UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CENTRO DE AQUICULTURA DA UNESP

AQUICULTURA, CONSERVAÇÃO E COMERCIALIZAÇÃO DE CAVALOS-MARINHOS NO CENÁRIO INTERNACIONAL

Felipe Pereira de Almeida Cohen

Jaboticabal, São Paulo

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Somewhere, something incredible is waiting to be known.

–Carl Sagan

SUMÁRIO

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Esse trabalho é dedicado a memória do meu avô, Heinz Cohen. Um grande exemplo de ética, caráter e honestidade.

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If I have seen further it is by standing on the shoulders of Giants.

–Isaac Newton

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I. RESUMO

Este trabalho teve por objetivo estudar o mercado, a produção e a comercialização de cavalos-marinhos com foco no atendimento da demanda mundial de forma mais sustentável e na conservação desses animais. Assim, a tese foi dividida em cinco artigos: (1) apresenta um panorama da pesquisa mundial no século XXI sobre cavalos-marinhos. Observou-se que os campos de pesquisa fundamentais para subsidiar decisões governamentais e que abordam as questões que afetam a produção, o comércio e a manutenção das populações naturais de cavalos-marinhos não vem sendo cobertos adequadamente. Cinco áreas foram identificadas como prioritárias para o avanço do conhecimento científico, atendendo à demanda e a conservação dos cavalos-marinhos. (2) Discute a comercialização e o mercado; mostra que o mercado é pouco conhecido e que as regulamentações internacionais para comércio de cavalos-marinhos estão sendo ineficientes na conservação. (3) Apresenta as principais oportunidades e limitações de se cultivar cavalos-marinhos em estuários em Manguezal como alternativa para produção de baixo custo. Observou-se que essa prática é bastante promissora, podendo aproveitar a disponibilidade local de reprodutores, água, espaço, alimento natural, mão de obra e gerar renda adicional para comunidades locais de forma mais sustentável. Alterações ambientais bruscas, predadores, obstrução das redes e escapes são as principais limitações. (4) Visa otimizar o transporte de animais vivos. Observou-se que *Hippocampus reidi* pode ser transportado em sacolas plásticas contendo 300 mL de água, com salinidade 15, com adição de substrato pequeno e preenchido com ar comprimido como uma opção barata e segura até 48 horas em temperaturas próximas de 21° C. (5) Analisa o uso de comunidades bacterianas no muco dos cavalos-marinhos como uma ferramenta para rastrear a origem dos animais vivos; foi demonstrado que esse método tem potencial, mas precisa ainda de mais pesquisas para ser implementado.

Palavras-chave: *Hippocampus*, Syngnathidae, bibliometria, cadeia produtiva, sustentabilidade, fisiologia, rastreabilidade

II. ABSTRACT

The aim of this work was to study the trade, the production and the commercialization of seahorses focusing on meeting world demand in a more sustainable way and on the conservation of these animals. Thus, this thesis was divided in five articles: (1) shows an overview of the seahorse research in the 21st century. It was observed that the key research fields necessary to support government decisions on addressing issues affecting the production, trade and maintenance of wild seahorses populations, have not been properly studied. Five knowledge gaps were identified as priorities for improving scientific knowledge, considering both world demand and seahorse conservation. (2) Discusses commercialization and trade; Shows that there is little information on trade and that international regulations of seahorse trade have been ineffective to promote their conservation. (3) Shows the main opportunities and constraints to produce seahorses in mangrove estuary as an alternative for low-cost aquaculture. It was observed that this system has great potential; it can rely on the availability of local broodstock, water, space, natural food, labour, and provide additional income for local communities in a more sustainable way. Sudden environmental changes, predators, net obstruction, and escapees are the main constraints of this system. (4) Aims to optimize transport of live seahorses. It was reported that *Hippocampus reidi* could be transported in plastic bags containing 300 mL of water, salinity 15, with addition of small substrate, and filled with compressed air as a cheap and safe option for up to 48 hours in temperatures close to 21 °C. (5) Analyses the use of bacterial communities in the mucus of seahorses as a traceability tool to trace the origin of live specimens; it was showed that this method has potential, but still needs more research to be implemented.

Keywords: *Hippocampus*, Syngnathidae, bibliometry, production chain, sustainability, physiology, shipping

III. INTRODUÇÃO GERAL

Cavalos-marinhos são peixes ósseos pertencentes à família Syngnathidae, juntamente com os peixes-cachimbo e os dragões-marinhos. Atualmente são reconhecidas 54 espécies, todas pertencentes ao gênero *Hippocampus* (*World Register of Marine Species* – disponível em: <http://www.marinespecies.org/>; acessado em março de 2016). No Brasil, são descritas três espécies: *H. reidi*, *H. erectus* e *H. patagônicos* (Silveira et al., 2014). Os cavalos-marinhos possuem uma morfologia única caracterizada pela postura ereta, ausência de nadadeira caudal e esqueleto revestido por placas ósseas (Foster e Vincente, 2004). Utilizam sua cauda preênsil para ficarem agarrados a substratos no fundo dos oceanos e estuários, como macroalgas, gramas-marinhas, esponjas, corais e raízes de mangue (Foster e Vincente, 2004; Kuiter, 2009). Além disso, os cavalos-marinhos possuem capacidade de camuflagem, com diferentes colorações e texturas, o que os tornam ótimos predadores por emboscada e dificulta a sua predação (Foster e Vincente, 2004). Porém, um dos maiores fascínios pelos cavalos-marinhos tem relação com sua biologia reprodutiva. Os machos possuem uma bolsa fechada para cuidado dos ovos até a liberação de pequenos juvenis de formato semelhante ao adulto, por vezes comparado à gravidez dos mamíferos (Stölting e Wilson, 2007). Todas essas peculiaridades tornam esses peixes carismáticos e capazes de cativar os seres humanos. Por isso, cavalos-marinhos são considerados potenciais espécies bandeiras para a conservação de ecossistemas marinhos (Shokri *et al.*, 2009; Vincente *et al.*, 2011).

Apesar de sua imagem ser usada como símbolo para conservação de espécies marinhas, os próprios cavalos-marinhos são vulneráveis à degradação ambiental e à coleta. A maioria das espécies vive em águas costeiras tropicais e temperadas (Foste e Vincent, 2004; Kuiter, 2009) que estão entre os habitats mais degradados por ações antrópicas (Pan *et al.*, 2013). A reduzida capacidade de natação dificulta que esses animais saiam a procura de melhores condições, ficando vulneráveis à degradação do habitat (Vincente *et al.*, 2011; Harasti, 2016) e a coletas. Milhões de cavalos-marinhos são coletados anualmente para serem vendidos secos para Medicina Tradicional Chinesa, artigos religiosos e decorativos (Rosa *et al.*, 2011; Foster *et al.*, 2016); e centenas de milhares são comercializados vivos para fins ornamentais (Foster *et al.*, 2016). Adicionalmente, são vítimas acidentais da pesca de arrasto para peixes e camarões (Rosa *et al.*, 2011; Foster *et al.*, 2016). No Brasil, as três espécies são classificadas como vulneráveis; é proibida a captura, armazenamento, transporte e comercialização de cavalos-marinhos sem autorização ou regulamentação dos órgãos federais competentes (Portaria MMA Nº 445, de 17 de dezembro de 2014). Apenas 11 espécies de cavalos-marinhos são consideradas "vulneráveis" e apenas 1 "ameaçada" de acordo com a lista vermelha da IUCN (IUCN, 2015-4). A falta de informações sobre a biologia, ecologia e mercado dos cavalos-marinhos é um dos estrangulamentos para avaliar o risco de extinção para a maioria das espécies.

Com o intuito de controlar o mercado dos cavalos-marinhos e evitar a sobre-exploração das espécies, todo o gênero *Hippocampus* foi inserido no Apendix II da Convenção sobre o Comércio Internacional de Espécies Ameaçadas (sigla em inglês para: CITES) em 2004 (CITES, 2004). Essa foi uma inclusão incomum porque há poucas espécies de peixes controladas pelo CITES. Além dos cavalos-marinhos, apenas os esturjões (Acipenseriformes) e as espécies *Holacanthus clarionensis* e *Cheilinus undulates* estão entre os peixes marinhos ósseos incluídos nesta lista. Assim, desde 2004, todas as transações internacionais de cavalo-marinho (vivo ou seco) devem ser autorizadas por autoridades governamentais e científicas do país de exportação, e os dados devem ser reportados e armazenados na base de dados da CITES. Essa base de dados possibilitou entender um pouco o mercado internacional desses animais.

Observou-se que os cavalos-marinhos são principalmente coletados da natureza em países em desenvolvimento no sudeste asiático e destinados a países mais desenvolvidos como a China (animais secos), EUA e União Europeia (animais vivos) (Foster *et al*., 2016). O mercado de espécies secas representa mais de 90% do total de cavalos-marinhos comercializados internacionalmente, sendo que as principais espécies são *H. trimaculatus*, *H. spinosissimus*, *H. kelloggi*, *H. kuda*, e *H. algiricus* (Foster *et al.,* 2016). As principais espécies comercializadas internacionalmente vivas são *H. kuda* e *H. reidi* (Foster *et al*., 2016). Porém, é preciso considerar que os dados da CITES são falhos, com grandes diferenças entre valores de importação e exportação (Foster *et al.*, 2016). Adicionalmente, a CITES não tem ferramentas para quantificar comércios domésticos e comércios ilegais, sendo essas suas principais limitações. Assim, essa falta de controle e crescimento de comércio ilegal desses animais tornam o mercado dos cavalos-marinhos difícil de ser analisado.

O desenvolvimento de cultivos de baixo custo é sugerido como uma alternativa mais sustentável para promover a conservação dos cavalos-marinhos (Cohen *et al*., 2017). Os cavalos-marinhos são comercializados para Medicina Tradicional Chinesa a mais de 600 anos (Vincent, 1996) e, recentemente, diversos estudos têm confirmado seus potenciais farmacológicos (Kumaravel et al., 2012; Chen et al., 2015). Esse comércio bem estabelecido por centenas de anos mostra que a demanda por cavalos-marinhos secos dificilmente reduzirá em um futuro próximo. Porém, apesar das espécies destinadas para o mercado seco serem as mais ameaçadas pelas coletas, são curiosamente as menos estudadas na aquicultura (Cohen *et al*., 2017). Um dos principais motivos para isso, é o fato de espécimes ornamentais atingirem maior valor de mercado (US$ 60-120/espécime) comparado com os secos (US$ 1-3/espécime), justificando o cultivo intensivo e tecnificado atualmente disponível para cavalos-marinhos (Koldewey e Martin-Smith, 2010; Olivotto *et al*., 2011). Assim, para viabilizar a produção em larga escala de cavalos-marinhos secos, é fundamental o desenvolvimento de cultivos de baixo custo, favorecendo a conservação desses animais e proporcionando uma atividade sustentável para comunidades litorâneas de baixa renda.

Entretanto, para estabelecer um sistema de produção perene, é necessário fortalecer toda a cadeia produtiva (Valenti e Moraes-Valenti, 2010). Isso inclui a distribuição e a certificação dos cavalos-marinhos. Os animais ornamentais são exportados vivos para o mundo inteiro em sacolas plásticas com água, tornando o transporte uma fase de risco e com custo elevado (Wood, 2001). Porém, faltam pesquisas para otimizar e uniformizar métodos de transporte para cavalos-marinhos vivos (Cohen *et al*., 2017). Portanto, o transporte é um elo crítico da cadeia produtiva dos cavalos-marinhos, que requer ainda muita pesquisa científica para reduzir os custos e garantir a entrega de animais saudáveis ao consumidor. Outro aspecto importante, é a garantia da procedência dos animais para que o consumidor saiba que está comprando organismos produzidos em sistemas mais sustentáveis. Isso agrega valor ao produto, principalmente se ele é produzido por comunidades costeiras de baixa renda ou em reservas extrativistas regulamentadas. Assim, a certificação e rastreabilidade são outros temas importantes que precisam ser estudados.

O atual sistema de certificação da cadeia produtiva de organismos ornamentais marinhos é ineficiente (Mathews Amos e Claussen, 2009). Um dos principais pontos de estrangulamento é a inexistência de um método apropriado para rastrear os animais. A demanda por um animal vivo, saudável e estético, associado à grande diversidade de espécies comercializadas em todo o mundo, torna os organismos ornamentais marinhos um produto difícil de ser rastreado (Cohen *et al*., 2013). Os métodos existentes para peixes de consumo humano são, na maioria das vezes, invasivos, precisam matar o animal e/ou comprometem sua estética. Em ampla revisão sobre o assunto, Cohen *et al*. (2013) apontam o método de análise do perfil de comunidades bacterianas associadas ao muco do peixe como um dos mais promissores para rastrear organismos ornamentais marinhos. O método é recente, ainda não foi aplicado para organismos ornamentais e, portanto, precisa de pesquisa científica para ser aprimorado e validado para diferentes espécies. Nenhum trabalho sobre a rastreabilidade de cavalos-marinhos foi encontrado na literatura técnica ou científica.

Assim, o cenário atual mostra os cavalos-marinhos com um mercado pouco conhecido e pesquisas focando somente em sistemas de aquicultura complexos que não atendem a necessidade de produção de animais secos. Além disso, o transporte de animais vivos é feito sem nenhuma base científica, com custos e riscos elevados e não há um método para conhecer a origem dos animais vendidos para a aquariofilia. Portanto, para promover a conservação desses animais e atender as necessidades humanas de forma mais sustentável, é fundamental conhecer melhor o mercado, desenvolver cultivos de baixo custo, otimizar o transporte e criar métodos de rastreabilidade. Assim, nesse trabalho estudamos três elos importantes da cadeia produtiva dos cavalos-marinhos, ou seja, o mercado, a produção, e a distribuição, que envolve a comercialização, o transporte e a rastreabilidade. Para isso, a tese foi dividida em cinco artigos. O primeiro artigo (V. Manuscrito I) é uma ampla revisão bibliográfica que apresenta um panorama da pesquisa mundial no século XXI sobre cavalos-marinhos. Cinco áreas foram identificadas como as mais importantes para o avanço do conhecimento científico, atendimento à demanda mundial e para a conservação dos cavalos-marinhos. Esse estudo foi fundamental para definir os temas que foram estudados nessa tese, que se inserem em quatro das cinco áreas estratégicas definidas na revisão, ou seja: Maximizar o Potencial de Uso dos Cavalos-Marinhos para Conservação de Ambientes Marinhos, Elucidar Aspectos Desconhecidos das Espécies mais Comercializadas, Desenvolvimento de Cultivo de Baixo Custo e Desenvolvimento de Métodos para a Rastreabilidade. O segundo artigo (VI. Manuscrito II) discute a comercialização e o mercado; mostra que o mercado é ainda pouco conhecido e que as regulamentações internacionais para o comércio de cavalos-marinhos estão sendo ineficientes na conservação desses animais. O terceiro artigo (VII. Manuscrito III) apresenta as principais oportunidades e limitações de se cultivar cavalos-marinhos em Manguezal como alternativa para produção de baixo custo e como forma de promover a conservação. O quarto artigo (VIII. Manuscrito IV) visou otimizar o transporte de animais vivos; analisou-se os principais fatores que interferem na fisiologia e bem-estar dos animais ou que podem reduzir o custo do transporte. Por fim, no quinto artigo (IX. Manuscrito V) é analisado o uso de comunidades bacterinas no muco dos cavalos-marinhos como uma ferramenta para rastrear a origem dos animais vivos de forma não invasiva. Todos os estudos tiveram autorização do ICMBio (nº41924-2 e 41924-3) e do comitê de ética da UNESP/CLP.

Objetivo geral:

Este trabalho teve como objetivo estudar aspectos importantes de três elos da cadeia produtiva dos cavalos-marinhos, que são o mercado, a produção e a comercialização, com foco no atendimento da demanda mundial e na conservação desses animais.

Objetivos específicos:

- Avaliar o mercado de cavalos-marinhos ao redor do mundo e analisar como as normas e regulamentações têm influenciado esse mercado e contribuído para a conservação dos cavalos-marinhos;

- Avaliar o potencial para a produção de cavalos-marinhos em sistemas simples e baratos, instalados em regiões de manguezal e operados por comunidades de baixa renda;
- Otimizar variáveis referentes ao transporte de cavalos-marinhos destinados ao mercado de peixes ornamentais;
- Avaliar a viabilidade do método do perfil de comunidades bacterianas associadas ao muco da pele de cavalos-marinhos para ser usado como ferramenta para conhecer a origem dos animais vivos.

IV. REFERÊNCIAS

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V. MANUSCRITO I

SEAHORSE AQUACULTURE, BIOLOGY AND CONSERVATION: KNOWLEDGE GAPS AND RESEARCH OPPORTUNITIES

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ABSTRACT

Seahorses are currently experiencing an unprecedented level of anthropogenic pressure promoted by habitat destruction and increasing fishing effort to supply premium markets. The present study provides an overview of the scientific literature on seahorses in the 21st century and critically discusses five major knowledge gaps and research opportunities to advance the state of the art on this research field. The average number of publications per year increased from 10 (2001-2002) to ~40 (2001-2015), the majority addressing issues on seahorse ecology, biology and aquaculture, with the most studied species being *Hippocampus kuda*, *H. guttulatus*, *H. reidi*, *H. abdominalis*, *H. erectus*, *H. hippocampus*, and *H. trimaculatus*. This study explores the opportunity of using seahorses as flagship species to foster mangrove conservation and decrease trawling fisheries. It also suggests that further scientific studies are needed to better understand and manage the populations of the most heavily traded seahorse species, as well as the need to monitor their vulnerability to emerging pollutants and climate change. Sustainable seahorse aquaculture can play an important role in seahorse conservation, as well as in the development of reliable traceability tools to fight the illegal trade of these highly priced organisms.

Keywords: bibliometry, seahorse trade, sustainability, sustainable aquaculture, marine ornamental species, threatened species.

INTRODUCTION

Marine habitats are exposed to an unprecedented level of anthropogenic pressure, including unsustainable fishing, degradation and loss of habitat, pollution and global climate change (Pan et al., 2013). For successful conservation actions and plans, the perception of marine conservation issues must raise awareness on a broader community level. Most threats to marine habitats will only be mitigated through a change in human behaviour through educational and awareness-raising actions, which has been the greatest challenge faced by conservationists (Wright et al., 2015).

Marketing tools have been successfully used to influence human behaviour in favour of conservation (Wright et al., 2015). A common marketing approach in biological conservation is the use of flagship species. Flagship species (*sensu* Heywood, 1995 and Walpole and Leader-Williams, 2002) are species that can be used as symbols in conservation campaigns to raise awareness and funding for specific conservation issues. The use of a particular species (or group of species) as flagship can benefit its conservation and the protection of its habitat and other associated species. Several features may qualify a species as a suitable flagship, as long as it appeals to the target audience, to the conservation issue being addressed and to the local context (Bowen-Jones and Entwistle, 2002; Home et al., 2009; Verissimo et al., 2011). Some charismatic features may favour the selection of a given species as flagship (Home et al., 2009). An example is the seahorses (Shokri et al., 2009; Vincent et al., 2011; Yasue et al., 2012), with their unique morphology similar to ponies and their reproduction mode, in which the males incubate the eggs in an abdominal chamber.

Currently, 54 extant species of seahorses are taxonomically recognized as valid, all within a single genus: *Hippocampus* (according to World Register of Marine Species (available at: <http://www.marinespecies.org/>; accessed in March 2016). Most of these species live in association with seagrasses and mangroves, as well as macroalgae, sponges and corals, generally within the shallow waters of tropical and temperate, and exceptionally in deeper habitats regions (Foster and Vincent, 2004; Kuiter, 2009). Seahorses are vulnerable species because of their habitat degradation (Vincent et al., 2011; Harasti, 2016) and also due to the collection of millions of specimens every year to supply the traditional Chinese medicine and, to a lower extent, the marine aquarium and curio trade (Vincent et al., 2011; Foster et al., 2016). Indeed, habitat degradation and the pressure arising from illegal, unreported and unregulated collection of seahorses worldwide prompted the inclusion of all *Hippocampus* species into CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) Appendix II in 2002 – a decision that became effective in 2004 (CITES, 2004).

The above rationale is the main driver that encourages scientists worldwide to investigate seahorses. The aim of this study was to provide a critical overview on the scientific literature addressing seahorses in the 21st century and discuss research opportunities and gaps of knowledge on these remarkable organisms.

DATA SURVEY

In March 2016, a survey was conducted using all databases of Thomson Reuters^{TM} Web of Science^{TM} (available at: http://apps.webofknowledge.com) to collect all references related to seahorses (Actinopterygii: Syngnathidae) published from 2001 to 2015. The term “seahorse” was used in the “Topic” field to conduct the search. Except for patents, all types of publications (scientific journals, book chapters, meetings abstracts, magazines, and short communications) were used for the analyses. A total of 796 references were retrieved, with each one of them being screened individually to determine whether they were within the scope of this study. Most of the excluded references were studies that used the word seahorse in a context other than the fish (e.g., seahorse extracellular flux analyzer).

Selected references were included in a gross table containing first author, title, year, journal, and country for both the first and last authors (as these are commonly the corresponding authors). If the first authors were affiliated with more than one country, the publication was considered as originating from both of them. The scientific name of the seahorse species studied was also annotated (according to the World Register of Marine Species – available at: <http://www.marinespecies.org/>; accessed in March 2016) and each reference was assigned to up to three different research fields. A total of 10 different research fields were selected to assign each reference: aquaculture, biology, conservation, ecology, morphology, pathology, pharmacology, physiology, taxonomy, and trade. The rationale employed to assign a given reference to a specific research field is detailed in Table 1. Whenever the publication did not detail the name of the species being addressed in the title or abstract, and it was not possible to gain access to the original text of the publication, it was registered the name as “Absent”. When studies addressed too many species (more than eight) or was a “broad scope” publication addressing seahorses in a generalist way (e.g., did not detail any species in particular) they were registered as “*Hippocampus* spp”.

Table 1. Criteria employed to assign each reference addressing seahorses retrieved from Thomson Reuters^{TM} Web of Science^{TM} (all databases) from 2001 to 2015 to a given research field.

Research fields	Criteria
Aquaculture	Refers to the culture of seahorse in captivity.
Biology	Refers to behavior, growth, reproductive biology, and mating system of seahorses.
Conservation	Refers to seahorse conservation.
Ecology	Refers to population, habitats, distribution, migration, abundance, population dynamics, and natural diet.
Morphology	Refers to the description and function of body parts, morphological development, muscles, and skeletons.
Pathology	Refers to bacteria, virus, fungus or any other pathogen isolated from seahorses.
Pharmacology	Refers to seahorse extract and compounds such as peptides, glycoproteins, and antioxidants.
Physiology	Refers to seahorse metabolism, biochemistry, and general physiology, including studies of ecotoxicology.
Taxonomy	Refers to species description and identification, including works with phylogeny.
Trade	Refers to the seahorse trade.

Overall scientific production on seahorses per year was used to assemble a frequency distribution histogram, which also included the cumulative frequency. To provide an overview of the most published topics on seahorses, all titles were plotted in a word cloud (program available at: <http://www.wordclouds.com>; accessed in March 2016). The principle of a word cloud is simple, in which the size of the word is related to its frequency of appearance in the titles of publications. To make the word cloud more informative, the four most repeated (and not informing) words and its derivatives were eliminated: "Hippocampus" (Genus), "Seahorse", "Syngnathidae", and "Species". Popular names, whenever followed by scientific names, were also eliminated.

The percentage of the most studied species and research fields was calculated, as well as the total number of publications produced per country, while also discriminating per research field. Because the percentage of countries to which the 1st and the last author were affiliated was similar (~88%), only the 1st author's country was used for analysis. Additional histograms of frequency distribution were made to highlight the top 10 journals publishing research on seahorses, as well as which seahorse species have been addressed in scientific literature across different research fields.

To end the critical review of scientific literature of seahorses during the period being covered (2001-2015), five knowledge gaps and research opportunities were identified and guidelines for future studies are presented.

SEAHORSE SCIENTIFIC LITERATURE PUBLISHED IN THE 21ST CENTURY

A total of 423 publications retrieved were relevant for this study and selected for analysis (see Table S1 on supplementary information). In spite of their recognised iconic character, the knowledge on biology of seahorses was scarce before the present century. Over the past 15 years (2001 to 2015) there has been an increase in the number of scientific publications addressing seahorses, with the average number of publications per year raising from 10 in 2001-2002 to ~40 in 2001-2015 (Fig. 1). This increase in scientific publications may be related to the inclusion of seahorses in CITES Appendix II. The need for scientific data to support regulation and management decisions may have promoted research effort towards seahorse-related topics. Therefore, the inclusion of seahorses in this list has contributed to their conservation (Foster et al., 2016), and scientific knowledge.

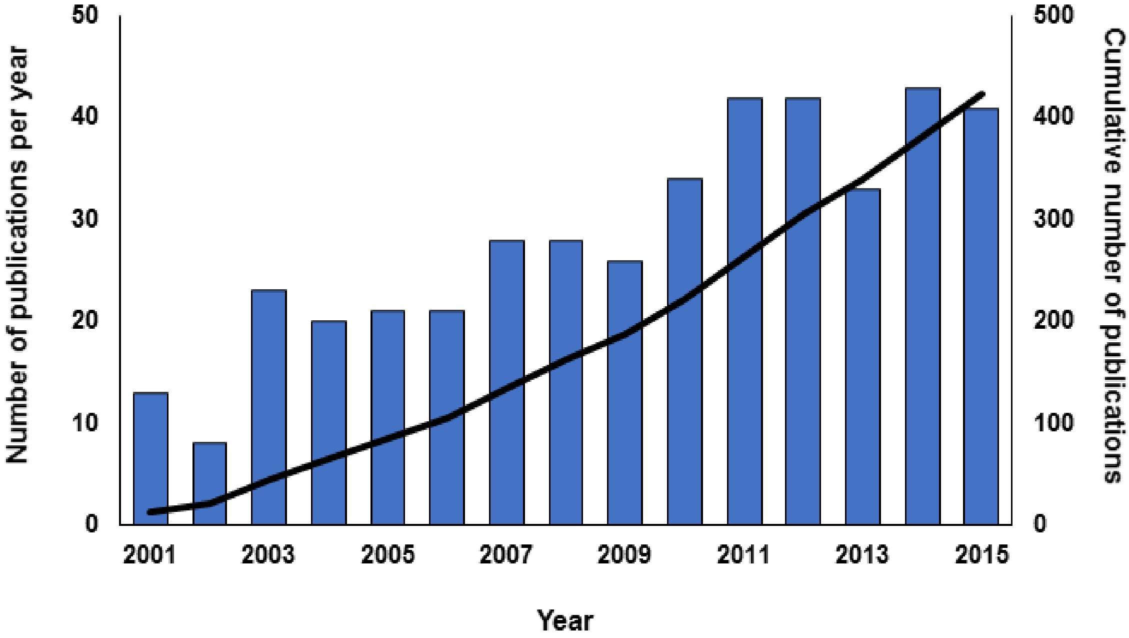

Figure 1. Number of scientific publications addressing seahorses retrieved from Thomson Reuters™ Web of Science™ (all databases) from 2001 to 2015. Bars show the number of publications per year and the line shows the cumulative number of publications.

Thirty-five species of *Hippocampus* were referenced in at least one of the publications selected for this study, which covers 65% of all extant species of seahorses. Nonetheless, most publications retrieved (76%) are focused on only seven species (~13% of all extant species) (Fig. 2). From these seven species, three are among the most heavily traded, either dried for traditional Chinese medicine (*H. trimaculatus* and *H. kuda*) or live for aquariums (*H. kuda* and *H. reidi*) (Foster et al., 2016). This finding shows a trade-driven research effort on these species. Seahorses were included in traditional Chinese medicine probably more than 600 years ago (Vincent, 1996). The trade of live seahorses for marine aquariums is more recent, dating to the early 1900's, with the beginning of the marine ornamental trade (Vincent, 1996; Wood, 2001). Trade regulation and management claims for scientific data, namely in the fields of ecology and biology (Fig. 2). Thus, it is expected that some of the heavily traded species to be among the ones most addressed by scientific research. Additionally, the increasing demand for knowledge on genus *Hippocampus* may prompt other countries to conduct research on species occurring in their national waters, even if not significantly traded (e.g., *H. erectus* in the USA, *H. guttulatus* and *H. hippocampus* in EU countries, and *H. abdominalis* in Australia).

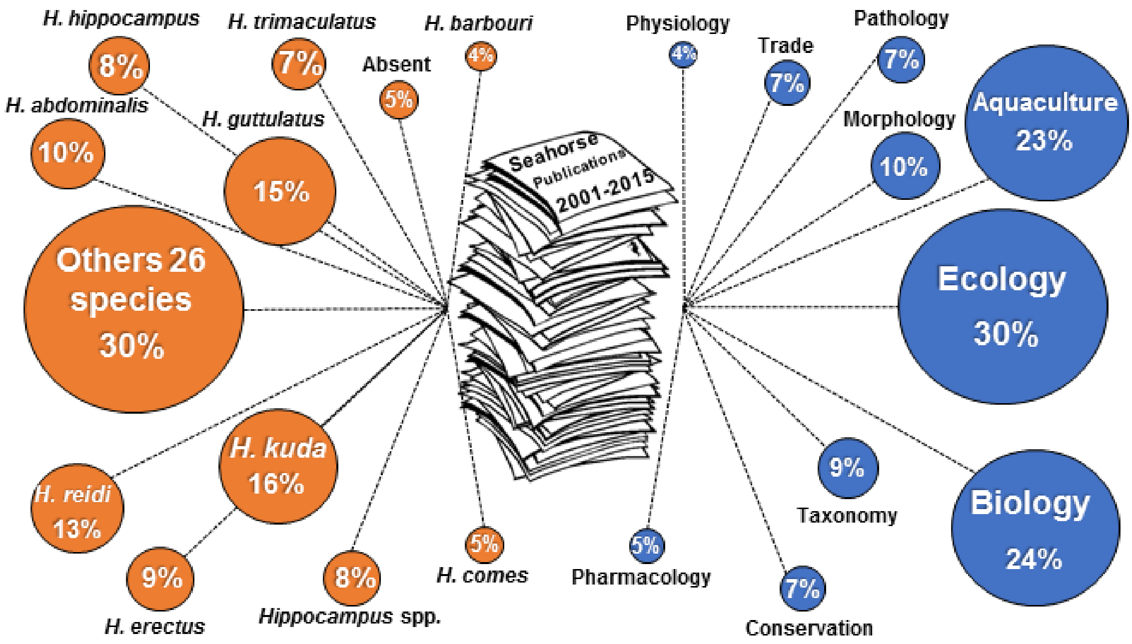

Figure 2. Percentage of scientific publications addressing each seahorse species (Orange – left) and the most studied research fields (Blue – right) based on data retrieved from 423 scientific publications using Thomson Reuters™ Web of Science™ (all databases) from 2001 to 2015. Note: some publications addressed more than one species and/or field.

The word cloud highlighted the most common words recorded on the titles of the publications selected for this study (Fig. 3). As expected, the names of the seven most studied seahorse species ranked among the most repeated words. Other words highlighted in the word cloud are mostly related to seahorse aquaculture and unique features. Among these, it is possible to see the words “growth”, “juvenile”, “feeding” “survival”, “development”, “fed”, “*Artemia*”, “cultured” and “diet”, which clearly refer to some of the bottlenecks in seahorse aquaculture (e.g., rearing of early life stages) (Koldewey and Martin-Smith, 2010; Olivotto et al., 2011). The growing awareness and concern for the global trade of seahorses and conservation were drivers fostering research reflected by words such as “population”, “conservation”, “trade”, “threatened” and “habitat”. The role of the male in brood care and pregnancy, along with the upright body position displayed by seahorses and their unusual flexibility – for a fish whose body is covered by bony plates – have inspired many studies reflected by the words “male”, “reproductive”, “pregnancy”, “pouch”, “morphological”, and "tail". Finally, words such as "genetic", "mitochondrial", "molecular", "microsatellite", and "evolution" were also commonly recorded among titles because of the increasing number of studies using genetic tools for seahorse taxonomy, evolution, population structure, and distribution.

Figure 3. Wordcloud detailing the most common words featured in the titles of scientific publications addressing seahorses selected for this study retrieved from Thomson Reuters^{TM} Web of Science^{TM} (all databases) from 2001 to 2015.

Based on the affiliation country of the first author, forty-three countries published at least one scientific publication addressing seahorse since 2001; however, nine countries accounted for more than 60% of all publications (Fig. 4). Canada and the USA produced the most in this century (Fig. 4). From a continental perspective, Europe represented 33% of all publications followed by Asia (26%), North America (21%), Oceania (10%), South America (8%), and Africa (1%). Publications on ecology, trade, and conservation were mostly from Canada (Fig. 4). This country held one of the head offices of the Project Seahorse, which is the largest seahorse conservation group in the world. Members of Project Seahorse have contributed a significant number of scientific publications addressing different species of *Hippocampus* (available at: <http://www.projectseahorse.org/>; accessed in March 2016). China was the leading country on publications addressing seahorse aquaculture, followed by Spain, the USA, and Australia (Fig. 4). Most scientific studies on seahorse morphology were from Belgium, followed by the USA (Fig. 4). Publications on pathology were mostly from Spain, followed by the USA and India (Fig. 4). Studies on pharmacology were performed only in Asia, where seahorses are used for medicinal purposes.

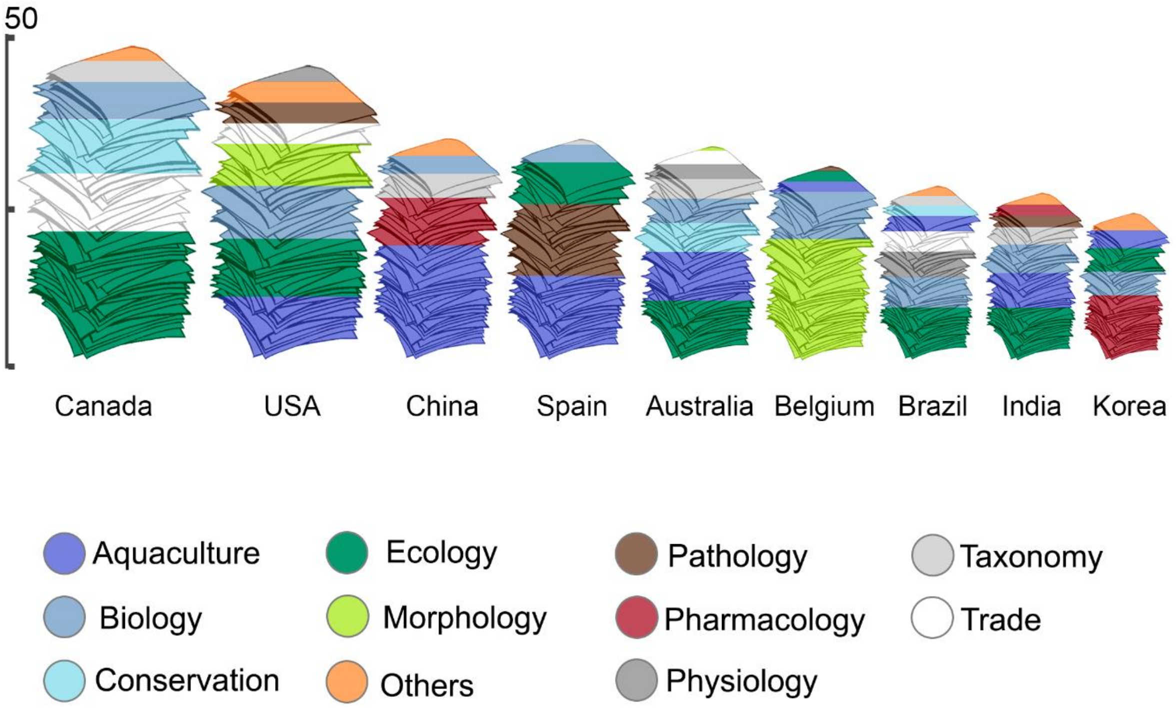

Figure 4. Number of scientific publications addressing seahorses ranked by country and their main field of study based on data retrieved from Thomson Reuters™ Web of Science™ (all databases) from 2001 to 2015.

The *Journal of Fish Biology* (Wiley-Blackwell) and *Aquaculture* (Elsevier Science B.V.) were the scientific journals, indexed in Thomson Reuters™ Web of Science™, which published more studies addressing seahorses in this century, accounting for 17% of all publications on this topic (Fig. 5). *Aquaculture, Journal of the World Aquaculture Society* (Wiley-Blackwell), and *Aquaculture Research* (Wiley-Blackwell) published 60% of all publications on seahorse aquaculture.

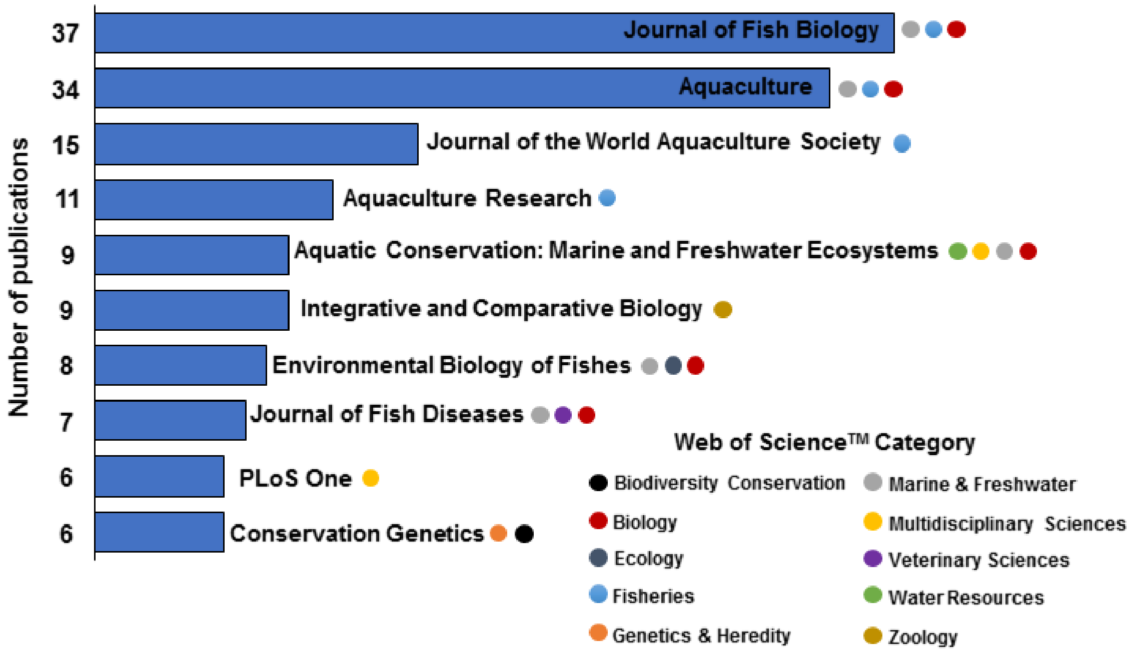

Figure 5. Top 10 scientific journals publishing scientific research on seahorses retrieved from Thomson Reuters^{TM} Web of Science^{TM} (all databases) from 2001 to 2015.

KNOWLEDGE GAPS AND RESEARCH OPPORTUNITIES

The critical analysis of the information retrieved from this survey revealed five major knowledge gaps and research opportunities that are essential to advance the state of the art on seahorse research: (1) Maximizing the potential of seahorses as flagship species for marine conservation; (2) Filling knowledge gaps on the most traded seahorse species; (3) Understanding the potential impact of emerging pollutants and climate change on seahorses; (4) Developing a sustainable low-cost aquaculture of seahorses; (5) Improving the traceability of traded seahorses to foster marine conservation.

Maximizing the potential of seahorses as flagship species for marine conservation

The unique morphology and reproduction makes seahorses charismatic animals. These features suggest that they could be good flagship species for marine conservation. Nonetheless, more than charismatic, a flagship species should fit a specific goal on conservation, in line with local context (Bowen-Jones and Entwistle, 2002; Home et al., 2009). According to Verissimo et al. (2011), flagship species are "*species used as the focus of a broader conservation marketing campaign based on its possession of one or more traits that appeal to the target audience."* Therefore, the question that should be investigated is whether the marketing strategies based on seahorse images are efficient enough to raise awareness on people for a specific conservation issue and allow the raising of enough funding to support it.

Seahorses inhabit many tropical and temperate shallow water habitats around the globe, including coral reefs, mangroves, and seagrass beds (Kuiter, 2009; Foster and Vincent, 2004). These areas are among the most affected areas in the sea, mainly through fishing, pollution, and tourism (Alongi, 2002; Hughes et al., 2003; Waycott et al., 2009). It has been shown that Syngnathids can be efficient flagship species for estuarine seagrass beds conservation, using as rationale that some additional species can also benefit from seahorse conservation (Shokri et al., 2009). Indeed, Project Seahorse also showed the effective use of seahorses as flagship species through the creation of some marine protected areas in central Philippines (Vincent et al., 2011). Nonetheless, further studies are still needed to link seahorses to their habitats and evaluate the true potential of them as flagship species, as well as on marketing strategies featuring these species. An urgent goal could be the use of seahorses to promote mangrove conservation. Mangroves are recognised as key marine habitats being amongst some of the most threatened tropical ecosystems (Alongi, 2002). Regions where mangroves are inhabited by seahorses (e.g., *H. reidi* in Brazil and *H. kuda* in Southeast Asia) (Foster and Vincent, 2004) should be associated with the species and studies on their potential as flagship to foster habitat conservation should be performed. Another goal that could benefit from seahorse image would be the conservation of coastal seabed through the reduction of destructive trawling fisheries (namely for shrimp). Study the feasibility of raising awareness of wild shrimp consumers by a "seahorse safe" label – developing and promoting fishing practices that do not harm seahorse populations. If costumers change their preference for a product originating from a more responsible fishing practices (e.g. "seahorse safe"), it can certainly affect the whole shrimp supply and value chain, and even benefit other marine species.

Filling knowledge gaps on the most traded seahorse species

Five seahorse species (*H. trimaculatus*, *H. spinosissimus*, *H. kelloggi*, *H. kuda*, and *H. algiricus*) account for more than 90% of the world trade of seahorses (Fig. 6), with most being traded as dried specimens collected from the wild (Foster et al., 2016). Except for *H. kuda*, scientific studies addressing these species are scarce (Fig. 6). On the other hand, *H. kuda* and *H. reidi*, species that are more well represented in the marine aquarium trade, have greatly been subject to scientific studies, namely in the field of aquaculture (Fig. 6). Even though not significantly traded internationally, *H. erectus*, *H. abdominalis*, *H. guttulatus*, and *H. hippocampus* are often referred as potential species for the marine aquarium trade (Koldewey and Martin-Smith, 2010) and have been widely studied (Fig. 6). Therefore, the aquarium trade has driven scientific research of seahorses, especially their aquaculture (Fig. 6). The higher prices fetched by live specimens, when compared to dried ones (Koldewey and Martin-Smith, 2010), may be the reason of this bias. In some way, it is puzzling that research efforts are not being target towards the most heavily traded species.

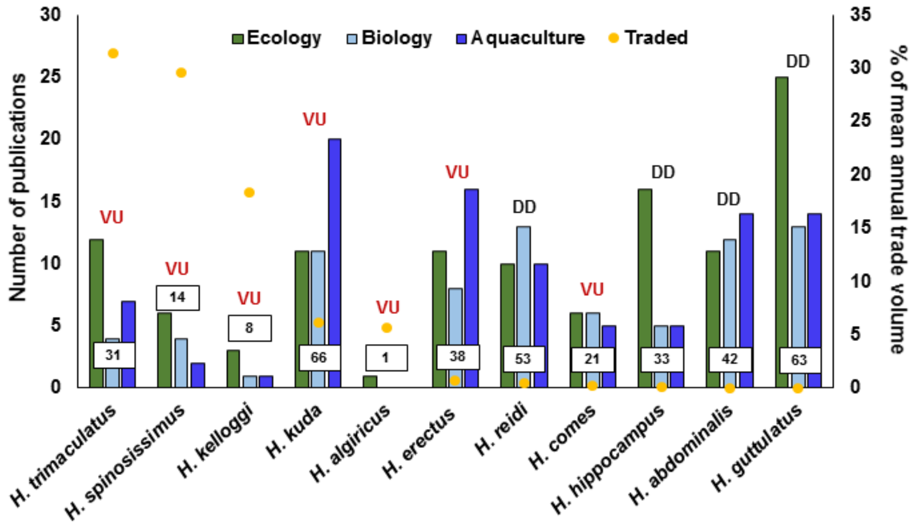

Figure 6. Number of scientific publications for the most studied and traded species of seahorses according to Thomson Reuters™ Web of Science™ (all databases) from 2001 to 2015. The risk of extinction for each species is indicated according to IUCN red list: VU = Vulnerable; and DD = Data Deficient. Total number of publication for each species – all studied fields included – is indicated in the boxes. Trade percentage was retrieved from Foster et al. (2016), which is an estimated mean of annual trade volume from CITES Trade Database (2004-2011).

Current aquaculture practices are not economically feasible for seahorses demanded by traditional Chinese medicine because of the low market price. Nevertheless, low-cost production of seahorse may be feasible and profitable (Fonseca et al., 2017). Thus, studies addressing the development of low-cost production systems should be prioritized. Additionally, studies addressing the population and fishery biology of the traded species should also be promoted to allow a better management of the fisheries, ensuring the maintenance of natural stocks and species conservation. Except for *H. algiricus* that is from West Africa, all other four most traded seahorse species are distributed across Southeast Asia (Foster and Vincent, 2004). Therefore, research focusing on the topics referred above (aquaculture and population biology) should be encouraged in Asian countries and be prioritized in international funding programs targeting marine conservation.

Transport is one of the highest costs in marine ornamental production, especially in countries where air shipping is necessary. Nevertheless, papers focus on transport of live seahorses are very scarce. An experiment conducted with *H. abdominalis* showed that it can tolerate extensive handling and confinement up to 35 hours of transportation (Wrigth et al., 2007). This shows a good opportunity to test the density during transport, since more animals in the same bag would significantly reduce freight. Cunha et al. (2011) showed that essential oil of *Lippia alba* can be an effective anesthetic for slight sedation and transport of *H. reidi*. No information on the effect of the micro environment inside bags is available. Seahorses are very low swimmers, have an efficient visual system, and use their camouflage for protection. Thus, the use of some specific colors background and inert substrates might reduce stress during transport, increasing survival, animal health and welfare. Studies on transportation of traded species are certainly an important avenue for new research. The optimization in this step may bring significant economic benefits and contribute to the animal welfare.

Some highly traded species lack of essential information for conservation. It is widely accepted that seahorse populations are threatened by overexploitation, bycatch, and generalized habitat degradation (Vincent et al., 2011; Harasti, 2016). Nonetheless, 67% of the 40 species included in the IUCN red list are classified as “Data Deficient” (IUCN, 2015-4). Only 11 species are categorised as “Vulnerable” and one as “Endangered” (*H. capensis*) (IUCN, 2015-4). Four of the seven most studied species are within the “Data Deficient” category (Fig. 6). Yet, most traded species are classified as “Vulnerable” (Fig. 6), probably based only on trade and fisheries quantification (Perry et al., 2010). The species *H. reidi* is highly traded for marine aquariums, but is classified as “Data Deficient” although many research efforts have been made to study this species. The “IUCN Seahorse, Pipefish and Stickleback Specialist Group” has developed significant efforts to provide information to improve the conservation of these fish (available at: https://iucn-seahorse.org; accessed in May 2016). Nonetheless, there are still knowledge gaps on growth, maximum size, longevity, reproduction biology, population structure and distribution, and population size of the most traded species. Overall, there are gaps on ecological and biological data from wild populations that needs to be overcome to promote regulations for a more sustainable fishery and conservation. Particularly, studies on time series in wild populations are imperative to ascertain the right assignment among IUCN categories and further conservation plans if required. Currently, there is not enough information to assess the endangerment status for most seahorse species.

Understanding the potential impact of emerging pollutants and climate change on seahorses

The low swimming capacity, small home range and preference for coastal habitats, enhance the vulnerability of seahorses to pollution (Vincent et al., 2011; Delunardo et al., 2013) and susceptibility to climate change (Faleiro et al., 2015). Nevertheless, seahorses may thrive in polluted areas (Tiralongo and Baldacconi, 2014) and even increase their populations (Correia et al., 2015). Exposure to crude oil has been studied in seahorses (Delunardo et al., 2013). The authors reported that crude oil can damage *H. reidi* cells, but that at an exposure of 10 ml/L during a 14-day period was not enough to induce severe gill damage (Delunardo et al., 2013). Others studies revealed that seahorses can bioaccumulate organochlorine pesticide and heavy metals (Nenciu et al., 2014; Zhang et al., 2016), a feature that coupled with their low motility suggests that these organisms can be good bioindicators (Delunardo et al., 2015). Nonetheless, pollution type and its extent may vary in marine habitats and generalizations may lead to pitfalls in decision-making. Thus, it is therefore important to study the effect of pollution on seahorse individuals and populations to understand in which scenarios they might affected. Additionally, studies should address the bioaccumulation on seahorses used for traditional Chinese medicine, as pollution could hamper any potential medical benefits or even pose a risk to human health. There is a knowledge gap in ecotoxicology assays evaluating the vulnerability of seahorses to emerging pollutions, such as nanoparticles, microplastic, and drugs.

Climate change can affect fish in different levels, from organisms, to populations, communities, and spatial ecosystems (Koenigstein et al., 2016). The main effects of climate change on oceans is the rising of water temperature, sea level, and acidification. Seahorse low motility might hamper migration from a changing environment, which would require adaptation to survive. The dependency of many seahorse species on adequate subtracts, mainly certain species of macroalgae and seagrass, would certainly be affected by the availability of anchoring elements and the composition, density and distribution of natural prey (strongly dependent on the type of vegetation) in temperature rising environments. Therefore, the biogeographical distribution of seahorse species could be altered (Planas et al., 2012). It was demonstrated that the combined effects of ocean warming and acidification negatively affect the behaviour and physiology of adult *H. guttulatus* (Faleiro et al., 2015). Further studies are therefore necessary to monitor the impact of climate change in seahorses because of their unique breeding strategy, and the broad distribution of the genus from temperate to tropical regions, including habitats where fish can be more resistant to climate change (e.g., estuaries) (Perry et al., 2015). Early life stages of some fish show abnormal calcification of otoliths (Munday et al., 2011) and skeleton (Pimentel et al., 2014) when exposed to acidification. The impact on seahorses should be investigated mainly because their bony plates are essential for protection against predation and their prehensile tail plays a key role in their stability in benthic substrates. Moreover, the paternal osmoregulation of pouch salinity in seahorses (Stölting and Wilson, 2007) is a feature that may also be affected under climate change scenarios and may negatively affect the offspring fitness.

Developing a sustainable low-cost aquaculture of seahorses

Although aquaculture of marine ornamental species is often presented as an option to relieve the collection of specimens from the wild, it can also drive negative environmental and social impacts (Tlusty, 2002). As an example, *H. reidi*, a West

Atlantic species that has been mostly cultured in Sri Lanka (Foster et al., 2016), a practice that may promote ecological issues through escapees (Vincent et al., 2011). The increase of captive bred seahorses in the trade (Foster et al., 2016) should make researchers consider the sustainability of these practices as a whole and not solely focus on the reduction of fishing effort targeting natural populations.

Most studies and commercial aquaculture practices of seahorses rely on the use of intensive monoculture systems, with animals being kept in aquariums or tanks under controlled water parameters and being totally depended on exogenous feeding to thrive. A system that depends exclusively on exogenous feeding might be inefficient for species that are difficult to feed, as seahorses have no stomach and this feature can reduce their ability to digest non-natural diets (Palma et al., 2014). Therefore, systems that could somehow allow the provisioning of natural food might be more sustainable than those currently used and even promote better results. An example is the cage-culture approach within an integrated multi-trophic aquaculture (IMTA) system. Some seahorse species can support a relatively high range of salinity (euryhaline) and temperature (eurythermal) (Hilomen-Garcia et al., 2003; Wong and Benzie, 2003; Curtis and Vincent, 2005; Lin et al., 2009; Hora et al., 2016), which makes them good candidates for cage-culture production in coastal areas, including coastal lagoons and estuaries. The natural growth of a periphyton-based community in the nets of grow-out cages, along with the natural flow-through of wild plankton, are suitable sources of natural food. Pilot trials have reported promising results during the grow-out of *H. reidi* in floating cages inside ponds destined for penaeid shrimp and oyster production (Fonseca et al., 2017). The authors reported a mean survival of ~80%, with seahorses attaining commercial size (7-8 cm) within approximately three months at a density of 80 ind.m^{-3} and without the input of any exogenous food. By growing *H. reidi* in an IMTA system already used to address the production of penaeid shrimp and oysters, seahorse aquaculture could be labelled as low-cost and economically feasible (Fonseca et al., 2017). Xu et al. (2010) have also shown that the integration of macroalgae (*Chaetomorpha* sp) grow-out in the production system increases survival and growth of juvenile *H. erectus*. Future studies addressing low-cost production systems should be supported, as this approach can also provide an opportunity to low-income coastal communities and make conservation efforts more perceptible at a local and regional scale. By enrolling local communities into such aquaculture practices, it can be possible to contribute towards a decrease of illegal, unregulated, and undeclared collection of seahorses from the wild and enhance environmental and social sustainability. Such enrolment would certainly require a simplification of the rearing system.

Improving the traceability of traded seahorses to foster marine conservation

Seahorses are the only group of marine ornamental fish traded to supply the aquarium industry that is currently included in CITES Appendix II (Vincent et al., 2014; Foster et al., 2016). This aspect puts seahorses in the forefront of trade regulations and management disputes. Nonetheless, there is still a substantial mismatch in species and volumes reported by CITES export and import records (Foster et al., 2016), with no method being currently available to confirm the origin of collection, nor to differentiate wild-caught from captive bred seahorses. Recently, the export of *H. algiricus*, one of the top five most traded species (Foster et al., 2016), was banned from Senegal and Guinea (Project Seahorse, 2016) and further restrictions can be anticipated to the trade of others seahorse species in the future. As captive cultured specimens are under less restricting regulations, these may be an alternative to fulfil demand. Nonetheless, without a reliable traceability toolbox, neither cultured specimens, nor those originating from sustainable collection, can be successfully discriminated from specimens illegally poached from the wild. Traceability is essential to enforce any conservation effort and avoid the collapse of their trade and the socio-economic impacts this scenario may pose (Cohen et al., 2013).

Before fine tuning traceability methods for seahorses, it is essential to identify the end market. Seahorses have two key and very distinct markets: the trade of millions of dried specimens for human consumption (traditional Chinese medicine) and the trade of thousands of live specimens for marine aquariums (Foster et al., 2016). Clearly, this dichotomy between markets requires different traceability methods and strategies for their implementation. The traceability of dried seahorses may be more easily achieved through the use of geochemical, biochemical, and molecular approaches already described for the seafood supply chain (Leal et al., 2015). Two studies highlighted the possibility to sample tissues from partial fin-clipping of seahorses for molecular and stable isotopes analysis (Valladares and

Planas, 2012; Woodall et al., 2012). Nonetheless, the drying process of specimens may affect the reliability of some of these methods and further studies are required to validate their use. The fatty acid profile is a promising tool for geographical traceability of seafood (Leal et al., 2015), and might be suitable to trace dried seahorse. Previous biochemical analysis showed significant difference on fatty acids composition among six seahorse species from the coast of China (Lin et al., 2008). Recently, Shen et al. (2016) developed and validated a sensitive and specific lipidomic protocol for the detection of phospholipids in dried seahorses. The authors were able to differentiate five wild species of dried seahorse based on phospholipid class. Therefore, future studies should investigate the reliability of this method to differentiate wild seahorses from captive bred ones, and to differentiate specimens from the same species originating from different origins (regions or farms). Concerning the trade of live seahorses to supply marine aquariums worldwide, the production of different colour morphs and shapes through hybridization might be a good way to differentiate captive bred seahorses. Two scientific studies reported interspecific hybridization in seahorses so far, one between male *H. algiricus* and female *H. hippocampus* (Otero-Ferrer et al., 2015), and other between male *H. erectus* and female *H. redi* (Ho et al., 2015). This method however poses environmental risks due to potential escapees (Cohen et al., 2013). The use of bacterial communities-based signatures present in fish mucus for their origin traceability has been addressed for marine fish in general (Leal et al., 2015) and marine ornamentals in particular (Cohen et al., 2013). The only study available to date on the phylogenetic characterization of bacterial communities associated with seahorses showed that the microbiological composition of the cutaneous mucus and that of both the surrounding seawater and the live food differ significantly (Balcázar et al., 2010). The low motility of seahorses may favour the use of this approach to differentiate wild populations, as well as wild and cultured specimens, in a non-invasive and non-destructive way. It is reasonable to assume that even in the wild, seahorses would stay in the same geographic area long enough to develop a local-specific bacterial signature in their mucus that may be used for traceability. With the advent of a reliable traceability method for seahorses, certification and eco-labelling could be implemented by CITES to trace animals throughout the whole supply chain, supporting a conscious and more sustainable trade.

CONCLUDING REMARKS

A multitude of factors may motivate researchers to study seahorses. Nonetheless, it is important to flag the paramount research fields to advance the state of the art to subsidize decision makers to address the issues affecting production, trade and upkeep the natural populations. This study highlights five knowledge gaps and research opportunities that can generate information to supply dry and live markets and promote seahorse conservation. Overall, a well-managed and sustainable trade of these emblematic marine organisms includes sustainable fisheries and aquaculture. Research should provide science-based information to develop a sustainable industry. This can contribute to marine conservation and foster socio-economic activities in developing regions.

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VI. MANUSCRITO II

CURRENT INTERNATIONAL REGULATIONS OF SEAHORSES TRADE ARE INEFFECTIVE TO PROMOTE CONSERVATION

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ABSTRACT

Millions of seahorses (*Hippocampus* spp) are collected every year from the wild mostly to supply the international trade of Traditional Chinese Medicine but also for marine aquariums. Since 2004, seahorses have been included in CITES Appendix II to regulate and record international transactions to avoid overexploitation. By analysing CITES Trade Database from 2004 to 2014, we highlight a significant decrease in the reports of dried and live seahorses between 2012 and 2014. This decrease is likely due to a recent ban of legal exports from key exporting countries and legal restrictions to exports imposed by international trade regulations. This scenario of a shortage of legal seahorse supply associated to a high demand, price inflation, and low trade control may intensify illegal, unregulated, and unreported fishing practices to supply unmet demand. Indeed, millions of seahorses have been smuggled in the past few years. Overall, we observed that the CITES Trade Database no longer reflects seahorse international trade. We anticipate that this scenario seriously threatens seahorse wild populations. To support seahorse conservation and local fisher communities it is paramount to control domestic and black markets, develop sustainable fisheries, and low-cost aquaculture practices for local species.

Key words: flagship species, protected fish, Syngnathidae, threatened fish.

INTRODUCTION

Seahorses (*Hippocampus* spp) are a group of bony fish that display a unique morphology and reproductive system. Their shape are similar to ponies and the males incubate the eggs in an abdominal chamber. These features make seahorses a potential flagship species for marine conservation (Shokri et al., 2009, Vincent et al., 2011, Yasue et al., 2012), but also contribute to extensive harvesting. Their limited swimming capacity and reduced productivity makes them vulnerable to overfishing and habitat degradation (Vincent et al., 2011, Harasti, 2016). More than 40 million dried seahorses are traded yearly to supply the Traditional Chinese Medicine, as well as folk remedies, magic-religious, talismans, decoration, and curio trade, whereas thousands of live specimens are traded for marine aquariums (Foster et al., 2016, Rosa et al., 2011, Zhang, 2015).

Seahorses were included in Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) in 2004 to regulate their international trade and avoid overexploitation (CITES, 2004). Since then, it is expected that all international transactions have been recorded by exporting and/or importing countries on the CITES Trade Database. Nevertheless, this database has a number of limitations and discrepancies (Blundell and Mascia, 2005), including significant divergences on seahorse import and export data (Foster et al., 2016), as well as a lack of domestic trade data. Despite these limitations, the listing of seahorses in CITES has favoured their conservation (Vincent et al., 2011, Vincent et al., 2014), promoted their scientific knowledge (Cohen et al., 2017), and created an opportunity to study and manage the international trade of these fish (Vincent et al., 2011, Foster et al., 2016).

The CITES Trade Database registers the amount of seahorses commercialized annually in the international market of dried and live seahorses per species and country. The most recent study using the CITES Trade Database analysed data ranging from 2004 to 2011 (Foster et al., 2016). Dried seahorses represented 90% of the total international trade reported, with *H. trimaculatus*, *H. spinosissimus*, *H. kelloggi*, *H. kuda*, and *H. algiricus* being the most exported species. Thailand and West Africa (Guinea and Senegal) were the main exporters of dried seahorse, mostly to China (including Hong Kong) (Foster et al., 2016). The same study refers that Vietnam, Sri Lanka, and Indonesia were the main exporters of live seahorses, mostly to the USA and the EU, with *H. kuda* and *H. reidi* being the most traded species. In the present study, we expanded the analysis by Foster et al. (2016), considering data entries on the CITES Trade Database ranging from 2004 to 2014 and obtaining some unofficial data in selected countries. The objectives were to explain the decreased trade in seahorses observed in CITES records after 2011, and to assess if data reported to the CITES are reliable and reveal an effective control of seahorses capture and commercialization worldwide.

MATERIAL AND METHODS

In December 2016, we collected all data available on the international trade of seahorses (*Hippocampus* spp.) on CITES Trade Database (available at: http://trade.cites.org/) ranging from 2004 to 2014. According to CITES Trade Database guidelines(UNEP-WCM, 2013), “the most recent year for which comprehensive trade statistics are available is normally two years before the current year”. Therefore, data from 2015 was considered incomplete and was not included in the present study. A total of 2960 trade records were recovered, each one containing the following information: year, importing and exporting countries, origin (only when the exporting country was not the country of origin of the seahorses being traded), imported quantity (value reported by the importing country), exported quantity (value reported by the exporting country), term (specimens, bodies, live, skeleton, derivatives, fingerlings, etc.), unit (Kg, g, L, mL, or specimens), and source (W: wild; C: captive-bred, F: captive-born, I: confiscated specimens, O: pre-convention species, R: ranched species, U: unknown). According to UNEP-WCMC (UNEP-WCM, 2013), specimens whose source is labelled as “F” refers to “animals born in captivity (F1 or subsequent generations) that do not fulfil the definition of 'bred in captivity' in Resolution Conf. 10.16 (Rev.)”. In the case of seahorses, this terminology refers to the capture of wild parents or pregnant males and grow-out of new-borns in captivity. This type of sourcing has a greater impact on wild populations when compared to specimens that are captive-bred (C), but has a lower impact when compared to wild-caught (W). Whereas “F” specimens are at times analysed along with “W” specimens (e.g., Foster et al. (2016)), herein “F”, “W” and “C” seahorses were analysed separately. All records identified as re-exports were excluded from any further analysis to avoid double counting. All entries considered valid were identified using the terms as “dried” or “live”, as detailed by Foster et al. (2016). Units expressed as “Kg” or “g” were converted to dried “specimens” using the conversion factors described by Evanson et al. (2011) and Foster et al. (2016). Individuals recorded as “live” were assumed to be “specimens”, thus, records of live seahorses reported as “Kg” or “g” were considered to be erroneous and were assumed to be “specimens”. All blank cells were considered as “specimens” (UNEP-WCM, 2013). Finally, we disaggregated the source of traded seahorses in three categories: wild (R and W), captive-bred (C), and captive-born (F) – all other categories and blank cells were excluded from the analysis.

We plotted a histogram with total import values per year, for both dried and live traded specimens. More than 100 different countries were involved in at least one seahorse transaction during the period covered in the present study (2004-2014). Nonetheless, more than 90% of the international trade for dried specimens was represented by Thailand, Guinea + Senegal, and China, including Hong Kong. For live specimens, Vietnam, Sri Lanka, Indonesia, USA, and the EU played the major role in the international trade. In this way, we highlighted these main territories in our analyses and aggregated all others in a single group termed as “others”. As the source of seahorses was directly related to its exporting country, we included source values (wild, captive-bred, and captive-born) in a line graph on the graphical representation of exporting countries. Values reported by exporting and importing countries rarely matched. Therefore, CITES Trade Database can be interpreted in many ways. We decided to use data reported by importing countries. Although this approach may underestimate the volume traded when compared to data reported by exporting countries or to a combination of both importing and exporting countries (Foster et al., 2016), our previous analyses showed that all these perspectives showed the same trend described herein.

In addition, information on domestic catch, aquaculture and trade were mined in scientific literature, magazines and newspapers. Some interviews with local people were also performed.

RESULTS AND DISCUSSION

Data retrieved from the CITES Trade Database indicate that the reported international commerce of seahorses experienced a significant decrease after 2011 for both dried (Fig. 1) and live (Fig. 2) specimens. This decrease was more abrupt for dried seahorses, compared to live specimens. The volume of dried seahorses imported from 2004 to 2011 averaged ~3.5 $\pm$ 0.4 million specimens.year^{-1} (mean $\pm$ 95% confidence interval) and decreased to below 800 thousand in 2012 and 50 thousand in 2014 (Fig. 1). The import volume reported for live seahorses from 2005 to 2012 averaged ~70.0 $\pm$ 9.6 thousand specimens.year^{-1} and decreased to below 40 thousand in 2013 and 30 thousand in 2014 (Fig. 2). Considering the contrasting nature of these two branches of the international trade supply chain (e.g., different exporter countries, species, and number of specimens), they are discussed separately in the following sections.

International trade of dried seahorses

Most of the observed decline in reported volumes of dried seahorses can be explained by Thailand's decision to implement a voluntary export quota for seahorses in 2012 (Foster, 2016). In 2012, Thailand exported less than 20% of its prior eight-year annual average. Thailand had been a major exporter of dried seahorses (Fig. 1A) to China, including Hong Kong (Fig. 1B). Thus, the reduction observed from 2012 onward explains a substantial part of the global drop observed in 2012-2014. CITES separates Hong Kong from China because Hong Kong is a Special Administrative Region of the People's Republic of China; however, it is likely that most seahorses landing in Hong Kong are latter distributed to mainland China. CITES have recently (2016) suspended all seahorse exports from Guinea and Senegal (CITES, 2016, Project Seahorse, 2016a). Thailand has announced the banishment of seahorse export to protect wild populations (Project Seahorse, 2016b, Foster, 2016), although an official CITES trade suspension is not in place as of 1 January 2017. Indeed, some fishers and traders have already indicated a decreasing availability of wild populations of seahorses in Thailand (Perry et al., 2010). Therefore, the main traditional exporters are legally stopping the supply of dried seahorses, causing a significant reduction in reported transactions in recent years. Similar restrictive actions were implemented by

India in 2001 and the Philippines in 2004, which were the main exporters of dried seahorse in the 1990s (Vincent et al., 2011).

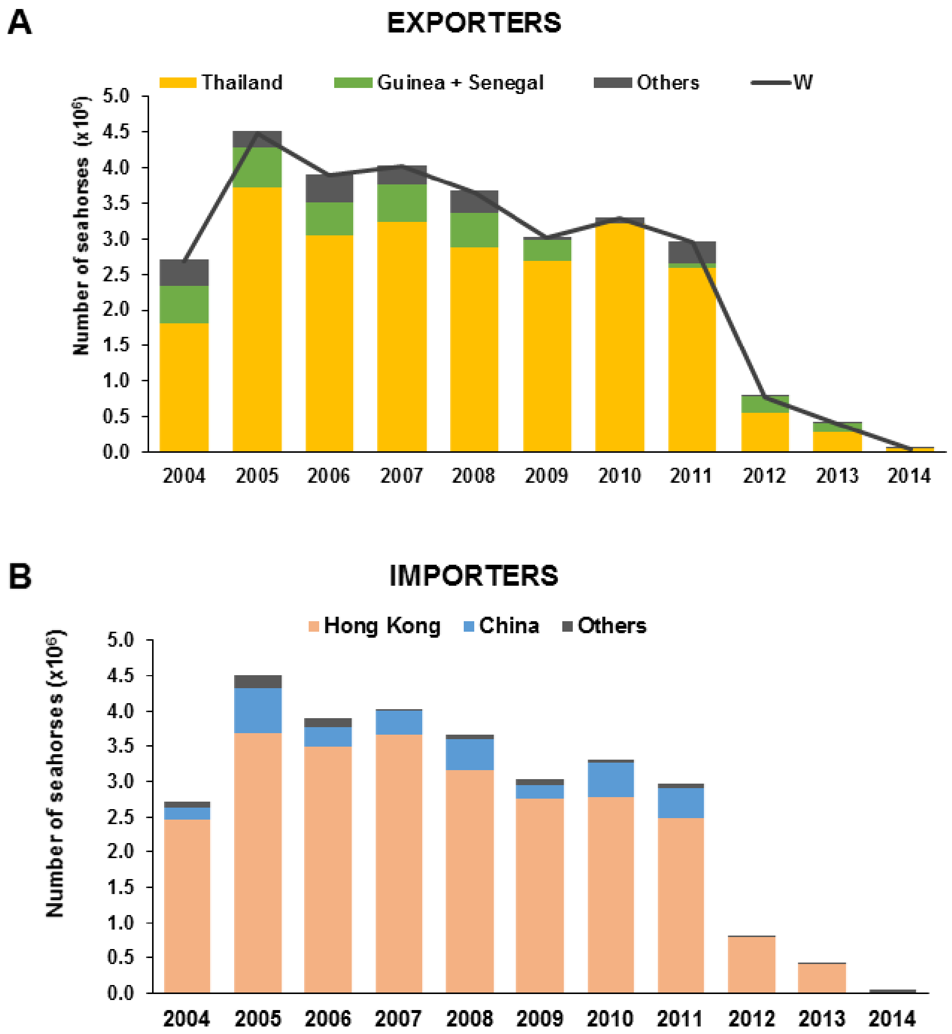

Figure 1. Number of dried seashores traded internationally. A: main exporting countries and source of traded specimens - collected from the wild (W). B: main importing territories. Hong Kong is a Special Administrative Region of the People's Republic of China. Data collected from CITES Trade Database.

Some traders in Hong Kong believe that CITES bureaucracies lead exporters to abandon the activity (Lam et al., 2016). This shortage of dry seahorses in the market may lead to increase domestic aquaculture in China (Lam et al., 2016) and in other importing countries. The domestic production is not subject to CITES regulations and is not reported to CITES Trade Database. Indeed, China/Hong Kong is the main importer (Fig. 1B) and is a leading country in seahorse aquaculture research (Cohen et al., 2017). Ho and Lin (Ho and Lin, 2015) reported a recent increase in seahorse aquaculture in China. Currently, there are more than 80 seahorse farms in China, which produce ~5 million specimens per year (Zhang, 2015). In addition, ~15 million of wild specimens are harvested in China every year. According to CITES Trade Database, there was no legal import of dried seahorses into China/Hong Kong in 2014 (Fig. 1B). Nonetheless, the local market consumes ~40 million of dried seahorses annually (Zhang, 2015) (Fig. 3), leading to a gap of ~20 million seahorses between supply and demand. This deficit is probably supplied by the black market, and shows that the decrease in legal reports does not indicate real decrease in the trade.

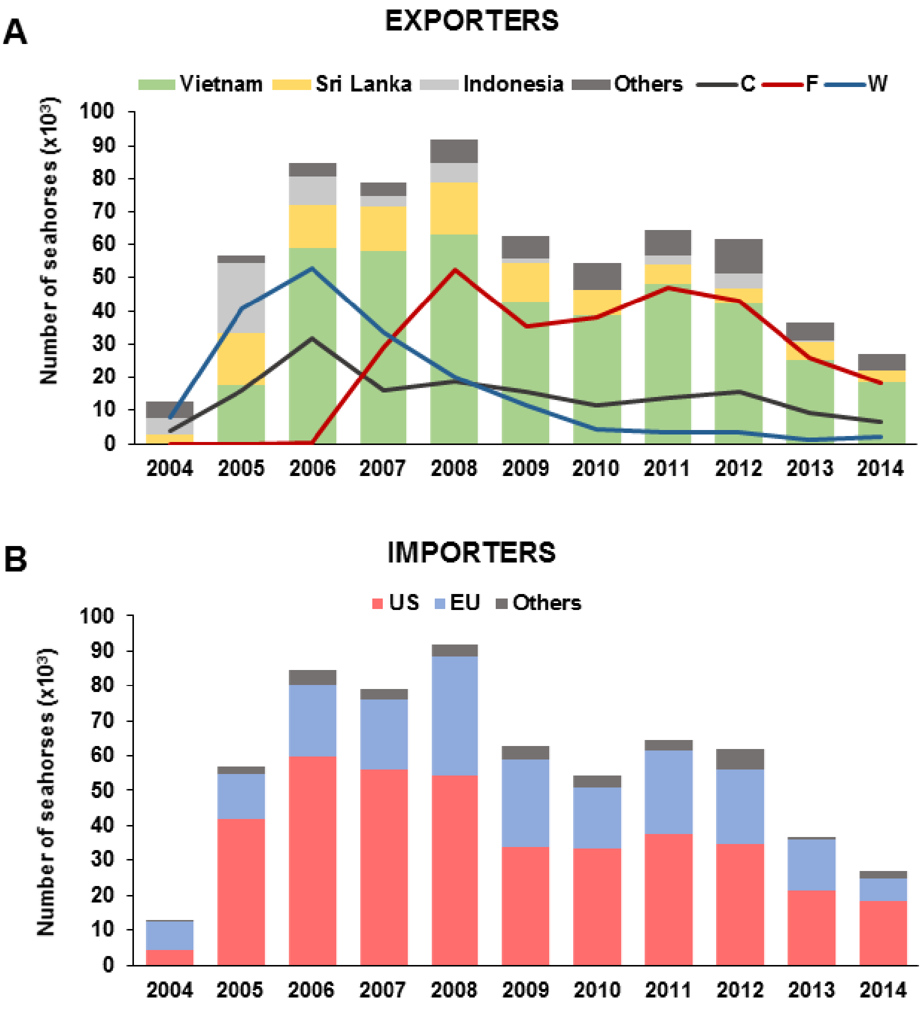

Figure 2. Number of live seashores traded internationally. A: main exporting countries and source of traded specimens - captive-bred (C), captive-born (F), and collected from the wild (W). B: main importing territories. Data collected from CITES Trade Database.

Considering the rationale detailed above, the trade of dried seahorses might be under pressure, as the decrease in the number of specimens being exported legally is not being matched by a proportional decrease in demand. The average price of dried seahorses in China has increased by about 20% in 2016 (for instance, US$ 3/specimen), likely due to a shortage in the legal supply and to numerous attempts to stop smuggling activity in China. Seahorses have been traded for Traditional Chinese Medicine for more than 600 years (Vincent, 1996), and recently, many studies have supported their medical properties (Kumaravel et al., 2012, Chen et al., 2015). In addition, government in China/Hong Kong has encouraged the development and exportation of TCM (Shanghaidaily.com, 2016). Thus, demand for seahorses will presumably remain high and even increase in near future. It is reasonable to assume that exporting restrictions alone will not protect seahorses if a significant demand persists in China and elsewhere. In fact, these restrictions may contribute to increase the illegal capture of wild seahorses worldwide and the legal exploitation in countries that have not yet limited their capture. The post-2014 CITES data do not support the assumption that legal exploitation is increasing in some countries. Thus, it can be presumed that illegal, unregulated and unreported (IUU) fishing practices are increasing. The decrease in legal supply may also encourage the exploration of new fishing areas and ultimately favour IUU fisheries. According to information from Wildlife Crime Control Bureau, India is smuggling a considerable amount of dried seahorses to Thailand (Vashishtha, 2014). As seahorse exports from India are illegal since 2001, these records will never be reported to CITES, thus, never enter the CITES Trade Database. Another impressive example that helps to perceive the dimension of the international black market for the trade of dried seahorses was the apprehension of eight million dried specimens in Lima (Peru) in 2016, which likely were going to be smuggled to Asia (Actiman, 2016). These eight million individuals in this single apprehension was alone greater than to the total number reported by CITES in a single year (Fig. 1a). Surprisingly, these specimens were apprehended in Peru, a country that has never been ranked as a major seahorse exporter in CITES Trade Database. Moreover, seahorse black markets were not exclusive from developing countries. In January 2017, more than two thousand dried specimens were illegally collected in Portugal, and seized in Spain (Planelles, 2017).A recent study reported that the genera *Hippocampus*, *Acipenser* and *Huso* accounted for almost 50% of all fish seizures entering the US (Petrossian et al., 2016). All of these genera are included in CITES appendix II (available at: https://cites.org/eng/app/appendices.php; latest access: December 2016). These are just a few examples highlighting the massive illegal trade of dried seahorses that persists worldwide, and that will possibly increase with the growing restrictions to the international transactions of seahorses.

Figure 3. Photo of a market in Guangzhou, China, selling thousands of dried seahorses in September 2016 (photo by Michael Tlusty).

Other domestic markets besides China trade a huge amount of dried specimens. For instance, a comprehensive survey estimated that about 1.2 million dried specimens were traded in Brazil annually in the past decade (Rosa et al., 2011). Domestic illegal trade may be hidden as accidental captures by the trawling fishery. It is reported that a substantial source of dried seahorses is a bycatch from shrimp or fish trawling (Perry et al., 2010, Rosa et al., 2011). This will continue to negatively affect wild populations even with stricter export restrictions in place (Foster et al., 2016). However, we have interviewed three researchers that have performed thousands of trawlings during the past 30 years in Brazil. All of them stated that seahorses were very sporadic in trawling catches and certainly cannot support regular commercialization (A. Fransozo; W. J. Cobo; R.C. Costa, 2017, personal communication). Thus, it may be possible that the seahorses declared as by-catch, really comes from illegal fisheries in coastal zones. Thus, regulating and monitoring domestic markets may be even more important than the global trade to best implement seahorse conservation.

All countries that are still legally allowed to export dried seahorses should aim to refine and enforce their regulations and management schemes to avoid new bans and depletion of wild populations. These procedures include the establishment of quotas and delimitation of fishing areas. Nevertheless, no action will be effective if black market persist. Therefore, countries should enforce domestic regulations to control black markets and create incentives and opportunities for fishing communities that relied on seahorse exploitation for income (Cisneros-Montemayor et al., 2016). Exporting countries, namely those in Asia and West Africa, should also invest in the development of low-cost aquaculture systems to produce the main local traded species (e.g., *H. trimaculatus*, *H. algiricus*, *H. spinosissimus*, *H. kuda*, *H. kelloggi*) to enhance environmental and social sustainability (Cohen et al., 2017). In China, seahorse culture costs have been significantly reduced through optimizing protocols, especially in disease control and live food supply. The progresses significantly improve the profit of cultured seahorses and make them competitive with wild collected ones. A Chinese Seahorse Aquaculture Association was stablished 2016 and one of its missions is pushing the government to obey CITES, to clean illegal market, and block illegal channels of seahorse trade.

One example of a successful low-cost production of seahorse is PRIMAR, an organic certificated farm located in an estuarine region in northeast Brazil. This enterprise is now (2017) legally producing seahorses (*H. reidi*) in cages using an integrated multi-trophic aquaculture (IMTA) approach along with shrimp, oysters, crabs and other local fish (Souza-Santos, 2014). All species are cultured within the same system and rely only on natural diets available in the environment, without the input of any exogenous food or artificial fertilization (Souza-Santos, 2014). Pilot experiments and commercial production have shown that seahorses achieved commercial size (~8 cm) within 90 days with up to 80% survival (Souza-Santos, 2014, Fonseca et al., 2017). The capacity to produce 12 thousand specimens in 402 m^2, per year, with a lower production cost, makes this system economically feasible even to supply dried specimens to local and international markets (Fonseca et al., 2017). The possibility to develop seahorse cage-culture in estuarine regions is a good opportunity for low-income communities that live in mangrove areas. Both *H. reidi* and *H. kuda* inhabit mangrove regions in Brazil and Southeast Asia, respectively (Rosa et al., 2002, Lourie et al., 2004). Therefore, these low-cost aquaculture systems should be encouraged to supply domestic and international demand as well as to promote seahorse conservation and social sustainability for communities that rely on seahorse production.

International trade of live seahorses

The trade in live seahorses peaked in 2008. Vietnam, Sri Lanka and Indonesia exported thousands of live seahorses (Fig. 2A), mainly to the USA and EU (Fig 2B). Some of the decrease may be explained by the national decisions to suspend export of all wild and captive-born (F) seahorses by Indonesia and Malaysia in 2011, and CITES-recommendations to ban trade of *H. kuda* from Viet Nam in 2013 (Foster, 2016). The USA and the EU have also developed significant research on the captive production of seahorses, focusing mainly on species used for marine aquariums (Cohen et al., 2017). In this way, it is possible that domestic production supported by aquaculture has increased in these two main importers of live seahorses. The high market price commanded by these organisms (for instance, US$ 60-120/specimen in the US) allows commercial breeders to employ costly intensive production systems. In addition, imported live seahorses may be more expensive to the final customer than those being domestically produced due to exporting expenses, as well as a higher bureaucratic load imposed by CITES and other regulations associated with their commercial transaction (Lam et al., 2016). In the long-term, these complex international regulations can significantly affect traders' interest in remaining exporting seahorses.

Aquaculture is the main source of seahorses destined for marine aquariums (both captive-bred and captive-born specimens; Fig. 2A). Since 2006, there has been an increase in the commercialization of live captive-born individuals and a decrease in the trade of live captive-bred and wild caught specimens. This dominance of captive-born specimens in the live seahorse trade (Fig. 2A) shows the potential to implement large-scale culture ventures. Captive-bred animals are usually under less regulation than wild conspecifics and could be a better opportunity for exporting countries to supply the international trade of live seahorses. Therefore, exporting countries in Asia and South America should intensify the development and implementation of low-cost aquaculture practices for local species demanded by the marine aquarium trade (e.g. *H. kuda*, *H. comes*, and *H. reidi*).

The culture of seahorses can also have negative environmental and social impacts (Tlusty, 2002). The environmental impacts includes the capture of wild specimens for reproduction (Koldewey and Martin-Smith, 2010) and the potential invasion of natural environment by exotic species (Tlusty, 2002). A potential social impact arising from seahorse aquaculture is the shifting of economic base, in which low-income coastal communities that rely on seahorses production in export countries lose their business opportunity due to the increasing domestic production in importing countries (Tlusty, 2002, Rhyne et al., 2014). Therefore, exporting and importing countries should do their best to safeguard that this increase in aquaculture production, for both dried and live seahorses, is in line with sustainable practices, both from an environmental and social perspective.

CONCLUSIONS

We have showed that the apparent decrease in the international trade of seahorses reported in CITES Trade Database is likely due to the end of legal exports from key exporting countries and the growing restrictions to the legal exports imposed by international trade regulation. This scenario prompted the expansion of domestic production in some importing countries and probably boosted the growth of the black market, both not under CITES regulations. Therefore, the low volume of animals traded in recent past years reported in CITES Trade Database does not reflect a real reduction in the capture and trade of seahorses. The CITES Trade Database no longer reflects the international trade of seahorses. In this way, current rules and regulations established to control the global capture and commercialization of seahorses may have failed to promote the conservation of these fish. This scenario of shortage in supply coupled with an increasing demand, along with price inflation and low trade control, is likely to intensify IUU fishing practices. In addition, domestic market in some countries may be very high. Therefore, we anticipate that this scenario seriously threatens the wild populations of seahorses. Every country involved in the trade, as well as those being part of current black market routes, should enforce control and regulation. Demand for this species will not likely diminish at any point in the future. As such, a sustainable production of dried seahorses that meets market demand is urgently needed and paramount to safeguard the conservation of these organisms. The development of sustainable fisheries and low-cost large-scale aquaculture systems to provide a suitable number of dried animals will provide the basis for seahorse conservation, including accounting for local livelihoods already involved in this trade.

ACKNOWLEDGMENTS

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VII. MANUSCRITO III

OPPORTUNITIES AND CONTRAINTS FOR DEVELOPING LOW-COST AQUACULTURE OF SEAHORSES IN MANGROVES

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ABSTRACT

Wild populations of seahorses are threatened by overexploitation and the increasing black market to supply the trade of dried specimens for Traditional Chinese Medicine. Intensive aquaculture systems, available for seahorses, are developed for producing pricey specimens for marine aquariums, and thus, are not suitable to solve the real threat facing their conservation. Therefore, our aim was to describe a first study on opportunities and constraints to develop low-cost aquaculture of seahorses in cages in mangrove estuaries. To observe the strengths and weakness of the system, we did three trials throughout one year in a sub-tropical mangrove in Brazil with a local traditional community. In this system, seahorses should grow-out relying only on wild plankton and periphyton available in the estuary. We discuss that the production of seahorse in mangrove has many opportunities to increase environmental and social sustainability. Nonetheless, we also addressed the main constraints of this production that should be overcome to stablish a reliable culture protocol, including the environmental instability of estuaries, net obstruction, predators, and escapees.

INTRODUCTION

Promote low-cost aquaculture systems for seahorses is crucial to improve seahorse conservation and to give economic and social opportunity for low-income coastal communities (Cohen *et al.* 2017, Cohen *et al.* unpublished, Manuscrito II). For many years, millions of seahorses have been collected from the wild and sold as dried specimens to supply the Traditional Chinese Medicine (TCM), folk remedies, magic-religious, talismans, decoration, curio trades (Vincent *et al.* 2011, Foster *et al.* 2016, Rosa *et al.* 2011) and for human food. In a lower extent, thousands of seahorses have been traded live for marine aquariums (Vincent *et al*. 2011, Foster *et al*. 2016). Although live specimens are mostly from captive sources, most specimens still rely on the capture of wild parents or pregnant males for grow-out of new-borns in captivity (Foster *et al*. 2016, Cohen *et al*. unpublished, Manuscrito II). Recent study showed that the international regulations of seahorse trade is currently inefficient to promote conservation, and that half of the demand for dried seahorses (~20 million specimens) have been probably supplied by black markets (Cohen *et al*. unpublished, Manuscrito II). About 1.2 million specimens/year may be sold in black market in Brazil during the past decade (Rosa *et al*. 2011). Nonetheless, the lower price that dried seahorses attain at final markets (for instance, US$ 1-3/specimen in China and Brazil) is incompatible with the current aquaculture systems for seahorses, which have been developed for priced specimens for aquariums (for instance, US$ 60-120/specimen in the US) (Koldewey and Martin-Smith 2010, Olivotto *et al.* 2011). Therefore, develop and implement a low-cost aquaculture is a top priority police to relieve collections of wild seahorses in near future.

Cage-culture in mangroves estuaries can be a great opportunity to develop low-cost aquaculture of seahorses (Cohen *et al*. unpublished, Manuscrito II). Production costs and feeding are the main constraints for the current aquaculture of seahorses. These fish have been produced in intensive systems, where they are kept in aquariums with fully controlled water parameters and dependent of exogenous feeding (Koldewey and Martin-Smith 2010). Aquaculture studies also focused most in these intensive systems, neglecting extensive farms (reviewed in Cohen *et al*. 2017). Intensive systems have high costs with facilities, electricity and feed and thus, it cannot supply dried seahorses for TCM and other uses in different countries. Recent study showed the economic feasibility of producing *H. reidi* in integrated multi-trophic (IMTA) systems without addition of exogenous food and artificial fertilization (Fonseca *et al.* 2017). In this example, seahorse juveniles grow in cages inside shrimp ponds and rely only on natural food available in the system. The same principle could be adapted to mangrove regions, where the cages could be installed directly in the estuary. Mangroves estuary have high primary productivity that boost zooplankton and benthos communities (Robertson and Blaber 1992), which are the main natural diets of seahorses (Foster and Vincent 2004). Therefore, a cage-culture in mangroves estuary would allow a supply of natural diet by the flow-through of wild plankton and by the development of periphyton communities inside the cage. Additionally, low-cost productions in mangroves could improve social sustainability because many families live in mangrove areas and need a low environmental impact economic activity to retain their style of life and improve their income.

Considering the above rational, low-cost aquaculture of seahorses is a new and urgent field that should be explored to supply the large market of dried seahorse and conserve the natural populations. Cage-culture in mangroves estuary has many theoretical reasons to achieve these goals and increase environmental, social and economic sustainability on the seahorse production chain. Nonetheless, no attempt has been reported yet, probably because of the difficulties inherent to set up aquaculture systems in mangroves due to geomorphic, hydrological and water quality instability as well as legal restrictions. Therefore, our aim was to describe a first study on opportunities and constraints to develop low-cost aquaculture of seahorses in cages in mangrove estuaries.

GENERAL PROCEDURES

We selected a subtropical mangrove region inhabited by a low-income community that rely on artisanal fishing and aquaculture as their main income source. Some local visits assisted by local people were performed to create a relationship of trust with the community, and to obtain information on site-characteristics, such as water movement, temperature and salinity, suspension matter, and depth. In early summer (2015), we set up four net-cages in the area for three weeks to adjust the structure and the location according to local conditions. Then, we stocked the cages with seahorses (*Hippocampus reidi*) three times to perform observations, once in the summer (rainy season) and twice in the winter (dry season) of 2015. This is the first part of a long-term project to implement low-cost seahorse aquaculture in this community.

Area of study and local community

The study was performed in a mangrove estuary located at the Extractive Reserve of Mandira in Cananéia, São Paulo – Brazil (Fig. 1), which is inhabited by the community “Quilombo do Mandira”. This reserve has approximately 1.175 hectares of Atlantic Forest, predominantly formed by mangroves (ICMBio 2010). Local climate is defined as “humid subtropical”, which rains year-round (precipitation above 2.000 mm/year) but have rain peaks and warmer temperatures during spring and summer (from October through March) (ICMBio 2010). This reserve is a protected area where the extractive community is allowed to fish, hunt, and collect raw materials for own consumption and even promote small-scale agriculture and aquaculture as commercial activities (ICMBio 2010). The community “Quilombo do Mandira” was founded at approximately one century and a half ago by descendants of slaves from African origin, and it is now formed by around 25 families in its seventh generation (available at: http://www.quilombosdoribeira.org.br/mandira/inicio; accessed in October 2016). For many years, they relied on the wild collection of oysters to supply local markets and, since 1997, they founded a cooperative to grow-out wild-collected oysters *in situ* (ICMBio 2010). Therefore, they collect wild individuals of approximately 50 mm and put it to grow-out in trays inside the estuary for approximately five months, until attain minimum commercial size (~80 mm). Farming oyster is the main activity of this community, followed by the handcraft made mostly by women. Although this culture system might be more sustainable than others extractive oyster productions (Machado *et al.* 2015), the income is lower than other employments in the city, which could cause an emigration from the younger generations to urban centres affecting the community structure and traditions (Bertolazzi 2013). This community is in similar conditions as many low-income coastal communities in developing countries that rely on artisanal fishing for food or ornamental markets. Therefore, the culture of seahorses might be an important additional income. This area is within the distribution range described for *H. reidi* (Floeter *et al.* 2003, Lourie *et al.* 2004), and local community reported that they found seahorses occasionally near oysters' trays and as bycatch in artisanal shrimp trawling.

Cages structure and installation

Four flouting cages of 1 m^3 each were installed in the estuary of the Extractive Reserve of Mandira. Cages structure was made of common and cheap PVC pipes, covered by a strong nylon net of 1 mm mesh. Two perpendicular plastic nets (1 m width and 0.5 m high each), with 5 mm mesh, were set inside the cages to function as substrate for seahorses to hold. Four rolled plastic nets, with 5 mm mesh, were also placed inside each cage to increase area for periphyton growth. The cages were rearranged and sewed by women in the community. This inclusion is important to promote community engagement and learning, as well as an opportunity for sexual equality in the production.

Before installing the cages, it was performed a prospection for selecting the site. The main factors relevant for site selection were the distance from the community, the depth, the water current, and the salinity. The system should be near the community and oyster production for easy access and maintenance. The depth should be deep enough to avoid the bottom of the cage to touch the floor in the lowest tide. The water current should be strong enough to allow water exchange in the cages without being too strong that could compromise the structure. The salinity should be between 10 to 36, which is the range tolerated by *H. reidi* juveniles (Hora *et al.* 2016, Melo-Valencia *et al.* 2013). During the three weeks of a practical attempt to install the system, the cages were submerged and carried away due to strong current and poor fixation. Therefore, within this period, we chose a better location (a small bay showed in Figure 1) and adjusted the structure fixation and buoyance.

The cages were installed in series, perpendicular to the water current (Fig. 2a, b). All cages were connected in a rope structure that was fixed in the bottom by four concrete piles (Fig. 2a). Mangrove substrate is very muddy; therefore, long piles (~2 m) were more efficient for fixation when compared to common weights – easily carried away with the current. The system buoyancy was made with four 5 L plastic bottles on each cage, and four 20 L plastic bottles above each pile (Fig. 2a, b). The amount of rope was just enough to allow the system to oscillate with the tide, considering the syzygy tide limits, so the cages would never be submerged and nether too loose in highest tides. The cages were installed 10 days prior to the beginning of the study to start the growth of periphyton.

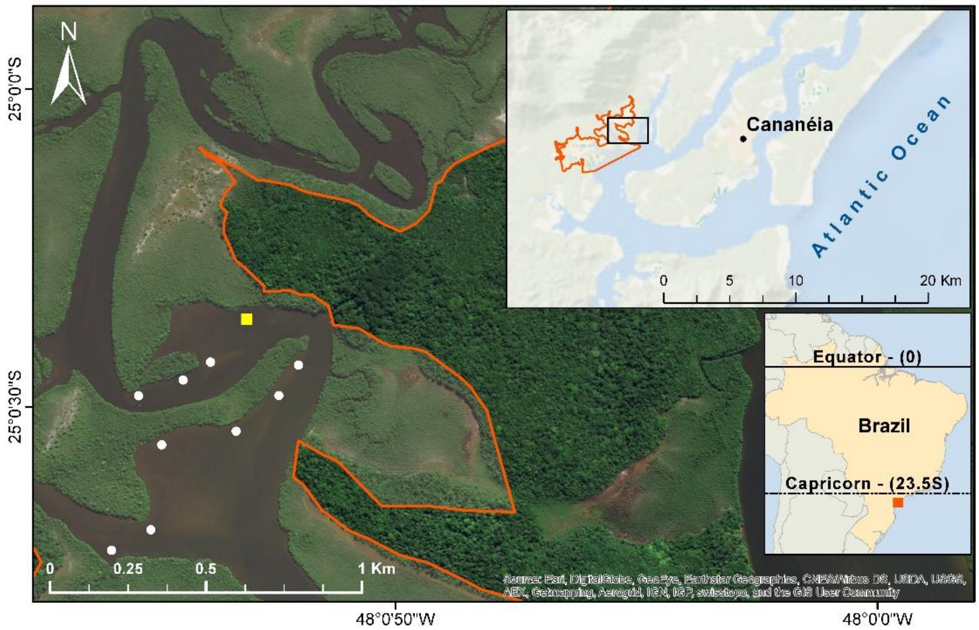

Figure 1. Location of the cage-culture of seahorse (*Hippocampus reidi*) in the mangrove at the Extractive Reserve of Mandira (red line) in Cananéia, São Paulo – Brazil. Yellow square: seahorse cages; white circles: oyster culture. The limit of Extractive Reserve of Mandira was obtained from ICMBio web site (available at: http://www.icmbio.gov.br/portal/unidadesdeconservacao/biomas-brasileiros/mata-atlantica/unidades-de-conservacao-mata-atlantica/2230-resex-mandira; accessed in October 2016). Map made with ArcMap^{TM} 10.2.

Stocking juveniles

Seahorses were reproduced and raised in laboratory until benthonic phase (juvenile II), and then, were stocked in the cages. Wild seahorse broodstock were collected in Ubatuba (São Paulo, Brazil). New-borns (juveniles I) were kept in a recirculation system with natural seawater in a temperature of 27 °C, salinity 28, and were fed twice a day with wild plankton. After ~15 days, most seahorses were in benthonic phase (juvenile II), and were packed and transported to the cages. *In situ*, we started the acclimatization process that was gradually and took approximately one hour. First, closed bags were put inside cages for 30 min to equalize temperature.

Then, bags were filled with small portions of natural water until salinity inside the bags equalize with the cage. We did one stock event in the summer and two in the winter. In the summer, 240 juveniles were equally divided in the cages in a density of 60 ind./m^3. Although water parameters were adequate during stocking, a high precipitation event dropped salinity below 10 during the culture and all seahorses died within few days. Then, we performed new attempts in the winter. In the first stock in winter, a total of 92 individuals were divided in the four cages, resulting a density of 23 ind./m^3 (19 days old). Twenty six days later, more 25 ind./m^3 from another cohort were added to each cage (13 days old). Thus, a total of 48 ind./m^3 was stocked in each cage during winter and spring.

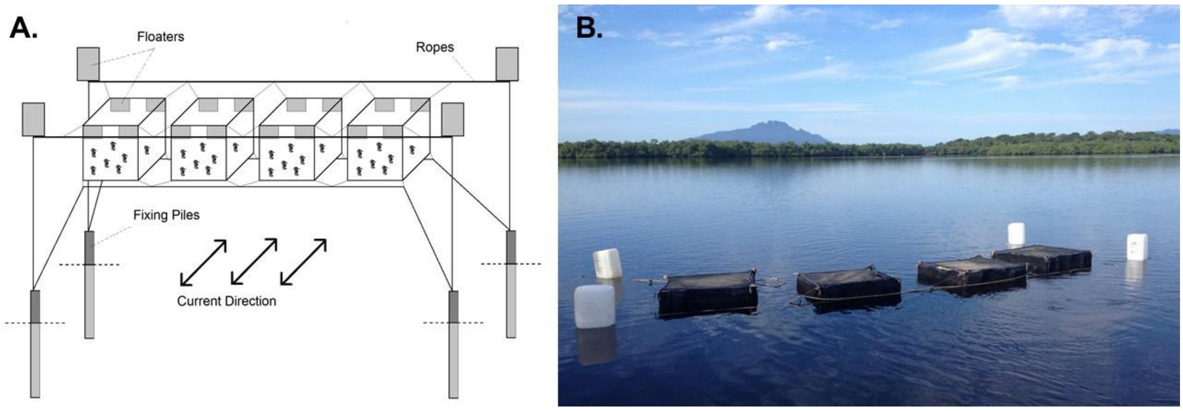

Figure 2. Cage-culture of seahorses (*Hippocampus reidi*) in the mangrove estuary at the Extractive Reserve of Mandira in Cananéia, São Paulo – Brazil. A: design of the system structure and foundation; B: photo of the cages.

Samplings and system maintenance

In the winter, we were able to kept seahorse in the cages for four months, enabling the assessment of seahorse growth, survival and environmental variables. For that, we measured seahorses, water parameters, sediment, and gas emission (CO_2 and CH_4) every two weeks approximately, in a sampling period of three consecutive days. Total length (TL) of seahorses was measured with the aid of an acrylic ruler from the top of the head to the tip of the stretched tail. Seahorses were difficult to spot because of their small size, low density in the cages and their camouflage capacity. Therefore, we measured as much seahorse as we could find in the cage without too much disturbance. Each TL datum was divided into its respective cohort. Individuals from older cohort (Cohort 1) were recognized by its bigger size and more developed body. A YSI Professional Plus® probe was used to measure water temperature, salinity and dissolved oxygen (DO) in the highest and lowest tides during sampling periods. All of these environmental parameters were measured inside each cage (replica) and outside, in a farther location for reference. A maximum minimum thermometer was installed in one cage to record the temperature limits among and within the sampling periods. Sediment was sampled using a Tripton collector set outside below the cage and in the bottom of the estuary in a farther location. To obtain the sediment dry mass, the samples were kept in a stove with air circulation at 60 °C until reach constant weight (~48h). Sedimentation rate for each sampling period was expressed as total dried mass (g)/m^2/day. To obtain the percentage of organic matter, the dried sediment was incinerated in the muffle for four hours at 550 °C, and allowed to cool in a desiccator before weighting. CO_2 and CH_4 emissions were sampled and measured by diffuse analysis as described in Matvienko *et al*. (2001) using a gas chromatograph TCD (Shimadzu GC-2014®). We measured gas emission inside two cages (replica) and in a farther location without culture interference once every 6 hours period for 24 consecutive hours (4:00–10:00; 10:00–16:00; 16:00–22:00; 22:00–4:00) in every sampling period. The mass of gases were expressed in CO_2 equivalent, according to IPCC guidelines (IPCC, 2007).

Cages were checked for integrity almost every day and were cleaned every other day by a person hired in local community. The cleaning consisted in brushing only the outside part of the front and backside of each cage (considering position in water current; Fig. 2a). The aim was to avoid net obstruction without harming possible periphyton growth inside the cage.

Data analysis

To evaluate growth of seahorses in these conditions, we fit the von Bertalanffy growth model to the length-at-age data with the Solver in Microsoft Excel, following procedures described in Harris (1998). The age was based on the birth in laboratory – the day that they were released by the male. We used a *t*-test for dependant samples to test if the water parameters and sedimentation from the cages would be significantly different ($\alpha < 0.05$) from the outside.

OPPORTUNITIES

Alternative for sustainable use of mangroves

Mangroves are large ecosystems distributed worldwide in tropical and subtropical regions, including territories that already trade seahorses, such as Southeast Asia, West Africa and South America. These ecosystems are plenty of water and space that can be used for aquaculture production. Nonetheless, the increase of intensive monoculture productions in mangroves around the world have been one of the main reasons why they are under threat (Spalding *et al.* 2010). Because of the uncontrolled use of resources and pollution, many countries have established legal frameworks to protect mangrove ecosystems, including Brazil, Mexico, Cambodia, Tanzania, and others (Spalding *et al*. 2010). If not properly managed, these legal restrictions could affect local communities that rely on mangrove resources for survival. Therefore, more sustainable productions, such as the culture of seahorses in cages, are good opportunities to support the conscious use of mangrove resources.

Availability of labour

Many families live in mangroves and need economic opportunities to obtain income, and thus, are good source of labour. The use of local labour and the development of local economy promote social sustainability in aquaculture systems (Valenti *et al.* 2011), which is important for a perennial activity. Nonetheless, to implement a new production dependent of local communities labour, it is fundamental to give support and to monitor the first few years. Traditional communities are not always open to begin new activities. The technology used should be very simple and not labour-intensive. People, who lives in mangrove and coastal areas generally does not like to be enrolled with rigid protocols or time schedules. In this study, we visit the community many times before installing the cages. These visits were important to create a bound of trust with the community. Additionally, we tried to involve the community in every step of the study, including teaching seahorse biology and training some members to install and maintain the cages. This strategy worked and in a few months, we have the engagement of many members of the community.

Occurrence of seahorse species

Mangroves are protected areas and thus, farms should focus on local species obtained from local populations. Another advantage on using local species is that they are adapted to survive in mangrove environment with daily fluctuations of salinity and temperature (Foster and Vincent 2004). At least two species of seahorses occurs in mangroves: *H. reidi* in Brazil, Caribbean and Florida; and *H. kuda* in Southeast Asia (Rosa *et al.* 2002, Lourie *et al*. 2004). Both species are largely traded for dried markets; *H. kuda* is traded mostly for TCM in Asia (Foster *et al*. 2016), and *H. reidi* for medicinal and religious uses in Brazil (Rosa *et al*. 2011). They are also the most internationally traded species for marine aquariums (Foster *et al*. 2016). The predominant species in the international TCM trade have been the *H. trimaculatus*, *H. spinosissimus*, *H. kelloggi*, *H. kuda*, and *H. algiricus* (Foster *et al*. 2016). Nonetheless, there is no scientific information supporting that these species are better fitted for TCM. In fact, other species have also been addressed in studies of biochemical composition for pharmacological use (reviewed in Chen *et al.* 2015). Therefore, we think that the dominance of these five species in TCM is because they are larger (>17 cm) and are harvest by the millions at a lower-cost. With this rational, *Hippocampus reidi* is also suitable for the international TCM trade because it is one of the biggest seahorses species (Lourie *et al*. 2004) and it is now produced at a lower cost in integrated multi-trophic aquaculture (IMTA) systems (Fonseca *et al*. 2017, Cohen *et al*. unpublished- Manuscrito II).

Obtaining initial broodstock surround the farms is important to avoid translocation of different populations, introduction of parasites and commensal microorganisms. Nevertheless, capture live animals in mangroves areas are not an easy task. Seahorses have good camouflage capacity and mangroves usually have low water transparency. In our study, we dive in 15 locations more than once to capture the initial animals from local populations, including places where they were reported by fishermen and local community, but we had no success. Thus, the availability of local broodstock is site-specific.

Availability of low-cost broodstock maintenance and juvenile production system

Before implementing production of seahorses on local communities in mangroves, it is important to find low-cost alternatives to maintain broodstock and to produce new-born juveniles until they could be stocked in cages for grow-out. A farm in Northeast Brazil, PRIMAR, is already maintaining seahorses' broodstock and developing nursery in a simple and low-cost production system that can be adapted for local communities in mangroves. Fonseca *et al.* (2017) reported a nursery phase of ~15 days with ~70% survival. Broodstock are kept in 2000-L polyethylene tanks, and are fed with small crustaceans and polychaetes collected from the oyster trays and shrimp ponds (Souza-Santos 2014, Fonseca *et al*. 2017). The new-born juveniles are kept in 200-L polyethylene barrel, and fed with wild zooplankton collected in shrimp ponds (Souza-Santos 2014, Fonseca *et al*. 2017). Both systems are static and require constant aeration and water exchanges. Although the maintenance of static systems is laborious and need water in abundance, it has low-cost and fits the reality and availability of resources of local communities in mangroves. In the case of "Quilombo do Mandira" community, they could easily collect plankton and small crustaceans (e.g. mysids) with a net in the estuary and when checking the oyster trays. Alternatively, they could install small traps or substrates in the oyster trays to improve collection of wild food for seahorses. Therefore, the main bottleneck to implement seahorse production in mangroves is to develop a protocol for grow-out in cages.

Availability of natural food

Seahorses do not eat commercial dried fish food and thus are difficult to feed them in aquariums. The absence of stomach may affects their digestion of non-natural diets (Palma *et al.* 2014). Producers offer live preys that are laborious and expensive (Woods and Valentino 2003), or frozen foods (e.g. mysids, copepods, shrimps and *Artemia*) to feed late juveniles and adults (Koldewey and Martin-Smith 2010). Nonetheless, some species/specimens does not adapt to eat frozen food, and the limited options of frozen food commercially available might not fulfil the nutrient requirements of seahorses. Furthermore, the low-digestive capacity of seahorses might require some active compounds of their prey (e.g. enzymes) to improve digestion, which could be lost in frozen process. The limited source of nutrient incorporated by seahorses in captivity may be the reason why many seahorse cultures rely on collection of wild broodstock to maintain production (Koldewey and Martin-Smith 2010, Olivotto *et al*. 2011). Therefore, installing cage-culture of seahorse in mangroves might take the opportunity to use natural food, which certainly improve seahorse health, reproduction performance and decrease production costs.

In general, mangrove waters are plenty of plankton and periphyton of different sizes (Robertson *et al.* 1992). Natural plankton is transported to the cages by water currents. In addition, it is very easy and cheap to capture organisms using plankton nets to supplement the food available in the water inside cages. Periphyton develops in the cage walls and can be enhanced using substrates inside cages. Some weeks after we installed the cages in the estuary, it was observed small amphipods, polychaetes and filamentous algae colonizing the cages nets. The availability of abundant natural food was observed in all trials performed in the present study.

Our measures and observation of seahorses during sampling periods enabled to report the growth of some individuals (Fig. 3). The juveniles were stocked with a mean total length (TL) of 1.8 cm ± 2.5 (cohort 1) and 1.5 cm ± 2.2 (cohort 2), and reached a mean TL of 7.2 cm ± 0.4 (cohort 1) and 5.9 cm ± 0.2 (cohort 2) after four and three months in grow-out, respectively. The constant and uniform growth (Fig. 4) is a good indicative of this system potential. Additionally, seahorses reached ~7 cm (7.7 cm the highest) in approximately fourth months, which is close from ~8 cm in less than four months described in the literature for others intensive (Hora and Joyeux 2009) and extensive systems (Fonseca *et al*. 2017). It must be also considered that this study was done during winter, and thus, it is reasonable to expect better growth in warmer temperatures.

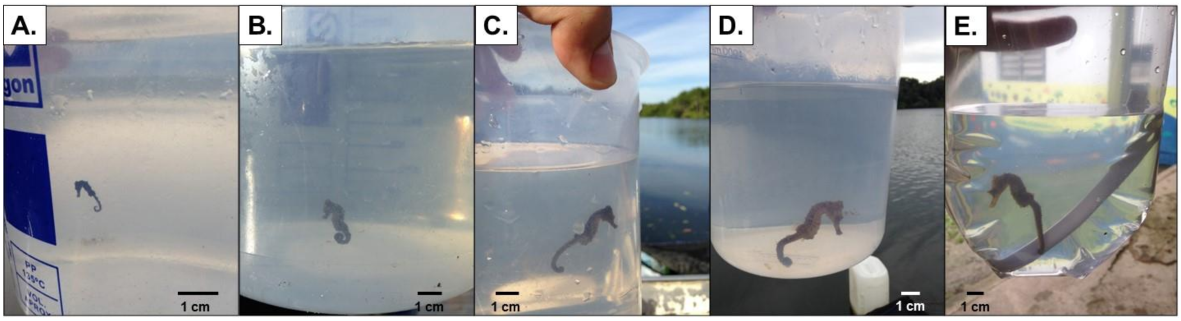

Figure 3. Seahorses (*Hippocampus reidi*) growth in cage-culture in the Extractive Reserve of Mandira in Cananéia, São Paulo – Brazil. A: specimen with 13 days old, recently stocked in the cages (1.3 cm); B: specimen with 39 days old (3.0 cm); C: specimen with 56 days old (4.5 cm); D: specimen with 104 days old (6.0 cm); E: specimen with 133 days old (7.3 cm).

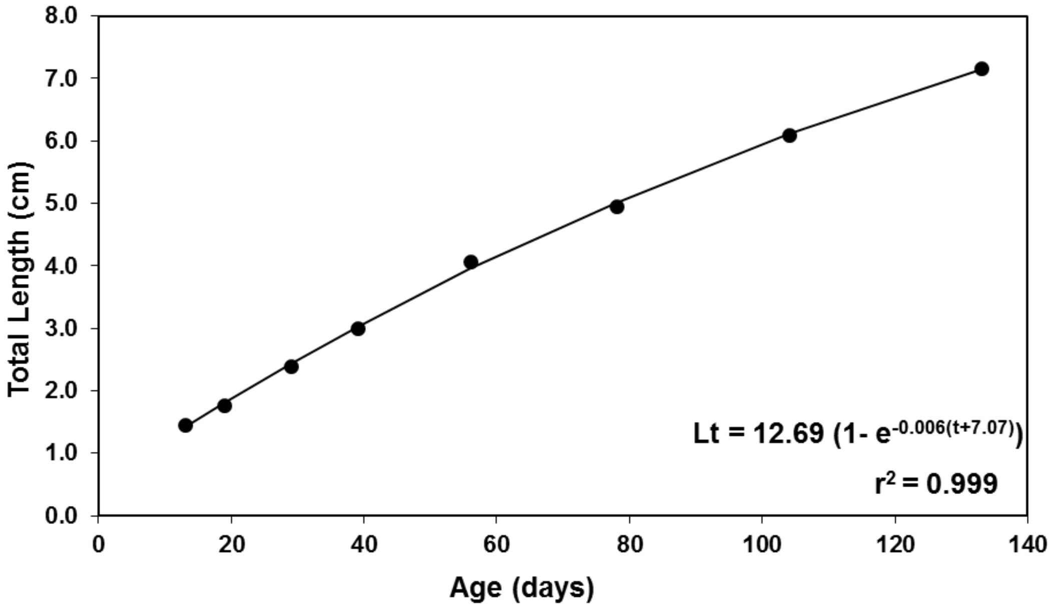

Figure 4. Seahorses (*Hippocampus reidi*) growth curve in cage-culture at the Extractive Reserve of Mandira in Cananéia, São Paulo – Brazil.

Periphyton production is dependent of nutrient availability, dissolved oxygen, light, substrate and fish density (van Dam *et al.* 2002, Baffico and Pedrozo 1996), which changes among regions and culture methods. Therefore, further studies should investigate the effect of stocking density, season of the year, and duration of production in the stability and composition of the periphyton community. If necessary, an option to increase periphyton in seahorse cage-culture in estuary would be to install some spare nets (or others substrates) outside the cages (e.g. oyster trays, in this community) and leave there to grow periphyton. Natural substrates such as bamboos or tree branches has shown better productivity of periphyton when compared to plastic or PVC (reviewed in van Dam *et al*. 2002). Then, it is possible to exchange regularly the substrates in the cages for those already colonized with periphyton. This would allow an additional input of food inside cages without adding much cost and labour. Additionally, cages could be installed more than 10 days prior to stock for better stablishing periphyton community.

Producing seahorses based on natural available diets also avoid environmental impacts caused by leftover food. Most sediment accumulated below intensive cage-culture systems are from leftover food. This accumulation of organic matter causes loss of benthos community, deteriorates water quality, and promotes the spread of diseases (reviewed in Buschmann *et al.* 2006). In this study, the sedimentation rate below the cages was significantly lower than the normal sedimentation in the bottom of the estuary (Tab. 1). This was expected because cages with small size mesh retain some sediment. Furthermore, we found no significant difference between the percentages of organic matter in sediment from below the cage and from the bottom of the estuary (Tab. 1), which is an indicative for good environmental sustainability. Additionally, promoting aquaculture systems without input of exogenous food might reduce greenhouse gas (GHG) emission, because the food production and its decomposition in the system are among the main source of GHG emission in aquaculture (Aqvaplan-niva and VGREEN). In this study, our results showed that the cages emitted less CO_2 and CH_4 than the estuary (Tab. 2). This may be because the periphyton community in the cages uses carbon to grow, and cages showed lower sediment accumulation when compared to the estuary, which are also source of GHG. Future studies should test if high stocking densities affect sedimentation below cages and GHG emission.

Table 1. Mean values of sedimentation and organic matter (OM) from below the cages and from the bottom of the estuary in a mangrove at Extractive Reserve of Mandira (Cananéia, São Paulo – Brazil). SD: Standard Deviation.

	Below the Cage				**Bottom of the Estuary**			
Sampling period	**Sedimentation ($g/m^2/day$)**	**SD**	**OM (%)**	**SD**	**Sedimentation ($g/m^2/day$)**	**SD**	**OM (%)**	**SD**
16-18/Jun	42	19	23.3	1.4	99	36	21.3	1.2
29/Jun - 01/Jul	69	3	21.8	5.1	96	14	23.5	1.7
14-16/Jul	28	5	27.5	3.5	83	46	22.4	1.9
27-29/Jul	55	35	21.6	4.5	35	1	24.7	0.3
11-13/Aug	41	9	24.5	1.2	55	18	26.0	2.4
Mean	**46^b**		**23.7**		**74^a**		**23.6**	

*Small letter indicate significant difference (P < 0.05) between means

Table 2. CO_2 equivalent (mg) emitted per square meter per day in a cage culture of seahorses *Hippocampus reidi* (Cage 1 and Cage 2) and outside the cages in a farther location (Outside) in the estuary in a mangrove at Extractive Reserve of Mandira (Cananéia, São Paulo – Brazil).

Samplig period	CO_2 Equivalent (mg)/m^2/day			
	Outside	Cage 1	Cage 2	Mean Cage
16-18/Jun	3838	228	1159	693
29/Jun - 01/Jul	1047	-876	378	-249
14-16/Jul	5690	1621	-203	709
27-29/Jul	1598	-368	388	10
11-13/Aug	1127	4026	982	2504
02-04/Sep	-771	1800	996	1398
Mean	**2088**	**1072**	**617**	**844**

Possibility to improve tourism and mangrove conservation

The culture of seahorses in mangroves by local communities can be a marketing strategy to explore tourism and foster mangrove conservation. Mangroves are one of the main threatened tropical ecosystems (Alongi 2002, Polidoro *et al.* 2010). Promoting conservation actions is particularly difficult for mangroves because the bad odour released from its anoxic and muddy soil gives the wrong impression of a polluted environment and covers its ecological importance. Seahorses, however, are charismatic animals with great potential to raise people's awareness over marine conservation issues (Vincent *et al*. 2011). Indeed, seahorses have been successfully used as flagship species to create protected areas in Philippines (Vincent *et al*. 2011) and may be used to improve conservation of others aquatic inhabitants in estuary regions (Yasue *et al.* 2012). Based on this, seahorses might be a good opportunity to advocate mangrove conservation (Cohen *et al*. 2017). Additionally, targeting tourism is a good opportunity because it does not require systems with high productivity. Tourism itself can bring higher net income than the seahorse sale. In the case of the "Quilombo do Mandira" community, they already receive tourists to visit their oyster production. The culture of seahorse could increase income from tourism directly by paying to visit the production and indirectly by consuming local food and handcraft. Therefore, promoting an ecological and base community tourism in the seahorse farm would allow additional revenue for producers, and would create an opportunity to raise funds and aware people about the conservation issues in mangroves.

CONSTRAINTS

Environmental conditions

Every outdoor production is dependent of environmental conditions. That is why many aquaculture systems are limited to a specific time of the year where environmental conditions are more stable and adequate to the species being farmed. Water conditions can be very unstable in tropical and subtropical estuaries. Parameters such as temperature, salinity and water movement depend on estuary location (e.g. tropical or sub-tropical regions), cage site (proximity to the sea and water current), and seasons of the year. Although *H. reidi* is adapted to daily fluctuations in temperature and salinity common in estuaries, wild specimens can do small migrations to search for suitable environment conditions (Foster and Vincent 2004). Nonetheless, individuals in cage-culture cannot migrate, and thus, producers should ensure appropriate water quality. Therefore, it is important to evaluate local conditions before starting the culture. When we first installed the cages to evaluate local conditions, the whole structure was carried away by an unexpected strong current. Thus, we moved the cages to a small-protected bay with lower water current and improved fixation with long concrete piles. In our first stocking on summer (rainy season), all seahorses died within a few days due to low salinity. Although this drop in salinity was resulted from an abnormal precipitation for the region, caution should be taken for cultures during the rainy season. Set up the cages close to the sea may help, but it would expose the structure to strong currents and probably will not avoid low salinity in cases of long time subjected to torrential rains.

We harvested the seahorses four months after the stocking of the first cohort in winter. At the end, only 18 seahorses survived, resulting in a mean survival of 9.4 % ± 2.1 (mean survival ± SD). All measured water parameters were within the range supported by *H. reidi* (Hora *et al*. 2016, Melo-Valencia *et al*. 2013). Temperature mean value measured for this period was 22.1 °C ± 1.4 (SD) (max. 25, min. 20) (Tab. 3). Salinity mean value measured was 24.9 ± 2.5 (SD) (max. 29.4 and min. 19.3)

(Tab. 4). Dissolved oxygen mean value measured was 82.3 % ± 12 (SD) 6.2 mg/L ± 0.8 (SD) (min. 4.8 mg/L) (Tab. 5). It is possible that an abrupt and punctual change in water parameters had occurred, although not measured, and caused mortality. In addition, other water quality parameter not monitored may had caused the mortality. Some other possibilities for this low survival is discussed below.

Table 3. Mean values of water temperature (°C) from inside the cages (Cages) and outside (Control) in a mangrove estuary at Extractive Reserve of Mandira (Cananéia, São Paulo – Brazil). SD: Standard Deviation.

Samplig period	**Temperature (°C)**					
	Cages	**SD**	**Max.**	**Min.**	**Control**	**SD**
09-11/Jun	23.6	0.6	24	23	23.8	0.6
16-18/Jun	20.9	0.9	23	20	20.9	1.0
29/Jun - 01/Jul	21.0	0.3	22	20	21.0	0.4
14-16/Jul	21.3	0.3	23	21	21.3	0.3
27-29/Jul	21.5	0.3	22	20	21.4	0.3
11-13/Aug	24.4	0.4	25	24	24.1	0.9
02-04/Sep	21.5	0.4	25	21	22.0	1.5
Mean	**22.1**	**1.4**	**23.4**	**21.1**	**22.1**	**1.4**

Table 4. Mean values of water salinity from inside the cages (Cages) and outside (Control) in a mangrove estuary at Extractive Reserve of Mandira (Cananéia, São Paulo – Brazil). SD: Standard Deviation.

Samplig period	**Salinity**					
	Cages	**SD**	**Max.**	**Min.**	**Control**	**SD**
09-11/Jun	25.2	0.4	25.9	24.6	25.1	0.8
16-18/Jun	25.5	2.4	29.4	23.1	25.9	2.5
29/Jun - 01/Jul	21.6	1.2	28.6	19.3	21.8	1.2
14-16/Jul	24.0	2.2	26.8	21.8	23.2	1.9
27-29/Jul	22.8	1.4	24.7	21.3	23.4	1.2
11-13/Aug	26.0	1.9	28.1	23.0	25.4	1.5
02-04/Sep	27.7	0.6	28.6	26.9	27.6	0.4
Mean	**24.9**	**2.5**	**27.4**	**22.8**	**25.0**	**2.4**

Table 5. Mean values of dissolved oxygen (DO) from inside the cages (Cages) and outside (Control) in a mangrove estuary at Extractive Reserve of Mandira (Cananéia, São Paulo – Brazil). SD: Standard Deviation.

Samplig period	DO (%)				DO (mg/L)				
	Cages	SD	Control	SD	Cages	SD	Min.	Control	SD
09-11/Jun	77.6	4.0	80.3	5.0	5.7	0.2	5.5	5.9	0.3
16-18/Jun	76.7	10.0	78.3	9.9	6.1	0.6	5.6	6.1	0.6
29/Jun - 01/Jul	78.9	7.4	79.1	7.3	6.2	0.6	5.5	6.2	0.6
14-16/Jul	85.3	15.6	87.3	15.1	6.6	1.1	5.2	6.7	1.1
27-29/Jul	91.0	8.0	95.8	8.4	7.1	0.5	6.6	7.4	0.4
11-13/Aug	90.7	18.4	90.5	17.0	6.6	1.2	4.8	6.6	1.2
02-04/Sep	74.3	5.1	75.1	4.7	5.6	0.4	5.1	5.6	0.3
Mean	**82.3**	**12**	**83.0**	**12**	**6.2**	**0.8**	**5.5**	**6.3**	**0.9**

Net obstruction

The water in mangrove estuaries is full of suspension matter and debris. Therefore, net obstruction is a concern because *H. reidi* juveniles require a cage with mesh size as small as 1 mm. Cleaning the cages might vary with the level of suspended material in the water column, which is site-specific, and seasonally because rain carry more material to water body. Net clogging causes decrease in dissolved oxygen inside cages, changes in temperature, and reduction in the flow-through of wild plankton. This would result in seahorse stress and mortality. In the present study, no significant difference (P > 0.05) was found between water parameters from inside the cages and outside (Tab. 3, 4 e 5). This indicates that brushing only the outside part of the front and backside of the cages, every other day avoids net obstruction. Although result may change in different sites and seasons, this management may be used as initial reference. Presumably, if cages stay for long periods into the water, they could be incrusted by algae and invertebrates that may not be removed with a brush. Therefore, long-term production might require occasional exchanges for clean spare cages, or moving animals to larger mesh-size cages as they grow. On both cases, it is important to install the cages in water few weeks prior to stock the seahorses for periphyton growth.

Predators

Adult seahorses have few natural predators. Their good camouflage capacity protects them against pour visual predators, and their skinny body covered by bony plates excludes them from the list of most appetizing fishes (Foster and Vincent 2004). Additionally, they usually inhabit shallow waters with dense vegetation, which hampers predation by large animals. Juveniles seahorses, however, are easy prey for most piscivorous and planktivorous organisms (Foster and Vincent 2004). Nonetheless, in culture, both adults and juveniles are vulnerable if predators enters in the cage.

Predators can enter the cages through the nets, as larvae or small juveniles, and from the top, especially birds by flying and fish by jumping. Larvae or juveniles of predators would not have enough time to grow and eat seahorses; however, they would compete for food. Covering the cages with nets avoid the entrance of larger predators from the top. In the present study, all cages were covered (Fig. 2b) and no other fish were found inside in any time. Nonetheless, we found many small crabs inside the cages, which were able to enter mostly when cages were opened for samplings and through small openings in the cover net. Although we did not find any injured seahorses, partial predation by crabs may be a treat to seahorses (Foster and Vincent 2004) and may had contributed for the low survival. Additionally, these crabs also compete for food (periphyton). Crabs are abundant in mangrove estuarine regions and can easily climb through nets. Thus, it is critical to control its invasion in cage-cultures. Some recommendations would be to seal the cages, avoid open the cages and when necessary, open for short time, manually remove spotted crabs, and carefully clean the cages after each production cycle. Additionally, rear some adults' seahorses together with juveniles may allow they to eat small predators without harming seahorse juveniles. This may be an important avenue for future studies.

Escapees

Escapees is a concern in every cage-culture worldwide. The main problems include losing production (cost for producers) and produce ecological imbalance (cost for society). In addition, escapee may cause introduction of exogenous species in the environment and changes in the pool genic of wild populations. Nonetheless, simple practices can be applied to minimise escapees' impacts, and even avoid them. Cages, floats and fixation structures should be compatible to local characteristics, mainly to support the larger water currents and storms. Rearing local species obtained from local populations avoid introducing exogenous species in the wild, including parasites and microorganisms and does not cause genetic disturbing. Ecological imbalance only occurs with a huge amount of seahorses added to environment, which can be avoid with good management practices. It is necessary to stablish a routine for cleaning, checking and maintaining all structures used in the culture, changing and repairing whenever required. In this study, we worked with a cheap and very simple structure. It showed suitable only for using in a small bay with low water current. We have dedicated all the time required for cleaning and checking procedures. No cage submersion, nether rupture or any structure damage was recorded during the winter trial. However, cages could have submerge during strong and high tides or storms, returning the same position after that; these events may not be perceived. Thus, the cover nets might had been forced to open, enabling escapees. This may explain the low survival observed at the end of the culture. Thus, using complete sealed cages is important to prevent escapees during storms or high tides.

CONCLUDING REMARKS

Cage-culture of seahorses in mangrove estuary can be a good alternative to produce low-cost seahorses and attend the huge demand of dry specimens. This production certainly will increase sustainability of the seahorse production chain and foster seahorse and mangrove conservation. Our field observations and discussed opportunities support the claim that producing seahorses in mangroves might be a reality in near future. Nonetheless, we addressed the main constraints of this production that should be overcome to stablish a reliable culture protocol. Future researches should analyse plankton and periphyton quantity and quality among different seasons of the year and different stocking density of seahorse juveniles to optimize production. Culturing individuals from different sizes in the same cage should also be investigated because it would possibly reduce dispute for the same sized food and larger seahorses could eat small predators. Assessing economic, environmental and social sustainability is other important avenue for future research to confirm that cage-culture of seahorses in mangroves is really a feasible and more sustainable system. The extensive culture of seahorses in land-based earthen ponds close to the coast also should be investigated as alternative to the intensive culture indoor, currently practiced.

ACKNOWLEDGMENTS

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VIII. MANUSCRITO IV

PACKING LIVE SEAHORSES FOR SHIPPING

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ABSTRACT

Shipping is a significant cost on production of marine ornamental species and a risk to the transported animal. In this study, we performed two transport experiments with seahorses *Hippocampus reidi* (~8 cm) to optimize packing methods. The first was a three-factor experiment to test density (1 ind./300 mL, 1 ind./450 mL, and 1 ind./600 mL), transit time (24 h and 48 h), and use of oxygen (pure oxygen and compressed air). The second was a two-factor experiment to test salinity (15, 25, and 35) and use of substrate. Survival was 100% in all treatments until 48 h after transport. The majority of seahorses showed normal feeding behaviour immediately after transport. No significance difference was found ($P > 0.05$) in weight-specific total ammonia nitrogen (TAN) excreted among treatments within the same transit time in the first experiment. Treatments with pure oxygen finished transport with over saturated DO, and treatments with compressed air for 48 h finished with DO saturation above 80%. Water with salinity 15 had significantly ($P < 0.05$) lower decrease in pH, followed by salinity 25 and 35. Lower salinity may have reduced breathing frequency of seahorse during transport. The presence of a substrate significantly ($P < 0.05$) decreased weight-specific TAN excreted, possibly due to stress reduction. Thus, *H. reidi* could be packed in density as low as 1 ind./300 mL for up to 48 h at 21 °C. The use of pure oxygen is not necessary when shipping for up to 48 h at 21 °C. Lower salinities and the use of substrate might provide better conditions on long time shipping and increase seahorse welfare without adding significant cost.

Keywords: physiology, stress, metabolism, aquaculture, trade, production chain

INTRODUCTION

Improving transport methods for seahorses could bring economic benefit for producers and increase animal welfare (Cohen *et al.*, 2017). Thousands of seahorses are traded live every year as marine ornamental species to supply aquarium industry (Foster *et al.*, 2016). As most marine ornamental species, seahorse trade also involves shipping specimens for long-distances among different countries. Nonetheless, shipping live aquatic animals for long distances in airplanes has a significant cost and high mortality risks (Lim *et al.*, 2007; Wabnitz *et al.*, 2003; Wood, 2001), making this step a fragile link in the production chain. Surprisingly, ornamental aquaculture researchers and enterprises have neglected the development of strategies on transport, focus mainly on culture protocols. To establish a perennial production system, it is essential to strength all steps involved in the production chain (Valenti, Moraes-Valenti, 2010). The international trade of live seahorses has experienced a change in the past few years, with most significant importing countries increasing their domestic production (Cohen *et al.*, unpublished, Manuscrito II). Therefore, to compete with local production on importing countries, exporters of live seahorses must optimize transport practices to guarantee good quality animals with minimum exportation costs. Additionally, standard transport methods are also important to reduces costs and improve animal quality and welfare on domestic trade.

Seahorses' morphology and biology are unique among fish and might require specific packing methods. Currently, there is no scientific study available on methods for packing seahorses for shipping. Traders have been using different methods based on personal experiences and on protocols developed for others marine species. Aquatic ornamental species are commonly shipped in closed plastic bags with one third water and two-thirds pure oxygen inside styrofoam boxes. The size of the bag, the density of individuals, and the volume of water depends on fish species and size. The water weight is much higher than the fish itself. Thus, the transportation cost is totally dependent to the amount of water used. Marine ornamental species are pricey products, and thus it is a common practice to pack them at a low density (Cole *et al.*, 1999; Lim *et al.*, 2007), usually only one specimen per bag. In bags with more than one animal, if one dies, the water quality decrease fast and could lead to total mortality. Seahorses' differences from others marine fish could be an opportunity to optimize packing methods. For example, seahorse up-right body position could allow a narrower bag for transport, decreasing weight and volume per specimen. Additionally, seahorse biology suggests that they could be packed in higher densities and without necessity to use pure oxygen. Seahorses are ambush predators, have low swim capacity, and remain attached to a substrate (Foster, Vincent, 2004). These characteristics suggest low metabolism and resistance to transportation. An experiment with *Hippocampus abdominalis* showed that this species have a fast recovery after transport, corroborating the hypotheses above (Wright *et al.*, 2007).

Shipping live fish should ensure enough oxygen and adequate water quality from packing to final destination. The transit time vary from few hours in domestic shipping to up 72 hours on international shipping (Cole *et al.*, 1999). During this time, the fish consume oxygen, release CO_2, and excrete ammonia. Air is more pressurized in closed bags than in atmosphere and the oxygen present in the headspace rapidly dissolve into the water according to the consumption. Thus, if enough amount are provided on packing, oxygen should not be a limiting factor (Berka, 1986). Nonetheless, the accumulation of CO_2 in closed conditions decrease water pH and may affect oxygen-carrying capacity of the hemoglobin, regardless on the oxygen availability (Berka, 1986; Lim *et al.*, 2007). Excreted ammonia can reach toxic levels depending on fish species, size, density, stress condition, and transit time. In low pH conditions, most ammonia nitrogen is in the form of ammonium ion (NH_4^+), which is less toxic than un-ionized (NH_3) due to its lower permeability in membranes (Boyd, 2015). Therefore, adding buffer solutions to keep pH alkaline during transport accumulates more toxic ammonia (NH_3), and thus, it is not recommended (Lim *et al.*, 2003). Hence, the main alternatives to avoid decrease in water quality during transport are removing ammonia and/or reducing fish excretion and oxygen consumption. Ammonia can be removed by using resins capable of adsorbing it, such as Zeolite (Berka, 1986; Lim *et al.*, 2007). Fish excretion and oxygen demand during transportation may be reduced by leaving fish without food for few days before packing, and reducing fish stress during transport. Starvation before packing is a common practice on live fish industry that reduces significantly the total ammonia excreted. Stress is close related to water quality. Low water quality is a major factor to induce stress in fish during transport (Portz *et al.*, 2006). Stressed fish excrete more ammonia, are more sensitive to ammonia toxicity and pathogens, have osmoregulatory dysfunction, and may consume more oxygen (Carneiro, Urbinati, 2001; Randall, Tsui, 2002), reducing fish welfare. Therefore, reducing fish stress largely improves transport conditions.

Fish stress may be reduced by using anaesthetics and/or applying better transport conditions for each species. Apparently, the use of essential oil of *Lippia alba* (Cunha *et al.*, 2011), MS-222 and clove oil (Pawar *et al.*, 2011) have potential to anesthetize seahorses. Nonetheless, there is a current lack on scientific knowledge on how to pack seahorses to reduce stress on shipping (Cohen *et al.*, 2017). Density, for example, is one of the most important factors that affect transport for both animal health (if density is higher than required) and production cost (if density is lower than necessary). Another example, specific for seahorses, is the use of substrate. Seahorses are benthonic, so the addition of a substrate during transport should not only decrease energy for fish stability, but may also decrease stress. Additionally, salinity may play an important role in controlling stress and oxygen consumption during transport. Adding salt in freshwater is very common to control osmoregulation dysfunctions or others physiologic disorder caused by stress when transporting live freshwater fish (Carneiro, Urbinati, 2001; Lim *et al.*, 2007; Long *et al.*, 1977; McDonald, Milligan, 1997). Therefore, the same rational might be true for saltwater fish when decreasing water salinity. Because *Hippocampus reidi* have an isosmotic point of approximately 12 (Hora *et al.*, 2016), transporting in salinities around 15 might reduce osmotic stress. Considering the above rationale, our aim was to test the effect of seahorse density, transit time, use of oxygen, salinity, and use of substrate on seahorse packing to improve transport conditions in attention to both animal welfare and shipping costs.

MATERIAL AND METHODS

We performed two experiments to test the effect of five factors on seahorse transportation. In the first experiment, we tested the effect of density, transit time (the period from packing to unpacking), and the use of pure oxygen. In the second experiment, we tested the effect of salinity and the use of substrate where seahorse could attach during transportation. On both experiments, it was used seahorses (*Hippocampus reidi*) bred in captivity with ~8 cm of total length (TL). Seahorses were kept for 24 hours without food before packing. After packing, bags with seahorses were placed inside closed styrofoam boxes (Fig. 1). These boxes were kept in a laboratory with controlled room temperature (~21 °C), and gently agitated for approximately five minutes every hour, except during 12 consecutive hours at night. This movement was to simulate a real shipping.

Figure 1. Photo of the closed plastic bags with seahorses (*Hippocampus reidi*) inside styrofoam box for transport experiment. The box remained closed during experiment.

Experiment 1: density, transit time, and oxygen

The effect of density, transit time, and oxygen were tested through a factorial experiment in a randomized block design with five replicates. Density was calculated as the number of individuals by volume of water. To test different densities, we used one specimen per bag and tested three different water volumes (300 mL, 450 mL and 600 mL). The tested transit times were 24 and 48 hours. The former represented a domestic shipping, whereas the latter an international shipping. To evaluate if the addition of pure oxygen inside the bags is necessary, we tested treatments with pure oxygen and with compressed air. We standardized the ratio water:air in the bags for 1:2. Considering a minimum water column of ~8.5 cm, and the pre-set ratio water:air, different bags had to be used for this experiment (Fig. 2). Therefore, there were 60 seahorses divided in five blocks with 12 treatments each – one styrofoam box per block. The water used for packing seahorses was a filtered natural seawater from the system they have been reared.

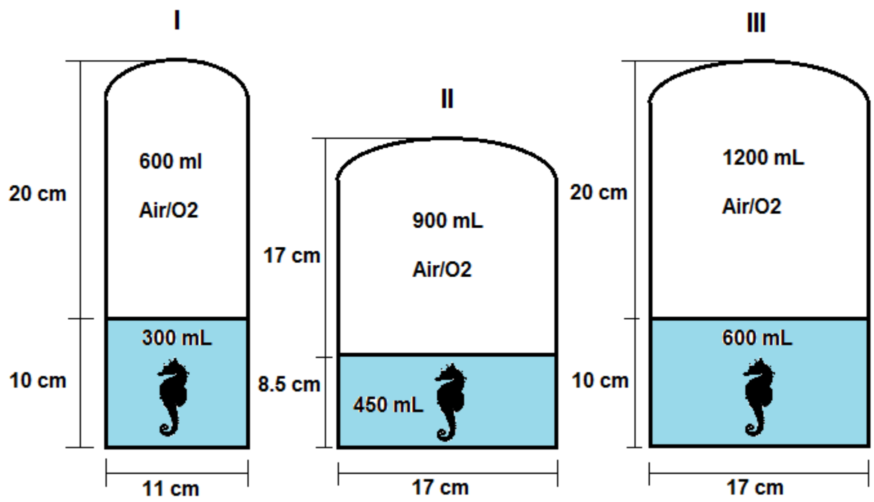

Figure 2. Schematic representation of the three densities (animals by volume) tested in the transport experiment with *Hippocampus reidi*.

Experiment 2: salinity and substrate

The effect of salinity and substrate were tested through a factorial experiment in a completely randomized design with five replicates. Based on preliminary results from experiment 1, it was used one specimen in a bag with 300 mL water and 600 mL of oxygen for 48 h. The salinities tested were 15, 25 and 35. To evaluate the use of substrate, it was tested treatments with and without substrate. The substrate used was a knot made of 20 cm hose (~0.5 cm diameter and ~5 g) (Fig. 3). Therefore, there were 30 seahorses in six treatments, all in the same styrofoam box. Because seahorses were in an aquarium with salinity 35 before experiment, we started acclimatization to the salinities 15 and 25 the day before packing. For that, seahorses were randomly separated in three aquariums (~30 L each) in a static system with aeration and controlled temperature (25 °C), and then, it was slowly dripped freshwater from reverse osmosis filter until it reached required salinities. This process took approximately 8 hours, and seahorses stayed in this system until being packed in the next morning (more 14 hours). No seahorse died in this process. In this experiment, the water used for packing was a new prepared water (mixture of filtered natural seawater with freshwater from reverse osmosis filter) with the same salinity and temperature from the systems they were.

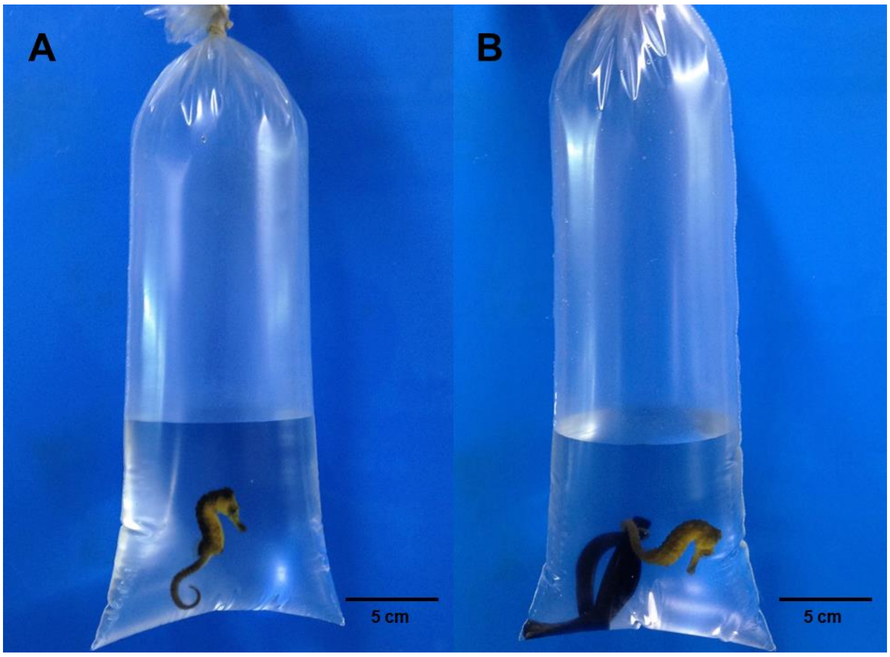

Figure 3. Photo of a transport experiment with seahorses (*Hippocampus reidi*) in bags with 300 mL water and 600 mL pure oxygen. A: treatment without substrate; B: treatment with substrate.

Data sampling

We measured seahorse total length and humid weight, and monitored survival until 48 hours after transport and feeding behaviour after transport. After unpacking, seahorses were measured with a ruler (d = 0.1 cm), and weighted with a scale MXX-212 Denver Instrument® (Max. 210g/d = 0.01g) to verify if by any chance some treatment was formed by larger or smaller animals, which would biased results. No significant difference ($P > 0.05$) was found on size and weight among treatments for both experiments. In experiment 1, the mean TL was 8.13 cm ± 1.35 (SD) and mean weight was 1.68 g ± 0.73 (SD). In experiment 2, the mean TL was 8.5 cm ± 0.67 (SD) and mean weight was 1.79 g ± 0.38 (SD). To observe survival until 48 hours after transport and monitor feeding behaviour, all seahorses were individually tagged with a coloured pendant in a nylon necklace, setting one colour for each treatment (Fig. 4), and acclimatized to a new system. This acclimatization consisted of joining seahorses from same treatments (and their remaining transport water) in 3 L beakers (one for each treatment) and slowly dripping water from the new system until temperature and pH equalized. This process took approximately one hour for both experiments. In this new system, it was set two treatments (10 seahorses) per aquarium (~30 L each), in a total of six aquariums for experiment 1 and three aquariums for experiment 2 (Fig. 5). For experiment 1, these aquariums were connected in a recirculated system, all sharing the same water for 48 hours. Because experiment 2 had treatments with different salinities, we change this system for static (no water sharing), and set each aquarium with the same tested salinity (15, 25 and 35). Seahorses remained in this system for 24 hours with aeration, temperature control (25 °C) and partial water changes. After this time, these aquariums were also connected in a recirculated system by slowly dripping water until all aquariums reached same salinity (35). Then, we monitored survival for more 24 hours with all treatments sharing the same water. The first few hours after transport are critical for ornamental animals, and any mortality in this phase could be related to transport conditions. On both experiments, we offered live food (Artemia adult and metanauplii) immediately after seahorses were introduced in the new system, and monitored feeding behaviour during 20 minutes of observation. Because most seahorses ate within this time, those that did not eat were considered more stressed.

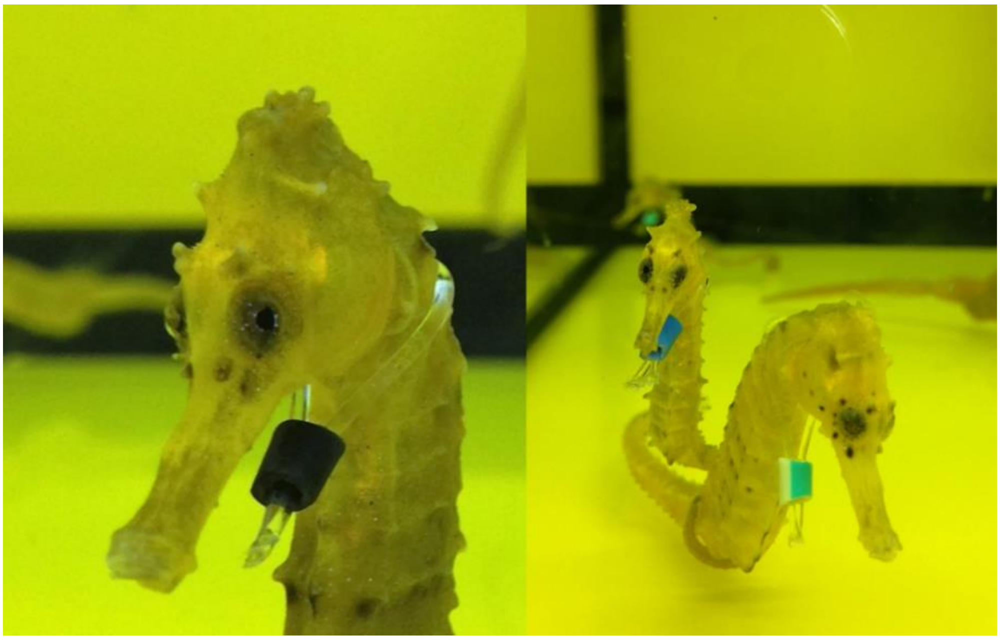

Figure 4. Seahorses *Hippocampus reidi* with coloured pendant in a nylon necklace used to differentiate treatments in the transport experiment.

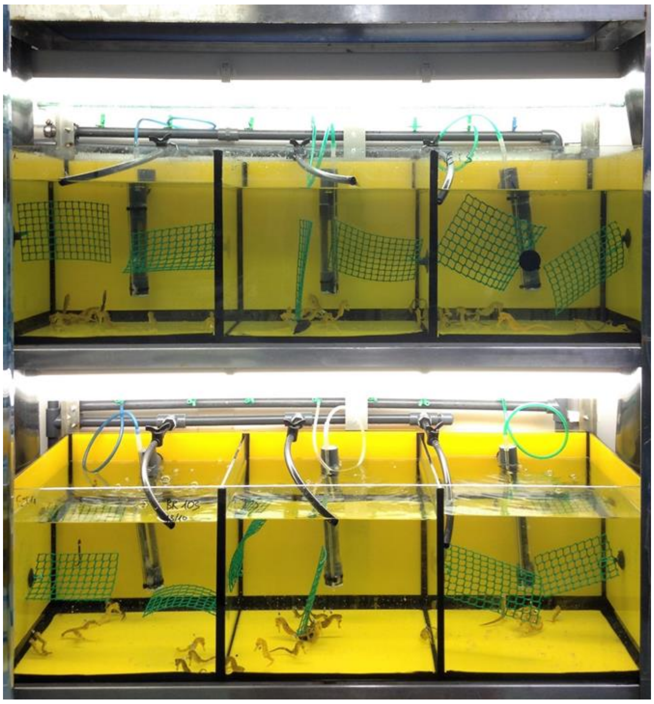

Figure 5. System used to monitor survival and feeding of seahorses *Hippocampus reidi* after the transport experiment. Each aquarium has approximately ~30 L. The systems can be set in recirculation or static configuration.

We measured parameters from initial water (packing water), water from the bags after transport (unpacking water), and water from the systems where seahorses were placed after transport (new system water). The measured parameters were temperature, dissolved oxygen (DO), pH, and total ammonia nitrogen (TAN). Temperature and DO were measured with a probe HI 98194 Multiparameter – Hanna®. The pH was measured with the pHmeter micropH 2001 – Crison® (probe Hack 5208). Total ammonia nitrogen was measured following standard methods described in Limnologisk Metodik (1992). The packing water and system water were measured for reference and control, and analysis showed that it were in good conditions to perform the experiments (Tab. 1).

Table 1. Water parameters from initial water used for packing seahorses (*Hippocampus reidi*), and water from the systems where seahorses were placed after transport (new system water). TAN: Total ammonia nitrogen; S15, S25 and S35: treatments with salinity 15 25; and 35.

	Packing Water				New System Water			
	Exp. 1	Exp. 2			Exp. 1	Exp. 2		
		S15	S25	S35		S15	S25	S35
Salinity	38	15	25	35	35	15	25	35
pH	7.82	7.94	8.23	8.41	7.99	7.77	7.92	8.00
Temperature	25.5	24.7	24.6	25.2	24.8	24.6	24.4	24.9
DO (mg.L^{-1})	6.40	7.60	6.98	6.71	6.30	7.44	7.09	6.61
DO (%)	95.0	99.4	96.8	98.3	95.5	99.3	99.9	100
TAN (µg.L^{-1})	20.9	10.5	8.0	29.7	4.2	56.3	59.6	98.8
Nitrite* (mg.L^{-1})	0	0	0	0	0	0	0	0
Nitrate* (mg.L^{-1})	< 5	< 5	< 5	< 5	< 5	< 5	< 5	< 5

*measured with a colorimetric test for marine aquarium

Data analysis

We tested decrease in pH (pH before transport minus pH after transport), weight-specific TAN excreted (individual total ammonia excreted divided by individual weight), and final DO concentration (DO after transport). For experiment 1, we also tested decrease in pH per hour (decrease in pH divided by the transit time), and weight-specific TAN excreted per hour. Because all treatments with pure oxygen showed oversaturated DO even after 48 hours, these values were beyond the standard curve of the probe, and thus, we considered it just as references and did not use for statistical analysis. For experiment 1, we applied three-way ANOVA to test interaction among the three factors. If no interaction was found, we continued the analysis by two-way ANOVA for factors density *vs* use of pure oxygen (3x2) within each transit time using the variables decrease in pH and weight-specific TAN excreted. For final DO concentration test (only in treatments with compressed air), we applied two-way ANOVA for factors density *vs* transit time (3x2) because the variability between treatments with oxygen and compressed air were higher than between transit time. For experiment 2, we applied two-way ANOVA for all variables. For both experiments, when significant differences were found, we applied the Tukey HSD as *post-hoc* test. The level of significance considered was 95%. Using values of final pH, temperature, salinity, and TAN, we estimated the concentration of un-ionized ammonia (NH_3) in the bags after transport based on methods described in Khoo *et al*. (1977), modified from Whitfield (1974).

RESULTS

Experiment 1: density, transit time, and oxygen

Survival was 100% until 48 hours after transport. Seahorses ate within less than 10 minutes after acclimatization, except for two animals in each of the three tested densities with compressed air for 48 h. Seahorses in these treatments (compressed air for 48 h) were visually more agitated. All seahorses were eating normally from the second day after transport. Temperature in the water after transport was 21.1 °C ± 0.2 (SD). Decrease in pH and weight-specific TAN excreted in treatments for 48 h were significantly ($P < 0.05$) higher than those for 24 h (Fig. 6A and 7A). When divided per hour, however, both decrease in pH and weight-specific TAN excreted in treatments for 48 h were significantly lower than in 24 h (Fig. 6B and 7B). Treatments with lower density (1 ind./600 mL) had significantly lower decrease in pH than others densities within 48 h (Fig. 6). Final mean pH values were 7.39 ± 0.21 (SD) (Min. 6.87) for treatments within 24 h, 7.37 ± 0.08 (SD) (Min. 7.25) for lower density (1 ind/600 mL) within 48 h, and 7.20 ± 0.10 (SD) (Min. 6.93) for other densities within 48 h. No significance difference was found ($P > 0.05$) in weight-specific TAN excreted among treatments within the same transit time (Fig. 7). The mean concentration of total estimated unionized ammonia was 0.001 $mg.L^{-1}$ ± 0.001 (SD) (Max. 0.002 $mg.L^{-1}$) for treatments on each transit time. Treatments with pure oxygen finished transport with over saturated DO, whereas treatments with compressed air finished with DO saturation above 80% (Fig. 8). Bags with higher densities (1 ind./300 mL) had significantly lower DO concentration after transport (Fig. 8).

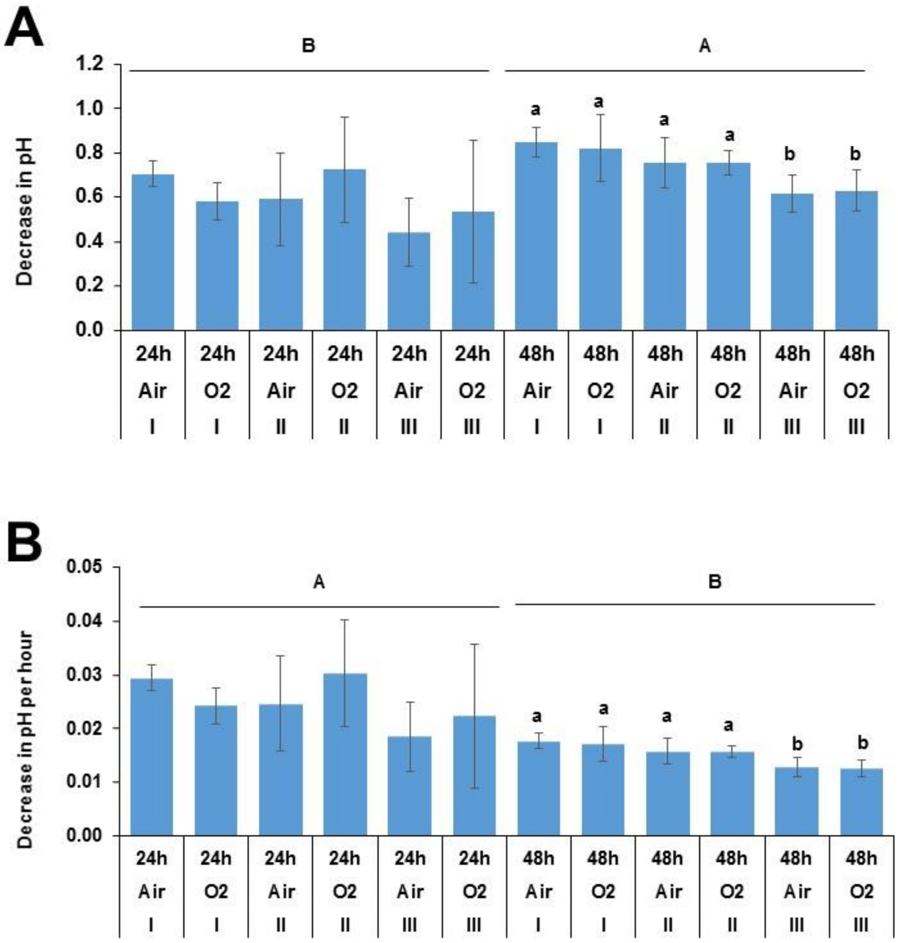

Figure 6. Decrease in pH (A) and decrease in pH per hour (B) in water used in a transport experiment with seahorses (*Hippocampus reidi*). Lines with upper and lower limits represent standard deviations for each treatment. Treatments are: different densities – 1 specimen in 300 mL (I), 450 mL (II), and 600 mL (III); bags filled with pure oxygen (O2) and compressed air (Air); and transit time of 24 h and 48 h. Letters indicate significant differences ($P < 0.05$) among treatments. No significant difference ($P > 0.05$) was found among treatments within transit time of 24 h.

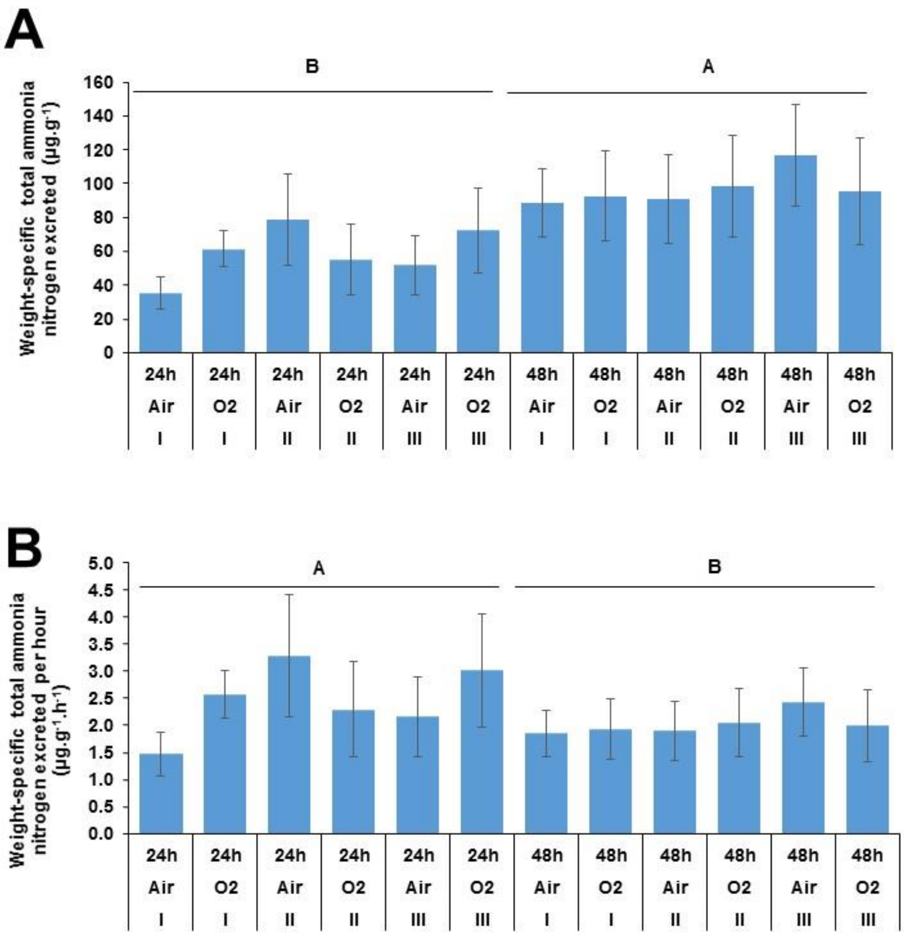

Figure 7. Weight-specific total ammonia nitrogen excreted (A), and weight-specific total ammonia nitrogen per hour (B) for seahorses (*Hippocampus reidi*) in a transport experiment. Lines with upper and lower limits represent standard deviations for each treatment. Treatments are: different densities – 1 specimen in 300 mL (I), 450 mL (II), and 600 mL (III); bags filled with pure oxygen (O2) and compressed air (Air); and transit time of 24 h and 48 h. Letters indicate significant differences ($P < 0.05$) among treatments. No significant difference ($P > 0.05$) was found among treatments within each transit time.

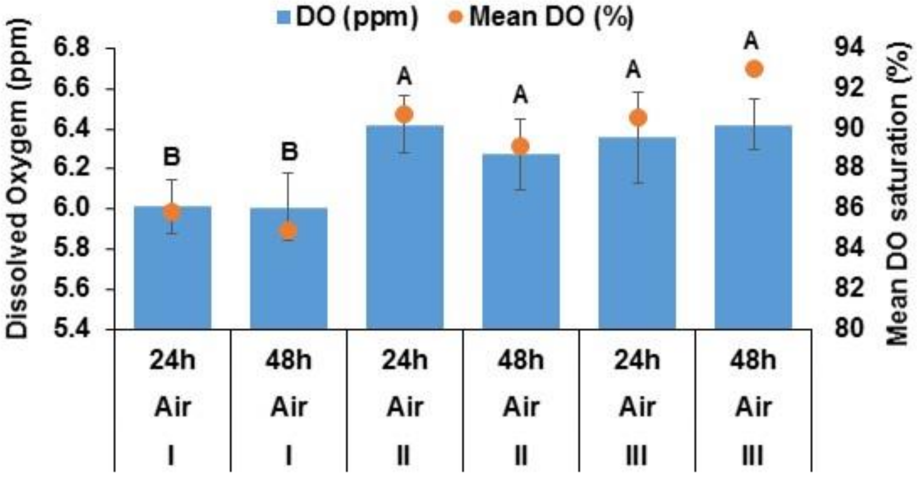

Figure 8. Concentration of dissolved oxygen in unpacking water in transport experiment with seahorses (*Hippocampus reidi*). Lines with upper and lower limits represent standard deviations for each treatment. Treatments are: different densities – 1 specimen in 300 mL (I), 450 mL (II), and 600 mL (III); bags filled with compressed air (Air); and transit time (24 h and 48 h). Letters indicate significant differences ($P < 0.05$) among treatments.

Experiment 2: salinity and substrate

Survival was 100% until 48 hours after transport. All seahorses ate within less than 5 minutes after acclimatization. Temperature in the water after transport was 21.0 °C ± 0.0 (SD). Water with salinity 15 had significantly lower decrease in pH, followed by salinity 25 and 35, respectively (Fig. 9A). Final mean pH values were 6.95 ± 0.04 (SD) (Min. 6.87) for salinity 15, 7.06 ± 0.06 (Min. 6.96) for salinity 25, and 7.13 ± 0.06 (Min. 7.03) for salinity 35. The presence of a substrate significantly decreased weight-specific TAN excreted (Fig. 9B). The mean total concentration of estimated unionized ammonia was 0.004 mg.L^{-1} ± 0.001 (SD) (Max. 0.005 mg.L^{-1}) in treatments with substrate, and 0.005 mg.L^{-1} ± 0.001 (SD) (Max. 0.007 mg.L^{-1}) in treatments without substrate. Concentration of DO was over saturated in all treatments after transport.

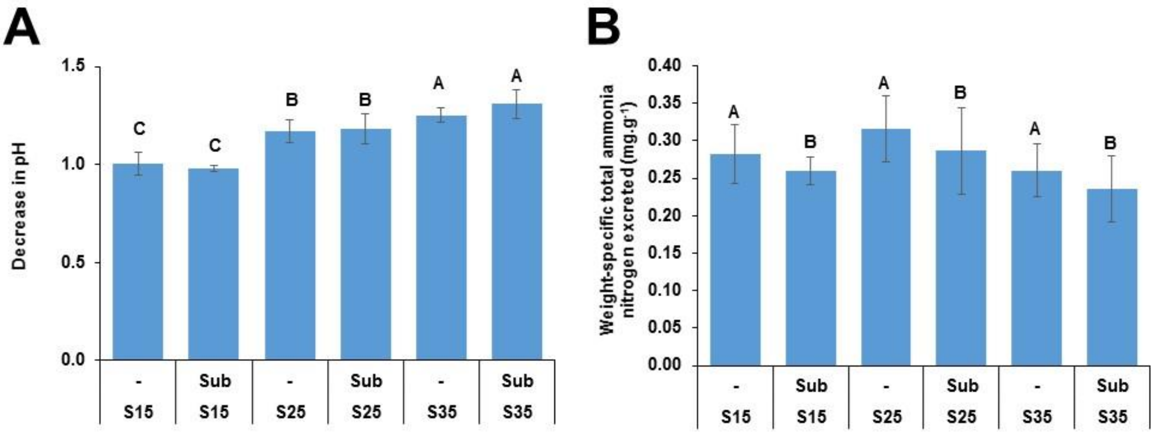

Figure 9. Decrease in pH (A) and weight-specific total ammonia nitrogen excreted (B) in transport experiment with seahorses (*Hippocampus reidi*). Lines with upper and lower limits represent standard deviations for each treatment. Treatments are: salinity 15 (S15), 25 (S25), and 35 (S35), with substrate (Sub) and without substrate (-). Letters indicate significant differences ($P < 0.05$) among treatments.

DISCUSSION

The 100% survival on both experiments with most specimens feeding normally immediately after transport suggests that seahorses *H. reidi* are very resistant to transport conditions in different densities, transit time, DO concentration, and salinities. They are also very resistant to changes in salinity because there was no mortality when we decrease salinity from 35 to 15 in eight hours. This tolerance to low salinity may be attributed for seahorses that occur in estuaries, such as *H. reidi* and *H. abdominalis* that thrive in salinities as low as 10 (Hora *et al.*, 2016; Martinez-Cardenas, Purser, 2016).

Treatments in lower density (1 ind./600 ml) had a significantly lower decrease in pH when transported for 48 h. During transport conditions, decrease in pH occurs due to the accumulations of CO_2 from fish breath. Because these treatments (1 ind./600 ml) had higher water volume per fish biomass, there was a lower concentration of dissolved CO_2 when compared to higher densities. Nonetheless, the mean pH in water from treatments with higher densities and longer transit time (48 h) was 7.2, which seems to be well tolerated by seahorses. Although a decrease in 0.5 unities in pH can affect *H. guttulatus* behaviour in many levels when exposed for one month (Faleiro *et al.*, 2015), no study reported seahorse resistance to acute exposure in lower pH. Water pH of 6.4 was recorded when simulating 12-h transport of adult *H. abdominalis* with mortality as low as 1% (Wright *et al.*, 2007). Additionally, in this study, *H. reidi* showed 100% survival and normal feeding behaviour immediately after transport, suggesting that they are resistant to this exposure (up to 48 h) to lower pH.

Animals that were transported for 48 h apparently decreased breathing frequency and metabolism after the first few hours. The highest decrease in pH and weight-specific TAN excreted in treatments transported for 48 h is a result of higher CO_2 and ammonia accumulations; however, when these variables were divided per hour, treatments in 48 h showed significantly lower rate of pH decrease and weight-specific TAN excreted (Fig. 6B and 7B). Probably, seahorse metabolism was more accelerated in the first 24 h due the stress for handling and packing the animals. This stress increased breathing frequency and ammonia excretion rate. After few hours, their stressed conditions might have returned to normal, resulting in the lower rate reported for treatments in 48 h. Curiously, the contrary was found when transporting clownfish (*Amphiprion ocellaris*); these fish increased ammonia excretion rate during shipping (Chow *et al.*, 1994). A study with *H. abdominalis* showed that seahorses can recovery fast from acute stressor caused by packing (Wright *et al.*, 2007). The stress recovery is a good characteristic to pack live seahorses because decreases water deterioration and allow packing in higher densities for longer time without affecting animal welfare.

Dissolved oxygen concentration did not affect breathing frequency and metabolism. No significant difference was fond on decrease in pH and weight-specific TAN excreted between treatments with compressed air and pure oxygen. This absence of effect showed that DO was not a limiting factor in this experiment. Treatments in higher densities with compressed air and longer transit time (48 h) showed significant lower DO concentration, but it was still above 80% of DO saturation. All treatments filled with pure oxygen finished transport with oversaturated DO oxygen. This showed that seahorse had very low oxygen consumption, even under possible stressed conditions caused by transport. Some animals from treatments of higher density (1 ind./300 mL) with compressed air for 48 h were apparently more stressed after the transport, but survival was 100% after transport and all seahorses were eating normally the day after transport.

Lower salinity may have reduced breathing frequency of seahorse *H. reidi* during transport. Salinity affected the decreasing in pH, with lower salinity resulting in lower decrease in pH (Fig. 9A). When fish are transported in water with salinity close to their isosmotic point, fish may reduce energetic cost for osmoregulation and better control osmoregulation dysfunctions caused by stress (Long *et al.*, 1977; McDonald, Milligan, 1997; Weirich, Tomasso, 1991). That is why adding salt in water is a common practice for shipping live freshwater fish (Lim *et al.*, 2007). Marine fish are hyposmotic in relation to their environment, and thus, reducing salinity of the transport water may reduce oxygen demand and improve transport conditions. Indeed, juveniles of cobia (*Rachycentron canadum*) showed better results when transported in water with salinity 12, possibly due to a decrease in its metabolic rate (Stieglitz *et al.*, 2012). Although water in treatments with salinity 15 had the lowest final pH (mean 6.95), they showed the lowest decrease in pH. This was because initial water used on these treatments had a slightly lower pH due to the mixture with freshwater (pH 7.0). The isosmotic point for *H. reidi* is around 12 (Hora *et al.*, 2016), and thus, our results support the hypothesis that transporting fish in water with salinity close to fish isosmotic point can reduce oxygen consumption. Nonetheless, the differences found between salinities were not too high, which suggests that seahorses *H. reidi* could be transported in any salinity from 15 to 35. Yet, this difference should not be neglected due to its possible significance in long time shipping.

The presence of a substrate may reduce stress of seahorses and increase animal welfare on transport. Treatments with substrate excreted significantly less ammonia, which can be a result of a lower stress compared to seahorses without substrate. Seahorses are benthonic fish that remain attached to different substrates, such as algae, sponges, corals, and mangrove roots (Foster, Vincent, 2004; Kuiter, 2009). It is reasonable to assume that seahorses attached in some substrates would be less stressed, especially considering that the box was agitated few times in this experiment. Box movements are inevitable during transport. Stressed fish have many physiological imbalances, which may result in more ammonia excretion (Randall, Tsui, 2002). Although the weight-specific TAN excreted showed a small difference between treatments with and without substrate, the use of substrate could increase this significance in long time shipping. Additionally, all seahorses with substrate held on it when packing and most were holding when unpacking after transport. This substrate added only 4 g (< 1.5 %) in the total package weight. Therefore, it seems that substrates are better for seahorse welfare and do not add much cost for shipping.

Considering both experiments, the low concentration of unionized ammonia after transport, the low oxygen consumption, and the tolerance to exposure to lower pH shows that seahorses could be shipped for long periods in closed plastic bags. Nonetheless, temperature and feeding are two important factors that could affect seahorse shipping and should be further studied. We did the experiments in a room with controlled temperature set in 21°C, which is within recommended temperature for transporting tropical fish, and it is compatible with cargo hold in aircraft (Froese, 1998; Lim *et al.*, 2007). Nonetheless, temperatures can change during shipping and would certainly affect oxygen consumption, gas solubility in water, and metabolic rate. Long time without eating might be also a problem when transporting seahorses for long distances. Seahorse have a low digestive capacity that requires constant feeding (Koldewey, Martin-Smith, 2010). In this study, some seahorses stayed 72 hours without eating (24 h preparation + 48 h transport). Although they were apparently healthy at the end of the experiment, shipping for longer time could be a problem. Seahorses seem to clean their digestive systems within few hours, and thus, it is not recommend starving for more than 24 h before packing. In fact, reducing time without feeding before transport could increase seahorse welfare without affecting water quality.

In conclusion, our results suggest that transporting one specimen of *H. reidi* (~8 cm) in 300 mL (narrow plastic bags) could be a cheap and safety option for international shipping up to 48 h in temperatures around 21 °C. Currently, there is no consistency on packing seahorses, with some traders reporting packing on densities as lower as 1 ind./L. Thus, our results might bring significant reduction on shipping cost. The use of pure oxygen is not necessary when shipping for up to 48 h in temperatures around 21 °C, but may be useful for longer shipping. *Hippocampus reidi* can be transported in salinity between 15 and 35, with or without substrate; however, lower salinities and the use of substrate might provide better conditions on long time shipping. Additionally, substrate seems to increase seahorse welfare without adding significant cost for transport, and thus, we recommend its regular use.

ACKNOWLEDGEMNTS

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IX. MANUSCRITO V

PRELIMINARY INSIGHTS ON THE MICROBIAL FINGERPRINTS OF SEAHORSE MUCUS – RELEVANCE FOR TRACEABILITY AND AQUACULTURE

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ABSTRACT

Developing a technique to trace the geographic origin of live seahorses is paramount to increase trade regulations and foster seahorse conservation. This is the first study to evaluate the suitability of using microbial fingerprints present in seahorse mucus to trace their origin. For that, bacterial 16S rDNA fragments were retrieved from seahorse mucus in a non-invasive way, and their profile (fingerprint) determined using denaturing gradient gel electrophoresis (DGGE). Microbial fingerprints were compared among seahorses; (1) originating from different geographic origins sampled at the same period; (2) originating from the same location but sampled one month apart; and (3) originating from the wild and after being stocked in captivity for 40 and 80 days. Similarities in microbial fingerprints were determined using hierarchical cluster analysis. Results showed that geographic location affected the microbial fingerprints of wild seahorses and that specimens sampled in the same location displayed a higher level of similarity. This finding supports that this methodological approach has potential to reveal local signatures and trace the origin of live seahorses. Microbial communities from wild seahorses varied over short-time periods, with this natural variability being an issue that may limit the comparison of specimens collected over long periods. Microbial fingerprints displayed by wild specimens shifted after 40 days in captivity, with a higher level of similarity being recorded for seahorses after 40 or 80 days in captivity, than when compared with those displayed in the wild. This stabilization of the microbial community under captive conditions shows the potential that bacterial fingerprints may hold for aquaculture, as these can be used as unique molecular signatures to trace seahorses to their production facility.

Keywords: ornamental, microbiological barcodes, bacterial communities

INTRODUCTION

Developing traceability protocols is crucial to foster seahorse conservation and increase trade sustainability. The global trade of dried and live seahorses is a multi-million dollars industry that targets several species (Foster *et al.*, 2016). In 2004, all species within genus *Hippocampus* were included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) to regulate the international trade and avoid the overexploitation of the stocks (CITES, 2004). Nonetheless, international regulations are still insufficient to promote seahorse conservation and have somehow contributed to the illegal trade of millions of specimens through black markets without any type of certification of origin (Cohen *et al.*, unpublished, manuscrito II). Therefore, it is important to improve trade regulations and promote sustainable fisheries and aquaculture practices that may contribute to protect wild seahorse populations (Cohen *et al.*, 2017; Cohen *et al.*, unpublished, manuscrito II). Nonetheless, a reliable method to trace the origin of traded specimens is essential to control the blurry supply chains trading seahorses and promote a sustainable use of these highly-priced marine resources (Cohen *et al.*, 2013; Cohen *et al.*, 2017).

Establishing a reliable traceability protocol to trace the geographic origin of seahorses is a difficult task. It is unlikely that a single methodological approach may suffice to implement a reliable traceability protocol to cover the whole trade of seahorses (Cohen *et al.*, 2017). Seahorses have two very distinct trades, one of dried specimens for Traditional Chinese Medicine, religion and curio; and other as live specimens for marine aquarium (Foster *et al.*, 2016; Rosa *et al.*, 2011), which hamper the implementation of a single traceability protocol. Some methods available to trace seafood for human consumption (reviewed in Leal *et al.*, 2015) may be suitable to trace the geographic origin of dried seahorses (Cohen *et al.*, 2017). However, tracing live seahorses traded to supply marine aquariums may be a more challenging task. The marine ornamentals industry demands pricey specimens focused on its healthy and aesthetic looks (e.g. colour, size and shape), which impair the use of most traceability methods available for seafood or larger live marine organisms (Cohen *et al.*, 2013).

Microbiological barcodes from fish mucus may be one of the best options to trace the geographic origin of marine ornamentals in a non-invasive and non-destructive way (Cohen *et al.*, 2013). This method consists on the amplification and analysis of the 16S rDNA fragment from the bacterial communities present in the fish mucus. The analysis is performed using non-culture dependent approaches, e.g., through a denaturing gradient gel electrophoresis (DGGE), to determine and compare the existence of significant differences in bacterial fingerprints that may be site-specific (Tatsadjieu *et al*., 2010). Previous works employing this technique have focused on the seafood production chain and reported their successful use to trace the geographic origin of fish from the wild (Smith *et al.*, 2009; Tatsadjieu *et al.*, 2010), as well as captive-cultured fish (Le Nguyen *et al.*, 2008). To date, no study has addressed the use of this methodological approach to trace the geographic origin on small-sized ornamental fish. Therefore, the aim of the present study was to perform a preliminary study to evaluate the suitability of using the microbial fingerprints of seahorse mucus to trace their geographic origin. For this purpose, the following null hypothesis were tested: (1) geographic location does not affect the microbial communities present in the mucus of wild seahorses; (2) microbial communities present in the mucus of wild seahorses do not present significant variations over short-time periods; (3) the stocking of wild seahorses in captivity does not promote significant variation on the microbial communities present in their mucus.

MATERIAL AND METHODS

Experimental design

Mucus from adult seahorses *Hippocampus reidi* was collected in four locations in Brazil Southeast Coast (Fig. 1). Location and sampling period was dependent on the null hypothesis being addressed and are detailed below.

To test the null hypothesis “geographic location does not affect the microbial communities present in the mucus of wild seahorses” mucus samples were collected from 11 seahorses sampled in three different islands of Rio de Janeiro (Fig 1; site 2, 3, and 4) in November 2015. All mucus samples were collected within two days: three samples from Ilha do Mantimento (Fig. 1; site 2), three from Ilha Comprida (Fig. 1; site 3), and five from Ilha do Cabo Frio (Fig. 1; site 4).

To test the null hypothesis "microbial communities present in the mucus of wild seahorses do not present significant variations over short-time periods" a new sampling of five seahorse mucus was done at Ilha do Mantimento (Fig.1; site 2), approximately one month (December 2015) after the first sampling event (at which the mucus from 3 seahorses were sampled; as the 3 seahorses sampled in November were not marked, it is not possible to know if any of the five seahorses sampled in December had been previously sampled as well).

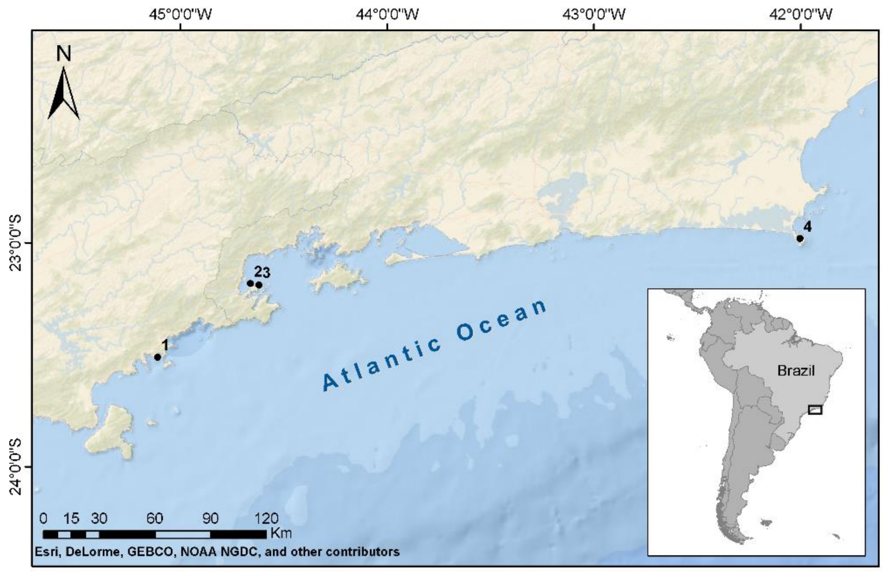

Figure 1. Map of site collections of seahorses *Hippocampus reidi* in Southeast Brazil. Site 1: Ubatuba, São Paulo; Site 2: Ilha do Mantimento, Rio de Janeiro; Site 3: Ilha Comprida, Rio de Janeiro; Site 4: Ilha do Cabro Frio, Rio de Janeiro. Map made with ArcMap^{TM} 10.2.

To test the null hypothesis "the stocking of wild seahorses in captivity does not promote significant variation on the microbial communities present in their mucus" the mucus of five wild seahorses was collected at Ubatuba, São Paulo (Fig. 1; site 1). Then, the same animals were transferred to the laboratory and further mucus samples were collected after 40 and 80 days in captivity. These five seahorses were kept in a recirculated system operated with natural seawater (collected in Santos, São Paulo, and ~ 200 km south from the place where the seahorses were collected). The system was formed by four 80-L polyethylene barrel connected in parallel to a filter tank (Fig. 2). The five seahorses were kept together in the same barrel, and no other animal was stocked in the system during this study. The filtration consisted of porous ceramics (biological filtration), a protein skimmer, two heaters (set at 27 °C) and an automatic osmoregulator dosing deionized freshwater to compensate for evaporation and keeping salinity stable at 28. Seahorses were fed twice a day *ad libitum* with frozen mysids, with uneaten food being siphoned from the tank to prevent decay in water quality.

Figure 2. Recirculated system employed to maintain wild-caught seahorses *Hippocampus reidi* in captivity to study microbial fingerprints in their mucus.

Field sample collection

Authorized researchers using SCUBA gear and disposable gloves (Fig. 3), one pair per each specimen sampled, manually collected all wild seahorses. This procedure was employed to avoid potential contamination of the microbial communities present in their mucus, with each diver putting on the gloves only when a seahorse was spotted. Mucus samples were collected from one seahorse at the time, with each specimen being removed from the water, slightly agitated to remove excess water and placed inside a disposable sterile sample bag (Labplas Twirl'EM™). Once inside the bag, the seahorse was gently rubbed from the outer side to release of mucus. This procedure was done as fast as possible, taking approximately 30 seconds per specimen, to minimize the stress induced to animals. Upon mucus collection, each specimen was immediately returned to the same place of collection. From each mucus sample collected, 200 μL were stocked in a sterilized centrifugal test tube (DNA and RNA free) with 1000 μL of phosphate buffered saline

(PBS) solution. Each tube was manually agitated and placed inside a thermic bag with ice for 24 h (maximum) before initiating microbial DNA extraction in the laboratory.

Figure 3. Manual collection of seahorses *Hippocampus reidi* using disposable gloves (one for each specimen) to avoid contamination on the microbial community in the mucus (photo by Suzana M. Ramineli).

DNA extraction, PCR amplification and DGGE analysis

Within 24 h after collection (maximum), bacterial DNA was extracted from each mucus sample using the QIAamp® mini DNA extraction kit (Qiagen, Hilden, Germany). The first extraction step consisted on centrifuging the samples (10000 g), adding a Tissue Lysis Buffer (QIAamp® kit) to stabilize bacterial cell and DNA, and perform cell lysis with proteinase K (QIAamp® kit). After this initial procedure, samples were frozen at -20 °C for further extraction. The final extraction consisted on adding Lysis Buffer (QIAamp®) kit and incubated at 70°C for 10 min. Then, it was added ethanol (96-100%) and transferred to spin columns (QIAamp® Kit) to clean the samples with Wash Buffers (QIAamp® Kit), and eluting the DNA with an Elution Buffer (QIAamp® Kit). At the end of the extraction procedure, microbial DNA present in the mucus of each seahorse sampled was concentrated in a 200-μL solution of eluent and buffer.

A nested polymerase chain reaction (PCR) was performed to amplify the V6–V8 region of bacterial 16S rDNA fragments. In the first PCR, bacterial primer pairs 27F/1492R were added to 1 μL of each sample (Weisburg *et al.*, 1991). In the second PCR, the bacterial primer pairs 984GC/1378R were added to 1 μL of the amplicons from the first PCR (Heuer *et al.*, 1997) allowing the preparation of fragments to perform denaturing gradient gel electrophoresis (DGGE). The PCR methodology employed was that described by Gomes *et al.* (2008). To validate the PCR methodology, an electrophoresis was performed by adding 5 μL of PCR mixtures (3 μL of amplicons from second PCR + 2 μL electrophoresis buffer) in an agarose gel at 2% using 1× Tris-Acetate-EDTA (TAE) at 90 V for 25 min (Pimentel *et al.*, 2016).

Denaturing gradient gel electrophoresis (DGGE)

Two DGGE analysis were performed according to procedures detailed in Pimentel *et al.* (2016). PCR amplicons from samples collected from Ilha do Mantimento, Ilha Comprida and Ilha do Cabo Frio (Fig. 1; site 2, 3 and 4) were used in the first DGGE analysis to compare the fingerprints of the bacterial communities present in the mucus of seahorses collected in different geographical location and at different periods (Ilha do Mantimento). In the second DGGE, PCR amplicons from mucus samples from seahorses collected from Ubatuba (Fig. 1; site 1) and subsequently sampled after being stocked in the laboratory for 40 and 80 days were used to compare their microbial fingerprints. Prior to DGGE analysis, DNA present in all samples was quantified using Qubit® fluorometric quantitation method (LIFE technologies). This quantification allowed to standardize the amount of DNA used for each DGGE analysis, which allows to have a more reliable comparison of band intensities. Approximately 130 and 77 ng of DNA were used per each sample in the first and second DGGE analysis, respectively.

Data processing and statistical analysis

Fingerprints formed by bacterial 16S rDNA fragments were analyzed using BioNumerics 6.6 (Applied Maths, Ghent, Belgium). This software processed the gel images to generate a matrix of intensity values for each band recorded. These values were processed to relative band intensity and transformed using log (x + 1), following the recommendations by Pimentel *et al.* (2016). Similarities in microbial fingerprint were determined using hierarchical cluster analysis through the group average method and the Bray–Curtis measure. Hierarchical cluster analysis were performed using PRIMER 6 (Primer-E Lta, Plymouth UK).

RESULTS AND DISCUSSION

Results showed that geographical location affects the microbial fingerprints present in the mucus of wild seahorses, while specimens within the same geographical location display higher similarities (Fig. 4 and Figure SI). Mucus collected from seahorses sampled in closer locations (e.g., Ilha do Mantimento and Ilha Comprida) display a higher level of similarity in their microbial fingerprints than when compared with those exhibited from specimens from a more distant location (e.g., Ilha do Cabo Frio) (Fig. 4). Although previous work reported that microbiological composition in seahorse mucus are significantly different from its surrounding water and live prey (Balcázar *et al.*, 2010), the difference in abiotic conditions and substrate may somehow affect the composition of the microbial communities present in seahorse mucus within different microhabitats. Therefore, the similarity found in the microbial fingerprints displayed by seahorses collected in Ilha do Mantimento and Ilha Comprida may be explained by the fact of these adjacent islands being subjected to more similar abiotic conditions (e.g. weather, currents, etc.); it is also possible that seahorses may sporadically migrate between these two islands, namely at their juvenile stage (dispersion). Previous works have confirmed that the bacterial profile displayed by fish mucus is a reliable approach to trace their geographical origin, such as for the marine fish *Merlangius merlangus* (Smith *et al.*, 2009) and of the freshwater fish *Oreochromis niloticus* (Tatsadjieu *et al*., 2010). Therefore, this higher similarity on microbial fingerprints among seahorses from the same location is a good indicative that this methodological approach has potential to reveal local signatures and allow the tracing of the place of origin of live seahorses.

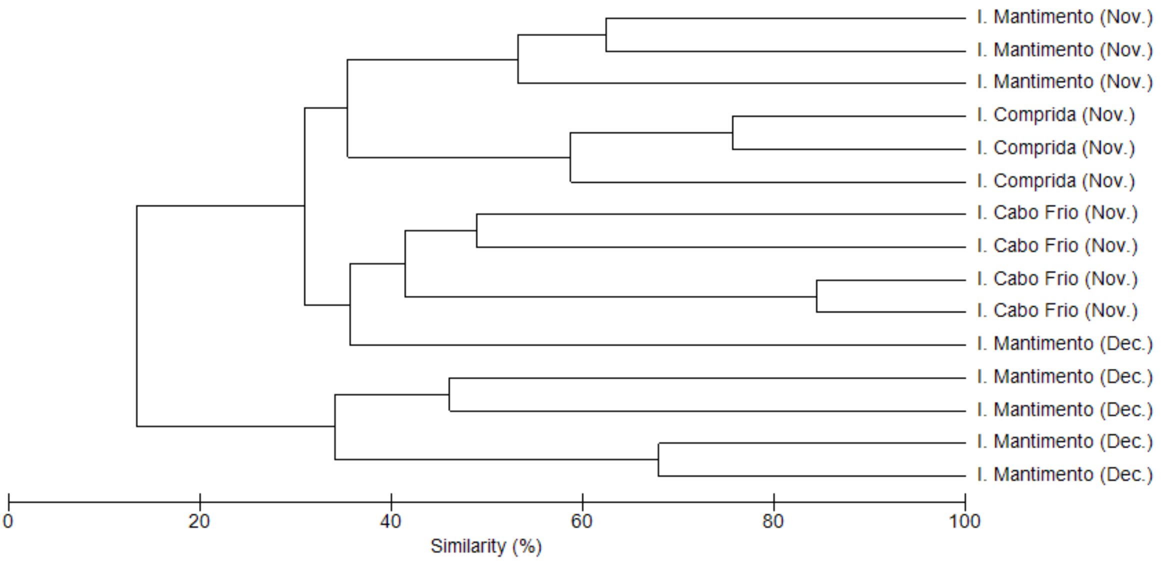

Figure 4. Cluster analysis of bacterial 16S rDNA fingerprints from microbial communities present in the mucus of seahorses *Hippocampus reidi* from three Islands in Southeast Brazil (Ilha do Mantimento, Ilha Comprida, and Ilha do Cabo Frio; Fig. 1) in November (Nov.) and December (Dec.) 2015.

The present study also confirmed that microbial communities present in the mucus of seahorses in the wild might vary over short-time periods (Fig. 4). Indeed, when comparing samples from Ilha do Mantimento collected in November and December, it was possible to confirm that microbial fingerprints changed from one month to the other; moreover, the differences recorded were higher in samples collected in the same location over two consecutive months than those found among specimens originating from different geographic regions (Fig. 4). It is likely that some environmental shifts may have significantly affected local conditions and consequently promoted a shift in the bacterial communities present in seahorse mucus within this period. The temporal variability recorded in the microbial fingerprint of fish mucus has been previously reported in freshwater fish sampled during rainy *vs.* dry season in wild lakes (Tatsadjieu *et al.*, 2010), as well as in earth ponds (Le Nguyen *et al.*, 2008). This variability in microbial fingerprints may limit the period during which the approach addressed herein can be used to trace the place of origin of wild seahorses. This constraint may be overcome if the place of origin of seahorses is accessed through the microbial fingerprinting shortly after their collection (e.g., up to one 1 week post-collection). Nonetheless, it is important to confirm if this temporal shift in microbial fingerprints occurs periodically (e.g., monthly) during the whole year or rather displays seasonal pattern. Periods during which environmental changes are less pronounced may contribute to a stabilization of the fingerprints displayed by the bacterial communities in seahorse mucus. Additionally, some bacterial bands recorded in the DGGE analysis using mucus of Pangasius remained stable throughout different seasons (Le Nguyen *et al.*, 2008). Therefore, further DNA sequencing studies may reveal if some bacteria taxa are more stable over a larger temporal scale than others and still allow a reliable tracing of origin for live seahorses.

The stocking of wild seahorse in captivity, even when placed in a recirculated system operated with natural seawater, promoted a shift in the bacterial communities associated with their mucus (Fig. 5 and Figure SII). Microbial fingerprints from wild specimens changed after only 40 days in captivity (Fig. 5). From collection to final costumer, marine ornamentals may pass through several aquarium systems in exporters, importers, wholesalers, and retailers (Cohen *et al.*, 2013). Therefore, our results showed that if specimens are stocked for about one month in a system throughout the supply chain, they might change the microbial signature of their mucus, thus limiting the chances to accurately trace their place of origin. Nonetheless, future studies should investigate if specimens originating from different geographical locations, and therefore displaying different microbial fingerprints, display similar microbial fingerprints after being stocked in captivity for at least 40 days in captivity. If this is not the case and a high level of differentiation persists in the microbial fingerprints of seahorses originating from different locations even after being stocked in captivity for long periods, the present approach may still be used to indicate if all specimens originate from the same location or not. Additionally, DNA sequencing studies can provide insights on which bacterial communities do not change when wild seahorses are transferred to captivity, and thus, allowing researchers to refine their analysis to pinpoint the place of origin of live seahorses by focusing on the molecular analysis of those bacteria.

The microbial fingerprints displayed by seahorse mucus after 80 days in captivity was similar to that displayed by specimens collected after 40 days (Fig. 5), revealing a potential stabilization of microbial communities in the mucus. This stabilization of microbial fingerprints was possibly due to the water conditions experienced by the seahorses in a closed recirculation aquaculture system indoors, as these are much more stable than in the wild or outdoor aquaculture systems. Therefore, it is possible that microbial fingerprints in seahorse mucus will remain stable after a few months in captivity. This stability in microbial communities under captive conditions was also reported for cultured soft corals (Pimentel *et al.*, 2016) and may hold the potential to establish a unique microbial signature specific for each production system. If different systems display different microbial signatures, in theory, it can be possible to trace the origin of traded specimens to different farms. Indeed, a previous study addressing the freshwater fish Pangasius was able to trace their farm of origin through the use of microbial fingerprints present in the mucus (Le Nguyen *et al.*, 2008). The possibility to trace captive seahorses to its producers would be paramount to support reliable certification programs and favour a more conscientious and sustainable trade (Cohen *et al.*, 2013; Cohen *et al.*, 2017). For example, certification agencies or programs, such as Seafood Watch, could inform end-costumers on which producer is following the most sustainable culture practices (Monterey Bay Aquarium; available at: http://www.montereybayaquarium.org/conservation-and-science/our-programs/seafood-watch; accessed in January 2017). The ability to distinguish farms would also be an incentive for producers to increase sustainability as a marketing strategy to enhance their income. Therefore, to validate this approach, future research should compare microbial fingerprints of wild-caught seahorses stocked in captivity with those displayed by specimens fully bred under captive conditions. Additionally, it would also be important to study microbial fingerprints on seahorse mucus from farms with different production systems, such as the use of closed and open systems.

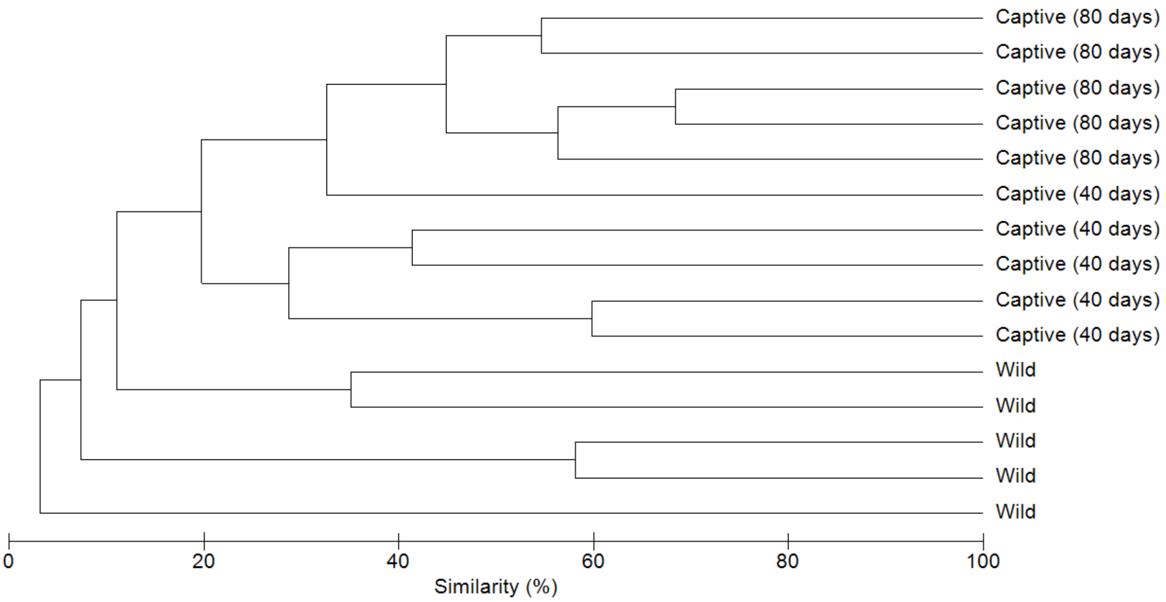

Figure 5. Cluster analysis of bacterial 16S rDNA fingerprints from microbial communities present in the mucus of seahorses *Hippocampus reidi* from the same specimens after 40 and 80 days in captivity.

Overall, the present study supports that microbial fingerprints displayed by seahorse mucus has potential to trace the geographic origin of live seahorses. This rationale may be used to trace the origin of specimens collected from the wild or farmed under controlled conditions. The variability displayed by microbial communities over a short-time period in the same geographic location in the wild, as well as their shift when transferred from wild to captive conditions are the main issues that need further research to support the use of this method for traceability. The reduced number of specimens employed in the present study may be considered as a potential limitation to achieve a more consistent analysis on the trends identified. Nonetheless, it worth highlighting that seahorses are a threatened group of animals and without a preliminary study, it would not have been ethically acceptable to collect a larger number of specimens from the wild. Considering the present results, future research may now advocate the need to increase the number of samples to be collected from wild seahorses to increase statistical power. Researchers may also now conscientiously choose to employ more expensive DNA sequencing techniques to gain an in-depth knowledge on which bacterial taxa play the most relevant role on the geographic and temporal variability displayed by fingerprints. The use of highthroughput sequencing techniques will also make possible to understand if and how microbial fingerprints of bacterial communities present in seahorse mucus can allow to discriminate between wild, captive raised and captive bred seahorses.

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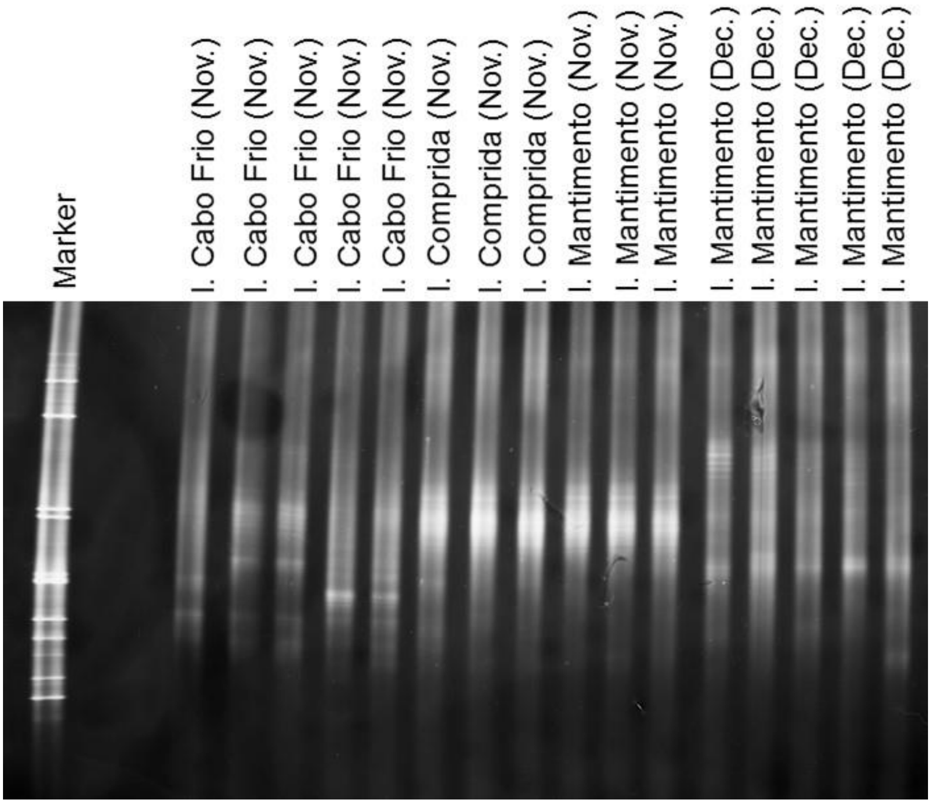
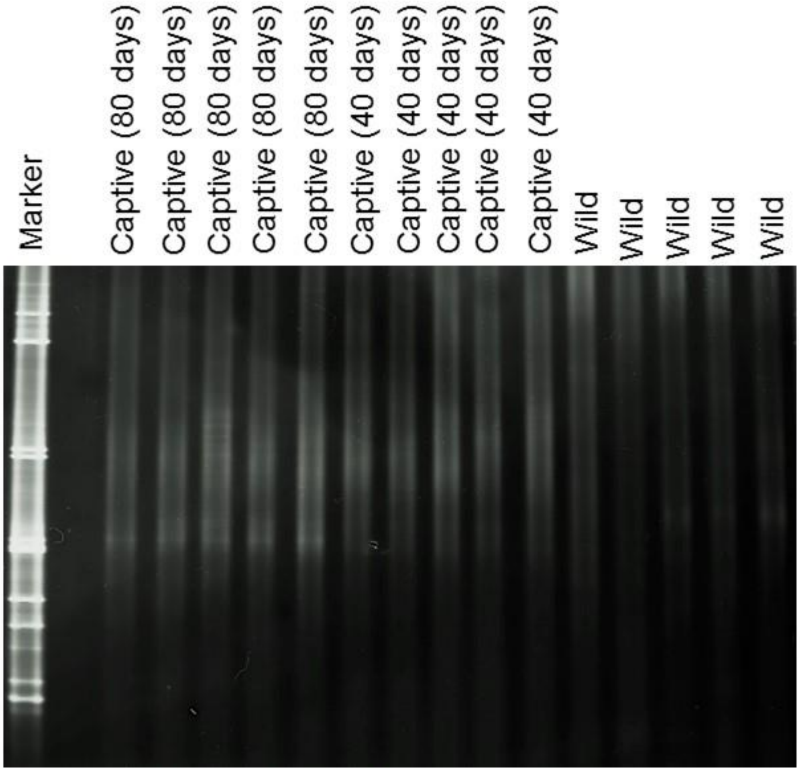
SUPPLEMENTARY FIGURES



Supplementary figure I. PCR-DGGE 16S rDNA banding fingerprints from microbial communities present in the mucus of seahorses *Hippocampus reidi* from three Islands in Southeast Brazil (Ilha do Mantimento, Ilha Comprida, and Ilha do Cabo Frio; Fig. 1) in November (Nov.) and December (Dec.) 2015.



Supplementary figure II. PCR-DGGE 16S rDNA banding fingerprints from microbial communities present in the mucus of seahorses *Hippocampus reidi* from the same specimens after 40 and 80 days in captivity.

X. CONSIDERÇÕES FINAIS

Uma infinidade de fatores tem motivado os pesquisadores a estudar os cavalos-marinhos. No entanto, observou-se que os campos de pesquisa fundamentais para subsidiar decisões governamentais e que abordam as questões que afetam a produção, o comércio e a manutenção das populações naturais de cavalos-marinhos não vem sendo cobertos adequadamente. Assim, no Manuscrito I, exploramos a oportunidade de usar os cavalos-marinhos como espécies bandeira para promover a conservação dos manguezais e diminuir as pescas de arrasto. Também sugerimos que são necessários mais estudos científicos para entender e gerenciar melhor as populações das espécies de cavalo-marinho mais comercializadas, bem como a necessidade de monitorar sua vulnerabilidade aos poluentes emergentes e às mudanças climáticas. Adicionalmente, destacamos que a aquicultura sustentável de cavalos-marinhos pode desempenhar um papel importante para atender a demanda mundial e na conservação de cavalos-marinhos, mas as pesquisas estão voltadas para sistemas complexos, sofisticados e caros, que não vão atender às demandas do grande mercado. Também destacamos a necessidade de pesquisas para desenvolver ferramentas de rastreabilidade fiáveis para combater o comércio ilegal destes organismos.

Desde 2012, ocorreu uma diminuição de mais de 90% de exportação de cavalos-marinhos, segundo os dos dados reportados pela CITES. Porém, essa redução é devido a proibições legais recentemente implantadas pelos principais países exportadores e às crescentes restrições impostas pela regulamentação do comércio internacional. No entanto, a baixa quantidade de animais comercializados nos últimos anos relatados na base de dados da CITES não reflete uma redução real na captura e comercialização de cavalos-marinhos. No Manuscrito II mostramos que ainda há demanda de milhões de cavalos-marinhos anualmente, especialmente secos, que veem sendo suprida por meio de pescas ilegais e mercados negros. Assim, as regras e regulamentos atuais estabelecidos para controlar a captura e comercialização global de cavalos-marinhos falharam em promover a conservação desses peixes. Este cenário ameaça seriamente as populações selvagens de cavalos-marinhos.

Uma das alternativas para aliviar essas populações selvagens é desenvolver protocolos de pescas sustentáveis e sistemas de aquicultura de grande escala e de baixo custo capazes de produzir um grande número de animais secos. Além de promover a conservação de cavalos-marinhos, essa medida também é uma opção de renda às populações locais já envolvidas neste comércio e pode inclusive servir para divulgar a conservação dos manguezais. No Manuscrito III, mostramos que o cultivo de cavalos-marinhos em tanques-rede em estuários pode ser uma boa alternativa para produzir cavalos-marinhos com baixo custo e atender a demanda de espécimes secos. Apesar de o Manguezal ser uma área protegida em todo o mundo, é fundamental proporcionar atividades de baixo impacto ambiental para a subsistência de comunidades locais. O cultivo de cavalos-marinhos em tanques-rede no mangue não utiliza alimentação alóctone e tem um baixo custo de implantação e manutenção. Além de diminuir os custos de produção, o cultivo embasado em alimentos naturais evita o excesso de nutrientes no sistema e proporciona uma dieta adequada aos cavalos-marinhos, resolvendo um grande gargalo no cultivo dessa espécie que é a falta de alimento seco adequado. Porém, alterações ambientais bruscas, predadores, obstrução das redes e escapes são as principais limitações desse sistema, e devem ser consideradas para cada local antes de sua implantação. Esse sistema tem potencial par favorecer o uso sustentável do Manguezal, e certamente aumentará a sustentabilidade da cadeia produtiva de cavalos-marinhos.

A otimização de práticas de transporte e de rastreabilidade são fundamentais para o fortalecimento da cadeia produtiva de cavalos-marinhos para fins ornamentais. Os cavalos-marinhos vivos são comercializados sem uma uniformidade no processo de embalagem e sem nenhum certificado de origem. Assim, no Manuscrito IV mostramos que cavalos-marinhos *H. reidi* são resistentes a condições de transporte em diferentes densidades, tempo de transporte, concentrações de oxigênio e salinidades. Podem ser transportados em 300 mL de água como uma opção barata e segura para transportes até 48 horas em temperaturas próximas de 21° C. O uso de oxigênio puro não é necessário quando o transporte é feito em até 48 h em temperaturas em torno de 21° C, mas pode ser útil para o transporte mais longo. Salinidades mais baixas e o uso de substrato podem proporcionar melhores condições fisiológicas e de bem-estar, sobretudo em transportes mais longos.

Com relação a rastreabilidade, no Manuscrito V reportamos que as impressões digitais microbianas no muco dos cavalos-marinhos têm potencial de rastrear a origem geográfica de cavalos-marinhos vivos. Este raciocínio pode ser utilizado para rastrear a origem dos espécimes coletados na natureza ou cultivados em condições controladas. A variabilidade exibida pelas comunidades microbianas em um curto período de tempo na mesma localização geográfica na natureza, bem como sua mudança quando transferidas de condições selvagens para cativeiro, são as principais questões que precisam de mais pesquisas para apoiar a utilização deste método para a rastreabilidade. Porém, esse trabalho forneceu suporte para que novas técnicas de sequenciamento de DNA sejam estudadas para aprofundar o conhecimento sobre quais táxons bacterianos desempenham o papel mais relevante na variabilidade geográfica e temporal exibida pelas impressões digitais microbianas. O uso de técnicas de sequenciamento também possibilitará compreender se e como as impressões digitais microbianas de comunidades bacterianas presentes no muco de cavalo-marinho podem permitir discriminar entre cavalos-marinhos nascidos em cativeiro dos criados em cativeiro (oriundos de populações selvagens).

Concluindo, o presente trabalho permitiu conhecer as principais áreas carentes de pesquisa científica para o desenvolvimento de métodos de produção e comercialização sustentáveis de cavalos marinhos, bem como sua conservação. Com base nessas informações, avançou-se na elucidação de alguns desses “gaps” mais importantes e demonstrou-se diversas áreas essenciais para as pesquisas futuras com vistas a gerar o conhecimento científico necessário para atender a demanda do mercado mundial e conservar as populações naturais desses peixes.