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Evaluation and effective use of artificial structures in seahorse habitat recovery

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Evaluation and effective use of artificial structures in seahorse habitat recovery

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DECLARAÇÃO DE AUTORIA DE TRABALHO

Evaluation and effective use of artificial structures in seahorse habitat recovery

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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ABSTRACT

With an increase in anthropogenic activities around the world, negative impacts affecting many species and the environment have also been rising. Greater attention has been given to the global rehabilitation of degraded habitats. Two seahorse species, the short-snouted seahorse (*Hippocampus hippocampus*) and the long-snouted seahorse (*H. guttulatus*), inhabit the shallow waters of the ria Formosa lagoon and have shown a substantial decrease in number due to rising anthropogenic impacts and their inherent vulnerability. This study consisted of two main objectives. The first was to design, test and select, under controlled conditions, a new Artificial Holdfast Unit (AHU) to be used in the wild. The second, was to deploy the selected AHU in the lagoon, in an attempt to contribute to the rehabilitation of the seahorses degraded habitats and help restore their numbers to earlier estimates. The three AHUs tested in the first study differed in material and shape. The first AHU used polyethylene strips attached to a mesh base; the second, used sisal rope attached to a mesh base; the third, consisted of an open cube made of welded iron. The sisal rope was later tested against an enclosed iron cube in the presence of two seahorse predators: the green crab and cuttlefish (*Carcinus maenas* and *Sepia officinalis*). 100 replicas of the selected AHU were constructed and consequently deployed in a selected Marine Protected Area (MPA) in the ria Formosa. Results showed that the deployed AHUs maintained their structural form and were able to offer a stable habitat for the seahorses in the lagoon. A significant difference was observed between the sisal rope and the other structures being tested, with more than 75% of the seahorses, choosing the natural fibre. After the deployment of the AHUs in the ria Formosa, the abundance of *H. guttulatus* continued to be significantly higher than *H. hippocampus* (P=0.04) and significant differences were found between the abundance of seahorses on the three monitorings (P=0.878). This study demonstrated that even in the presence of previously successfully tested polyethylene AHUs, seahorses preferred the newly designed sisal rope structure. The natural fibres of this AHU were also preferred by the seahorses when common predators were present and maintained its structure efficiently when deployed in the ria Formosa.

Keywords: *H. hippocampus*, *H. guttulatus*, artificial structures, natural fibre, habitat restoration

RESUMO

Com o aumento das atividades antrópicas em todo o mundo, os impactos negativos que afetam muitas das espécies e o seu meio ambiente, também aumentaram. A contínua sobre-exploração das áreas de maior produtividade tem levado ao aumento da poluição, degradação do habitat e perda da biodiversidade. As áreas marinhas costeiras, incluindo estuários e lagoas costeiras, são por isso alguns dos ecossistemas mais vulneráveis e afetados por estas atividades e onde em muitos desses locais, os cavalos-marinhos são parte integrante desses ecossistemas. Por via da contínua degradação ambiental, tem sido dada uma maior atenção à reabilitação global desses habitats. Os cavalos marinhos têm características morfológicas únicas, caracterizando-se por terem baixa mobilidade, distribuição dispersa, baixa fecundidade, cuidados parentais prolongados e uma elevada fidelidade aos seus habitats. Por via dessas características, e por viverem em zonas costeiras de baixa profundidade, os cavalos-marinhos são globalmente suscetíveis a atividades antropogénicas, incluido a degradação ambiental, sobre-pesca, captura acessória em artes de pesca, e captura para o comércio ilegal de animais selvagens (principalmente para a medicina tradicional chinesa) e poluição aquática. As duas espécies europeias de cavalos-marinhos, o cavalo marinho de focinho curto (*Hippocampus hippocampus*) e o cavalo marinho de focinho comprido (*H. guttulatus*), ocorrem na ria Formosa onde a sua abundância tem sofrido uma diminuição substancial devido aos crescentes impactos antropogénicos que ai se verificam. No início dos anos 2000, a ria Formosa albergava uma enorme população de cavalos marinhos, reportada como sendo a mais elevada no mundo, no entanto, apenas alguns anos volvidos, foi constatado um declínio massivo, com uma diminuição na abundância destas espécies de 94% para o *H. guttulatus* e de 73% para o *H. hippocampus*. Foram identificadas várias causas de origem antropogénica, e que no seu conjunto foram tidas como sendo responsáveis por esse declínio. No entanto, identificou-se como maior ameaça e como causa prevalente a degradação dos habitats. Presentemente, as populações de cavalos marinhos na ria Formosa ainda se mantêm em níveis de abundância baixos, por via dos fatores antropogénicos acima mencionados, e que ainda ocorrem. Assim, este estudo enquadrou dois objetivos principais, onde o primeiro, foi projetar e testar sob condições controladas, novas unidades artificias de abrigo (UAAs), e mediante os resultados obtidos, selecionar uma, que melhor se adaptasse a ser utilizada em ambiente natural. O segundo, foi proceder à colocação da UAA selecionada em ambiente natural, numa tentativa de contribuir para a reabilitação dos habitats degradados de cavalos-marinhos, de forma a ajudar à sua reabilitação e contribuir para a recuperação destas espécies. Na primeira

experiência, foram testadas três UAAs diferentes, quer em termos de materiais, quer de forma. Na primeira UAA (S1) foram utilizadas cordas de polietileno presas a uma base de malha de arame; na segunda (S2), foi utilizada corda de sisal presa a uma mesma base de malha de arame; e uma terceira (S3), que consistia num cubo aberto de ferro soldado com corda de sisal mais finas, como abrigo interno. Os resultados obtidos, demonstraram que mesmo na presença da UAA (S1), a qual tinha já sido testada com sucesso em estudos anteriores, os cavalos-marinhos observados preferiram a nova estrutura S2 composta por corda de sisal. Verificou-se que uma percentagem significativamente maior ($p < 0,05$) (81,3%) dos cavalos-marinhos preferiu a UAA S2, enquanto que as UAAs S1 e S3 mostraram ter uma preferência muito inferior e semelhante (6,8% para ambas as UAAs). A UAA S2 foi posteriormente comparada com uma versão alterada da UAA S3, com a colocação de uma rede de malha larga (10cm) para avaliar se uma melhoria no design da UAA S3 modificaria a preferência de escolha dos cavalos-marinhos em observação na presença, e ausência de potenciais predadores, o caranguejo verde (*Carcinus maenas*) e o choco (*Sepia officinalis*) (testados separadamente). A UAA S2 foi novamente preferida pelos cavalos-marinhos, quer na ausência de potenciais predadores quer na sua presença, avaliada individualmente. A UAA S2 foi preferida por 78%, 82% e 75% dos cavalos-marinhos observados, valores que revelaram não ser significativamente diferentes ($p > 0,05$) entre os ensaios. Numa segunda fase do estudo, e com base nos resultados anteriores, 100 réplicas da UAA S2 (1×1 mt cada) foram construídas e posteriormente colocadas numa área selecionada dentro do perímetro de uma das duas Áreas Marinhas Protegidas (AMPs) agora existentes na ria Formosa e onde anteriormente existia apenas uma cobertura de fundo escassa (<10% cobertura). As UAAs foram colocadas num padrão pré-definido resultando numa área de cobertura de 100 m². Os resultados mostraram que as UAAs implantadas mantiveram a sua forma estrutural e foram capazes de providenciar um habitat estável para as duas espécies de cavalos-marinhos. As novas UAAs levaram a um aumento significativo ($p < 0,05$) na abundância de cavalos-marinhos de 0,05 animais por m², antes da colocação das UAAs, para 0,07 animais por m² após sua implantação. Também foi observado que em condições naturais, a abundância de *H. guttulatus* continuou a ser significativamente maior do que *H. hippocampus* ($P = 0,04$) e que a percentagem geral de re-avistamento foi, no entanto, baixa, pois apenas 4,3% dos animais foram re-avistados nos dois momentos de amostragem efetuados. Numa primeira fase deste estudo, verificou-se que mesmo na presença de UAAs de polietileno previamente testadas com sucesso, os cavalos-marinhos observados preferiram as novas estruturas de fibras naturais, e que essa preferência prevaleceu mesmo na presença de potenciais predadores, sendo esses fatores, claros indicadores da sua adequabilidade em programa de requalificação que

requeiram a sua utilização. Globalmente, os resultados obtidos mostram uma melhoria no desenho das UAAs a serem utilizadas em condições naturais para a recuperação de habitats naturais dos cavalos marinhos na ria Formosa, uma vez que a sua utilização conduziu a um aumento da sua abundância. A eficácia destas UAAs, ajudou a providenciar um habitat adequado para estas espécies e outras que com elas coabitam, podendo por isso, a sua utilização vir a ser considerada em futuras ações de conservação.

Palavras-chave: *H. hippocampus*, *H. guttulatus*, ria Formosa, unidades artificias de abrigo, degradação ambiental

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LIST OF ABBREVIATIONS

AHU- Artificial Holdfast Unit
MPA – Marine Protected Area

CHAPTER 1: GENERAL INTRODUCTION

In the last 20 years, considerable attention has been given to the regeneration of degraded habitats and the effects of anthropogenic activities on natural ecosystems (Curtis *et al*., 2007). Tourism, coastal development, aquaculture and fishing are just some of the pressures which are leading to habitat degradation as well as the alteration of the species habitat structure (Correia *et al*., 2018; Curtis *et al*., 2007). The major cause for species extinction worldwide is in fact considered to be habitat destruction (Pimm and Raven, 2000), this plays a considerable role in the abundance of marine species (Cunha *et al*., 2013).

Marine coastal areas such as estuaries and lagoons, are some of the most vulnerable ecosystems affected by anthropogenic activities. The continuous over exploitation of these areas has led to increased pollution, habitat degradation and biodiversity loss. (Hughes *et al*., 2003; Lotze and Milewski, 2004). For this reason, a number of studies have been carried out in order to discover the best solution for effective habitat recovery in particularly threatened environments around the world (Alongi, 2002; Cunha *et al*., 2013; Nyström *et al*., 2000; Waycott *et al*., 2009).

Currently, there are 46 recognized species of seahorses (IUCN, 2021) all included in the *Hippocampu*s genus of the Syngnathidae family, which includes more than 230 living species being grouped with pipefishes, pipehorses and seadragons.

Seahorses have unique morphological characteristics, inhabit shallow temperate and tropical waters and can be found among corals, algae and seagrasses as well as open sandy bottoms (Lourie *et al*., 2004). Seahorse populations are under threat worldwide due to by-catch, over-exploitation, illegal catch and habitat degradation (Vincent, 1996).

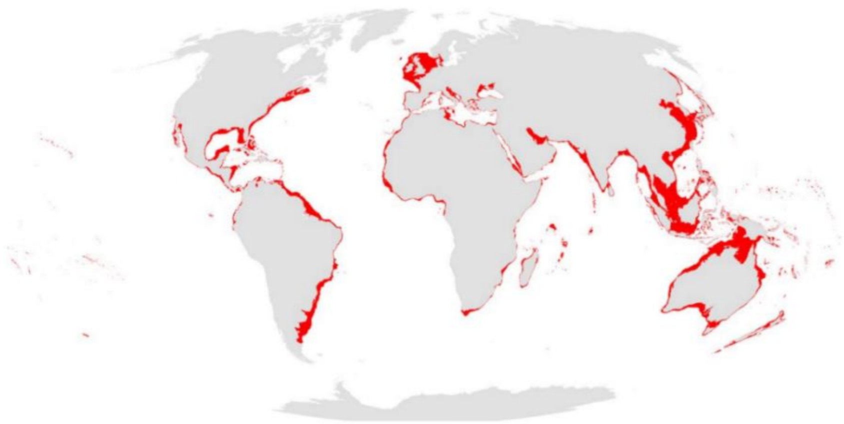

Figure 1.1 Worldwide distribution of seahorse population (Zhang and Vincent, 2018).

The basal morphotype for Syngnathidae is an elongated body, positioned horizontally in the water column when swimming. The seahorse morphotype is similar, except that they position their body vertically, their head has a tilted position with respect to the body and they have a prehensile tail (Teske and Beheregaray, 2009)

Seahorses differentiate themselves by their distinct morphology, which originated in the Early Oligocene, 34 million years ago after the collision of the Australian and the Eurasian plate. Geological changes led to the lowering of sea levels and formation of shallow-water habitats with seagrass beds, where seahorses may have developed their particular upright posture (Hall, 1998; Wilson, 2013). By positioning their body this way, seahorses would have had more ease of manoeuvrability and camouflage opportunities within the seagrass (Teske and Beheregaray, 2009).

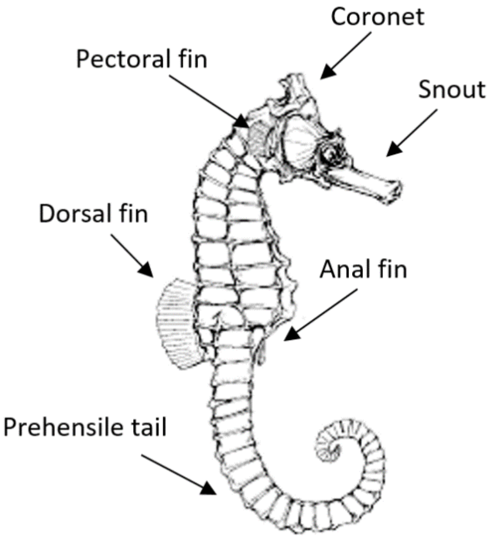

Figure 1.2 Basic seahorse morphology.

As well as numerous other species, seahorses play an important ecological role in the correct functioning of the ecosystem (Foster and Vincent, 2004). They make use of their grasping finless tail (Lourie *et al*., 2004) as a means of holding onto different holdfasts from seagrasses and macroalgae, to corals and artificial structures (Correia *et al*., 2015). In this way, they are able to remain static under strong tidal and ocean currents, protect themselves from predators as well as avoid being swept away, since they are known to have poor swimming abilities (Foster and Vincent, 2004).

Seahorses are characterized by low-mobility, sparse distribution, low-fecundity, lengthy parental care and high site-fidelity (Foster and Vincent, 2004). Due to these attributes and because they inhabit shallow coastal waters, seahorses around the world are particularly susceptible to anthropogenic activities such as habitat degradation, overfishing, by-catch, illegal wildlife trade (mostly traditional Chinese medicine) and ocean pollution (Bell *et al*., 2003; Foster and Vincent, 2004).

Of the 46 presently recognized seahorse species, only two can be found in the ria Formosa Natural Park in the South of Portugal (Figure 1.3): the long-snouted seahorse (*Hippocampus guttulatus*) and the short-snouted seahorse (*H. hippocampus*) (Boisseau and Lemenn, 1967; Correia *et al*., 2018; Lourie *et al*., 1999). These species contribute to the natural equilibrium needed in this coastal ecosystem (Gamito, 2008) and can be recognized by their snout size, abdomen, skin colouration and adult body size (Curtis and Vincent, 2006). In fact, *H. guttulatus* has been reported to have a total length of 23cm, much longer than *H. hippocampus,* which generally reaches 15cm (Lourie *et al*., 1999). The two species have overlapping areas of distribution in the shallow estuarine lagoon of the ria Formosa. However, they have shown to have slightly distinct habitat preferences with *H. guttulatus* being found in shallow waters and high habitat complexity, whilst *H. hippocampus* is found in deeper waters with low holdfast availability and in less complex habitats (Correia, 2015; Curtis and Vincent 2005; Gristina *et al*. 2015; Woodall *et al*. 2018).

Figure 1.3 The two seahorse species inhabiting the ria Formosa. *Hippocampus guttulatus* (left) and *Hippocampus hippocampus* (right).

In the early 2000’s, the ria Formosa supported a very dense population of seahorses (Curtis and Vincent, 2005), however, just a few years later, Caldwell and Vincent (2012), reported a massive decline with a 94% decrease in *H. guttulatus* and a 73% decrease in *H. hippocampus*. Several anthropogenic causes were identified as being responsible for this massive decline, with the greatest threat being habitat degradation (Foster and Vincent, 2004).

The ria Formosa lagoon is a highly productive ecosystem, which supports a high abundance of marine life (Erzini *et al*., 2002) and behaves as a nursery for numerous species (Newton *et al*., 2003). Located in the South of Portugal and extending for 55km in length (Gamito and Erzini, 2005), the ria Formosa is a system of saltmarshes and mudflats which are separated from the ocean by an island system acting as barriers (Ceia *et al*., 2010). It has an average depth of 3m, a temperature that varies between 12 ˚C and 28˚C, where only 14% of the lagoon is always submerged (Newton *et al*., 2003; Ribeiro *et al*., 2006).

Figure 1.4 Map of the Iberian Peninsula, showing the location of the ria Formosa, in the South of Portugal.

Most lagoons are subject to natural disturbances as well as human exploitation (Gamito, 2008). In recent years, increasing levels of anthropogenic activities have accelerated the loss of seagrass beds in the ria Formosa (Guimarães *et al*., 2012), thereby affecting species distribution and density. Seagrasses, such as *Zostera noltii* in the ria Formosa, may be considered a keystone habitat, where the highest species richness is usually found (Heck and Valentine, 2006). The 75% reduction in the distribution of *Z. noltii* in the last 20 years, is recognized as being a serious threat to the *Hippocampus* species (Harasti, 2016) and has led to a greater interest in the development of seagrass restoration and rehabilitation projects (Cunha and Serrão, 2009).

In addition to the aforementioned worldwide anthropogenic disturbances affecting marine ecosystems, the ria Formosa is burdened with economic activity: fisheries, boating, anchoring, tourism, aquaculture development and clam farming (Duarte, 2002; Almeida *et al*., 2008). The clam beds, for example, are established in the intertidal area. They are prepared by destroying the *Z. noltii* meadows and covering the area with terrestrial sediment (Amaral, 2008; Carvalho *et al*., 2006).

Following the destruction of vast areas of seagrass beds, Artificial Holdfast Units (AHUs) have been tested in different forms in many countries and have proven to be a beneficial tool for conservation (Correia *et al*., 2015; Curtis *et al*., 2007; Kenyon *et al*., 1999),

These experiments utilized structures such as simple swimming barrier nets (Hellyer *et al*., 2011), aluminium cages known as "Seahorse Hotels" (Simpson, 2020) and seagrass-like holdfasts (Correia *et al*., 2013; Correia *et al*., 2015). Although both the "Seahorse Hotels" and the swimming barrier nets were successful in hosting the seahorses of their study areas (Hellyer *et al*., 2011; Simpson *et al*., 2020), these structures had no resemblance to the species usual natural habitats. In the ria Formosa, on the other hand, seagrass-like artificial holdfasts, designed to tolerate the hydrodynamic characteristics of the lagoon, have already been tested (Correia *et al*., 2013) and showed successful results in temporarily providing a suitable habitat for many different species.

Although AHUs are still considered a subject of debate (Hauser *et al*., 2006; Vega Fernandez *et al*., 2009), they have shown to be an effective way to replace the damaged natural ecosystem, provide a new habitat and maintain balance in the marine environment (Shahbudin *et al*., 2011). Artificial habitats have demonstrated a very high potential for being a powerful conservation tool for seahorses (Simpson *et al*., 2020) and it is therefore very important to keep widening knowledge to find the best version of these structures, which can support the affected species and expand their habitats. This study aimed at two main objectives. The first was to design, test and select, under controlled conditions, a new Artificial Holdfast Unit (AHU) to be used in the wild. The second, was to deploy the selected AHU in the lagoon, in an attempt to contribute to the rehabilitation of the seahorses degraded habitats and help restore their numbers to earlier estimates.

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CHAPTER 2:

DESIGN EVALUATION AND USEFULNESS OF ARTIFICIAL STRUCTURES IN THE REHABILITATION OF SEAHORSE DEGRADED HABITATS

2.1 Abstract

Increasing worldwide anthropogenic activities in aquatic ecosystems are leading to habitat loss and consequent decline of aquatic species including the seahorses. One species inhabiting this area is the *Hippocampus hippocampus,* which has unfortunately shown a decrease in the last decades due to numerous anthropogenic impacts. Illegal bottom trawling and dredging activities are two of many which have influenced the rate of habitat degradation. It has been suggested that artificial structures may be an important tool for habitat enrichment in degraded habitats. In this study, the effectiveness of three different Artificial Holdfast Units (AHUs) were tested in two experiments in a controlled environment for the duration of 7 weeks. The three AHUs tested in the first experiment differed in material and shape. The first AHU used polyethylene strips attached to a mesh base; the second, used sisal rope attached to a mesh base; These both mimicked *Codium* spp. Algae present in the ria Formosa. The third, consisted of an open cube made of welded iron. In the second experiment, the sisal rope was tested against an enclosed iron cube in the presence of two common seahorse predators, the green crab (*Carcinus maenas*) and cuttlefish (*Sepia officinalis*), in order to analyse which structure they were inclined to select for anchorage and safety when under threat. A significant difference ($P<0.05$) was observed between the sisal rope and the other structures being tested, with more than 75% of the seahorses choosing the natural fibre. Past studies have shown that polyethylene AHUs were very successful in seahorse habitat restoration. This study demonstrated that even in the presence of previously successfully tested polyethylene AHUs, seahorses preferred the newly designed sisal rope structure. The seahorse preference to the AHU built with natural fibres also prevailed in the presence of predators. Overall, these results show an improvement of AHU design to be used in natural conditions.

Keywords: Artificial Holdfast Units, habitat restoration, holdfast preference, *H. hippocampus*, *H. guttulatus*

2.2 Introduction

Seahorses, as other marine fish species, are an integrant part of the marine biodiversity and ecosystem function (Foster and Vincent, 2004) and are as recognized flagship species (Shokri Gladstone and Jelbart, 2009). Their study and driven conclusions can be considered representative of their entire ecosystem and be used as a guideline for the creation of conservation and management programs for those ecosystems.

Seahorses are considered as so, due to their unique life-history characterized by a sparse distribution, low mobility, site fidelity, small home ranges, low fecundity, mate fidelity and lengthy parental care to small broods, which might render them vulnerable to anthropogenic impacts. These may include fishing, illegal wildlife trade, ocean pollution and habitat destruction through bottom trawling (Foster and Vincent, 2004; Newton *et al*., 2003). In addition, seahorses belong to the Syngnatidae family and inhabit shallow coastal areas worldwide, where anthropogenic impacts tend to be most frequent and severe (Bell *et al*., 2003). These constraints help to explain why 12 seahorse species are listed as 'Vulnerable', 2 as 'Endangered' on the 2021 IUCN Red List of Threatened Species, 17 as 'Data Deficient', one as "Near Threatened" and 10 as "Least Concern", which still reflects a substantial gap in knowledge of a heavily exploited fish group, such as seahorses.

Depending on where they are found, seahorses may have natural predators, and predation may somehow be minimized if they can inhabit well preserved habitats. With a degraded habitat, seahorses may be more exposed to predation therefore, it is important to test artificial AHUs that these species may be compelled to choose as shelter in the presence of predators.

The ria Formosa is a shallow estuarine lagoon located in the south of Portugal (Figure 2.1). It is a particularly biodiverse ecosystem, an important nursery ground for many commercial fish species and is inhabited by two species of seahorse: *Hippocampus guttulatus* and *H. hippocampus* (Correia *et al*., 2018).

Figure 2.1 The ria Formosa Natural Park, South of Portugal.

The ria Formosa lagoon, had a very dense population of seahorses in the early 2000's (Curtis and Vincent, 2005), in recent years however, there has been a massive decline with a 94% decrease in *H. guttulatus* and a 73% decrease in *H. hippocampus* (Caldwell and Vincent, 2012).

Anthropogenic activities have led to an extensive loss of seagrass habitats (Cunha *et al*., 2013), leading to a greater interest in the development of seagrass restoration and rehabilitation projects (Cunha *et al*., 2009). Seahorses make use of their distinctive morphology as a means of holding onto different holdfasts from seagrasses to mangrove roots, corals and artificial structures (Correia *et al*., 2015). The lack of natural holdfasts could be a major reason for the decrease in the number of seahorses in the ria (Correia *et al*., 2013), with a 75% reduction in the distribution of *Zostera noltii* in the last 20 years (Cunha *et al*., 2013).

Seahorses are flagship species and excellent indicators of the ecosystems health. For all the reasons aforementioned, a number of studies have been made regarding the beneficial role of artificial structures on different species of seahorses around the world. Artificial seagrass structures have been tested and used in many countries (such as South Africa, Portugal, Australia) (Claassens *et al*., 2018; Correia *et al*., 2013; Simpson *et al*., 2020), utilizing structures from simple swimming barrier nets (Hellyer *et al*., 2011), to aluminium cages (Simpson *et al*., 2020) and seagrass-like holdfasts (Correia *et al*., 2013).

Results showed that the artificial structures supported a large population of threatened seahorses (*H. whitei*) in areas where they had decreased due to habitat loss and also showed that breeding occurred in the "Seahorse Hotels" during the experiment (Simpson, 2020).

An artificial nylon structure had already been tested, based on natural preferences, and resulted successful in past experiments with *H. guttulatus* (Correia, 2013) in the ria Formosa. The study provided preliminary data and promising results on an approach to designing artificial holdfasts for seahorses in low complexity damaged or depleted areas (Correia, 2013).

Although still a controversial topic (Hauser *et al*., 2006; Vega Fernandez *et al*., 2009), artificial habitats have demonstrated to be a powerful conservation tool for seahorses (Simpson *et al*., 2020). They are also seen as a solution for the regeneration of degraded habitats, encouraging seahorse settlement and prosperity (Curtis *et al*., 2007). This study aimed to test different designs of Artificial Holdfast Units (AHUs) in order to discover the one preferred by the seahorses, with and without the presence of predators, and contribute to seahorse habitat recovery in the ria Formosa.

2.3 Material and Methods

Two different experiments, each consisting of 2 trials, were carried out throughout this study. The first experiment was performed to determine which AHU design was preferred by the seahorses. The second experiment tested if, when in need of greater protection in the presence of predators, the same AHU was preferred by the seahorses,

2.3.1 Artificial holdfast units (AHUs)

Three different types of Artificial Holdfast Units (AHUs) were assembled and tested. Each unit had a 50x50cm base but differed in shape and material (Figure 2.2). Structure 1 (S1) was composed of a wire base coated with plastic, to which 36 polyethylene strips (each with 40cm in length and 1.6 cm Ø) were attached vertically to the base in a 6x6 design. The inferior ends of the strips were melted in order to glue them to the base, whilst the superior ends were left loose. Structure 2 (S2) obeyed to a similar design, but natural fibre ropes (made of sisal with same length and diameter) were used instead. In these structures a piece of cork was then applied around the top end in order to avoid the expansion of the rope when submerged in water and assist with floatation; the lower end of the rope was attached to the base with a plastic cable tie. Structure 3 (S3) consisted of a 50x50 cm open cube, made of welded iron (0.5 cm Ø) with 4 sisal ropes crossing in the centre and knotted to the inside corners of the cube. The choice of the iron cube was based on structures called the “Seahorse Hotels”, aluminium cages enclosed by steel, which were tested in Australia (Simpson, 2020).

After the first experiment indicated that S2 and S3 were the preferred structures of the seahorses, the iron cube (S3), was slightly modified by being enclosed with a metal grid covered with polypropylene plastic (Figure S2.1). The 2 structures were once again tested in experiment 1 (trial 2), and later, also with the introduction of seahorse predators (*Carcinus maenas* and *Sepia officinalis*), in experiment 2 (trials 1 and 2).

Figure 2.2 A representation of the 3 initial AHUs tested. From left to right: S1, S2, S3.

2.3.2 Experimental design

During the study, a similar experiment design was used in all trials. Two replicate fiberglass tanks (9 m³ capacity and 2.5m bottom diameter) assembled in a flow-through system were utilized. The seahorses needed for each trial were selected and kept in a rearing tank before use. In each experiment, 10 seahorses (*H. hippocampus*) at a 1:1 gender ratio (5 males and 5 females) were assembled into each of the replicate tanks. Each seahorse was tagged using a soft braided fishing line necklace with a specific bead colour for individual recognition (Table S2.1). After the termination of each observational period, the seahorses were taken out of the tanks and the necklace tags were removed. Water quality parameters (oxygen level and temperature) were monitored each morning prior to the video recordings in order to ensure proper environmental quality for the seahorses; they were stable throughout the experimental period with an average of 94.3% dissolved O_2 and 19.5°C respectively. The general procedures used were identical for each of the 4 experiments (Figure S2.2), which are explained below. In all experiments, seahorses were daily fed live mysids (*Mesopodopsis slabberi*).

2.3.3 Experiment 1 - trial 1

A total of 40 seahorses were used and randomly selected for each of the 4 replicate tests carried out over a 14 day period. The experiment was performed using 2 replicate tanks (Tank A and B). 10 seahorses were placed in each tank together with the 3 structures (S1, S2, S3) positioned equidistant from each other. The experiment was carried out for 7 days. The same experiment design was then repeated for a further 7 days, in the 2 same tanks, with a new set of 20 seahorses.

2.3.4 Experiment 1- trial 2

A total of 40 seahorses, were used (10 per tank) and randomly selected for each of the 4 replicate tests carried out over two consecutive periods of 7 days. The experiment was performed using 2 replicate tanks according to the design described above. Two AHUs were tested: the sisal rope structure and the enclosed cube.

2.3.5 Experiment 2 - trial 1

A total of 20 seahorses were used (10 per tank at a 1:1 gender ratio) and randomly selected for each of the 2 replicate tests carried out over a period of 7 days, with two replicate tanks (A and B). The seahorses were placed in the controlled tanks with the same two structures as experiment 1 (trial 2). 4 green crabs (*C. maenas*) were individually placed into small open-top fully transparent plexiglass tanks. The daily placement and removal of the crabs enabled to capture the seahorses' reaction when a predator was introduced.

2.3.6 Experiment 2 - trial 2

A total of 20 seahorses were used (10 per tank at a 1:1 gender ratio) and randomly selected for each of the 2 replicate tests carried out over a period of 7 days, with one tank available. The seahorses were placed in a controlled tank with the same two structures as experiment 1 (trail 2) and experiment 2 (trial 1), along with 4 cuttlefish (*S. officinalis*). Each cuttlefish was placed in the same open-top transparent plexiglass tanks, this time covered by 1 cm Ø mesh to present the predators escape and then lowered into the tanks. Seahorses were fed with live mysids and cuttlefish were fed with shrimp every other day. The same 20 seahorses used in the first trial of experiment 2, were used again for the second trial. By re-utilising the same seahorses, the reaction of individuals to different predators could also be noted.

2.3.7 Camera recordings

Each morning a short-term recording was made to capture the position of each individual seahorse in the tank. A long-term fixed camera was then placed in the tank for 1 hour. The seahorses were then fed at the start of the recording period in order to check if the feeding behaviour induced any changes in the AHU use. At the end of the hour, another short-term recording was made to capture the position of each individual which may have changed during that period.

The same pattern of video recordings was made every day for all the 49 days of the duration of the experiments in the controlled tanks. To obtain the best results, videos were recorded daily, at the same time. Videos were later analysed to track individual seahorse activity during the observational period to evaluate seahorse displacement during the previous non-recorded time frame.

2.3.8 Statistical analysis

Seahorse holdfast preference in experiment 1 (trial 2) and experiment 2 (trial 1 and 2), was compared using a one-way ANOVA. Tukey's post-hoc test was consequently used to identify whether there were any differences in the use of the structures and if so, which ones.

2.4 Results

2.4.1 Holdfast preference experiment 1 trial 1

The holdfast preference in each of the 4 replicate observations for the first trial of experiment 1, were thoroughly analysed. A significant difference was found in the holdfast preference ($p>0.05$). Through the combined video analysis (short and long term), it was therefore possible to identify the holdfast preference of all seahorses under observation. Seahorses showed a distinct preference for the sisal rope (S2) with 81.25% choosing this holdfast, with the other options showing a much lower and similar preference (Figure 2.3).

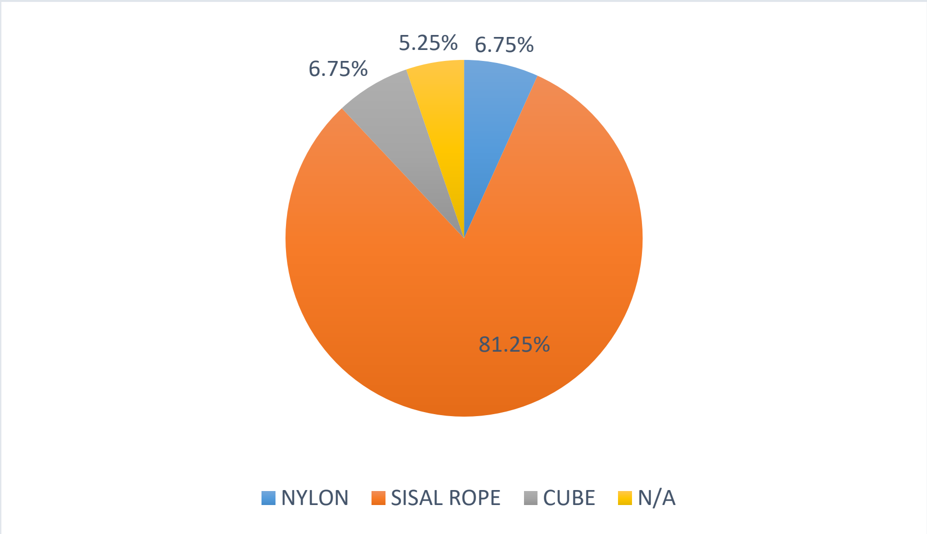

Figure 2.3 Seahorse holdfast preference (%) for experiment 1 trial 1. (S1-nylon rope, S2-sisal rope, S3- iron cube, S4-not attached).

2.4.2 Seahorse individual movement

Tags with different colour combinations were used to distinguish each seahorse (e.g. Pink and Red, Pink and Green) and those can be seen as abbreviations in the X axis (e.g. PR, PG) (Figure 2.4). To visually represent the seahorse preference, each tested structure was given a different colour in order to make the seahorse preferences more visual (Figure 2.4). When a seahorse was not attached to any of the three structures during the short-term video, it was classified as not attached (N/A). Although most seahorses chose the sisal rope structure, different types of movements were observed. In Figure 2.4 c, the Pink and Red seahorse (PR) as well as the Pink and Dark Blue (PDB) seahorse in Figure 2.4 d, showed almost continuous movement between all the structures in the tank. On the contrary, in Figure 2.4 d, four individual seahorses (PR, PG, PLB, YLB) only ever chose the rope structure throughout the 7 days of the experiment.

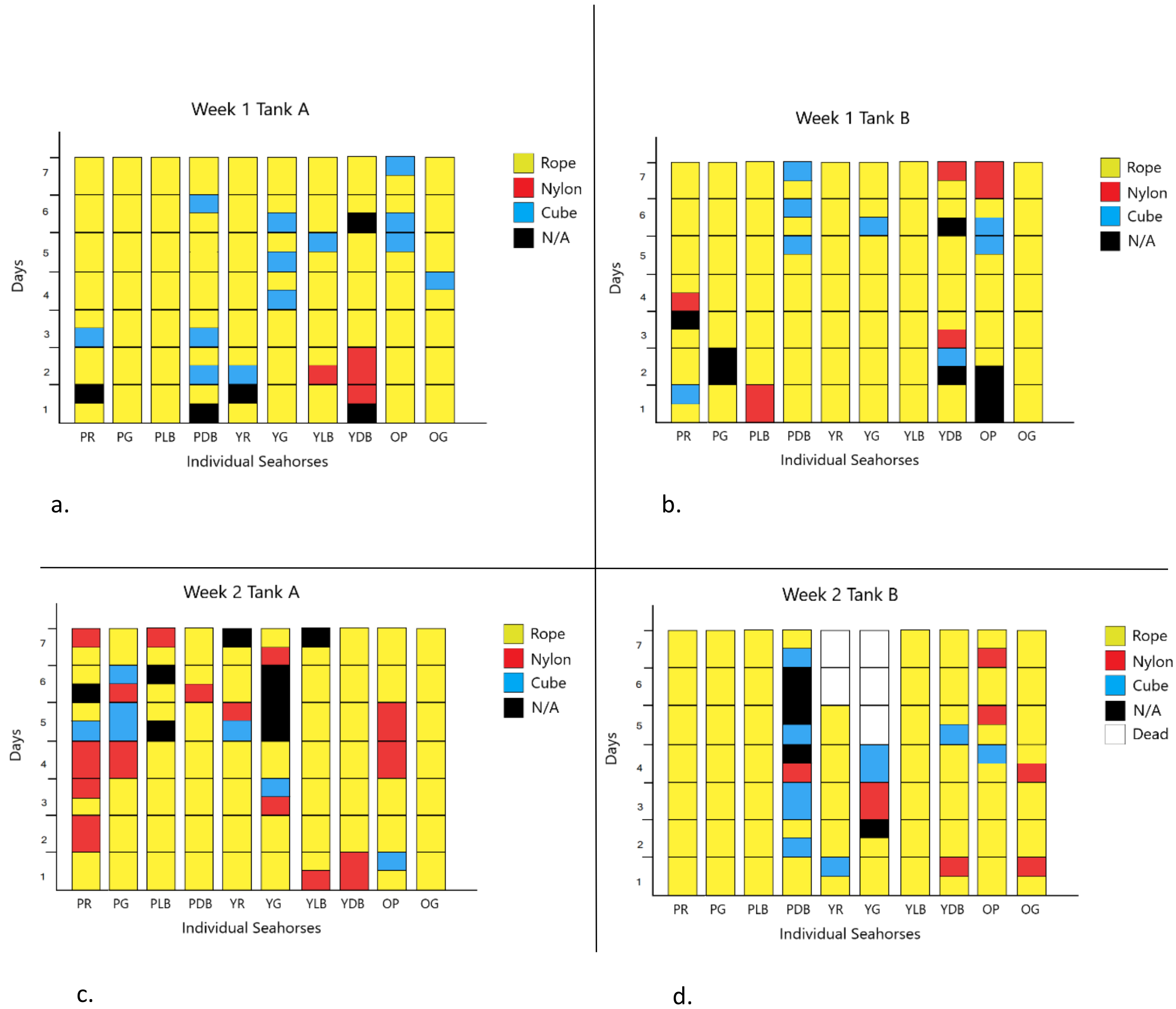

Figure 2.4 (a., b., c., d.). Visual representation of individual seahorse movement over the 7 days of experiment 1 trial 1. Colour codes shown on the X axis.

The observed seahorses could have spent their 7 days moving and settling on a combination of structures. In this experiment 27.5% of seahorses settled on S2 only, 10% on S1+S2, 22.5% on S2+S3 and 32.5% S1+S2+S3. This represents an evident preference to the S2 (sisal rope) structure and some inherent movement of the seahorses to less preferred structures. A one-way ANOVA showed that there was a significant difference between the 3 tested structures.

2.4.3 Gender holdfast preference

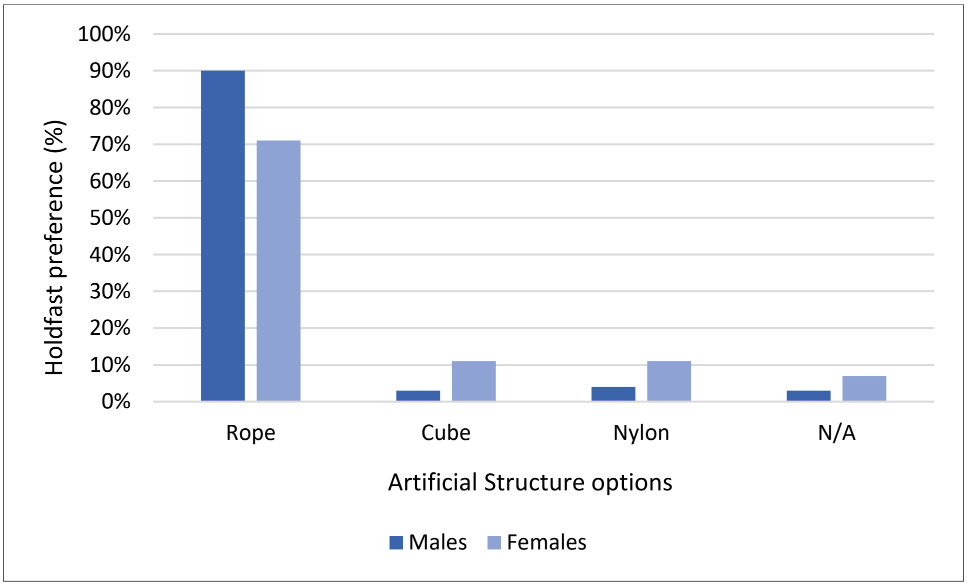

Figure 2.5: Holdfast preference (%) by gender in experiment 1 trial 1.

The holdfast preference was also analysed by looking at the genders. Figure 2.5 shows that 90% of males preferred the sisal rope against 71% of females. The females seemed to overall distribute themselves also on the other two available structures as well as often swimming around the tank. It may also be noted that 11% of females preferred the iron cube against only 3% of the males choosing this structure. Through statistical analysis, using a two-way ANOVA, statistical difference was observed in holdfast preference between genders ($p>0.05$).

2.4.4 Final average holdfast preference

Average holdfast preference for experiment 1 (trial 2) and experiment 2 (trial 1 and 2) is represented in the figure below (Figure 2.6), where the preference for the sisal rope structure varied from 78% to 82% and 75%. When also compared with the 81.3% sisal rope preference obtained from the first trial of experiment 1, no significant differences ($p<0.05$) were observed.

The percentage of seahorses choosing the iron cube structure ranged between 9 and 19% and those not attached to any of the three tested AHUs ranged between 5 and 9%. Using Tukey's test, a significant difference was seen between the sisal rope and the cube.

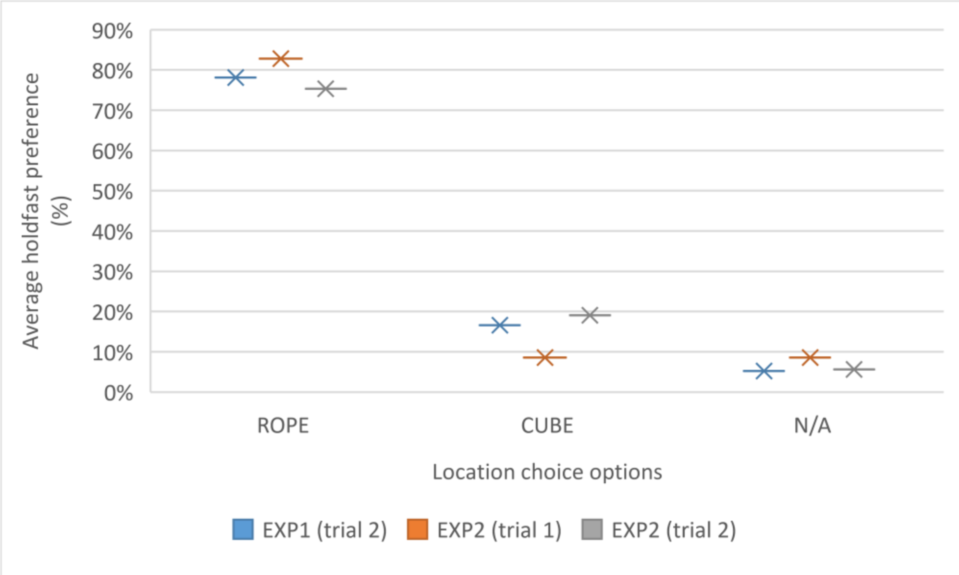

Figure 2.6 Average holdfast preference for seahorses in experiment 1 trial 2 and experiment 2 (trials 1 and 2).

2.5 Discussion

The seahorses' need for a holdfast or any other kind of structure to hold onto is very important for their survival in the wild (Newton *et al*., 2003). Seahorses in the ria Formosa have been seen to utilize both macroalgae (*Codium* spp.) and seagrasses (*Z. noltii*, *Z. marina*, *Cymodocea nodosa*) as means to hold onto, in order to remain static under strong currents (Correia *et al*., 2015; Cunha *et al*., 2009). Natural changes in the ria's dynamics as well as anthropogenic activities such as dredging and bottom trawling (Correia *et al*., 2013), may be leading to habitat loss (Curtis *et al*., 2007). *H. hippocampus*, which showed lower demand for complex habitats compared to *H. guttulatus* (Correia *et al*., 2015), was used in controlled tanks to test the versatility of different Artificial Holdfast Units (AHUs) differing in shape, size and material.

Finding the best way to study animal behaviour without causing disturbance has always been a challenge. Small underwater cameras were chosen to be used in this study after being proved to be a reliable way to undertake seahorse behavioural assessments (Claassens and Hodgson, 2018) without affecting their behaviour. The artificial structures designed for this experiment were based on a number of different factors including camouflage, predation, protection, stability and seahorse preference. Artificial structures used in Australia, Portugal and South

Africa (Correia *et al*., 2013; Claassens *et al*., 2018; Simpson *et al*., 2020), under different circumstances and with different seahorse species, were a source of inspiration for the structures selected in this study.

The nylon structure had already been tested, based on natural preferences, and resulted successful in past experiments with *H. guttulatus* (Correia *et al*., 2013; Correia *et al*., 2015) and both *H. guttulatus* and *H. hippocampus* (Correia *et al*., 2015). The structure was designed to mimic macroalgae species where they were seen to be grasping on. This AHU was seen to be more stable, enabling successful camouflage, hunting and protection from prey (Correia *et al*., 2013). It was therefore utilized in this study as a form of comparison to another relatively similar structure (S2).

The sisal rope mimicked the *Codium* spp. macroalgae present in the ria Formosa lagoon. The vertical holdfasts, attached to a base whilst moving in the water column, simulated natural seagrass blades whilst also helping decrease bottom currents (Correia *et al*., 2015). The thickness of the sisal rope seemed to provide a dense and stable shelter for the seahorses, giving them a major sense of protection; most seahorses in the experiment, in fact, showed a much greater preference for this structure. The major and most relevant difference between S1 and S2 was in their materials. They were both relatively soft holdfasts but S1 was made with artificial materials whilst S2 was made with natural fibre. The latter was chosen to be tested in order to be more ecological and find the most sustainable and environment-friendly solution for the seahorses and the natural environment as a whole. Moreover, to our knowledge, it appears to be the first time that natural fibres were being tested for their effectiveness in substituting a natural habitat for seahorses in the ria Formosa.

The choice of the iron cube was based on structures called the "Seahorse Hotels", tested by Simpson *et al*. (2020), aluminium cages enclosed by steel, which were tested in Australia (Simpson *et al*., 2020). Results showed that the structures supported a large population of threatened seahorses (*H. whitei*) in areas where they had decreased due to habitat loss and also showed that breeding occurred in the "Seahorse Hotels" during the experiment (Simpson *et al*., 2020). Although different, in this experiment some seahorses showed a form of interest in the iron cube, possibly, seeing it as a potential habitat. Simpsons' "Seahorse Hotels" demonstrated that seahorse *H. whitei* will inhabit artificial structures if in lack of a natural habitat (Simpson *et al*., 2020), however, they differed slightly in material, shape and size from the Iron cubes which were used in this experiment. The lack or presence of an external covering, the possibility of choosing another artificial structure and being a different species, may have influenced the seahorses' preferences shown over the course of the experiments.

To the present, seahorse natural predation at adult stages was not clearly identified in the ria Formosa. It is however arguable that predation exists in the lagoon, thus some of the potential seahorses' main predators were introduced in the controlled tanks for the second experiment (trials 1 and 2). Predation risks can affect species behaviours, abilities and morphologies over time (Kleiber *et al*., 2011). All species of the Syngnathidae family have evolved characteristics that help them to hide from predators: in fact, they can camouflage by blending in and matching their body colour to that of their surroundings, or by imitating the shape of other organisms (Livingston., K., 1986; Ruxton *et al*., 2004). Seahorses also present morphological characteristics such as *H. guttulatus*, being characterized by bony plates with additional spines (Lourie, 2004), making them quite difficult to swallow (Harris *et al*., 2008). Seahorses have been found in the stomachs of large pelagic fish (Jordan and Gilbert, 1882) as well as a loggerhead sea turtle (Burke *et al*., 1993). However, among 135 reports of predation on Sygnathids, it was demonstrated that more than 50% of predators were shown to be Teleosts followed by seabirds (Kleiber *et al*., 2011). High predation was seen to coincide with high densities of Sygnathids, suggesting that most of the predators are opportunistic feeders (Kleiber *et al*., 2011).

Regardless of the introduction of both green crabs and cuttlefish in the controlled tanks, the seahorses did not show any specific behaviour other than showing a continued preference for the sisal rope (S2) instead of the modified "Seahorse Hotels" cube structure (S3). This preference can be supported by experiment 1 as well as previous studies (Correia *et al*., 2013), which demonstrated the "*Codium*-like" structure to be the most stable one, able to manage the hydro dynamicity of the Ria Formosa and give the seahorses the highest feeling of protection.

With experiment 1 testing the best structure for the seahorses, the sisal rope continued to be utilized in the subsequent experiments and the seahorses continued to show a significant preference for this natural fibre. They were seen to attach most frequently to the lower section of the rope, as shown in the past with *H. guttulatus* (Correia *et al*., 2013), where a denser habitat was available and preferable. Although the final and major choice of the seahorses seemed to fall onto the Sisal rope (S2), the long-term videos did show that they were continuously moving between all three structures suggesting a possible holdfast versatility for *H. hippocampus*. With the introduction of predators, seahorses once again showed preference for the sisal rope structure, which obviously can provide that feeling of protection and security to this species.

2.6 Conclusion

It is important to keep in mind that although *H. hippocampus* has an overlapping area of distribution with *H. guttulatus* and that the two species have similar life histories, their habitat use and preferred choices have shown to differ due to differences in their morphology (Curtis and Vincent, 2005). The former has shown to be found in shallow waters rich with holdfasts, whilst the latter has shown to be found in deeper and rather bare waters (Correia *et al*., 2015; Curtis and Vincent, 2005; Gristina *et al*, 2015; Woodall *et al*, 2018;). This study showed that *H. hippocampus* may be able to survive on newly introduced holdfasts and significantly preferred the artificial holdfast unit made out of natural fibre (sisal rope – S2) rather than the previously tested artificial nylon structures. In conclusion, with natural habitats in continuous decline and seahorses being so vulnerable due to their low mobility, low fecundity and sparse distributions (Foster and Vincent, 2004), the need for novel solutions to support threatened species worldwide is important and needs to be brought to attention (Lai *et al*., 2015; Travis, 2003). This study provided significant results and confirmation that the sisal rope Artificial Holdfast Unit can be deployed and further tested in the ria Formosa Natural Park, in order to support depleted and underpopulated areas, as part of a wider restoration strategy.

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2.8 Supplementary material

2.8.1 Figures

Figure S. 2.1 The modified and final version of S3.

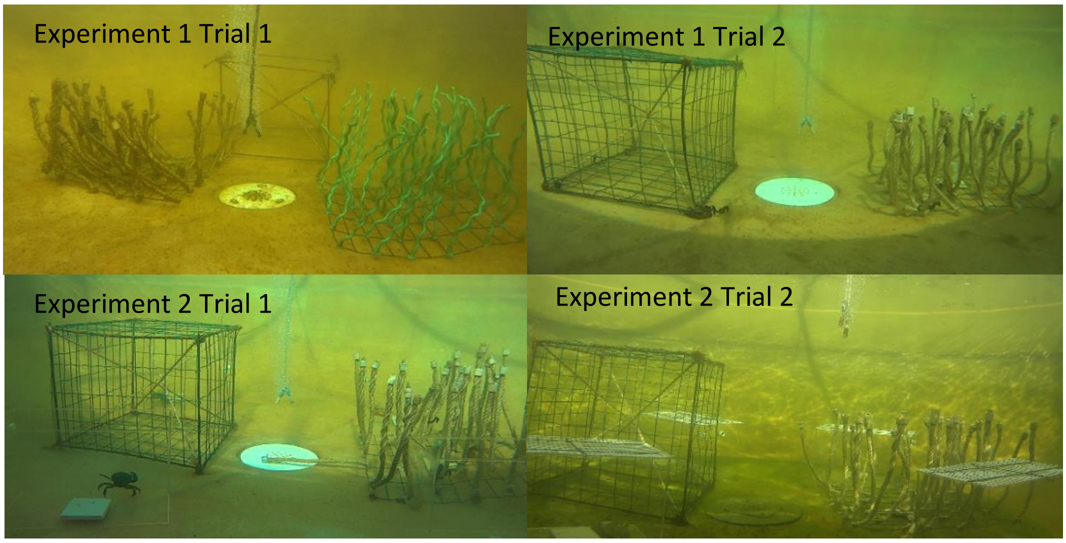

Figure S 2.2 Underwater view of experiments 1 and 2.

2.8.2 Tables

Table S2.1 The specific bead colours given to the relative sex in all 4 experiments.

Yellow & Red	Male
Yellow & Green	Female
Yellow & Light-blue	Male
Yellow & Dark-blue	Female
Pink & Red	Female
Pink & Green	Male
Pink & Light-blue	Male
Pink & Dark-blue	Female
Orange & Green	Male
Orange & Pink	Female

CHAPTER 3:

EVALUATION OF SEAHORSE HABITAT REQUALIFICATION USING NATURAL FIBRE HOLDFAST UNITS IN THE RIA FORMOSA

3.1 Abstract

Increasing anthropogenic activities in the ria Formosa are leading to habitat loss and consequent decline of the population of seahorses (*Hippocampus hippocampus* and *H. guttulatus*). Illegal bottom trawling, coastal construction and dredging activities are some of many, which have influenced the rate of habitat degradation in the area. Previously tested Artificial Holdfast Units (AHUs) have resulted in finding an effective artificial holdfast for one of the species of interest (*H. hippocampus*). With an increasing necessity for habitat restoration and seahorse conservation, 100 AHUs, made from natural sisal ropes attached to a 1x1m (1m²) mesh base, were deployed in a Marine Protected Area (MPA) in the ria Formosa lagoon. The structures were placed in a pre-planned pattern over an area of 100 m². Results showed that the new AHUs were successful and led to a noticeable rise in the number of seahorses. Their abundance per m² increased from 0.05, in absence of the AHUs, to 0.07 after their deployment in the ria. Significant differences were found between the seahorses' abundance on the different dives (P=0.878) and the recapture percentage was 4.3%, with the abundance of *H. guttulatus* being significantly higher than *H. hippocampus* (P=0.04). In this study, where natural fibre structures were for the first time tested for their effectiveness in substituting a natural habitat for seahorses in the ria Formosa, their use led to an increase in seahorse abundance. The effectiveness of the sisal rope AHU, which retained its structural form, helped to provide a suitable habitat for the seahorses in the ria Formosa and are therefore deemed to be applicable in future conservation actions.

Keywords: *H. hippocampus*, *H. guttulatus*, habitat restoration, seahorse conservation

3.2 Introduction

Seahorses comprise one genus (*Hippocampus*) of the Sygnathidae family. They make up an important part in the natural flow of the marine biodiversity and ecosystem (Foster and Vincent, 2004). Their sparse distribution, low mobility and site fidelity are just some of the unique characteristics that may make them more exposed and vulnerable to all kinds of natural and anthropogenic impacts (Foster and Vincent, 2004). By inhabiting shallow coastal areas, they are in fact particularly affected by overfishing, habitat degradation, bycatch and illegal wildlife trade (Foster and Vincent, 2004; Bell *et al*., 2003)

The ria Formosa, in the South of Portugal, is a shallow estuarine lagoon that extends 55km in length (Gamito and Erzini, 2005) and has a highly productive ecosystem (Ribeiro *et al*., 2006). As most coastal lagoons do, the ria Formosa plays an important ecological role for coastal zone ecosystems (Clark, 1998) but the coastal development and periodic dredging to maintain navigation channels have, over the years, been destroying the natural habitat (Cunha *et al*., 2013).

The aforementioned factors have led to the disappearance of vast areas of seagrass beds, therefore affecting the distribution and density of marine species in the area (Guimaraes *et al*., 2012). In the last 20 years in the ria Formosa, *Zostera noltii* has shown a reduction of 75% (Cunha *et al*., 2013) and *H. hippocampus* and *H. guttulatus* have shown a decrease of respectively 73% and 94% (Caldwell and Vincent, 2012). A combination of all the above mentioned factors has led to a greater and growing involvement in the development of artificial seagrass restoration projects for the long-term conservation of marine biodiversity (Cunha *et al*., 2009).

Although AHUs are still considered a subject of debate (Vega Fernandez *et al*., 2009; Hauser *et al*., 2006), they are thought to be a solution for the regeneration of degraded habitats, encouraging seahorse settlement and prosperity (Curtis *et al*., 2007). Different forms of Artificial Holdfast Units(AHUs) have been tested around the world (Correia *et* al., 2013; Correia *et* al., 2015; Curtis *et al*., 2007; Kenyon *et al*., 1999) with structures ranging from swimming nets, metal cubes (Hellyer *et al*., 2011), to aluminium cages (Simpson *et al*., 2020) and seagrass-like holdfasts (Correia *et al*., 2013). These all resulted in effectively replacing the damaged natural ecosystem and providing the species with a new marine habitat (Shahbudin *et al*., 2011).

With marine ecosystems being subjected to negative impacts, Marine Protected Areas (MPAs) are increasingly being implemented in different parts of the world to reduce negative interventions, prevent anthropogenic impacts, further habitat degradation and help restore the health of the oceans (Jones, 2002).

The ria Formosa is home to two MPAs, which were created to preserve and support the population of seahorses inhabiting the lagoon. An MPA in particular, (Figure 3.1), was chosen to be the location for this study where the Artificial Holdfast Units (AHUs) were deployed.

Figure 3.1 The ria Formosa lagoon with the deployment site location (upper photo) and the chosen Marine Protected Area where the AHUs were placed (lower photo).

Materials used to build artificial structures to be deployed in the natural environment need to comply with environmental adequateness to minimize the introduction of extremely long lasting artificial material. Therefore, the use of natural fibres to be provided as artificial holdfast is the advisable best case scenario. This study aimed to test the effectiveness of natural fibre Artificial Holdfast Unit (AHU) on habitat restoration in the ria Formosa, in order to assist in seahorse habitat recovery and to support the conservation of these species in this lagoon.

3.3 Material and Methods

3.3.1 Artificial Holdfast Unit (AHU)

The selection and construction of the AHUs used in this study were an output of the previous experiment (Chapter 2), where various holdfasts, differing in material, shape and size, were tested in controlled conditions.

The sisal rope Artificial Holdfast Unit (AHU) (Figure 3.2) provided the best results and were chosen for this study, to be used and deployed in the ria Formosa. The structures were manually constructed over the course of a month: they consisted of a 1x1m (1m²) wire base coated with plastic (with 10 x 10 cm grid gaps) and 25 sisal ropes of length 40cm each. A piece of cork was applied around the top end of the rope in order to ensure long-term floatability when submerged in water as well as a metal lace; to prevent the cork from slipping off and to avoid rope expansion and its breakdown. Finally, the lower end of the rope was attached to the base with a cable tie.

Figure 3.2 The sisal rope Artificial Holdfast Unit.

3.3.2 Site description and structure deployment

The study was conducted in an MPA in the ria Formosa lagoon (N36° 59.585'; W7° 54.065'), 4km SE of the Faro marina (Figure 3.1).

A total of 100 AHUs were constructed and later deployed in the selected MPA settled in 10 groups of 10 AHUs arranged in a 2x5m design (Figure 3.3). Each group of 10 was then placed in a pre-planned pattern at 10m and 20m distances from each other at a depth ranging between 3.4m and 4.5m. The structures were positioned in two parallel rows at 5m distance from each other (Figure 3.3), with row B being placed 5m North of row A. In order to avoid them being dragged away by currents, each AHU was secured to the bottom substrate with plastic covered metal pins. These AHUs were deployed at the end of May 2021 and were monitored on a monthly basis until August 2021.

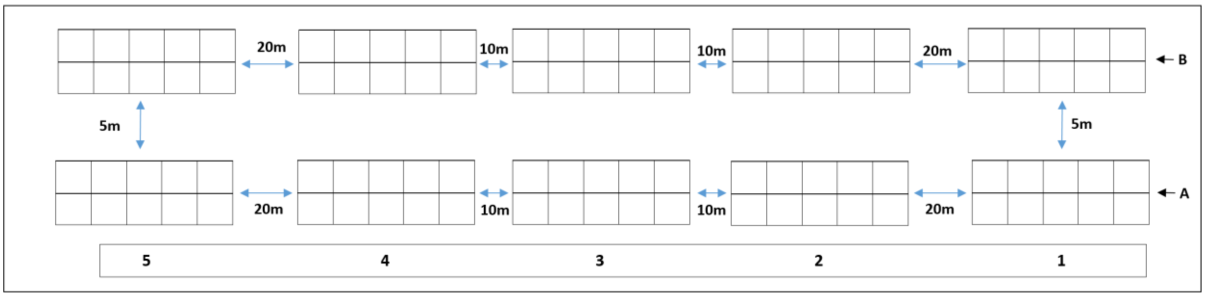

Figure 3.3 Deployment plan of the AHUs in the Marine Protected Area.

3.3.3 Data collection

Data was collected during every dive carried out between May and August (Tables S3.1, S3.2, S3.3)

During the first monitoring (May 25th), data was collected according to the protocol by Correia *et al.* (2016) where two 30m belt transects were placed in parallel and 2m wide strips on each side were surveyed covering a total area of 240m², before deploying the AHUs in order to monitor the study area for seahorse abundance. Later the same day, the AHUs were placed in the MPA following the pattern detailed in Figure 3.3, over an area of 100m². The structures were then left to settle before the second dive was carried out in the first week of July and the third at the start of August.

The collected data included different elements, such as: gender, size, species, water temperature and depth. In addition to these elements, each animal was photographed for future photo identification. The photographs were analysed using a computer software algorithm, I3S®Contour3.0. to help determine the movement and patterns of previously registered seahorses as well as noting any physical changes (eg. Pregnancies, injuries).

3.3.4 Statistical analysis

Gender differences for *H. guttulatus* and *H. hippocampus* were analysed using a one-way ANOVA. The number of seahorses encountered on the dives were tested for statistical differences using a one-way ANOVA.

3.4 Results

3.4.1 Pre-AHU deployment

In the first monitoring, before the deployment of the AHUs, 33% of seahorses had chosen *Ascidia* and various shells as their natural holdfast, 17% chose *Codium* spp. and the remaining 17% selected other algae species (Figure 3.4). The substrate of the study area consisted predominantly of mud and holdfast availability (composed of *Ascidia*, shells, *Codium* spp. And algae), was found with a patchiness and was less than 10% of the bottom coverage, with water temperature ranging from 16 °C to 21 °C, increasing through the course of sampling.

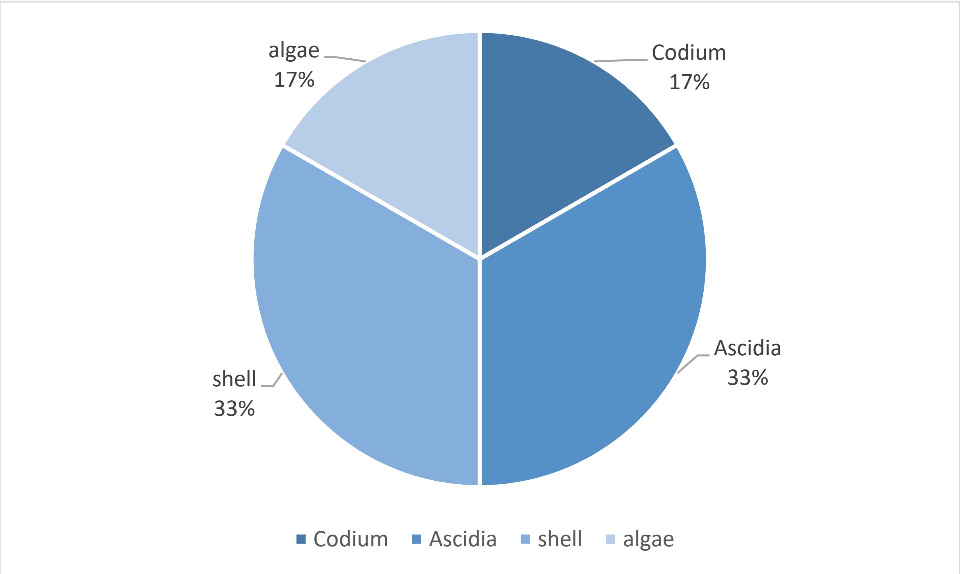

Figure 3.4 The natural holdfasts chosen by seahorses in the study area before the introduction of the AHUs.

3.4.2 Seahorse monitoring

The bar charts below show the number of seahorses, genders and species encountered on the dives. As the first monitoring, carried out before the deployment of the AHUs covered an area of 240 m², and the AHUs covered a 100 m² area, seahorse abundance was calculated accordingly.

In the first monitoring, carried out before the deployment of the AHUs, 12 seahorses were recorded representing a seahorse abundance of 0.05 per m², with the majority being *H. guttulatus*. After the AHU deployment, 7 seahorses were recorded in July and 4 during August representing a seahorse abundance of 0.07 and 0.04 animals per m², respectively (Figures 3.5 and 3.6). The lower seahorse abundance was therefore observed in August, coinciding with an extensive coverage of the AHU's by the bryozoan *Zoobotryon verticillatum*, which minimized the available habitat.

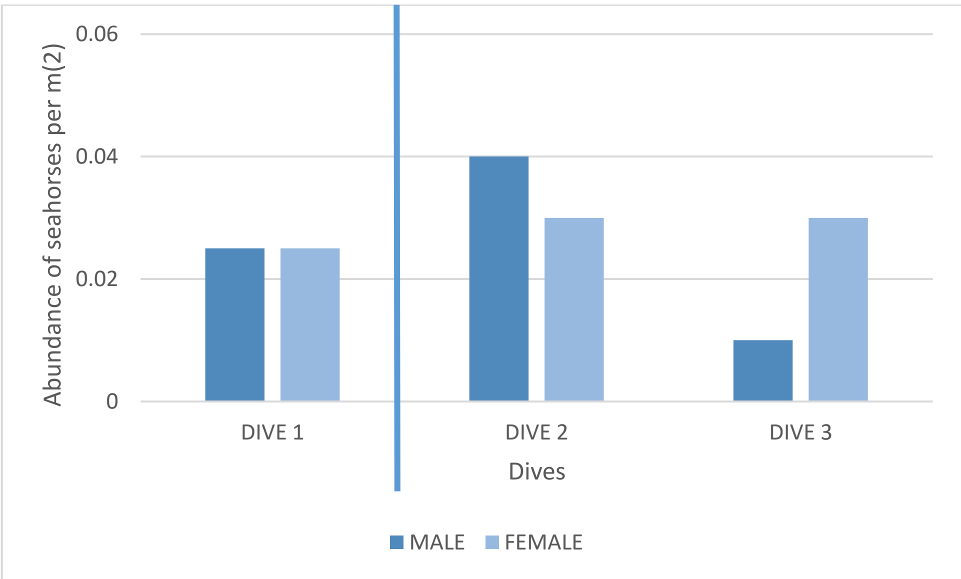

Figure 3.5. Seahorse abundance (gender/m²), both before and after the AHUs deployment. The vertical line separates the dives carried out before and after the AHUs deployment.

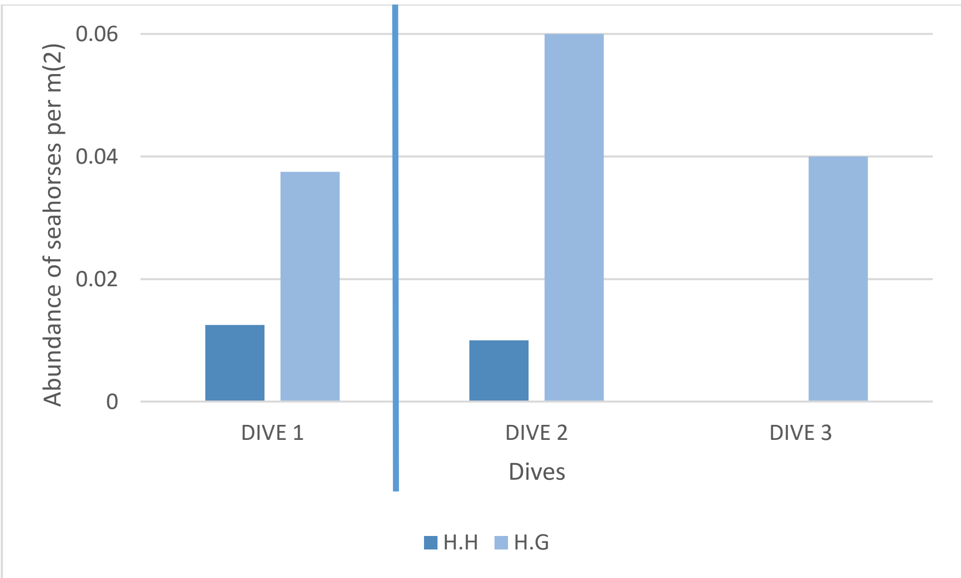

Figure 3.6 Seahorse abundance (species/m²), both before and after the AHUs deployment. The vertical line separates the dives carried out before and after the AHUs deployment.

Throughout the 4 month study period both seahorse species were found utilizing the AHUs, however, the abundance of *H. guttulatus* was significantly higher than *H. hippocampus* (P=0.04). No significant differences in gender were found for *H. guttulatus* and *H. hippocampus* (P=0.85 and P=0.56, respectively). However, significant differences were found between the seahorses' abundance on the three dives (P=0.878).

Individual seahorse length ranged between 7.4cm and 16.4cm. The average lengths of males and females on each dive showed that the highest number of larger males were encountered during the first monitoring (15.98±0.26), whilst on the second monitoring, the length of the females increased (14.8± 0.16) and there was no significant difference between the length of females and males in the last 2 dives (Figure 3.7)

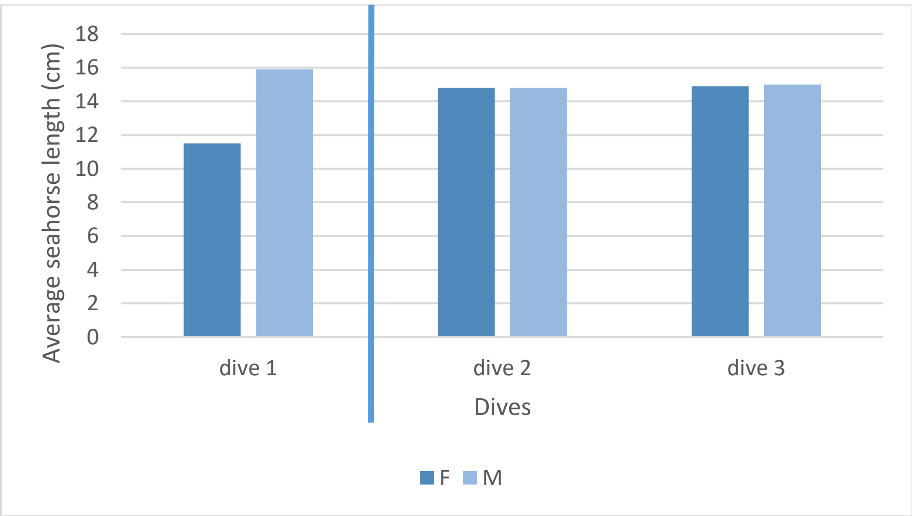

Figure 3.7 Average female and male seahorse length (cm) on each monitoring.

3.4.3 Photo-identification

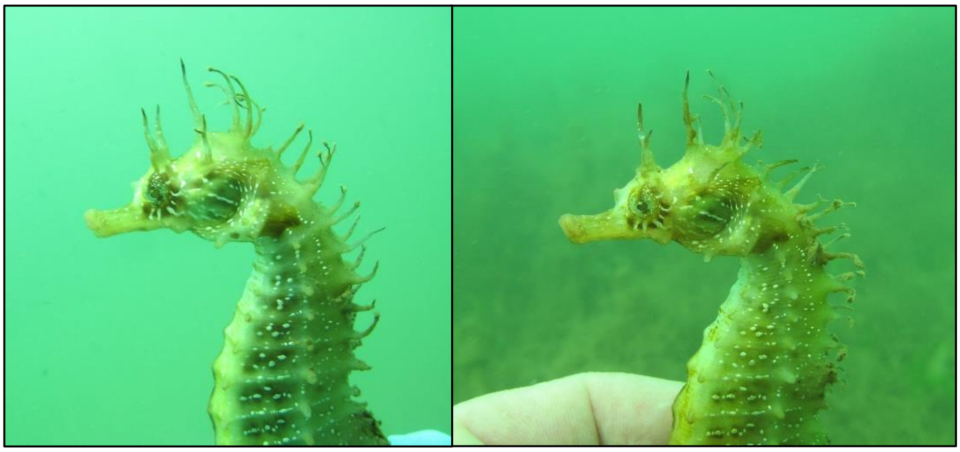

Figure 3.8 The seahorse individual that was re-sighted.

The recapture percentage, ie., percentage of seahorses re-sighted at least once in a sampling period was 4.3%.

3.5 Discussion

Artificial structures have been used to improve degraded habitats for various marine organisms around the world (Kenyon *et al*., 1999; Shahbudin *et al*., 2011; Lee *et al*., 2001). In Australia, some have been constructed in an attempt to support seahorse populations in particular, ranging from swimming barrier nets (Hellyer *et al*., 2011) to aluminium cages (Simpson *et al*., 2020). In the ria Formosa, seagrass-like structures, designed to fit the hydrodynamic characteristics of the ria, have already been tested (Correia *et al*., 2013) and showed successful results in temporarily providing a suitable habitat. Overall, these artificial structures have shown to be effective in supporting species and helping in maintaining the balance of a marine environment with a weakened natural habitat (Shahbudin *et al*., 2011). Although artificial structures are still a subject of debate (Vega Fernandez *et al*., 2009; Hauser *et al*., 2006), they have the potential to provide an improved habitat for seahorses, promote their settlement and contribute to a wider restoration project (Correia *et al*., 2015).

The causes for the 94% and 73% decrease in *H. guttulatus* and *H. hippocampus* respectively (Caldwell and Vincent, 2012) were not fully clarified. However, activities such as fishing, boating, anchoring, illegal catch, aquaculture development and clam farming (Duarte, 2002; Almeida *et al*., 2008) have shown to contribute to this decrease, with the decline of holdfast availability being one of the major factors to be considered (Correia *et al*., 2015). Presently, the seahorse abundance in the ria Formosa is estimated to be even lower, with a projected overall reduction of 96% compared to the start of the century (Palma, unpublished data). The seahorses' particular morphology renders them vulnerable to human activities as well as natural impacts such as strong currents (Foster and Vincent, 2004). The ria Formosa lagoon, of 10,000 ha (Newton *et al*., 2003), includes large areas of salt marshes and sand flats (Dias and Sousa, 2009) with the average depth of the navigable channels being 6 m and most areas being less than 2 m in depth (Salles *et al*., 2005). The high level of hydrodynamics caused by tidal activity within the lagoon, increases the need for stable holdfasts which seahorses rely on for survival (Curtis and Vincent, 2005). Slower water currents, on the other hand, may allow seahorses to move freely without too many risks and disperse (Correia *et al*., 2018).

Seahorse species in the ria Formosa have shown to utilize both macroalgae (*Codium* spp) and seagrasses (*Z. noltii*, *C. nodosa*) as a means to hold onto (Correia *et al*., 2015; Cunha *et al*., 2009). Previous studies have shown that the most useful artificial holdfasts were those which mimicked *Codium* spp (Correia *et al*., 2013), such as nylon vertical strips (Correia *et al*., 2015).

However, this study made a more ecological choice by utilising sisal rope to replace the nylon holdfast previously tested. Natural fibres were chosen in the hope of finding the most environmental-friendly and effective solution for seahorse habitat restoration and the marine environment as a whole. In fact, the sisal rope holdfasts successfully simulated the natural seagrass blades of the ria and provided a dense and stable habitat.

Past studies have shown that *H. guttulatus* was the most abundant seahorse in the ria Formosa, with the presence of *H. hippocampus* being much lower in an approximate 10 to 1 occurrence (Curtis and Vincent, 2005; Caldwell and Vincent, 2012). This situation was once again confirmed in this study, where a much larger number of *H. guttulatus* individuals were found prior and later in the AHUs. Therefore, the overall abundance of these two species on the deployed AHUs were in proportion to their abundances in natural situations.

Seasonal variation in seahorse abundances has already been observed in the past (Correia *et al*., 2015). The season and the presence of Bryozoa could also have been a factor affecting the low abundance of both species on the AHUs in the last monitoring. The Bryozoa (*Z. verticillatum*) thrives in the summer months and covers large areas regardless of the habitat structure, including the AHUs during the course of the experiment. In fact, it has been seen that in precious studies, bryozoan decreased until complete disappearance in October (Correia et al., 2015), allowing seahorse abundances to increase again. Therefore, in this study, the high abundance of Bryozoa, covering most of the AHUs, made it difficult for seahorses to grab onto the structures, possibly leading to a decrease in total seahorse numbers during the summer months. This factor may have also affected the recapture percentage that occurred during the first 4 months of the study.

In past studies (Curtis and Vincent 2005; Caldwell and Vincent 2012; Correia *et al*., 2018), an overall decrease in seahorse density was seen in correlation with higher temperatures. *H. guttulatus* abundance was seen to decrease during May to July and then increase from August to December (Correia *et al.*, 2018). The decrease in seahorse abundance, as well as the disappearance of larger male individuals, occurred during the species' breeding season (Correia *et al*., 2018); in fact, with *H. guttulatus* being a serial monogamous species (Woodall *et al*,, 2011), the search for mates during those months could be another reason for the decrease in their abundance (Correia *et al*., 2018). It is very important to take into consideration all these factors, in order to avoid bias due to seasonal changes and its effect on local seahorse populations. For this reason, the study is a screenshot of an ongoing project which will continue over a period of 12 months, in order to retain the shifting trends in one area throughout the 4 different seasons.

A Marine Protected Area is defined as "any area of littoral or sublittoral terrain, which has been reserved by law or other effective means to protect part or all of the enclosed environment" (Kelleher and Kenchington, 1992). Whilst the assemblages of seahorses in non-protected areas may render them a lot more vulnerable to anthropogenic events, the placement of AHUs in an MPA might contribute a lot more to the support of seahorse population (Correia *et al*., 2015).

The deployment of AHUs may lead to seahorse aggregations. These aggregations are generally considered a positive outcome but unfortunately may also contribute to the seahorses' vulnerability, in particular to bottom trawling and dredging (Correia *et al*., 2015). In this study however, the fact that the AHUs were deployed in an MPA, where negative interventions are minimized but not always absent, may have reduced this risk and acted in combination providing a safe and reliable habitat, thus contributing to increase abundance of these species in the ria Formosa.

3.6 Conclusion

The sparse distribution and low mobility of seahorses, makes them vulnerable to anthropogenic as well as natural impacts, leading to a huge decrease in seahorse abundance (Foster and Vincent, 2004; Caldwell and Vincent, 2012). Although significant decreases in *H. guttulatus* densities have been noticed since 2008 (Caldwell and Vincent, 2012), the greatest population size for these two species of seahorses was recorded back in the early 2000's (Curtis and Vincent, 2005). Seahorses have shown high adaptability over the years (Correia *et al*., 2018), however, preferences for particular holdfasts have become more noticeable. With this study, to the best of our knowledge, it was the first time that natural fibre materials were tested for their effectiveness in the rehabilitation of degraded natural habitat for seahorses in the ria Formosa.

The AHUs constructed and deployed for this experiment, retained their structural form, helped to reduce the effects of the hydrodynamicity in the ria Formosa and caused an increase in the abundance of seahorses found in the study area. Water currents and seasonal variations, makes it very important to asses whether seasonal events may be having an effect on seahorse abundances in the ria Formosa. For this reason, this study is an initial screenshot of a 12 months study, designed to avoid any biased results.

3.7 References

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3.8 Supplementary material

3.8.1 Figures

Figure S3.1 The deployed AHUs on site covered with Bryozoa.

3.8.2 Tables

Table S3.1 Data from first monitoring (before deployment of AHUs).

Species	M/F	Size	Tº	Depth	Holdfast	%Coverage	Substrate
HH	F	12.3	16	3.7	Codium	30% Shell	Mud
HG	F	12.1	16	3.9	Ascidia	40% Ascidia	Mud
HH	F	8.5	16	4.3	Ascidia	40% Ascidia	Mud
HH	F	7.4	16	4.3	Ascidia	40% Ascidia	Mud
HG	M	16.4	16	4.3	Ascidia	40% Ascidia	Mud
HG	M	16.2	16	4.2	Shell	40% Shell	Mud
HG	M	15.8	16	4.3	Shell	40% Shell	Mud
HG	M	15.7	16	4.4	Codium	30% Shell	Mud
HG	M	16.1	16	4.2	Algae	30% Algae	Mud
HG	F	14.4	16	4.2	Shell	40% Shell	Mud
HG	M	15.7	16	4.2	Shell	40% Shell	Mud
HG	F	13.8	16	4.3	Algae	30% Algae	Mud

Table S3.2 Data from second monitoring (after deployment of AHUs).

Species	M/F	Size	Tº	Depth	Holdfast	%Coverage	Substrate	
HH	M	12.3	20	3.9	ST2 A	-	Mud	
HG	M	15.7	20	3.9	ST2 A	-	Mud	Preg
HG	M	15.3	20	4.5	ST4 B	-	Mud	Preg
HG	F	14.6	20	4.3	ST5 A	-	Mud	
HG	M	16.1	20	4.3	ST1 B	-	Mud	Preg
HG	F	14.8	20	4.4	ST1 B	-	Mud	
HG	F	15	20	4.2	ST3 B	-	Mud	

Table S3.3 Data from third monitoring (after deployment of AHUs).

Species	M/F	Size	Tº	Depth	Holdfast	%Coverage	Substrate
HG	F	14.2	21	3.9	ST3 B	-	Mud
HG	M	15	21	4	ST5 B	-	Mud
HG	F	14.8	21	3.9	ST5 B	-	Mud
HG	F	15.7	21	3.4	ST3 A	-	Mud