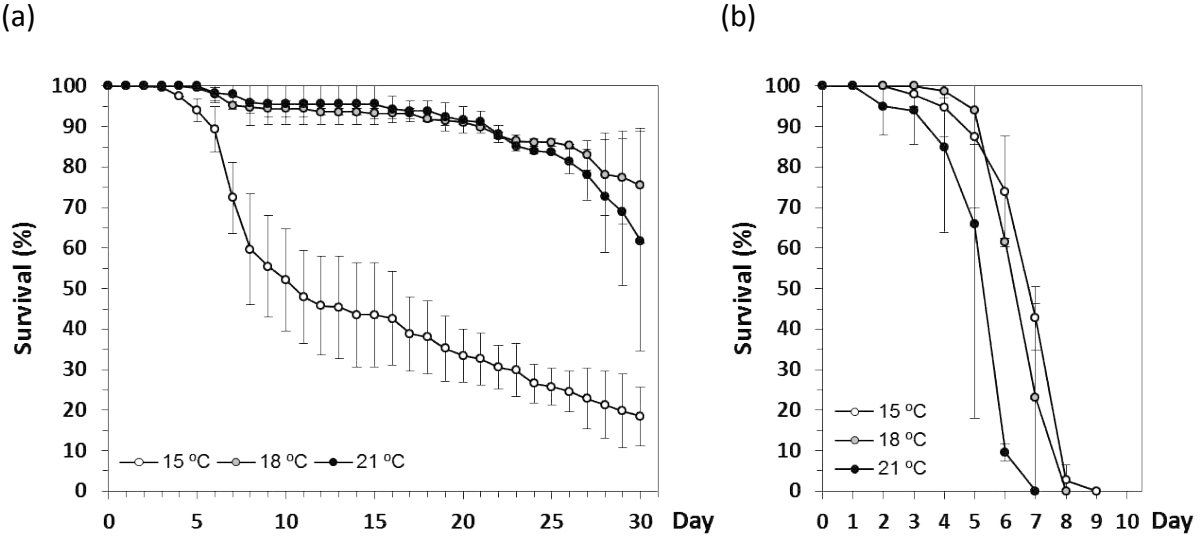


Figure 3.1. Survival (%) in fed (a) and starved (b) juvenile seahorses *Hippocampus guttulatus* under the three temperature conditions (15, 18 and 21 °C) over each experimental period. Mean and SD of two batches are indicated by vertical bars.

Seahorses maintained at 18 °C had a higher C:N ratios compared to the other temperature conditions, in both fed and starved seahorses (Table 3.1.). Water temperature and age had a significant effect on $\delta^{13}C$ values in seahorses ($F_{2,9}$ = 6.41, p = 0.02 and $F_{2,9}$ = 20.80, p < 0.001, respectively). At the onset of the experiment, juvenile seahorses had a $\delta^{13}C$ value of -15.45 ± 0.42 and a $\delta^{15}N$ value of 10.83 ± 1.05. At day 5 in the feeding experiment, a decrease in both $\delta^{13}C$ and $\delta^{15}N$ was detected, especially at 21 °C (-16.74 ± 1.37 and 7.25 ± 1.19, respectively) (Fig. 3.2.). From day 5 onwards, the trends of carbon and nitrogen stable isotopes in relation to temperature level were different. Isotopic values of carbon showed a progressive drop until the end of the experiment (day 30), particularly at 18 and 21 °C (Fig. 3.2a.). Isotopic values of nitrogen showed a different trend. A steady decrease was observed at 15 °C until the end of the experiment, contrary to the increase observed at 18 °C after day 5; at 21 °C a small decrease was observed until day 15 followed by an increase at day 30 (Fig. 3.2b.). Regardless of the not significant temperature effect ($F_{2,9}$ = 0.29, p = 0.75), a certain influence of temperature was noticed on $\delta^{15}N$ values. Also, $\delta^{15}N$ values changed with age ($F_{2,9}$ = 4.83, p = 0.04). The small increase observed for $\delta^{13}C$ in 5 days old starved juveniles at all experimental temperature levels was opposed to the sharp decrease observed at the same age in fed juveniles (Fig. 3.2a.). However, nitrogen isotopic values in starving juveniles were maintained constant (21 °C) or decreased slightly (15 °C and 18 °C). That pattern was similar to that find in fed juveniles but the drop was considerably lower (Fig. 3.2b.).

When $\delta^{13}C$ and $\delta^{15}N$ values were modelled as a function of effective-day degrees ($D°_{eff}$), the isotopic composition in fed and unfed seahorses suggest a major developmental progress at 21 °C (Fig. 3.2.).

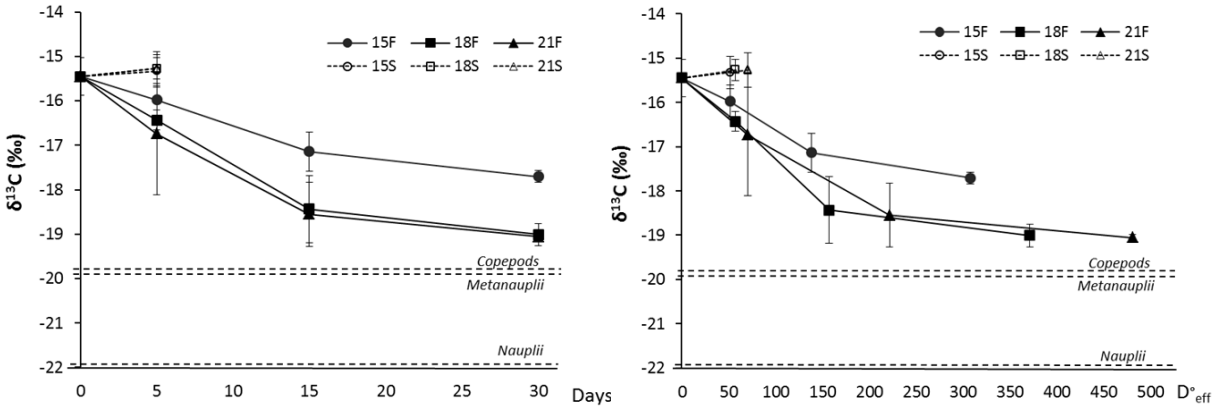
3.4. Discussion

3.4.1. Fed seahorses

Carbon and nitrogen isotopic values were affected by temperature level, age and feeding status of seahorse juveniles. For carbon, the progressive decrease observed in fed seahorses from birth until the end of the experiment indicates a clear $\delta^{13}C$ shift towards diet isotopic values, especially under the most active feeding conditions (18 and 21 °C). This finding suggests a more efficient assimilation of the diet in juveniles at warmer temperatures. Similarly, the $\delta^{15}N$ values of fed seahorses were reduced at day 5, especially at 21 °C, when compared to the initial values in newborns. That trend indicated a shift towards the isotope values of the dietary source (copepods) in such early developmental period. These results can be interpreted as a better assimilation of prey (copepods) in juveniles grown at warmer temperatures.

Values of $\delta^{15}N$ in *Artemia nauplii* and *metanauplii* supplied after day 5 were much higher than in copepods. Hence, an increase in $\delta^{15}N$ values would be expected in juvenile seahorses from day 5 as a result of *Artemia* intake and assimilation. The progressive increase in $\delta^{15}N$ in juveniles beyond day 5 at warmer temperatures also suggests more efficient prey assimilation in juveniles feeding at 18 and 21 °C.

(a)



(b)

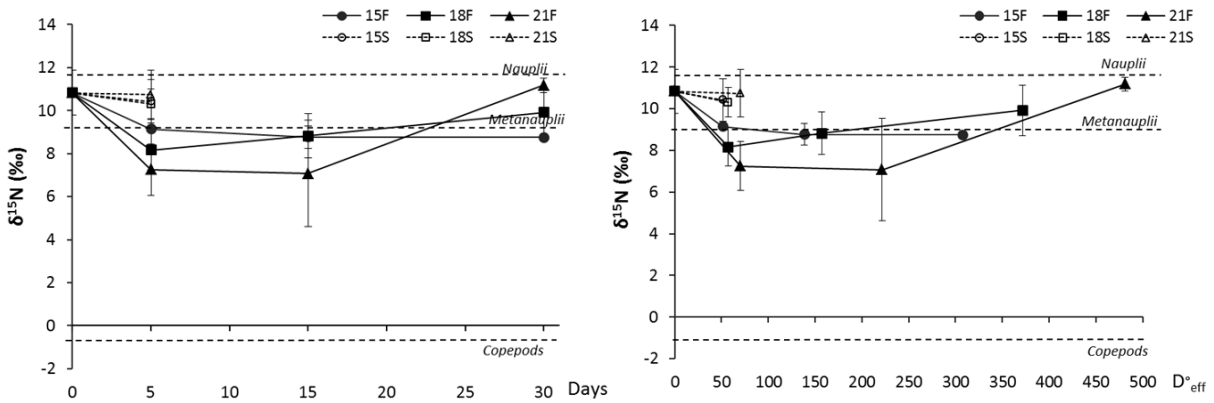


Figure 3.2. Changes in δ^{13}C (a) and δ^{15}N (b) values (‰) in juvenile seahorses *Hippocampus guttulatus* under feeding (solid lines, F) and starving (dotted lines, S) regimes at the three temperature conditions (15, 18 and 21 °C). Data are provided for chronological time (days) and effective day-degrees ($D°_{eff}$). Horizontal dotted lines represent isotopic composition of food. Mean and SD of two batches are indicated by vertical bars.

In accordance with the statement that the isotopic composition of a consumer must approach to that of the food, as a result of nutrient incorporation (DeNiro and Epstein, 1978), it is very likely that juveniles were able to digest and assimilate the offered food from the onset of feeding but at different levels depending on the age and temperature. Major physiological processes and higher metabolism occur at warmer temperatures (Fry, 1971; Schmidt-Nielsen, 1997). Hence, higher changes in both carbon and nitrogen isotopic composition at 18 and 21 °C when compared to 15 °C would be expected in the present study. Witting et al. (2004) estimated that isotopic composition changes in summer flounder (*Paralichthys dentatus*) after a diet shift, occurred generally faster at warmer temperatures. Similar temperature effects on carbon and nitrogen isotopic turnover rates were described by Bosley et al. (2002) in winter flounder (*Pseudopleuronectes americanus*). Also, it has been reported that the changes in carbon isotopic composition exhibited by red drum larvae (*Sciaenops ocellatus*) were slightly faster at warmer temperature (Herzka and Holt, 2000). However, temperature effects on carbon and nitrogen stable isotopes might differ among

species, as reported by Barnes et al. (2007), who pointed out lower δ^{15}N values of the European sea bass (*Dicentrarchus labrax)* reared at warmer temperatures, but lower δ^{13}C values at cooler temperatures. Growth rates in fish (Hidalgo et al., 1987; Houde, 1989; Hart et al., 1996; Dou et al., 2005), and specifically in seahorses (Wong and Benzie, 2003; Lin et al., 2008; Planas et al., 2012) are directly related to temperature level. In addition to the fact that changes in stable isotope values indicated assimilation of nutrients by seahorses in the present study, growth rates also confirm a higher performance of juveniles grown at warmer temperature levels in terms of digestion, assimilation and tissue formation. C:N ratios reflect the amount of fat reserves in tissues being used to assess the physiological conditions of animals (Post et al., 2007). The ratio increases due to the assimilation of exogenous nutrients so that C:N values are higher in animals with better physiological conditions. Although the highest growth in our study was achieved at 21 °C, C:N ratios of juveniles suggest a better condition for those reared at 18 °C. This suggestion is also supported by the highest Fulton's condition factor achieved at 18 °C.

Chronological time (days) is not a temperature-independent index of development but effective day-degrees ($D°_{eff}$) represents a temperature-independent scale to describe physiological features in early larval stages of seahorses (Planas et al., 2012). When effective day-degrees were used as developmental index, seahorses fed at 21 °C showed an advanced developmental stage with respect to lower temperatures, probably due to an increase of physiological processes at 21 °C. We propose temperature levels of about 18 °C or slightly higher for the development and growth of the seahorse *H. guttulatus*. At that temperature, higher survivals, best physiological conditions and more efficient food assimilation would be met.

3.4.2. Starving seahorses

Juveniles deprived of food are dependent exclusively on their own reserves, specifically through the mobilization of endogenous reserves in tissues due to the absence of yolk reserves, to sustain metabolic and energetic demands. Under such condition, the ratio of anabolism to catabolism changes leading to a shift in isotopic composition. The increase in ^{13}C values of starving animals would be explained by a preferential use of the lighter carbon isotope ^{12}C during the catabolism of lipids, proteins and carbohydrates (DeNiro and Epstein, 1978). In the same way, tissues of starving animals become enriched in ^{15}N because animals are forced to fabricate their own amino acids pool by transamination from tissue proteins. Furthermore, the catabolised and excreted lighter nitrogen ^{14}N is not replaced by dietary protein (Hobson et al., 1993).

Some studies carried out in a variety of fishes focused on the effects of starvation in the isotopic composition of fish but the results were rather variable among species. A small increase in δ^{13}C values of unfed common carp (*Cyprinus carpio*) and whitefish (*Coregonus lavuretus)* larvae was observed by Schlechtriem et al. (2004) and Schlechtriem et al. (2005), respectively. Also, a slight increase in δ^{13}C values was described in starved pacu (*Piaractus mesopotamicus*) larvae whereas δ^{15}N values were practically constant (Jomori et al., 2008). Starvation significantly affected the isotopic composition of Nile tilapia (*Oreochromis niloticus*). Starving Nile tilapia showed higher δ^{15}N values than fish fed but did not differ in δ^{13}C values (Gaye-Siessegger et al., 2007). In red drum (*Sciaenops ocellatus*) larvae, Herzka and Holt (2000) reported a not significant relationship between isotopic composition and food deprivation in a 4-days period.

The documented increase in $\delta^{13}C$ values due to starvation in fishes is in agreement with the small increase achieved in the present study in starving seahorses, with a negligible effect of temperature levels. On the contrary, the small decrease observed in $\delta^{15}N$ as a result of starvation from birth until day 5 has not been reported before in other species. The highest weight loss observed at 21 °C was very likely due to a higher metabolic activity and lower energetic efficiency compared to juveniles kept at 18 °C and 15 °C. As a consequence, starving seahorses maintained at 21 °C would consume faster their own reserves, showing the lowest condition index (K_F = 1.01 ± 0.04). Juveniles deprived of food progressively lost weight until day 5, but increased in length at the expense of the consumption of endogenous reserves. A weight loss also occurred in juveniles during the first 5 days of feeding at 15 and 18 °C, whereas a slight weight gain was observed at 21 °C. As reported previously in seahorses, low food availability or late prey capture success is accompanied by a progressive decrease in weight and energetic status (Sheng et al., 2007; Blanco et al., 2011).

Despite seahorses are completely developed, active swimmers and hunters immediately after male's pouch release, they are exclusively dependent on exogenous feeding due the lack of yolk reserves. Hence, their tolerance to starvation would rely on the onset of prey capture and prey availability. Our findings suggest juvenile seahorses can stand for certain food deprivation, as was described in other seahorse species (Sheng et al., 2007). Moreover, it was demonstrated that resistance of *H. guttulatus* juveniles to starvation is inversely dependent on temperature level. This fact would have ecological implications on wild seahorse populations. Juvenile seahorses developing at lower temperatures would be less dependent on food availability during the initial planktonic period, enhancing the survival rate under adverse food availability conditions. Furthermore, our findings would contribute to the interpretation of some biological and ecological features, such as the geographical distribution and the duration of the breeding season of the *H. guttulatus* seahorse (Planas et al., 2012). In the natural environment, *H. guttulatus* seahorses seem to be adapted to different temperature levels along their distribution from Canary Islands to British Isles (Lourie et al., 1999). The extension of the breeding season depends on the region considered and coincides with the warmer period of the year, when primary and secondary production is maximal (Arbones et al., 2008; Curtis, 2007). Considering that water temperature would act as a decisive factor in food assimilation efficiency and in the early development (growth and survival) of wild *H. guttulatus* seahorses, it would be expected that *H. guttulatus* populations inhabiting regions with temperatures of about 18 °C or higher would result enhanced under optimal prey availability when compared to other from colder waters, as suggested by Planas et al. (2012).

Our results provide new insights for the understanding of food assimilation processes in young *H. guttulatus* seahorses under different water temperatures. It was demonstrated that temperature contributes to the variation in carbon and nitrogen stable isotopes values of seahorse juveniles. Therefore, the interpretation of stable isotope data would be misleading when temperature is not taken into account in comparative studies about diet composition or trophic level estimations. Further experiments need to be conducted to assess this aspect in other seahorse species and to help in the interpretation of food assimilation in early developmental stages of these fishes.

Chapter 4

Prey assimilation in first feeding seahorses evidenced by stable carbon isotopes

Prey assimilation in first feeding seahorses evidenced by stable carbon isotopes

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4.1. Introduction

The increasing demand of seahorses for traditional Chinese medicine, aquarium trade and curious is mostly supplied by wild-caught seahorses, leading to overexploitation and negative impact on natural populations. In this context, seahorse culture represents an alternative to reduce fishing pressure on wild stocks (Vincent, 1996; Lourie et al., 1999). Global interest in captive breeding of seahorses has increased in recent years to satisfy aquarium trade demand (Payne and Rippingale, 2000; Woods, 2000b; Job et al. 2002; Lin et al., 2008; Olivotto et al., 2011; Planas et al., 2012). However, seahorse rearing is a relatively recent activity that faces many culture problems that need to be conveniently addressed (Wilson and Vincent, 1998; Foster and Vincent, 2004; Koldewey and Martin-Smith, 2010; Olivotto et al., 2011). In particular, the low survival in the early life stages of juveniles is one of the main bottlenecks.

Recent research studies have focused much effort on studying factors affecting survival and growth of juvenile seahorses, mainly on prey type preferences, prey enrichment, temperature, light regime and culture density (Payne and Rippingale, 2000; Woods, 2000b; Job et al. 2002; Wong and Benzie, 2003; Woods, 2003a, b; Lin et al., 2008; Olivotto et al., 2008; Murugan et al., 2009; Otero et al., 2010; Blanco et al., 2011; Palma et al., 2011; Planas et al., 2012). Feeding issues are considered one of the most decisive factors in the initial survival of juveniles, especially regarding nutritional requirements, feeding efficiency and digestive capabilities (Payne and Rippingale, 2000, Olivotto et al., 2008, Planas et al., 2009; Blanco et al. 2014).

In the rearing of seahorses, juvenile seahorses are generally fed on rotifers and *Artemia*, depending on the species and the mouth size at birth. *Artemia* (*nauplii* and *metanauplii*) is easy to produce in large quantities but need to be enriched to enhance its nutritional quality. For some species, feeding of juveniles only on *Artemia nauplii* has resulted in poor survivals, probably due to their poor digestibility (Payne and Rippingale, 2000; Planas et al., 2009). In such cases, the initial supply of copepods for a few days is a valid alternative to *Artemia* since survival and growth rates result significantly enhanced (Payne and Rippingale, 2000, Olivotto et al., 2008, Murugan et al., 2009, Blanco et al., 2011). In spite of recent improvements in the rearing procedures and successful progress in survival rates in reared seahorses, nutrient assimilation in first feeding juveniles is still unknown mainly due to difficulties in the quantification of feed intake, absorption and utilization of nutrients. In this regards, studies should be undertaken to understand nutrient processes and to establish the most adequate diet for a successful rearing in early life stages of seahorses.

Faeces collection, direct gut content examination and physiological approaches are indirect techniques generally applied in studies related to the nutritional assessment in fish larvae (Govoni et al., 1986; Cunha and Planas, 1999; Tudela et al., 2002). However, those methodologies are difficult to apply in small specimens at early life stages. In addition, these methods have some limitations since digested soft prey tissues are neither identifiable nor quantifiable. In such cases, direct tracing of dietary components becomes essential to achieve more precise data research. Radioactive isotopes have been used as reliable tracers to measure ingestion and assimilation rates (Koven et al., 1998; Morais et al., 2004, 2005). However, their use is often impractical in nature because of health threats and potential environmental contamination. Alternatively, enriched stable isotopes have been recently applied to trace nutrients in fish larvae (Conceição et al., 2001; Rønnestad et al., 2001; Verschoor et al., 2005). The limitation of this approach is related to both the use of live prey and the difficulties of including chemical or isotopic markers on these preys.

Stable isotopes represent natural tracers that allow the direct determination of ingestion and assimilation rates in organisms. The application of stable isotopes as a nutritional tool in aquaculture represents a powerful method to assess the incorporation of nutrients from the diet into consumers (Schlechtriem et al., 2004; Gamboa-Delgado et al., 2008; Le Vay and Gamboa-Delgado, 2011) since the isotopic composition of animal tissues is related to that of the consumer's diet, with a slight variation named trophic discrimination (DeNiro and Epstein, 1978, 1981). The long-snouted seahorse *Hippocampus guttulatus* Cuvier 1829 is an European species whose rearing has been recently studied (Faleiro et al., 2008; Palma et al., 2008; Planas et al., 2008b, 2009, 2012; Blanco et al., 2011; Valladares and Planas, 2011). As in many other seahorse species, significant improvements on husbandry and rearing techniques have been achieved. However, the knowledge on essential issues is still lacking, especially on those related to feeding and nutrition and their effects on initial mortalities in juveniles. Due to the high importance of survival optimization in early juveniles and the scarce knowledge on feeding and nutrition, and considering the limitations of other methodologies available, we used the stable isotope approach to trace the assimilation of two life preys (*Artemia* and copepods) in early juveniles of *H. guttulatus* to assess the feeding and assimilation of food when (1) initially feeding on *Artemia* or copepods, and (2) shifting feeding from copepods to *Artemia*. The research on this aspect of seahorse culture is advisable to understand their feeding requirements. This study represents the first attempt to investigate nutrient assimilation in a seahorse species using stable isotopes.

4.2. Material and methods

H. guttulatus juveniles were obtained from the broodstock maintained in *ad hoc* aquaria (Planas et al., 2008b) at the Instituto de Investigaciones Marinas (IIM-CSIC), in Vigo (Spain). Adult seahorses were kept under an annual temperature cycle ranging from 15 °C in winter to 19 °C in summer (± 0.5 °C) and a natural photoperiod cycle (16L:8D in June-July; 10L:14D in December-January) (Planas et al., 2010, 2013). Pumped seawater was filtered (5 µm) and UV treated, with a 10-15% water exchange rate per day. Water quality was checked periodically for $N0_2$, $N0_3$ and NH_4/NH_3 content (0 mg l^{-1}) by using Sera Test Kits. Salinity and pH levels were 38 ± 1 ppt and 8.1 ± 0.1, respectively. Captive adult seahorses were fed twice

per day *ad libitum* on enriched adult *Artemia* (EG, Inve, Spain), supplemented with captured Mysidacea (*Leptomysis* sp. and *Siriella* sp.).

A batch of 624 juveniles was released by a male kept in captivity for 8 months. Immediately after birth, the juveniles were randomly distributed (143-144 juveniles aquarium^{-1}) into four 30 L aquaria. The rearing system was submitted to a constant 16L:8D photoperiod regime supplied by 20 W fluorescent lamps (Power Glo), a temperature of 20 ± 1 °C, a continuous inflow flux of 700 ml min^{-1} and gentle aeration in the upper part of the water column (Blanco et al., 2014).

Experiment 1: *Artemia vs.* copepods feeding

Juveniles were fed *ad libitum* until day 20 on two different feeding regimes (2 aquaria per treatment) consisting on:

- *Artemia* regime: *Artemia nauplii* from days 0 to 10 (1 *Artemia* ml^{-1}) and a mixture of *Artemia nauplii* and *metanauplii* (1:1, 1 *Artemia* ml^{-1}) from days 11 to 20.
- Copepods regime: a mixture of the calanoid *Acartia tonsa* and the harpacticoid *Tisbe* sp. (0.6 copepods ml^{-1}) from days 0 to 20.

Experiment 2: Shifting of feeding regime

Thirty 20 days old juveniles from each aquarium submitted to copepods regime in Experiment 1 were transferred to two additional aquaria and fed on *Artemia nauplii* and *metanauplii* until day 30. The remaining juveniles in the copepods regime aquaria (20 individuals aquarium^{-1}) were maintained on copepods until day 30.

Artemia metanauplii were enriched on a mixture of the microalgae *Phaeodactylum tricornutum* ($1.6 \cdot 10^7$ cells ml^{-1}), Spirulina (KF Iber Frost, Spain) and Red Pepper (Bernaqua, Belgium). Copepods were cultivated on a mixture of the microalgae *Isochrysis galbana* (10^7 cells ml^{-1}) and *Rhodomonas lens* (16^7 cells ml^{-1}).

At the onset of Experiment 1 and prior to first feeding, 50 individuals were sampled to determine initial carbon isotope values, weight and length. Also, samples of *Artemia* (*nauplii* and *metanauplii*) and copepods were collected at different days, rinsed with distilled water and kept frozen at -80 °C for isotopic determination. Seahorse juveniles were also randomly collected before feeding time from each aquarium at different ages (1, 2, 3, 5, 8, 11, 15, 20, 25 and 30 days) for stable carbon isotope analysis, weight and length. Sampled seahorses were anesthetised with MS222 (0.1 g l^{-1}), transferred individually to Petri dishes and photographed for standard length (SL) measurements. The excess of water was removed and the seahorses weighted on a Sartorius microbalance (± 0.01 mg). Then, seahorses were rinsed with distilled water and frozen at -80 °C. Standard length of juveniles was measured as head + trunk + tail length (curved measurement), following Lourie et al. (1999). Measurements were made on digital images using image processing software (NIS Elements, Nikon). Daily mortalities were recorded throughout the feeding experiment. Live food and whole body of seahorses were lyophilised, homogenised and sub-samples of 1 mg were weighted into tin capsules.

Analysis of stable carbon isotope and elemental composition were performed at Servizos de Apoio á Investigación (SAI) of the University of A Coruña. Samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112

elemental analyser (ThermoFinnigan, Italy) coupled to a Deltaplus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface. Carbon stable isotope abundance was expressed as $\delta^{13}C$ parts per thousand (‰) relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air, according to the following equation: $\delta^{13}C$ (‰) = [($R_{Sample}/R_{Reference}$) – 1], where R is the corresponding ratio $^{13}C/^{12}C$. As part of an analytical batch run, a set of international reference materials for $\delta^{13}C$ (NBS 22, IAEA-CH-6, USGS-24) were analysed. The precision (standard deviation) for the analysis of $\delta^{13}C$ of the laboratory standard (acetanilide) was ± 0.15 ‰ (1-sigma, n=10). The amount of carbon relative to the amount of nitrogen present in seahorses (C:N ratio) was also determined at different times as an indicator of nutritional variations due to endogenous and exogenous nutrient utilization.

Variables were checked for normality using the Shapiro–Wilk test. Growth parameters and survival between feeding regimes were compared by means of Student's *t*-tests. SPSS v.15.0 software was used to perform the statistical analyses at a significant level of $p < 0.05$.

Seahorse rearing and manipulation practices were conducted in compliance with all bioethics standards of the Spanish Government and approved by the Bioethics Committee of CSIC.

4.3. Results

Growth of juvenile seahorses fed the different feeding regimes is represented in Fig. 4.1. In Experiment 1, mean dry weight in 20 days old juveniles fed on copepods (6.20 ± 1.07 mg) was significantly higher than in those fed on *Artemia* (4.42 ± 1.36 mg) (*t*-test = 2.97, p = 0.011). Similarly, the mean standard length was also significantly higher in the former (30.8 ± 1.9 and 27.96 ± 2.3 mm, respectively) (*t*-test = 2.84, p = 0.014). In Experiment 2, the dietary shift from copepods to *Artemia* initiated at day 20 resulted in a lower, but not significant (*t*-test = 1.74, p = 0.112), mean dry weight in 30 days old juveniles (7.46 ± 3.27 mg) in relation to those from the copepods feeding regime (10.80 ± 3.34 mg). Not significant differences were neither noticed in mean standard lengths at day 30 between both feeding regimes (35.29 ± 3.4 and 35.08 ± 2.2 mm, respectively) (*t*-test = -0.85, p = 0.41). Differences in growth between feeding regimes in Experiments 1 and 2 were clearly noticed considering weight gain rather than size gain (Table 4.1.).

In Experiment 1, survivals at day 20 were significantly different between feeding regimes (*t*-test = 48.91, $p < 0.001$). Seahorses fed on *Artemia* exhibited very low survivals (7.87 ± 1.12 %) compared to those from the copepods diet (98.66 ± 2.36 %) (Fig. 4.2a.). After the dietary shift from copepods to *Artemia* in Experiment 2, the average survival from days 20 to 30 decreased from 98.3 to 91.2 %, whereas no mortalities occurred in juveniles that continue feeding on copepods (Fig. 4.2b.). However, differences in survival among feeding regimes were not significantly different (*t*-test = 3.62, p = 0.07).

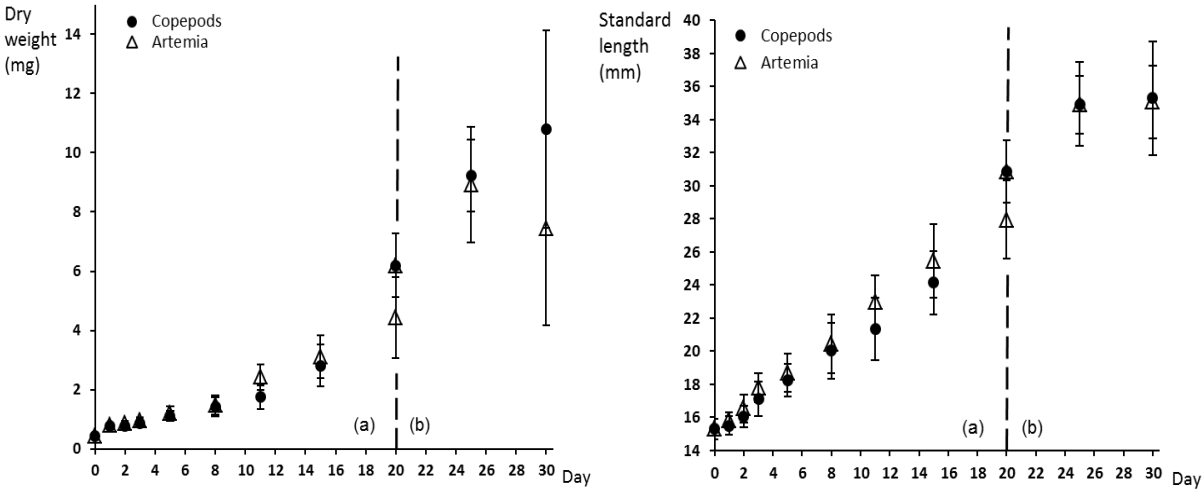


Figure 4.1. Growth (dry weight and standard length) of seahorses *Hippocampus guttulatus* fed on copepods or *Artemia* over the experimental period. Vertical dotted line separate Experiment 1 (a) from Experiment 2 (b). Means and standard deviations are represented.

Table 4.1. Survival, individual dry weight (DW), standard length (SL) and percentage of weight and size gain (WG; SG) of 0, 20 and 30 days old seahorses *Hippocampus guttulatus* fed on two feeding regimes at the end of each experimental period (means ± SD).

Diet	Day	Survival (%)	DW (mg)	SL (mm)	WG (%)	SG (%)
Initial	0	100 ± 0	0.44 ± 0.03	15.3 ± 0.6		
Experiment 1: Days 0 - 20						
Copepods	20	98.3 ± 2.4	6.20 ± 1.07	30.8 ± 1.9	1409	202
Artemia	20	7.8 ± 1.1	4.42 ± 1.36	27.96 ± 2.3	1005	183
Experiment 2: Days 20 - 30						
Copepods	30	98.3 ± 2.4	10.80 ± 3.34	35.29 ± 3.4	2455	231
Artemia	30	91.2 ± 1.5	7.46 ± 3.27	35.08 ± 2.2	1695	229

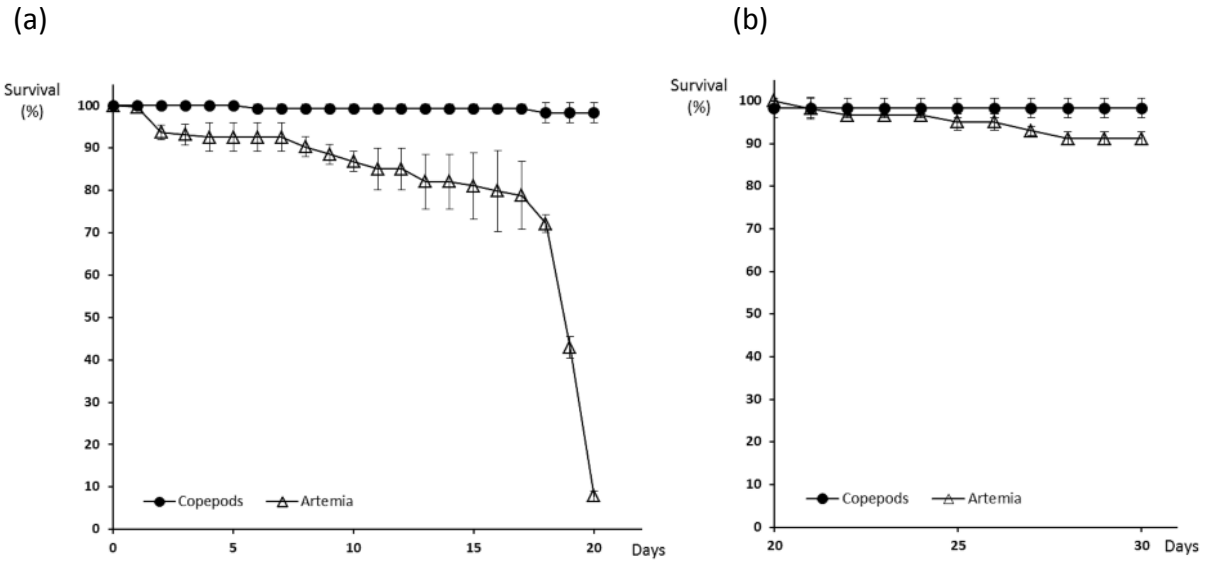


Figure 4.2. Survival (%) in juvenile seahorses *Hippocampus guttulatus* fed on copepods or *Artemia* over each experimental period (a: Experiment 1; b: Experiment 2). Means and standard deviations are represented.

Considering stable isotopes in prey, δ^{13}C values in copepods and *Artemia nauplii* were identical (-20.6 ‰) and lower than in *Artemia metanauplii* (-18.5 ‰). C:N ratios in *Artemia nauplii* and *metanauplii* were rather similar (5.2 and 4.7, respectively), compared to the ratio obtained for copepods (3.9). The evolution of δ^{13}C values in seahorse juveniles in Experiments 1 and 2 is shown in Fig. 4.3. In Experiment 1, the initial δ^{13}C value in seahorse newborns was -18.9 ‰. In the following 48 h, an increase was noticed for δ^{13}C values in both feeding regimes but C:N ratios dropped in both treatments (Fig. 4.4.). Thereafter, δ^{13}C values changed on a different way depending on the feeding regime. In juveniles fed on copepods, δ^{13}C values until day 20 were rather stable, ranging from -19.4 to -18.6 ‰. Significant changes in δ^{13}C values at days 5 and 8 (Fig. 4.3a.) has a great effect on C:N ratios at those ages (Fig. 4.4.). In Experiment 2, δ^{13}C values in seahorses remained steady from days 20 to 30 (-19.4 ± 0.1 ‰) (Fig. 4.3b.). A similar stable trend was found until day 10 in juveniles fed on *Artemia nauplii* (-18.7 ± 0.1 ‰) (Fig. 4.3a.). Since day 10, δ^{13}C values increased progressively until day 20; at the same time the C:N ratio decreased sharply (Fig. 4.4.). This δ^{13}C increase reflects the switch to the isotopically enriched new diet (*Artemia metanauplii*) added at day 11. Similarly, the diet shifting from copepods to *Artemia* diet caused an increase of δ^{13}C values in Experiment 2.

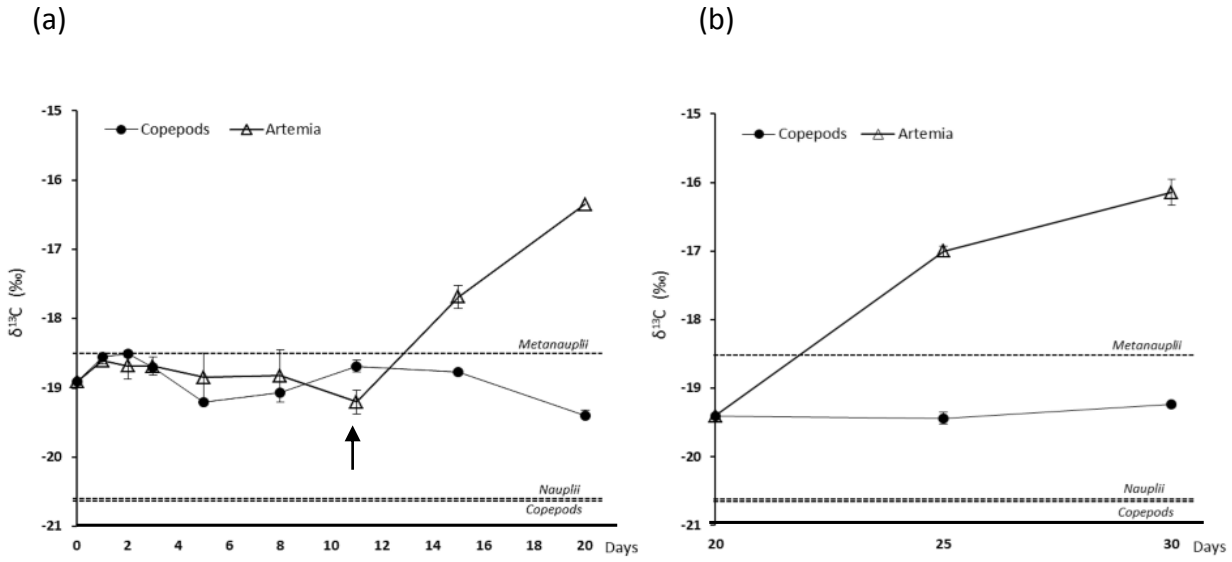


Figure 4.3. Changes in δ^{13}C values in juvenile seahorses *Hippocampus guttulatus* fed on copepods or *Artemia* over each experimental period (a: Experiment 1; b: Experiment 2). Horizontal dotted lines represent isotopic composition of food. Black arrow represents the start of the addition of *Artemia metanauplii* (a). Means and standard deviations are represented.

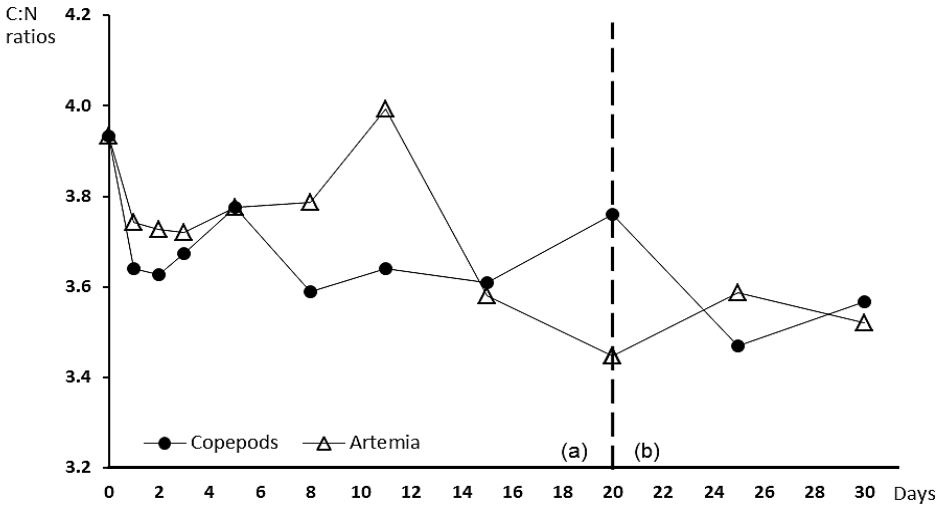


Figure 4.4. C:N ratios in juvenile seahorses *Hippocampus guttulatus* fed on copepods or *Artemia* over the experimental period. Vertical dotted line separate Experiment 1 (a) from Experiment 2 (b).

4.4. Discussion

At the end of Experiment 1 (day 20), the growth of seahorse juveniles fed on copepods was significantly higher than those from *Artemia* regime. Similarly, in Experiment 2 higher growth was achieved when juveniles were maintained on a copepods feeding regime instead of *Artemia*. Regarding the survival, juveniles fed on *Artemia* suffered high mortalities in Experiment 1. After the dietary shift from copepods to *Artemia* in Experiment 2, the survival showed a slightly decreased, whereas no mortalities occurred in juveniles maintained on the copepods feeding regime. The higher survival and growth observed in copepods regime is in agreement with previous studies on the rearing of several seahorse species. Payne and Rippingale (2000) reported that copepods offered to *H. subelongatus* seahorses resulted in higher survival and growth compared to seahorses fed on *Artemia*. Olivotto et al. (2008) pointed out that *Artemia* might not be an optimal prey in the rearing of *H. reidi* juveniles, since survival and growth in juveniles fed exclusively on that prey were lower than in seahorses fed on a combined diet including copepods. These results are consistent with the higher survival rates reported by Murugan et al. (2009) when juveniles of *H. trimaculatus* were reared using copepods instead of *Artemia*. Survival and growth rates were also improved when copepods were offered as the unique prey in the first feeding of *H. guttulatus* (Blanco et al., 2011).

Adequate growth performances and survivals in fish larvae are related to an efficient assimilation of food. From this study results, it seems that copepods are more efficiently assimilated *H. guttulatus* juveniles than *Artemia* since growth and survival were clearly improved when copepods were offered, especially in early development. Furthermore, it is feasible that a better digestibility of ingested copepods enhanced their assimilation and hence provided the required nutrients for tissue growth, resulting in higher survival of juveniles. On the contrary, the lower assimilation of *Artemia*, probably due to its lower digestibility (Payne and Rippingale, 2000; Planas et al., 2009), might limit its contribution to tissue growth of young juveniles, promoting high mortalities (Experiment 1).

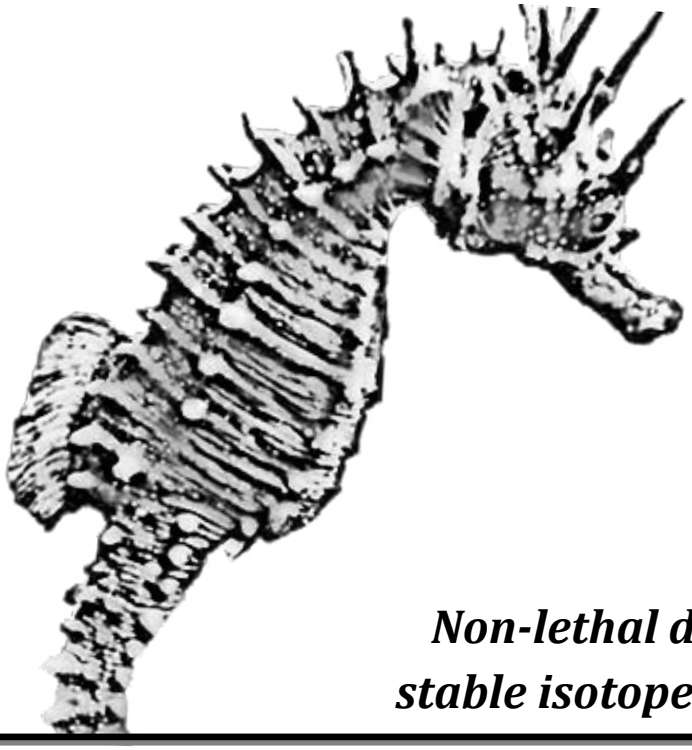
Carbon isotopic values ($\delta^{13}C$) are used to evaluate assimilation occurrence as the isotopic composition of the carbon consumed by an individual must represent the isotopic composition of the carbon assimilated into tissues (DeNiro and Epstein, 1978). Hence, the $\delta^{13}C$ changes toward the values of the corresponding diet exhibited by juvenile seahorses indicate that the food offered was assimilated constantly from day 2 until the end of the Experiment 1. The increase after day 11 of $\delta^{13}C$ values in juveniles fed on *Artemia* Experiment 1 was expected due to the isotopically enriched $\delta^{13}C$ values of the *Artemia metanauplii* added at day 11 and allowed as to confirm the correct assimilation of *Artemia*. The significant change in $\delta^{13}C$ values after the diet shift from copepods to *Artemia* in Experiment 2 also reflected the adequate assimilation of the new diet. These findings are in accordance with other studies suggesting that the relative incorporation efficiency of diet nutrients may affect the carbon isotope ratios in fish tissues. Schlechtriem et al. (2004) reported that $\delta^{13}C$ values in common carp (*Cyprinus carpio*) larvae fed on two types of nematodes were influenced by the stable carbon isotope values of the food, indicating that the larvae assimilated nutrients from the nematodes. Food assimilation in pacu *Piaractus mesopotamicus* larvae was also verified through the changes in the isotopic signatures of the larvae (Jomori et al. 2008).

The fact that assimilated nutrients were used to synthesise organic tissue for growth of seahorse juveniles is also supported by the values of C:N ratios, which reflect the amount

of fat reserves in tissues. The relative abundance of different stable isotopes in tissues is consequence of their different behaviour in chemical reactions. For example, the lighter isotope of carbon (^{12}C) tends to form weaker bonds and to react faster than the heavier isotope of carbon (^{13}C). A decrease in carbon and nitrogen content apparently reflects the consumption of endogenous lipid reserves. In this context, the isotopic enrichment occurs because lipids are usually less enriched in ^{13}C whereas an increase in carbon and nitrogen content might indicate the assimilation of exogenous nutrients along with the isotopic depletion (DeNiro and Epstein, 1978). Juvenile seahorses are active swimmers that start feeding immediately after birth due to the lack of yolk reserves. The decline in C:N ratios observed in Experiment 1 over the first 2 days in both feeding regimes (copepods and *Artemia*) followed the opposite trend of $\delta^{13}C$ values, reflecting the use of endogenous lipid reserves. Then, although active foraging and ingestion was observed since the onset of first feeding (Experiment 1), it is very likely that the successful initial digestion of prey and assimilation of nutrients be delayed for at least 2 days as indicated by both the increase of C:N ratios and the depletion in $\delta^{13}C$ at days 3 in copepods regime and 5 in *Artemia* regime. The efficient assimilation of *Artemia* after the shifting of the diet in Experiment 2, can be also confirmed by the significant increase in $\delta^{13}C$ along with the decrease in C:N ratios. Similar changes in C:N ratios and $\delta^{13}C$ values in Senegalese sole (*Solea senegalensis*) larvae and postlarvae were described by Gamboa-Delgado et al. (2008).

Although digestion and assimilation of *Artemia* might occur, the low nutritional quality of that prey would limit its contribution to tissue growth in juveniles, promoting higher mortalities, as clearly noticed in Experiment 1. The presence of poorly digested *Artemia* described in faeces of early developing *H. guttulatus* juveniles evidences its low digestibility (Planas et al., 2009). Limited growth and survival rates supplying *Artemia* was consistently reported in juveniles of *H. guttulatus* (Blanco, 2014) and other seahorse species (Job et al. 2002). Conversely, copepods would provide essential nutrients with higher nutritional quality, promoting higher survival and growth rates. Considering the fact that the diet of juvenile seahorses in their natural environment is primarily based on copepods (Tipton and Bell, 1988; Teixeira and Musick, 2001; Castro et al. 2008), it is reasonable to consider copepods as a more adequate diet than *Artemia* for juvenile seahorses.

The present study demonstrates that the consumption and assimilation of preys by juvenile *H. guttulatus* seahorses could be traced using stable carbon isotopes. Considering the whole results achieved, the addition of copepods in the diet during the first feeding days is highly recommended for the early rearing of juvenile seahorses. Within an ecological context, our findings can also contribute to the understanding of the feeding dynamics in wild seahorse populations.



Chapter 5

Non-lethal dorsal fin sampling for stable isotope analysis in seahorses

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Non-lethal dorsal fin sampling for stable isotope analysis in seahorses

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5.1. Introduction

The study of the trophic ecology in fishes by means of stable isotope analysis (SIA) has been extensively used over the last two decades (Hobson and Welch, 1992; Cabana and Rasmussen, 1996; Jennings et al., 1997; Frediksen, 2003; Vizzini and Mazzola, 2009), since isotopic composition of a consumer's tissue can be correlated with that in the diet (DeNiro and Epstein, 1978; 1981). Nitrogen and carbon stable isotopes are commonly used to study food webs, as nitrogen stable isotope ratios ($^{15}N/^{14}N$) are an indicator of a consumer's trophic position, while carbon stable isotope ratios ($^{13}C/^{12}C$) indicate potential sources of food consumed (Peterson and Fry, 1987; Hobson and Welch, 1992).

Generally, the sampling of fish tissues (muscle, liver, heart, etc.) for stable isotope analysis (SIA) requires the sacrifice of the animal (Hobson and Welch, 1992; Cabana and Rasmussen, 1996; Jennings et al., 1997; Frediksen, 2003; Vizzini and Mazzola, 2009). The use of a non-lethal sampling to measure stable isotope values in studies with threatened or endangered species, such as seahorses (included in the IUCN Red List Category and Criteria) (IUCN 2011), would be more than suitable as an alternative to lethal sampling procedures. Furthermore, it would allow the study of food webs in seahorses without affecting wild populations, which has a high conservation value.

Fin-clipping is a non-lethal sampling method that requires minimal equipment, handling time, and training. It has been widely used in fisheries and research for identification, contaminant analysis and genetics analysis purposes (Gunnes and Refstie, 1980; Wilson and Donaldson, 1998; Helstsley et al., 2005). In recent years, fin tissue sampling has become a useful non-lethal tool used in SIA of fish (Jardine et al., 2005; Kelly et al., 2006; Sanderson et al., 2009; Jardine et al., 2011) instead of lethal sampled tissues. In seahorses, fin-clipping has also been used to obtain tissue for genetic analysis and has been shown to have no significant effects on survival (Kvarnemo et al., 2000; Lourie, 2003; Pardo et al., 2007). This sampling procedure can also be advisable for SIA in seahorses due to seahorse's capacity for fin regeneration, in around 1–2 months (Planas et al., 2008b). Therefore, fin-clipping could be an adequate non-lethal sampling method for stable isotope analysis in seahorses.

In seahorses, the limited availability of tissue obtained from fin-clipping makes necessary a previous assessment of sample size to evaluate its specific use in SIA. In addition, comparisons of stable isotope values of different tissues should be performed to assess differences among seahorse tissues because isotope values can show variability among tissues due to isotopic fractionation occurring in different tissues (DeNiro and Epstein, 1978; 1981; Pinnegar and Polunin, 1999). Previous studies performed in other fish

species (e.g. slimy sculpin, atlantic salmon, brook trout) have demonstrated that stable isotope values of fin and muscle tissues are correlated (Jardine et al., 2005; Kelly et al., 2006; Jardine et al., 2011), supporting the use of fin tissue as a convenient sample for food web studies using stable isotope analysis.

The aim of the study was to establish a sampling and analysis procedure to ensure accurate and reproducible analysis of stable isotopes (δ^{13}C and δ^{15}N) in tissues of adult seahorse *Hippocampus guttulatus*. Considering the conservation concern of seahorses, the main objective of this study was to determine the suitability of a non-lethal sampling procedure (fin tissue) and compare it to the use of lethal tissue sampling (liver or muscle). Firstly, three types of tissue (muscle, liver and fin) were compared for SIA in order to assess the adequacy of fin tissue in further studies. Secondly, the effects of lipid extraction on the carbon and nitrogen stable isotope values in seahorse tissues (muscle, liver and fin) were evaluated, as it is known that the lipid content in tissues can potentially affect carbon stable isotope values (DeNiro and Epstein, 1978; Pinnegar and Polunin, 1999). Finally, the dependency of the isotope values in dorsal fin samples on the sample size of the fin was evaluated. As an application in the field, we provide results of stable isotopes in wild seahorses of the Galician coast (NW Spain).

5.2. Materials and methods

All tissue samples used in this study were taken from six freshly deceased seahorses *H. guttulatus* of the broodstock maintained at the Instituto de Investigaciones Marinas (CSIC) (Vigo, NW Spain). The analysed seahorses did not show evidence of disease nor external or internal lesion. The diet of the captive seahorses consisted of adult enriched *Artemia* (EG, Inve, Spain), with a δ^{13}C value of -19.31 ‰ and a δ^{15}N value of 3.79 ‰, offered *ad libitum* twice daily, over a 2-year period.

Seahorses were frozen immediately after dead and stored at -20 ºC until processing. Three types of tissue were analysed: muscle, liver and whole dorsal fin (n = 6 per each tissue type). Muscle and liver tissue are the most common tissues used to obtain long-term or short-term, respectively, dietary information by stable isotopes analysis (SIA). Muscle has a low-medium lipid content, while liver has high lipid content. Dorsal fin samples were selected to assess their adequacy for trophic ecology studies of seahorses. Muscle and liver samples require the sacrifice of the fish, whereas fin-clipping is a non-lethal sampling procedure. The tissues were removed from each seahorse for lipid extraction assessment and tissues comparison. Each sample was freeze-dried and split into two similar subsamples. One of the subsamples was submitted to lipid extraction following a modification of the procedure described by Bligh and Dyer (1959) (Fernández-Reiríz et al., 1989). Lipids were first extracted with chloroform/methanol (1:2) and after centrifugation (3,246 x g), the lipids of the resulting sediment were extracted again with chloroform/methanol (2:1). Finally, both supernatants were washed with chloroform/methanol/water (8:4:3) (Folch et al., 1957). Total lipids content was quantified gravimetrically according to Herbes and Allen (1983). Both subsamples, with or without lipids, were submitted to SIA. Whole dorsal fins (n = 6) were also taken and cut off into three sections differing in size (from smaller to larger: DF1, DF2, and DF3) (Fig. 5.1.). The surface of each section was measured from digital photographs using image processing software (NIS Elements, Nikon). Samples were rinsed with distilled water, frozen, freeze-dried, and stored at -20 ºC until further analysis.

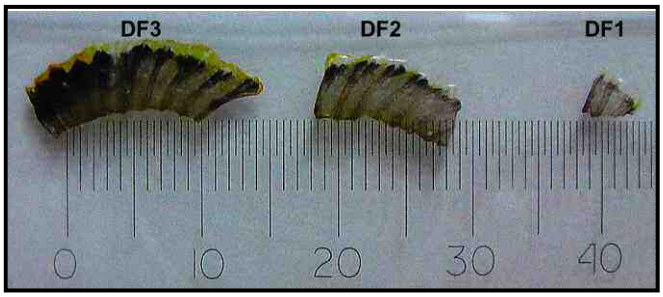


Figure 5.1. Sections with different sizes (from smaller to larger: DF1, DF2, and DF3) of dorsal fin tissue of seahorse *Hippocampus guttulatus.*

In tissue processing, muscle and liver samples were ground to a powder, whereas dorsal fin samples were cut off with scissors into small pieces, except small portions of dorsal fin (DF1), which were used intact. Tissue samples were taken and weighted into tin capsules (1 mg of muscle and liver; 0.2–1 mg of dorsal fin). The samples were analysed for stable carbon and nitrogen isotopes using an elemental analyser FlashEA 1112 connected to a Thermo-Finnigan MAT 253 mass spectrometer (CACTI, Universidade de Vigo), with an analytic precision of ±0.04 ‰ for C and ±0.10 ‰ for N (n = 10). Stable isotope values were expressed in conventional delta notation (δ) as parts per thousand (‰) according to the following equation:

$$\delta X = [(R_{SAMPLE}/R_{STANDARD}) - 1]$$

where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. Peedee Belemnite (PDB) and atmospheric nitrogen (AIR) were used as reference material for carbon and nitrogen, respectively. Standards of acetanilide, sulfate ammonia, urea, sucrose, and polyethylene were used for system calibration and weighted accordingly to samples weight variability.

Stable isotope values were checked for normality using the Shapiro–Wilk test. Paired t tests were applied to assess differences in $\delta^{13}C$ and $\delta^{15}N$ values between lipid extracted samples and non-lipid extracted samples of muscle, liver, and dorsal fin tissues. A repeated measures ANOVA test was applied to check for differences among tissues. When significant differences were found among tissues ($p < 0.05$), a Bonferroni post hoc test was applied. Relationships between weight and isotope values of dorsal fin were analysed using linear regressions. All the analyses were performed using the statistical package SPSS v.15.0.

5.3. Results

5.3.1. Tissue comparisons

Tissue comparisons were made using $\delta^{15}N$ values of non-lipid extracted tissues of dorsal fin, liver, and muscle, $\delta^{13}C$ values of non-lipid extracted dorsal fin tissue, and $\delta^{13}C$ values of lipid extracted samples of liver and muscle. $\delta^{15}N$ values in muscle (11.35 ± 0.53) were slightly higher but not significantly different (ANOVA, $F_{2,4} = 0.62$, $p = 0.580$) than $\delta^{15}N$ values in dorsal fin and liver (11.05 ± 0.62, 10.67 ± 1.39, respectively). For $\delta^{13}C$, significant differences were found among tissues (ANOVA, $F_{2,4} = 63.81$, $p < 0.05$) (-19.27 ± 0.60 in liver, -17.04 ± 1.07 in dorsal fin, and -17.59 ± 1.61 in muscle) (Table 5.1.), although differences were only significant between dorsal fin and liver tissue (Bonferroni post hoc test, $p < 0.05$). The relationship among tissues for both $\delta^{15}N$ and $\delta^{13}C$ values is provided in Fig. 5.2.

Table 5.1. Values of δ^{15}N and δ^{13}C (‰) and elemental composition in C and N (dry weight %) in dorsal fin, liver and muscle tissues of six adult seahorses *Hippocampus guttulatus.*

	Dorsal Fin		Liver		Muscle		Dorsal Fin		Liver		Muscle	
	δ^{15}N	N	δ^{15}N	N	δ^{15}N	N	δ^{13}C	C	δ^{13}C	C	δ^{13}C	C
	9.94	13.02	11.19	4.87	10.74	14.78	-18.81	41.96	-20.17	49.03	-19.66	40.38
	11.71	11.76	12.21	7.14	11.27	13.75	-17.87	40.64	-19.51	47.54	-19.46	47.24
	11.19	12.21	11.39	3.81	10.8	12.41	-16.41	38.8	-19.51	53.16	-16.92	50.6
	10.81	12.37	8.73	3.58	11.76	13.02	-16.54	40.48	-18.4	46.54	-16.98	46.1
	11.26	15.66	11.34	3.47	12.08	14.55	-16.6	56.32	-19	51.56	-16.89	46.95
	11.38	12.91	9.16	4.41	11.44	12.46	-15.99	42.36	-17.9	45.57	-15.64	45.6
Mean	11.05	12.99	10.67	4.55	11.35	13.50	-17.04	43.43	-19.08	48.90	-17.59	46.15
SD	0.62	1.39	1.39	1.38	0.53	1.03	1.07	6.44	0.83	2.96	1.61	3.32
Minimum	9.94	11.76	8.73	3.47	10.74	12.41	-18.81	38.8	-20.17	45.57	-19.66	40.38
Maximum	11.71	15.66	12.21	7.14	12.08	14.78	-15.99	56.32	-18.4	53.16	-15.64	50.6

Mean ± SD, minimum and maximum values are provided for each tissue. See text for further details

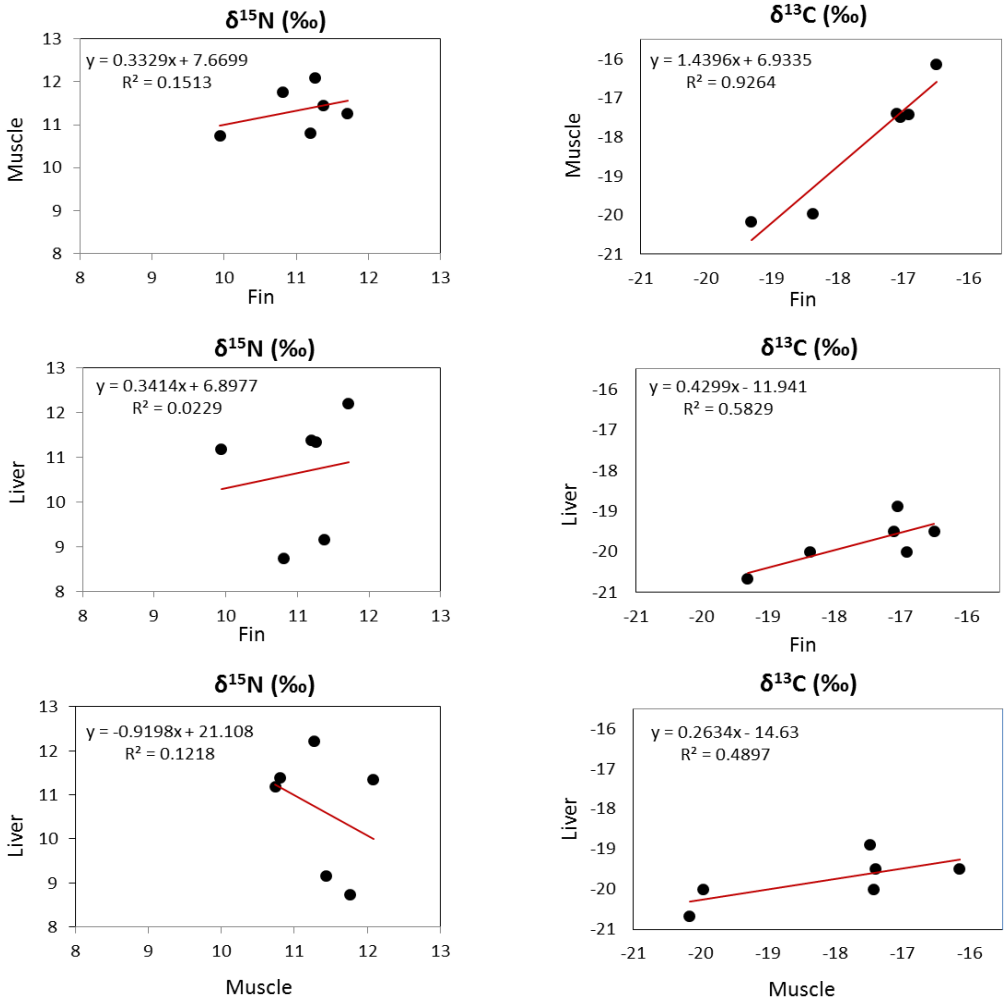


Figure 5.2. Relationship between δ^{15}N and δ^{13}C values and tissues (fin, liver, and muscle) in adult seahorses *Hippocampus guttulatus*. Liver and muscle tissues were lipid extracted for δ^{13}C.

5.3.2. Lipid extraction

The total lipids content (%) and $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SD, ‰) in lipid extracted and non-lipid extracted tissues are summarised in Table 5.2. Lipid extraction was found to cause no difference in $\delta^{15}N$ and $\delta^{13}C$ values of the dorsal fin; neither did it significantly affect $\delta^{15}N$ values in muscle and liver. However, $\delta^{13}C$ values in both muscle and liver were significantly affected by lipid extraction (Student paired t test, $p < 0.05$), with an increase after lipid extraction of 0.55 ± 1.61 ‰ for muscle and 2.56 ± 0.72 ‰ for liver.

Table 5.2. Lipid content (% dry weight) and $\delta^{13}C$ and $\delta^{15}N$ values (mean ± SD) in dorsal fin, liver and muscle tissues (n = 6) of adult seahorses *Hippocampus guttulatus* submitted or not to lipid extraction.

Tissue	Lipids	$\delta^{13}C$				$\delta^{15}N$			
		Non-lipid extraction	Lipid extraction	t	p	Non-lipid extraction	Lipid extraction	t	p
Dorsal fin	2.6 ± 2.3	-18.24 ± 0.63	-17.98 ± 0.53	-1.50	0.21	10.80 ± 0.36	10.90 ± 0.44	-0.48	0.66
Muscle	7.1 ± 4.0	-18.14 ± 1.62	-17.59 ± 1.60	-6.59	0	11.35 ± 0.53	11.96 ± 0.89	-2.05	0.1
Liver	58.5 ± 16.2	-21.64 ± 0.61	-19.08 ± 0.83	-19.1	<0.001	10.67 ± 1.39	10.67 ± 0.50	-0	1

Statistic *t* and level of significance *p* of the student paired *t* test analysis are provided

5.3.3. Dorsal fin size

The mean size of the fin clips sampled was 19.99 ± 9.10 mm^2 in the small section DF1, 64.63 ± 16.15 mm^2 in the intermediate section DF2, and 94.50 ± 20.73 mm^2 in the big section DF3. The minimum and maximum fin size analysed were 12.74 mm^2 (DF1) and 119.29 mm^2 (DF3), respectively. These portions corresponded to 0.21 and 2.15 mg dry weight, respectively. The isotope values were found to be independent of the size of fin analysed (Linear regression, $F_{1,17} = 2.22$, $p = 0.15$, $F_{1,17} = 0.009$, $p = 0.92$, for N and C respectively) (Fig. 5.3). Average values of $\delta^{15}N$ for portions DF1, DF2, and DF3 were 11.05 ± 0.62, 10.64 ± 0.12, and 11.63 ± 0.42, respectively, whereas mean values of $\delta^{13}C$ were -17.04 ± 1.07, -16.75 ± 1.07, and -16.96 ± 1.27.

For comparative purposes with the values of stable isotopes in the three tissues analysed in the present study from captive seahorses, the values of $\delta^{13}C$ and $\delta^{15}N$ in fin samples of wild seahorses *H. guttulatus* captured at four different sites in the Galician coast (NW Spain) are shown in Fig. 5.4.

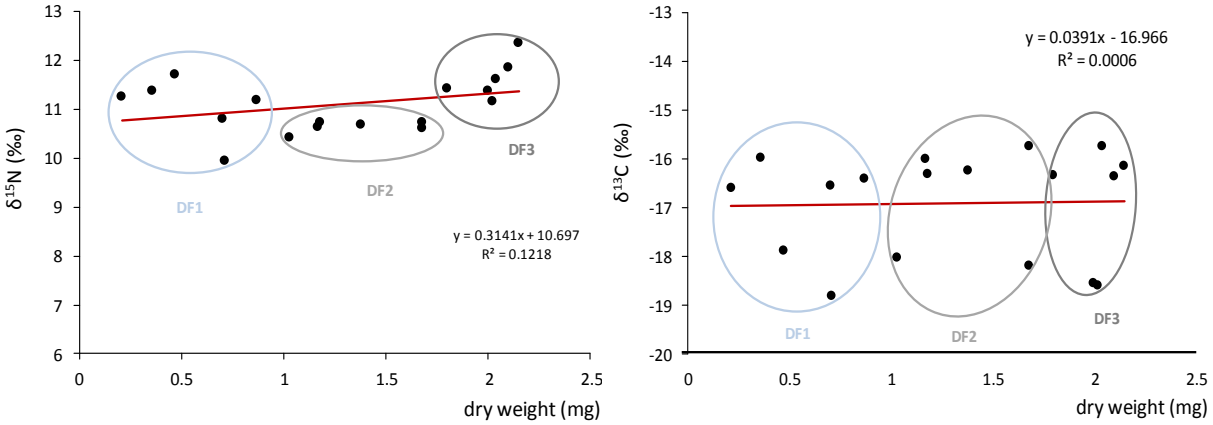


Figure 5.3. Relationship between dry weight (mg) of non-lipid extracted dorsal fin samples (n = 6) and δ^{15}N and δ^{13}C values of dorsal fin in adult seahorses *Hippocampus guttulatus*. DF1 small portions (n = 6), DF2 medium portions (n = 6), DF3 large portions (n = 6).

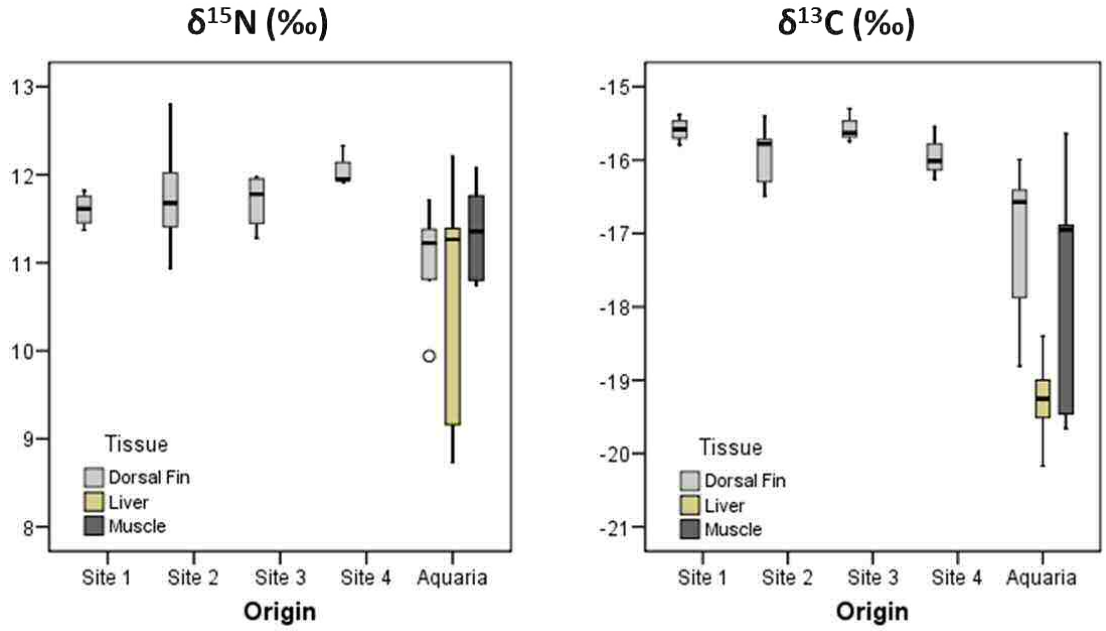


Figure 5.4. Boxplot of δ^{15}N and δ^{13}C values in dorsal fin samples from wild seahorses *Hippocampus guttulatus* captured at four different sites in the Galician coast (NW Spain)—Site 1 (n = 4), Site 2 (n = 11), Site 3 (n = 4), Site 4 (n = 3). Values of δ^{15}N and δ^{13}C from three different tissues (dorsal fin, liver and muscle) of captive *Hippocampus guttulatus* seahorses (n = 6) are provided for comparative purposes.

5.4. Discussion

5.4.1. Tissue comparison

Despite the slightly higher δ^{15}N values found in muscle, the values encountered in the three tissues analysed (muscle, liver, and dorsal fin) were not significantly different. McCarthy and Waldron (2000) also reported equivalent values in fin and muscle tissue for δ^{15}N in brown trout. Similar results were also reported by Kelly et al. (2006) in slimy sculpin, although a correction factor was applied by these authors. On the contrary, a significant enrichment in δ^{15}N was pointed out in muscle relative to fin tissue in salmon (Jardine et al., 2005; Sanderson et al., 2009). Pinnegar and Polunin (1999) suggested that differences in δ^{15}N among different tissues could be due to their composition in amino acids. The similarity of δ^{15}N values in the three tissues analysed in this study suggests that all three tissues are suitable for SIA, although dorsal fin would be recommended in alive adult seahorses as a non-lethal method.

Lipid rich tissues, such as muscle and especially liver, have lower δ^{13}C values than other tissues, because lipids tend to be more δ^{13}C depleted (DeNiro and Epstein, 1978; Pinnegar and Polunin, 1999). Unexpectedly, lipid extraction did not reduce differences in δ^{13}C values between dorsal fin and liver. As for δ^{15}N, differences among tissues in δ^{13}C values have been attributed to the amino acid composition in tissues (DeNiro and Epstein, 1978). The δ^{13}C values of seahorse dorsal fin tissue, however, were found to be similar to those in muscle, similarly to previous studies in brown trout (McCarthy and Waldron, 2000), Atlantic salmon (Jardine et al., 2005) and tropical fishes (Jardine et al., 2011). Muscle tissue has a slow turnover rate that provides more information over time about the diet when compared to tissues with fast isotopic turnover rate, such as liver (Hobson and Welch, 1992). The similarity between δ^{15}N and δ^{13}C values of *H. guttulatus* dorsal fin and muscle tissue suggests that both tissues are adequate for SIA to provide dietary information in a relatively long term. In food web studies, the analysis of δ^{15}N and δ^{13}C in dorsal fin tissue would constitute a simple and nonlethal sampling procedure providing long-term information on the feeding habits in seahorses.

5.4.2. Lipid extraction

Compared to other biochemical components, lipids are depleted in δ^{13}C due to lipid synthesis (DeNiro and Epstein, 1978). Hence, the variability of lipid content in different tissues significantly influences δ^{13}C values in the tissue (DeNiro and Epstein, 1978; Pinnegar and Polunin, 1999). For this reason, tissues submitted to SIA frequently undergo lipid extraction increasing the reliability of the results. Although the effects of lipid extraction in fish tissues have been reported by several authors (Pinnegar and Polunin, 1999; Sotiropoulos et al., 2004; Sweeting et al., 2006; Logan et al., 2008), the results achieved are contradictory. No previous studies had been carried out in seahorses, and we considered necessary to determine the effects of lipid removal on the quantification of stable isotope in seahorse tissues.

According to the results obtained from liver and muscle analysis, the differences found in δ^{13}C values between lipid extracted and non-lipid extracted tissues agree with previous studies in fish (Pinnegar and Polunin, 1999; Sotiropoulos et al., 2004; Sweeting et al., 2006; Logan et al., 2008). Those differences can be explained by the lipid content in muscle (7.1 %

dry weight) and especially in liver (58.5 % dry weight). Therefore, lipid removal seems to be necessary in the analysis of $\delta^{13}C$ in both muscle and liver of seahorses. Conversely, lipid extraction did not affect $\delta^{15}N$ values in either muscle or liver. Similar results were attained by Logan et al. (2008) in salmon, perch, and herring. Some studies have reported significant differences in $\delta^{15}N$ values associated with lipid extraction in muscle and liver tissues of fish (Sotiropoulos et al., 2004; Sweeting et al., 2006). These findings were related to the solvent effect. Therefore, in some cases, the analysis of stable isotopes would require a preliminary lipid extraction in the samples depending on the type of isotope considered, C or N.

Regarding dorsal fin, this tissue is composed by a mixture of bone, muscle, and cartilage, containing 2.6 % dry weight of lipids. As expected, due to this very low lipid content, lipid removal had no effect on stable isotope values. Consequently, lipid removal in dorsal fin tissue of seahorses would not be necessary to perform SIA. Our results agree with Post et al. (2007), who reported that for aquatic animals, it is not necessary to account for lipids in samples when lipid content is consistently low (< 5 % lipids; C:N < 3.5), which is the case of fin samples (2.6 % lipids; C:N = 3.3).

5.4.3. Dorsal fin size

The minimum amount of C and N required for SIA with the analytical equipment used in the present study was 20 and 50 µg, respectively. This requirement was fully satisfied with the smaller section DF1, whose mean content in C and N was 69.98 and 230.10 µg, respectively. Consequently, small sections of dorsal fin with 19.99 ± 9.10 mm^2 surface or 0.21 mg dry weight were perfectly adequate for $\delta^{15}N$ and $\delta^{13}C$ analysis. The surface of this section sample is equivalent to 8.66 % of total dorsal fin surface.

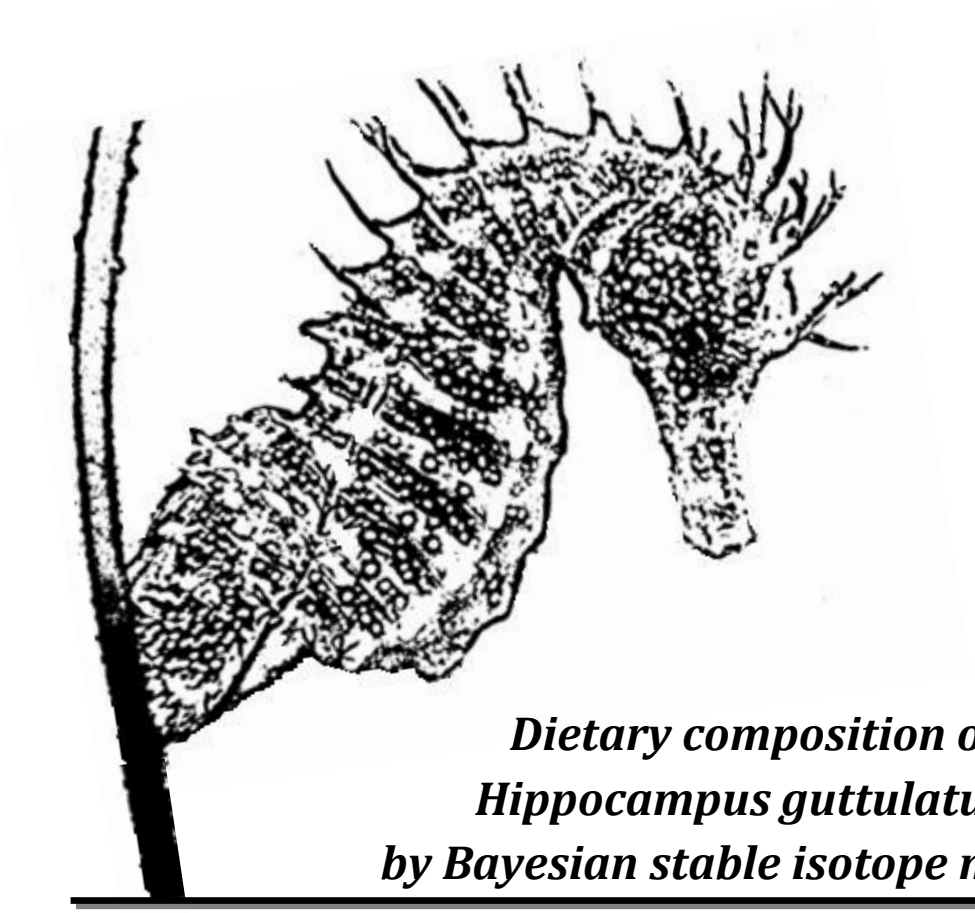
According to our results, fin-clipping in seahorses has important advantages over the use of other tissues: (1) it is a non-lethal sampling procedure, (2) it does not require lipid removal in the tissue, (3) the fin is regenerated in 1–2 months (Planas et al., 2008b), allowing multiple fin clips on the same seahorse over time, and (4) it has no effect on growth, survival or ability to swim (unpub. data).

Our study was performed in adult seahorses, measuring >15 cm in total length and the results achieved here cannot be extrapolated to juveniles or newborns, where the full body must be analysed. Further studies would be necessary to assess the application of fin-clipping to SIA according to the age of seahorses.

We consider that fin-clipping is an alternative to muscle tissue for SIA in *H. guttulatus*, a species with conservation concern and very low population densities. Due to imperative legal limitations (the capture of seahorses was not allowed for sacrifice) in the availability of seahorses, the number of samples available for SIA in this study was very low and restricted to naturally dead animals. Sanderson et al. (2009) pointed out that fin-clipping use provides a useful tracer for ecologists (e.g. to determine dietary sources) and has been found in wild seahorses at the Galician coast with a relatively low intra-site variability of the isotopic composition. A high number of samples would be necessary to assess a more precise quantification in all aspects of the analysis performed. In spite of this, we consider that the analysis of fin resulted in values equivalent to those of muscle tissue. Average $\delta^{15}N$ (range, 9.94–11.71 ‰ in fin and 10.74–12.08 ‰ in non-lipid extracted muscle) and $\delta^{13}C$ (range, -18.81 to -15.99 ‰ in fin and -19.66 to -17.59 ‰ in lipid extracted muscle) values in fin differed from muscle by 0.30 and 0.55 ‰, respectively. This variation corresponds to 2.7 ‰ for $\delta^{15}N$ and 3.25 ‰ for $\delta^{13}C$, which is much lower than the variation encountered when

comparing adult seahorses from the wild with adult seahorses from the laboratory (Fig. 5.4). Sanderson et al. (2009) demonstrated that analysing fins for δ^{15}N and δ^{13}C in *Oncorhynchus tshawytscha* and *O. mykiss* would produce results equivalent to those using muscle tissue and that fin δ^{15}N and δ^{13}C mimic those of muscle tissue in both time and space. These authors also pointed out that if there is no specific need to quantify isotopes using muscle tissue, muscle and fin tissues are equally powerful, and suggested that new projects can simply collect fin tissue throughout the project duration.

Our results provide a framework for using dorsal fin tissue in the measurement of δ^{15}N and δ^{13}C in seahorses. We propose fin-clipping as a standard non-lethal sampling method in future stable isotopes studies (SIA) with seahorses, avoiding the use of lethal techniques.



Chapter 6

Dietary composition of the seahorse Hippocampus guttulatus determined by Bayesian stable isotope mixing models

Dietary composition of the seahorse Hippocampus guttulatus determined by Bayesian stable isotope mixing models

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6.1. Introduction

Knowledge of the feeding habits of endangered species is crucial for their suitable conservation to ensure their population persistence in the wild (Foster and Vincent, 2004). In general, species feeding on a wide range of prey items (generalists) are more likely to be resilient to exploitation and habitat disturbance than species feeding on one or a few preferred prey items (specialists) because of their greater capability to adapt (Vázquez and Simberloff, 2002; Pratchett et al., 2004; Wilson et al., 2006). Thus, identifying the prey preferences of target species may allow determining their degree of vulnerability and applying appropriate management actions.

Seahorses (*Hippocampus* spp.) are characterised by sparse distribution, low mobility, small home ranges, low fecundity and long parental care. Due to their life history characteristics, seahorses are particularly vulnerable to overfishing or other disruptions such as habitat degradation, main factors that contribute to the decrease of many wild seahorse populations (Vincent, 1996; Lourie et al., 1999). As a result of the state of seahorse populations, all seahorse species were included on the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species in 1996. The charismatic and attractive traits of seahorses make them valuable as a flagship species to promote their conservation. Despite the increasing worldwide concern over the conservation status of seahorses, there is a current lack of detailed information about their feeding ecology, as well as other life history characteristics, which restricts the effectiveness of management strategies (Curtis and Vincent, 2006).

All seahorse species are ambush predators, feeding primarily on live crustaceans, especially amphipods and decapods; however, information on specific dietary composition is limited to a few species (*H. erectus*, Teixeira and Musick, 2001; *H. abdominalis*, Woods, 2002; *H. breviceps* and *H. subelongatus*, Kendrick and Hyndes, 2005; *H. patagonicus*, Storero and González, 2008; *H. hippocampus* and *H. guttulatus*, Gurkan et al., 2011). The diet variability among adult seahorse species can be explained by their snout morphology, individual size, method of predation, or availability and abundance of prey in the ecosystem (Tipton and Bell, 1988; Kendrick and Hyndes, 2005; Woods, 2002). Hence, feeding habits can vary for each seahorse species and population, which may become more or less vulnerable to environmental changes.

Seahorse's diet has been inferred by traditional methods (gut content analysis, field and laboratory observations), which are usually difficult to apply or even inappropriate for these threatened species due to mortality associated with destructive sampling. Prey information from gut contents is often difficult to identify if a relatively large sample size is not well collected, which is time-consuming and present potential threats for endangered species. This issue is based on that these more traditional methods only provide information on recently food ingested (snapshot), since each sample represents only a specific feeding event, and may not reflect long-term dietary patterns. Another disadvantage associated with these methods is the bias towards prey items that are more resistant to digestion.

Alternatively, stable isotopes analysis of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) is commonly applied for dietary analysis based on the fact that consumers incorporate the isotopic composition of the resources on which they feed, with a consistent trophic discrimination at each trophic level, so that a consumer's diet can be inferred (DeNiro and Epstein, 1978, 1981). Unlike more traditional dietary methods, stable isotope analysis therefore provides information of assimilated food sources over longer time periods, ranging from days to months depending on the tissue analysed (Tieszen et al., 1983; Fry, 1988, Hobson et al., 1995). Although the benefits of stable isotope approaches have caused an extensive use to study dietary preferences of fish (Hobson et al., 2002; Melville and Connolly, 2003; França et al., 2011; Galván et al., 2012; Chiaradia et al., 2014), dietary studies of seahorses based on stable isotopes are lacking in the literature yet. One reason for this lack might be the predominant use of lethal techniques for sampling of fish tissues (e.g. muscle, liver) for stable isotope analysis (Hobson and Welch, 1992; Cabana and Rasmussen, 1996; Jennings et al., 1997; Frediksen, 2003; Vizzini and Mazzola, 2009), since non-lethal sampling methods and suitability of alternative tissues (e.g. fin, scales) must be previously tested for routine application. Then, when dietary studies involve threatened species, such as seahorses, traditional sampling methods are inappropriate and non-lethal methodologies are needed (Kelly et al., 2006; Sanderson et al., 2009; Jardine et al., 2011; Valladares and Planas, 2012). Fin tissue sampling (fin-clipping) has been demonstrated an advisable non-lethal tool for stable isotopes analysis in adult seahorses, providing equivalent isotopic information than muscle tissue (Valladares and Planas, 2012). Within the conservation framework, this procedure allows to determine feeding habits of seahorses reducing the impact on the population under study.

The long-snouted seahorse (*Hippocampus guttulatus*) Cuvier 1829 is one of the European seahorse species occurring in the Mediterranean Sea and the Northeastern Atlantic Ocean (Lourie et al., 1999; Garrick-Maidment, 2004; Curtis and Vincent, 2005). Habitat loss or degradation and by-catch fisheries are mainly considered the potential threats on wild *H. guttulatus* populations and it has been reported a progressive population decline or disappearance in many areas in the last decades (*pers. comm., pers. obs.*), for instance, in Galicia (Northwest Spain). Furthermore, available biological and ecological information on the species *H. guttulatus* is considerable scarce. Concerning feeding habits, a limited number of gut content studies have documented that its natural diet is composed mainly on amphipods, mysidaceans and decapods (d'Entremont, 2002; Kitsos et al., 2008; Gurkan et al., 2011). Due to the insufficient information available to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status, this species has been listed as 'Data Deficient' species since 2003 (IUCN, 2014), modified from their previous 'Vulnerable' status in 1996.

Given the dearth of information on feeding habits of *H. guttulatus* seahorses in the wild, this work focused on assessing the dietary composition of this European seahorse inhabiting coastal waters of Galician using Bayesian stable isotope mixing models. The aim of this work was to assess temporal and spatial variations in food sources use by three seahorse populations along the Galician coast. This will increase our understanding of *H. guttulatus* seahorse feeding ecology to further support conservation actions on wild populations of this endangered species.

6.2. Material and methods

Wild seahorses *H. guttulatus* were collected from winter 2010 to summer 2012 in three locations along the Galician coast (NW Iberian Peninsula): Toralla (42°12'5''N; 8°47'56''W), Bueu (42°19'52''N; 8°46'40''W), and Ribeira (42°33'45''N; 8°59'15''W) (Fig. 6.1.). Toralla and Bueu localities are characterised by sandy or rocky bottom with patches of macroalgae (*Sargassum muticum*, *Dictyota* sp., *Chondrus crispus* and *Ulva* sp.) and seagrass beds (*Zostera marina*). On the contrary, Ribeira is mainly dominated by muddy substrate and anthropogenic debris with accumulations of *Ulva* sp. and mussels.

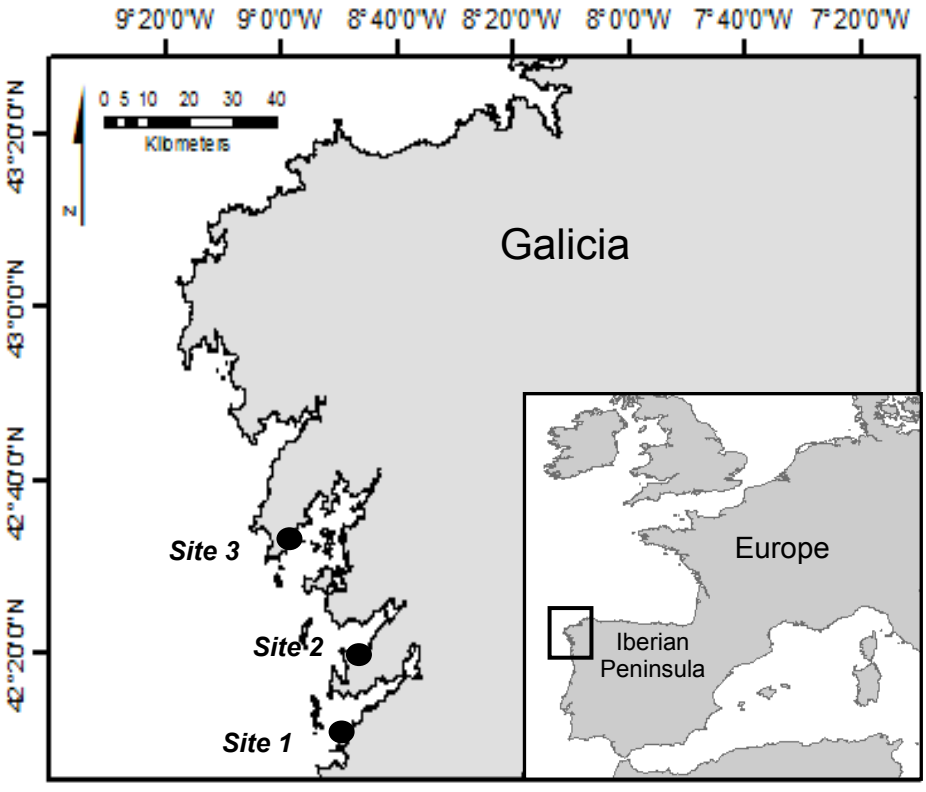


Figure 6.1. Map of the area and sampling locations in Galicia (NW Iberian Peninsula). Site 1: Toralla, Site 2: Bueu, Site 3: Ribeira.

Seahorses were hand-caught by Scuba diving in shallow waters (<10 meters in depth), placed in plastic bags filled with surrounding seawater and taken them out of the water for biological parameters measurements and sampling. Handling time was kept at a minimum to minimize their stress. Then, seahorses were immediately released to their original site. For each seahorse encountered sex, sexual maturity, reproductive status (males) and weight were recorded. Individuals were also photographed by placing them carefully over a gridded plate for standard length (SL) measurements following Lourie et al. (1999). Morphometric measurements were made on digital images using image processing software (NIS Elements, Nikon). A small sample of the dorsal fin was taken non-invasively from each individual as described by Valladares and Planas (2012) and kept at –20 ºC for further stable isotopes analysis.

Along the two years survey from each site where seahorses were collected, main potential preys (benthic invertebrates) were collected at several time periods. Benthic invertebrates were picked out from the vegetation randomly hand collected and stored at –20 ºC before stable isotope analysis. Prey items were identified to the lowest taxonomic level possible.

Prior to stable isotope analyses, all samples were washed in distilled water and freeze-dried. Neither lipid extraction nor acid washing was considered. Dorsal fin samples and homogenised prey samples (whole body) were weighted (0.2-1 mg) into tin capsules on a Sartorius microbalance MC210P (± 0.01 mg). Carbon and nitrogen isotopic analyses were conducted at *Servizos de Apoio á Investigación* (SAI), University of A Coruña (Spain). Stable isotope ratios of samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyser (ThermoFinnigan, Milan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Bremen, Germany) through a Conflo II interface.

Stable isotope abundances are expressed in conventional delta notation (δ) as parts per thousand (‰) relative to VPDB (Vienna Pee Dee Belemnite) and Atmospheric Air, according to the following equation: $\delta X = [(R_{Sample} / R_{Reference}) – 1]$, where X is ^{13}C or ^{15}N and R is the corresponding ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$, respectively. As part of an analytical batch run, a set of international reference materials for $\delta^{15}N$ (IAEA-N-1, IAEA-N-2, IAEA-NO-3) and $\delta^{13}C$ (NBS 22, IAEA-CH-6, USGS24) were analysed. The precision (standard deviation) for the analysis of $\delta^{13}C$ and $\delta^{15}N$ of the laboratory standard (acetanilide) was ± 0.15 ‰ (1-sigma, n=10).

The relative contributions of various food sources to a consumer's overall diet can be calculated using mathemathical mixing models approaches (Phillips, 2001). Several programs have been developed recently to improve the accuracy of these mixing models (Phillips and Gregg, 2001; Phillips and Gregg, 2003; Moore and Semmens, 2008; Parnell et al., 2010; Stock and Semmens, 2013). Among them, Bayesian stable isotope mixing models have the potential to provide more robust results by incorporating the uncertainties associated with multiple sources, discrimination factors and isotope values (Moore and Semmens, 2008; Parnell et al., 2010). The trophic discrimination factor is assumed to be about 3-4 ‰ for $\delta^{15}N$ and 0-1 ‰ for $\delta^{13}C$ between trophic levels (Post, 2002; McCutchan et al., 2003; Boecklen et al., 2011).

We employed a Bayesian stable isotope mixing model (MixSIAR, Stock and Semmens, 2013) to estimate the relative contributions of the prey items to the diet of *H. guttulatus* seahorses. Potential dietary sources or preys were grouped in five taxonomic categories, which included Amphipoda Gammaridea (thereafter Gammaridea), Amphipoda Caprellidea

(thereafter Caprellidea), Caridea, Mysidae and Annelida. Within taxonomic groups, particular species were most prevalent. The most frequent species of gammarids were *Ampithoe ramondi*, *Dexamine spinosa* and *Gammarella fucicola*. The main caprellids were the species *Phtisica marina*. Caridean shrimps were mostly composed of *Hippolyte* sp. and *Thoralus cranchii*. *Leptomysis* sp. and *Siriella* sp. were the dominant mysidaceans. *Platynereis* sp. was the most prevalent Annelida. Preys from each group had a mean size of about 5.1 mm (Gammaridea), 5.7 mm (Caprellidea), 8.1 mm (Caridea), 10.5 mm (Mysidae) and 28.7 mm (Annelida). Gammaridea, Caprellidea and Caridea are found amongst algae. Meanwhile, Mysidae are pelagic typically living near the bottom and Annelida included typical errant worms living in muddy sand. These crustaceans groups were the most dominant preys found in the sampling sites, considered the most likely prey items to include in the mixing model based on previous knowledge of *H. guttulatus* seahorse diet (d'Entremont, 2002; Kitsos et al., 2008; Gurkan et al., 2011) (Table 6.1.). We compared two models, considering the prey type Annelida (model I) or not (model II) since this food source was relatively predominant in the study area and likely to be consumed by seahorse species, however, in a very low proportion (Teixeira and Musick, 2001; Woods, 2002; Kitsos et al., 2008). The Bayesian mixing models were run using stable isotope data from seahorses as the consumer (raw data by site), prey groups as the sources (mean ± SD data by site) and the discrimination factors of 1 ± 1.3 ‰ for δ^{13}C and 3.4 ± 1 ‰ for δ^{15}N (Post, 2002; Sweeting et al., 2007a, b). The models were run with Markov Chain Monte Carlo (MCMC) parameters of 3 chains of 300,000 iterations, a burn-in phase of 200,000, and a thinning of 100. Individuals as a random effect and only one error term (process error) were included in the model. The model generates posterior probability distribution that can be described by average estimates of the source contributions and their credible interval associated (Bayesian confidence intervals). Convergence and diagnostic statistics were performed using the Gelman-Rubin test.

One-way ANOVA analysis was used to examine the differences in the isotopic composition between reproductive status of male seahorses. We considered breeding period (active: May-October, null: November-April) to analyse seasonal differences. Multivariate analysis of variance (MANOVA) was applied to examine the effects of site, sex and breeding period on carbon and nitrogen isotope values of seahorses. When significant differences were observed with MANOVAs, multiple comparisons were performed with Tukey's HSD post hoc tests. Relationships between individual seahorse length and isotope values were analysed using linear regressions. Carbon and nitrogen isotope values of the potential sources were compared with multivariate analysis of variance (MANOVA), considering site and prey group as factors.

Variables were checked for normality and homogeneity of variances with a Shapiro–Wilk and Levene's tests. SPSS v.15.0 software was used to perform the statistical analyses at a significant level of $p < 0.05$.

Table 6.1. Relative dietary importance (frequency of occurrence) of the relevant prey groups identified from gut contents of seahorse species.

Species	Prey group	Frequency of occurence (%)	Locality	Reference
H. abdominalis	Copepoda	7	Wellington Harbour, New Zealand	Woods, 2002
	Gammaridae	13		
	Caprellidae	25		
	Ischyroceridae	18		
	Caridea	47		
	Mysidacea	20		
	Isopoda	7		
	Annelida	< 2		
H. breviceps	Copepoda	52	Port of Fremantle, SW Australia	Kendrick and Hyndes, 2005
	Gammaridae	95		
	Caprellidae	45		
	Ostracoda	22		
	Isopoda	30		
	Decapoda larvae	< 2		
	Annelida	< 2		
H. erectus	Copepoda	11	Chesapeake Bay, Virginia (US)	Teixeira and Musick, 2001
	Amphitoidae	40		
	Gammaridae	36		
	Caprellidae	35		
	Mysidacea	< 1		
	Isopoda	< 1		
	Palaemonidae	< 4		
	Annelida	< 3		
H. guttulatus	Copepoda	17	Ria Formosa, Portugal	D'Entremont, 2002
	Amphipoda	86		
	Decapoda	76		
	Isopoda	57		
	Gastropoda	42		
	Ostracoda	23		
H. guttulatus	Amphipoda	88	Aegean Sea, Greece	Kitsos et al., 2008
	Decapoda			
	Anomura	63		
	Mysidacea	42		
	Copepoda	< 1		
	Isopoda	< 1		
H. guttulatus	Copepoda	23	Aegean Sea, Turkey	Gurkan et al., 2011
	Amphipoda	47		
	Mysidacea	85		
	Decapoda larvae	100		
	Gastropoda	20		
	Isopoda	15		
H. hippocampus	Amphipoda	73	Aegean Sea, Greece	Kitsos et al., 2008
	Decapoda			
	Anomura	21		
	Mysidacea	26		
	Cumacea	5		
	Ostracoda	5		
	Annelida	5		

Species	Prey group	Frequency of occurence (%)	Locality	Reference
H. hippocampus	Copepoda	5	Aegean Sea, Turkey	Gurkan et al., 2011
	Amphipoda	21		
	Mysidacea	16		
	Decapoda larvae	26		
	Ostracoda	15		
H. patagonicus	Gammaridae	83-100	Patagonia, Argentina	Storero and González, 2008
	Caprellidae	100		
	Brachyura	52-61		
H. reidi	Copepoda	91	Paraíba, NE Brazil	Castro et al., 2008
	Nematoda	86		
	Caridea	14		
	Amphipoda	12		
	Ostracoda	10		
	Annelida	< 3		
	Isopoda	< 1		
H. zosterae	Copepoda	90-100	Tampa Bay, Florida	Tipton and Bell, 1988
	Amphipoda	37		
	Ostracoda	23		
	Caridea	< 3		

6.3. Results

During the two-year study period, a total of 132 seahorses were sampled. Number of seahorses, standard length and isotopic compositions for each sex and site are summarised in Table 6.2.

We found no significant differences between the reproductive status of males (pregnant or not) for δ^{15}N ($F_{1, 24}$ = 0.40, p = 0.53; $F_{1, 18}$ = 2.41, p = 0.14, in Sites 2 and 3, respectively) or for δ^{13}C ($F_{1, 24}$ = 1.72, p = 0.20; $F_{1, 18}$ = 0.18, p = 0.67, in Sites 2 and 3, respectively). No pregnant males were found in Site 1.

When both stable isotope of carbon and nitrogen were assessed together, the factor breeding period did not have a significant effect on the isotopic composition of seahorses (MANOVA, Wilks, $F_{1, 128}$ = 0.36, p = 0.70). Significant differences on the isotopic composition of seahorses were caused by sex and site (MANOVA, Wilks, $F_{1, 128}$ = 7.83, p < 0.001; $F_{2, 128}$ = 9.34, p < 0.001, respectively). Post hoc comparisons showed that Site 3 had higher δ^{15}N and δ^{13}C values than Sites 2 and 1 (Table 6.2.).

No significant seasonal differences were observed in the isotopic composition of the potential preys collected over the sampling periods. Therefore, we pooled prey samples among collection periods for the subsequent statistical analysis (i.e. mixing models). Isotopic compositions for each prey group from the three sites are summarised in Table 6.3. Site and prey group had significant effects on the isotopic composition of prey sources (MANOVA, Wilks, $F_{2, 134}$ = 4.8, p < 0.001; $F_{4, 134}$ = 27.6, p < 0.001, respectively).

Table 6.2. Number of *Hippocampus guttulatus* seahorses sampled from three locations of the Galician coast, mean (± SD) and standard length range (SL, mm). Site 1: Toralla, Site 2: Bueu, Site 3: Ribeira. Mean stable carbon and nitrogen isotope values (± SD) of seahorses at the three locations.

	Site 1	Site 2	Site 3
Females	22	21	30
mean SL (mm)	186 ± 20.9	177 ± 16.1	191 ± 16.2
SL range (mm)	142 - 227	146 - 216	166 - 221
$\delta^{15}N$	11.8 ± 0.6	11.8 ± 0.5	12.4 ± 0.7
$\delta^{13}C$	-15.1 ± 0.7	-16.0 ± 0.4	-15.2 ± 0.8
Males	16	24	19
pregnant	0	4	3
no pregnant	16	20	16
mean SL (mm)	186 ± 24.9	176 ± 15.2	195 ± 17.9
SL range (mm)	141 - 239	151 - 208	146 - 222
$\delta^{15}N$	11.6 ± 1.0	11.5 ± 0.6	12.5 ± 0.6
$\delta^{13}C$	-16.5 ± 0.9	-16.4 ± 0.4	-15.5 ± 0.4
Total	38	45	49
$\delta^{15}N$	11.7 ± 0.8	11.7 ± 0.9	12.4 ± 0.7
$\delta^{13}C$	-16.1 ± 0.8	-16.3 ± 0.6	-15.4 ± 0.7

Table 6.3. Mean stable carbon and nitrogen isotope values (± SD) of the five prey groups at the three locations. Site 1: Toralla, Site 2: Bueu, Site 3: Ribeira.

	Site 1	Site 2	Site 3
$\delta^{15}N$			
Gammaridea	8.4 ± 0.6	9.6 ± 0.4	8.8 ± 0.8
Caprellidea	8.2 ± 0.8	8.8 ± 0.8	9.0 ± 1.0
Caridea	9.4 ± 0.7	9.4 ± 0.7	9.8 ± 0.8
Mysidae	10.6 ± 0.3	10.6 ± 0.3	10.6 ± 0.3
Annelida	8.4 ± 0.6	9.6 ± 0.4	8.8 ± 0.8
$\delta^{13}C$			
Gammaridea	-18.2 ± 1.1	-17.6 ± 0.9	-18.4 ± 3.0
Caprellidea	-16.6 ± 1.1	-16.4 ± 1.0	-15.7 ± 0.3
Caridea	-16.8 ± 1.1	-16.5 ± 0.7	-16.7 ± 0.5
Mysidae	-18.1 ± 0.6	-18.1 ± 0.6	-18.1 ± 0.6
Annelida	-19.9 ± 1.3	-20.8 ± 1.9	-21.6 ± 4.4

Mean isotope values and their variances for the 132 seahorses sampled and for preys from the three sites were plotted in an isospace plot (Fig. 6.2.). The estimated contribution of the prey groups, presented below, are given in terms of the median (50 % quantiles) of the posterior source contributions and expressed as percentages. The mixing model estimated that Caprellidea represented the highest relative contribution to *H. guttulatus* diet (42 %) for the overall population, both considering and not the prey Annelida into the model. Gammaridea and Caridea contributed a maximum of 25 % to the diet in both models. Mysidae accounted for 5.9 % (model I) and 8.1 % (model II). The contribution of Annelida (model I) was the lowest (3.5 %) to the *H. guttulatus* diet (Fig. 6.3.). The variation in seahorse diet was driven mostly by site compared to individuals, based on the variance parameters (σ_{site} = 0.4, $\sigma_{individual}$ = 0.1). Accordingly, diet proportion by site showed differential contributions of Gammaridea and Caridea to the seahorse diet. The second dominant prey group in Site 3 was Caridea (27.4 % in model I and II), whereas Gammaridea was in Site 1 (24.3 % and 28.7 % in model I and II, respectively) and Site 2 (29.9 % and 32.4 % in model I and II, respectively). Caprellidea represented the dominant prey group in the three sites (up to 49.1 % in model I and 48.4 % in model II), and Mysidae the less dominant (up to 5.5 % in model I and 8 % in model II). (Fig. 6.3.).

Linear regressions between seahorse length and isotope values were significant (Site 1 δ^{15}N: $F_{1,36}$ = 22.2, p < 0.001, δ^{13}C: $F_{1,36}$ = 14.2, p = 0.001; Site 2 δ^{13}C: $F_{1,41}$ = 10.1; p = 0.003; Site 3 δ^{15}N: $F_{1,44}$ = 12.9, p = 0.001, δ^{13}C: $F_{1,44}$ = 14.2; p < 0.001), except for δ^{15}N in Site 2 ($F_{1,41}$ = 0.3, p = 0.6). Regressions between seahorse length and the median contribution estimates of each prey showed that seahorses with smaller standard length are more likely to prey on Caprellidea and Gammaridea than Caridea compared to larger seahorses that showed the opposite feeding pattern.

6.4. Discussion

The prey preferences described for *H. guttulatus* represent a novel contribution towards a greater understanding of the feeding ecology of this endangered seahorse.
The Bayesian isotope mixing model results showed that Caprellidea is the primary food source for *H. guttulatus*, followed by Gammaridea and Caridea on these marine systems. The large proportion of these prey groups suggest that they are consistent preys in the diet, being consumed regularly. In contrast, the low proportion of Mysidae and Annelida indicates that they are not essential dietary components for seahorses, and might be occasionally consumed.

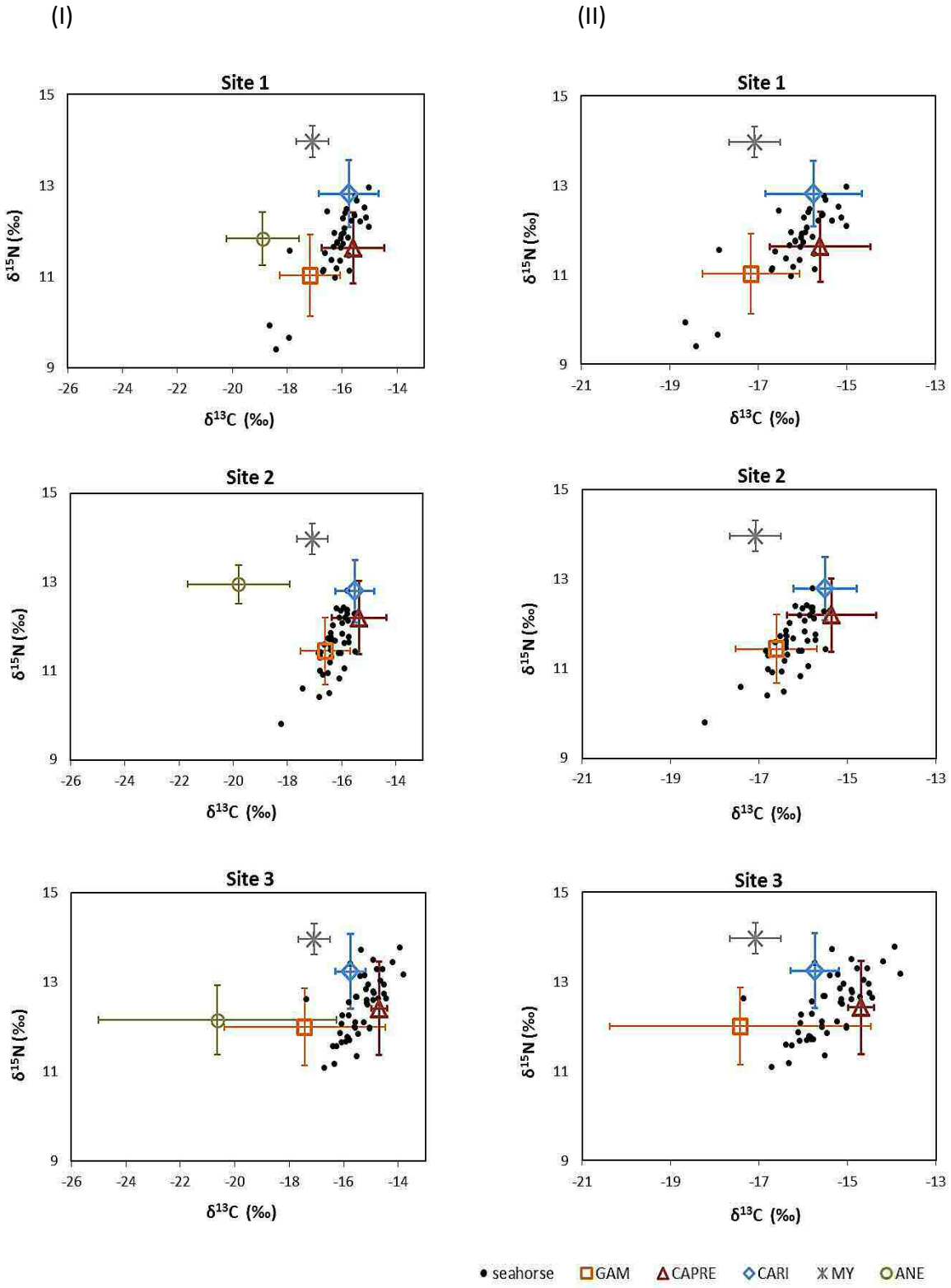
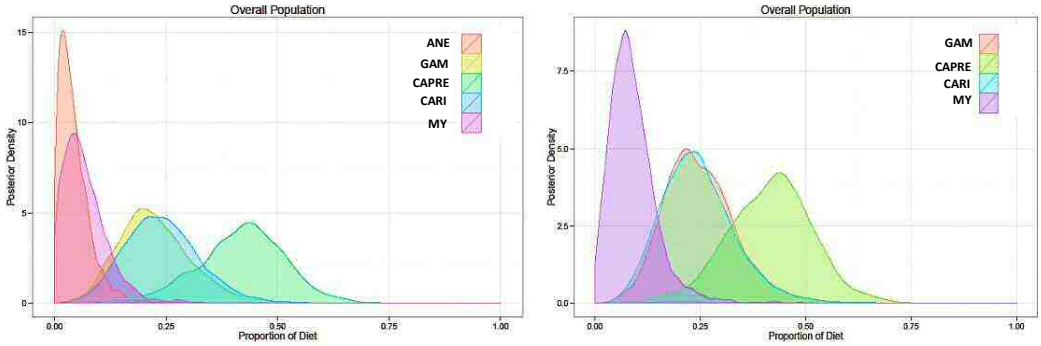


Figure 6.2. Stable isotope bi-plots of individual *Hippocampus guttulatus* seahorses (n = 132) and mean prey groups by site for model I (I, with Annelida) and model II (II, without Annelida). Prey data has been adjusted by discrimination factors (1 ‰ for δ^{13}C and 3.4 ‰ for δ^{15}N). Error bars indicate standard deviations. Site 1: Toralla, Site 2: Bueu, Site 3: Ribeira.

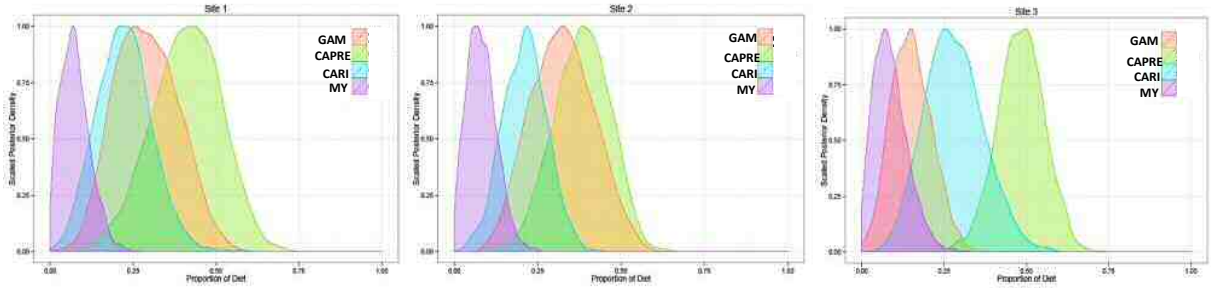
(a)

(I) (II)



(b)

(I)



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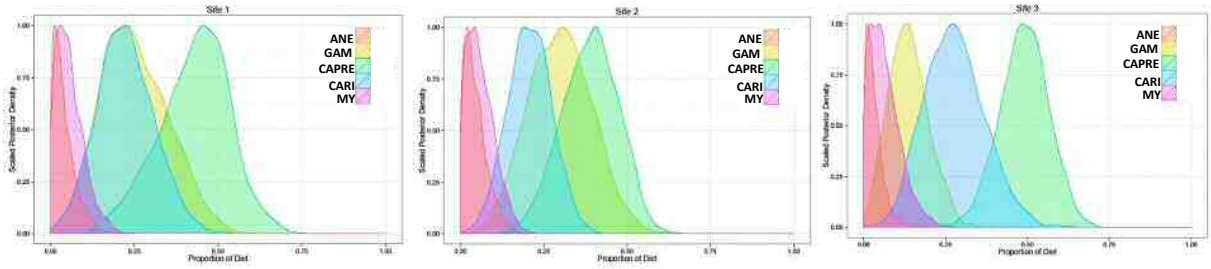


Figure 6.3. MixSIAR results showing the posterior density distributions of proportional contributions of the prey groups to the diet of *Hippocampus guttulatus* seahorses from (a) the overall population and (b) the three locations of the Galician coast (Site 1: Toralla, Site 2: Bueu, Site 3: Ribeira). (I) model I: five prey groups including Annelida, (II) model II: four preys groups without Annelida. GAM: Gammaridea, CAPRE: Caprellidea, CARI: Caridea, MY: Mysidae, ANE: Annelida.

Prey selectivity of seahorses can be explained by prey features (e.g. availability, abundance, habitat and behaviour) and predation behaviour, other than capture effort (Tipton and Bell, 1988; Kendrick and Hyndes, 2005; Woods, 2002). The unexpected low proportion of Mysidae in the diet given their high frequency in all sites reflects that method of predation and/or prey habitat and behaviour, rather than abundance of prey, would influence the prey choice of *H. guttulatus.* The relatively reduced swimming ability and low motion of seahorses would limit their success in capturing fast swimming preys such as mysids (Kendrick and Hyndes, 2005). During the feeding process, seahorses usually grasp the vegetation or holdfast (any structure used as attachment sites) with their prehensile tail and wait until prey approaches the mouth (Lourie et al., 1999; Curtis and Vincent, 2005). Therefore, Mysidae might represent a less accessible prey for *H. guttulatus* as these prey form aggregates in the water column and remain in constant movement. Although gut content analyses have revealed that Mysidae are an important dietary component for *H. guttulatus* in other geographical areas (d'Entremont, 2002; Kitsos et al., 2008; Gurkan et al., 2011) (Table 6.1.), it resulted to be a more secondary prey in our study region when the dietary composition was analysed with stable isotope approaches. Information from gut contents represents the food items that seahorses ingested immediately before collection, while stable isotopes analysed in dorsal fin tissue provide longer-time information. Thus, Mysidae identified from gut contents would reflect specific feeding events dealing to overestimation of this prey in the overall diet of seahorses, e.g. up to 85 % frequency of occurrence reported by Gurkan et al., 2011.

Considering the foraging behaviour of seahorses, *H. guttulatus* would hunt effectively on benthic and less mobile crustaceans (Kendrick and Hyndes, 2005). Caprellidea, Gammaridea and Caridea are commonly associated with vegetation, which is the habitat preferentially occupied by *H. guttulatus* (Curtis and Vincent, 2005; Planas et al., 2008). In this situation, life habits of these preys make them accessible and vulnerable to seahorse's predation. The major contribution of these three benthic preys to the diet of *H. guttulatus* possibly reflects the sedentary feeding behaviour of this species. Storero and González (2008) described that seahorses are likely to ingest not only the most accessible but also the most abundant prey in the natural environment. However, the different preference within the available benthic preys shown by *H. guttulatus* in the present study could not be only related to prey abundance, as Caprellidea was the main food source but less frequent than Gammaridea and Caridea in the three sites. One reason to explain this fact might be a quantitative underestimation of Caprellidea items, which could be attributable to the difficulty in detecting this small prey among the algae. However, these differences could also be caused by the degree of prey digestion and assimilation, as suggested by Phillips et al. (2014). In feeding trials with *H. guttulatus*, Corse et al. (2014) reported that the time between prey ingestion of the large prey (*Palaemonetes*) and its detection in faeces was shorter than smaller prey (Mysidae) suggesting different degrees of prey digestion by seahorses. Thus, a potential higher effective digestibility and assimilation of caprellids with fewer indigestive hard parts, compared to other prey such as caridean shrimps, would explain their major contribution to *H. guttulatus* diet. However, digestive capabilities in adult seahorses need to be further investigated.

In the case of Annelida, their very low proportion in the diet of *H. guttulatus* was expected given the low preference of this prey identified previously in stomach content studies of wild seahorses (Teixeira and Musick, 2001; Woods, 2002; Kendrick and Hyndes, 2005; Kitsos et al., 2008) (Table 6.1.). Although seahorses have been observed feeding on

sediment capturing either benthic or infaunal preys (Curtis and Vincent, 2005; Storero and González, 2008), the consumption of annelids may occur occasionally and hence can be considered as a minor dietary item or a secondary prey for seahorses.

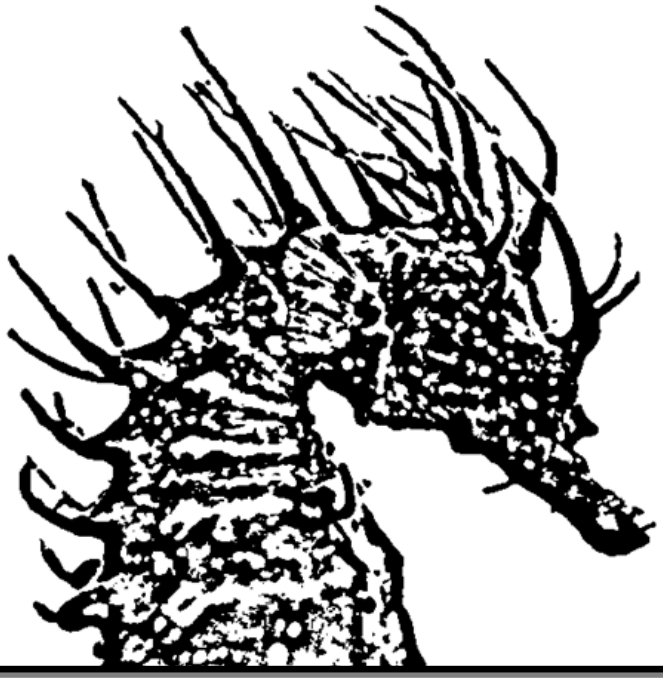
Our data revealed that benthic crustaceans clearly predominate over other prey types in the diet of *H. guttulatus*. The high proportion of Amphipoda (Caprellidea and Gammaridea) and Caridea in the diet of *H. guttulatus* is in accordance with previous dietary studies based on gut contents undertaken on this species (d'Entremont, 2002; Kitsos et al., 2008; Gurkan et al., 2011), as well as for other seahorse species (Teixeira and Musick, 2001; Woods, 2002; Kendrick and Hyndes, 2005; Storero and González, 2008) (Table 6.1.). As described by Curtis and Vincent (2005), feeding habits may explain the preference of *H. guttulatus* in occupying vegetated habitats where they can forage on these benthic crustaceans, instead of open areas where might be difficult to find benthic crustaceans or capture mobile preys such as mysidaceans due to their low mobility speed.

Besides the differences in diet attributed to feeding behaviour of seahorse, other factors (e.g. sex, reproductive status, spatial and seasonal differences) might be involved (Woods, 2002; Curtis and Vincent, 2005). When comparing the diet composition of *H. guttulatus* among the three studied sites, the dietary preferences changed according to the different habitat characteristics of each site. Differences in seahorse diet among sites were specifically related to Gammaridea and Caridea; the average estimated contribution of the former was greater in Sites 1 and 2, whereas Caridea seem to be higher consumed than Gammaridea in Site 3. Since both prey groups were similarly predominant in all sites, these differences would suggest differential habitat use by seahorses within each site, which may reflect different prey prevalence and hence to different isotopic composition in seahorses. Gammaridea has been described as the dominant taxonomic group associate to *Zostera* beds located in the same geographic area that our study (Gestoso, 2014). In Sites 1 and 2, where both *Zostera* beds and macroalgae are present, seahorses could prefer the seagrass beds to foragewhere Gammaridea would be more present than Caridea. On the contrary, feeding events of seahorses in Site 3, absence of *Zostera* beds, would occur in certain macroalgae habitats where Caridea rather than Gammaridea would be dominant. In the same way, different foraging habitat by females and males could explain the significant effect of sex on the variability in the diet of *H. guttulatus*. Sexual differences in diet exist depending on the seahorse species considered (d'Entremont, 2002; Castro et al., 2008) or not (Gurkan et al., 2001; Woods, 2002; Storero and González, 2008). Factors inherent to both males and females would also explain such differences. The period of male pregnancy and female eggs production in seahorses represents a high energetic/nutritional cost with probably distinct energetic demands (Masonjones, 2001; Teixeira and Musick, 2001; Planas et al., 2010), which may be reflected in different prey selection by males and females. In this regard, we could expect variation in the isotopic composition of *H. guttulatus* considering both reproductive status of males (pregnant and no-pregnant) and breeding period. However, the effect of these reproductive factors in carbon and nitrogen isotope values of *H. guttulatus* was not significant, suggesting similar feeding patterns in males and females along the year.

Prey selection in terms of size undergoes changes throughout seahorses' ontogeny, with larger preys preferentially selected with growth (Tipton and Bell, 1988; Teixeira and Musick, 2001; Woods, 2002). A similar feeding pattern was also found for *H. guttulatus*, smaller seahorses would prey more on smaller crustaceans (Gammaridea and Caprellidea) than on Caridea. The particular feeding strategy of seahorses would be a possible reason to

explain this trend. With no teeth and a digestive tract without a differentiated stomach (Foster and Vincent, 2004), prey items must be generally swallowed whole, passing rapidly along the digestive tract. Hence, seahorses would commonly feed on preys small enough to fit into their mouths.

Stable isotope approaches provided a more accurate quantification of prey contribution to seahorse's diet. Our findings would contribute to the knowledge on feeding patterns of the seahorse *H. guttulatus* in the wild, providing relevant data for conservation management of this endangered species. In particular, a deep knowledge of seahorse feeding patterns will contribute to understand their vulnerability to habitat loss and degradation (Foster and Vincent, 2004; Storero and González, 2008), one of the main factors related with the decrease of many wild seahorse populations (Lourie et al., 1999). One of the main consequences of habitat disruptions is the reduction of resources availability, specifically the fauna associated to the vegetation, which may affect species both direct and indirectly (Vázquez and Simberloff, 2002). In the case of seahorses, the disappearance of vegetated habitat would alter the relative abundance of their main prey source (benthic crustaceans). As a result, seahorse populations could be indirectly affected. Conservation actions should be addressed to maintain vegetated habitats to ensure benthic crustaceans availability and to satisfy the feeding optimum requirements of seahorses.



Chapter 7

Conclusions

Conclusions

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1. It was demonstrated that water temperature acts as a decisive factor in food assimilation efficiency and in the early development (growth and survival) of *H. guttulatus* seahorses.

2. Within the range of 15 - 21 °C, 30 days old juvenile seahorses showed the highest growth rate in the warmest condition (21 °C), and the highest survival at 18 °C. C:N ratios and the Fulton's condition factor suggest a better condition for juveniles reared at 18 °C.

3. Changes in stable isotope values (δ^{13}C and δ^{15}N) towards diet isotopic values indicated a more efficient assimilation of nutrients by juveniles maintained at warmer temperatures (18 and 21 °C).

4. A temperature level of about 18 °C or slightly higher is proposed for the optimum early development and growth of the seahorse *H. guttulatus*. It would be expected that *H. guttulatus* wild populations under optimal prey availability inhabiting regions with temperatures of about 18 °C or higher would result enhanced when compared to others from colder waters.

5. Juvenile seahorses could support certain food deprivation in a manner that would be inversely dependent on temperature level (Longer survivals at 15 °C and lowest Fulton's condition factor at 21 °C). This fact would have ecological implications on wild seahorse populations since juvenile seahorses developing at lower temperatures would be less dependent on food availability during the initial planktonic period.

6. Consumption and assimilation of preys by *H. guttulatus* juveniles was effectively traced using stable carbon isotopes. δ^{13}C values in juvenile seahorses were influenced by the carbon stable isotope values of the corresponding diet (copepods or *Artemia*).

7. The better digestibility of copepods compared to *Artemia* would enhance their assimilation and hence would provide better feeding conditions for tissue growth, resulting in a higher survival of juveniles when fed on copepods.

8. Copepods are highly recommended for the early rearing of juvenile *H. guttulatus* seahorses to optimise survival and growth rates.

9. Fin-clipping sampling has been demonstrated an advisable non-lethal tool for stable isotopes analysis in alive adult seahorses providing equivalent isotopic information than muscle tissue.

10. Dorsal fin sampling is a reliable non-lethal technique for stable isotopes studies in seahorses avoiding the sacrifice of the fish, allowing the assessment of feeding habits of seahorses, and reducing the impact on the population under study.

11. The dietary composition of wild *H. guttulatus* seahorses inhabiting coastal waters in Galicia was determined for the first time using Bayesian stable isotope mixing models.

12. Benthic crustaceans clearly predominate over other prey types in the natural diet of *H. guttulatus*. Caprellidea would represent the primary food source, followed by Gammaridea and Caridea. Mysidae and Annelida would not be essential dietary components.

13. Breeding did not have a significant effect on the isotopic composition of seahorses, suggesting a similar feeding pattern along the year.

14. Among the three seahorse wild populations studied, spatial differences in diet were specifically related to Gammaridea and Caridea; the contribution of Gammaridea to the diet was greater in Toralla and Bueu, whereas Caridea were highly consumed in Ribeira. These findings are very likely supported by differences in the habitat characteristics of the studied sites.

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