UNIVERSIDADE DE LISBOA

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DEPARTAMENTO DE BIOLOGIA ANIMAL

A NEW HOME FOR THE LONG-SNOUTED SEAHORSE,

Hippocampus guttulatus:

BREEDING IN CAPTIVITY TO PRESERVE IN THE WILD

Filipa Faleiro de Jesus

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Tese orientada por:

Professor Doutor Luís Narciso

Professor Doutor José Paula

Man cannot discover new oceans unless he has
the courage to lose sight of the shore.

Andre Gide

Agradecimentos

Mais do que um doutoramento em Biologia, este foi sem dúvida um doutoramento em Vida! Uma viagem por mares nunca antes navegados… Onde se procuram certezas e se encontram incertezas… Onde se vencem limites, se fortalecem fragilidades e se superam impossíveis… Uma viagem de descoberta, de conhecimento, de aprendizagem, de crescimento… Uma viagem… Uma viagem que, sem dúvida, não teria sido possível sem a vossa existência …

Ao Luís…
Por me guiar com sabedoria e amizade por mares de dúvida e incerteza…
Mas principalmente por ter ajudado uma pequena "ostra" a abrir-se ao mundo…

Ao Zé…
Pela sua pequena mas grande contribuição…
Por ter aceitado trocar um "banquete de marisco" por um saudável "peixe grelhado"…

Aos colegas de gabinete (Beluga inclusive)…
Por terem sido uma lufada de ar fresco…
E muito muito mais…

Aos professores e colegas do Laboratório Marítimo da Guia…
Pelos muitos e bons momentos partilhados naquele forte à beira mar (e arredores)…

Aos amigos de toda a vida…
E aos amigos de novas vidas…
Em terras lusitanas ou além mares…
Por simplesmente fazerem parte da minha vida…

À minha família…
Por serem parte de mim…
O meu porto seguro…
O meu farol em noites de nevoeiro…

Ao meu eterno Matias…
Por simplesmente lá ter estado…
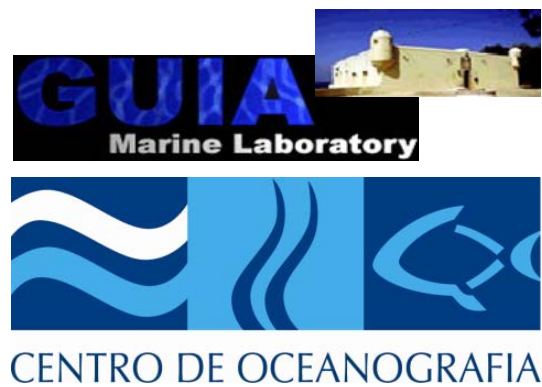Em cada palavra escrita, pensada, imaginada…

Por fim, aos meus cavalos-marinhos…

Por terem sido uma fonte de inspiração e de boa sorte…

Um muito obrigada a todos… por tudo!

Que a inspiração e boa sorte dos cavalos-marinhos vos acompanhem em cada viagem… sempre!

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Abstract

Habitat degradation and intensive exploitation are threatening seahorse populations worldwide. Captive breeding may be essential to replace harvesting of natural populations and provide an alternative source of seahorses for commercial trade and supplementation programs in the wild. The present investigation evaluates the potential of *Hippocampus guttulatus* culture as a tool for seahorse conservation. The main goals of this study were to optimize reproduction and juvenile rearing in captivity, and to evaluate the effects of captive breeding on the reproductive success of the species. Breeding the long-snouted seahorse in captivity proved to be a challenging but promising goal to achieve. High water flow conditions, great water columns depths, large holdfast availability, low stocking densities, balanced sex ratios, large size mates and diets rich in essential fatty acids were important to improve seahorse husbandry and reproduction. *Hippocampus guttulatus* juveniles were born at an exceptionally nutritionally depleted state and presented low survival rates in the first month of life. The high monounsaturated fat content and low docosahexaenoic acid level of *Artemia* nauplii proved to be unsuitable to fulfill the high polyunsaturated requirements of the juveniles. Optimization of the rearing system and prey quality may however improve juvenile survival. Captive breeding had a strong impact on the reproductive performance of the long-snouted seahorse, decreasing the number, size, condition and nutritional reserves of the juveniles and, consequently, their ability to survive and to tolerate stressful conditions. The poor reproductive performance and low survival rates of captive seahorses may jeopardize the economic viability of commercial production and the success of supplementation programs in the wild, but should not be discourage. Further research that incorporates the knowledge acquired in this investigation is expected to improve the success of *H. guttulatus* culture and to allow developing a suitable protocol for this species.

Keywords: *Hippocampus guttulatus*; seahorse; captive breeding; reproduction; juvenile culture; conservation.

Resumo

A degradação dos habitats naturais e a intensa exploração das populações estão a ameaçar as espécies de cavalos-marinhos em todo o mundo. As características únicas destes peixes tornam-nos particularmente sensíveis à exploração e a perturbações do seu ambiente natural e, por isso, um importante e iminente foco para conservação. Em 2002, o declínio global das populações de cavalos-marinhos levou à sua inclusão na Lista Vermelha das Espécies Ameaçadas da IUCN (União Internacional para a Conservação da Natureza) e no Apêndice II da CITES (Convenção sobre o Comércio Internacional das Espécies da Fauna e da Flora Selvagens Ameaçadas de Extinção).

Tendo em conta a crescente exploração e o acentuado declínio das populações naturais, tornou-se essencial agir no sentido de reduzir a pressão sobre os stocks naturais e preservar a sustentabilidade das populações de cavalos-marinhos. Conjuntamente com a preservação dos habitats naturais e a restrição da captura de espécimes selvagens, a produção de cavalos-marinhos em cativeiro assume também um papel essencial para a conservação das espécies. Para além de poder constituir uma fonte alternativa à exploração das populações naturais e assegurar, pelo menos em parte, as exigências dos mercados globais, o cultivo de cavalos-marinhos poderá ainda constituir uma fonte de animais para os programas de reintrodução na natureza, caso uma estratégia de último recurso se torne necessária para a conservação destas espécies.

O elevado valor conservacionista dos cavalos-marinhos, aliado ao seu elevado valor comercial, têm despertado um interesse global crescente no desenvolvimento de protocolos de cultivo que permitam fechar o ciclo de vida das espécies em cativeiro. Embora o cultivo destes peixes tenha revelado inúmeros desafios ao longo do tempo, a difusão de estudos e experiências de cultivo nos últimos anos tem permitido aprofundar o conhecimento da biologia destes peixes e superar muitos dos desafios. Contudo, existem ainda importantes lacunas no cultivo de juvenis, em particular no que diz respeito à satisfação dos requisitos nutricionais. Actualmente, existem já algumas espécies de cavalos-marinhos produzidas com sucesso em cativeiro, que asseguram parte da procura global para aquariofilia, mas para outras existe ainda um longo caminho pela frente.

O cavalo-marinho de focinho comprido, *Hippocampus guttulatus*, é uma das duas espécies de cavalos-marinhos presentes em águas europeias. Esta espécie destacou-se em meados de 2000 por apresentar uma das populações mais densas do mundo, a população da Ria Formosa no sul de Portugal. *Hippocampus guttulatus* é comercializado para os mercados de aquariofilia e de recordações, estando actualmente classificado como Indeterminado na Lista Vermelha das Espécies Ameaçadas da IUCN. Actualmente, a sustentabilidade da população da Ria Formosa já está também em risco, tendo-se observado um decréscimo populacional de cerca de 85% em menos de 10 anos.

Até ao início da presente investigação, pouco se conhecia sobre esta espécie de cavalo-marinho. Este estudo constitui uma primeira abordagem ao cultivo de *H. guttulatus*, pretendendo optimizar a produção desta espécie em cativeiro e avaliar o seu potencial para a conservação das populações naturais. Os principais objectivos desta investigação foram: 1) optimizar as condições de manutenção

e reprodução de cavalos-marinhos em cativeiro com base no comportamento dos adultos; 2) avaliar o potencial reprodutivo dos progenitores e o efeito do tamanho parental no sucesso reprodutivo da espécie; 3) determinar os requisitos nutricionais de cavalos-marinhos adultos e juvenis com base no perfil de ácidos gordos dos oócitos e na dinâmica lipídica durante o desenvolvimento embrionário; 4) optimizar o cultivo de juvenis e avaliar a qualidade nutricional de *Artemia* como dieta inicial para cavalos-marinhos recém-nascidos; 5) avaliar as consequências do cultivo em cativeiro para o sucesso reprodutivo da espécie e capacidade de sobrevivência dos juvenis.

O cultivo de *H. guttulatus* revelou ser um objectivo desafiante mas possível de atingir. A reprodução desta espécie em cativeiro foi possível em sistemas de cultivo específicos, com condições ambientais adequadas e boa qualidade de água. Fluxos de água elevados, grande disponibilidade de substrato e densidades reduzidas mostraram ser condições preferenciais para a manutenção de cavalos-marinhos em cativeiro. Embora não se tenham detectado efeitos significativos no bem-estar dos animais, densidades elevadas e poucos substratos afectaram o comportamento social da espécie, tendo aumentado os ritmos de actividade e o tamanho dos grupos.

No que diz respeito à reprodução, colunas de água elevadas, densidades reduzidas e proporções de sexos equilibradas demonstraram ser essenciais para diminuir a competição sexual e aumentar o sucesso reprodutivo da espécie. Uma coluna de água de 90 cm (i.e., cinco vezes a altura máxima da espécie) permitiu que os cavalos-marinhos se reproduzissem, mas não foi suficiente para evitar que a parada nupcial fosse interrompida quando o casal atingia a superfície da água. Densidades elevadas e proporções de sexos desequilibradas levaram a uma maior competição sexual e à interrupção da cópula, pelo que o isolamento dos casais (que constituem a base da estrutura social desta espécie em cativeiro) poderá ser vantajoso.

Tendo em conta que o sucesso reprodutivo da espécie foi determinado pelo tamanho dos reprodutores, a selecção de fêmeas e machos maiores poderá aumentar significativamente o desempenho reprodutivo desta espécie em cativeiro. Fêmeas maiores produziram ovos maiores com conteúdos nutricionais mais elevados. Machos maiores apresentaram bolsas de incubação mais volumosas e deram origem a juvenis maiores. Ambos os sexos demonstraram ter um papel decisivo no tamanho dos juvenis. Os machos conseguiram alcançar um aumento de 30% e 15% no peso e comprimento dos juvenis, respectivamente, ao diminuírem a densidade de juvenis no interior da bolsa. Esta variação no tamanho de juvenis de proles diferentes mas dos mesmos progenitores demonstrou que os machos têm uma capacidade de incubação limitada que obriga a um compromisso entre o número e o tamanho dos juvenis no interior da bolsa.

O desenvolvimento de uma dieta equilibrada que preencha os requisitos nutricionais dos progenitores e juvenis é essencial para reproduzir e cultivar com sucesso esta espécie em cativeiro. Tendo em conta que a composição nutricional dos oócitos pode ser um bom indicador dos requisitos nutricionais dos reprodutores e dos juvenis recém-nascidos, o perfil de ácidos gordos dos oócitos de *H. guttulatus* revelou que esta espécie tem elevados requisitos de ácidos gordos polinsaturados (PUFA), particularmente dos ácidos gordos essenciais DHA (ácido docosahexaenóico) e EPA (ácido

eicosapentaenóico), e uma menor necessidade de ácidos gordos monoinsaturados (MUFA). Devido ao longo desenvolvimento embrionário dos cavalos-marinhos, os juvenis nasceram num estado nutricional altamente carenciado, exigindo por isso uma dieta nutricionalmente rica e equilibrada.

Hippocampus guttulatus recém-nascidos demonstraram ser bastante vulneráveis às condições de cultivo, apresentando taxas de sobrevivência reduzidas (15.0-26.7%) durante o primeiro mês de vida. O sistema de cultivo utilizado não foi o mais adequado para juvenis de cavalos-marinhos, tendo permitido a ingestão de bolhas de ar. Por outro lado, os náuplios de *Artemia* provaram ser um alimento nutricionalmente inadequado para os juvenis desta espécie, incluindo os náuplios enriquecidos. O elevado conteúdo em MUFA e o reduzido nível de DHA desta presa não preencheram os elevados requisitos em PUFA dos juvenis, em particular de DHA. O enriquecimento de *Artemia* com ácidos gordos essenciais melhorou o perfil nutricional dos náuplios mas, tendo em conta os grandes constrangimentos nutricionais desta presa, a utilização de outras dietas vivas alternativas poderá aumentar significativamente as taxas de sobrevivência. A optimização do sistema e protocolos de cultivo irão certamente permitir melhorar o sucesso de cultivo de *H. guttulatus*.

A manutenção de cavalos-marinhos em cativeiro teve um grande impacto no seu desempenho reprodutivo. O ambiente artificial foi responsável por diminuir o número, tamanho, condição e reservas nutricionais dos juvenis, bem como a sua capacidade de sobreviver e de tolerar condições de stress. O pior desempenho dos reprodutores e as reduzidas taxas de sobrevivência dos juvenis em cativeiro podem constituir uma forte ameaça à viabilidade económica do cultivo de *H. guttulatus*, bem como ao sucesso dos programas de reintrodução no meio natural, mas não devem contudo ser desencorajadores. Este estudo constituiu uma primeira abordagem ao cultivo de *H. guttulatus*, deixando ainda um longo caminho de investigação pela frente até fechar o ciclo de vida da espécie e obter a primeira geração de cavalos-marinhos em cativeiro. Como primeira abordagem, os resultados sugerem um desfecho promissor e encorajam estudos futuros que corrijam os erros e problemas detectados ao longo desta investigação e que descubram novas formas de melhorar o sucesso de cultivo desta espécie.

A produção de *H. guttulatus* em cativeiro poderá vir a tornar-se um projecto técnica e economicamente viável a curto prazo. Tendo em conta os elevados valores atingidos pelos cavalos-marinhos no mercado de aquariofilia, é expectável que a optimização do protocolo de cultivo desta espécie permita criar uma fonte de cavalos-marinhos alternativa à exploração das populações naturais e comercialmente rentável, providenciando uma nova oportunidade de investimento e desenvolvimento económico, particularmente para as comunidades piscatórias que se vêem obrigadas a deixar de explorar as populações naturais de cavalos-marinhos.

Em contrapartida, a produção de *H. guttulatus* para programas de reintrodução na natureza não é nem deve ser, pelo menos para já, uma opção. A suplementação de populações deve ser adoptada apenas como último recurso e em situações extremas. Ate lá, outras medidas conservativas devem ser consideradas e implementadas. A captura de cavalos-marinhos e a degradação dos seus habitats naturais constituem as principais ameaças à sustentabilidade desta espécie e, por isso, nenhum

programa de conservação será bem sucedido sem antes eliminar as ameaças reais. A conservação de *H. guttulatus* terá, por isso, maiores hipóteses de sucesso se integrar uma abordagem multifacetada que combine investigação biológica, restrição da captura e do comércio, preservação dos habitats naturais, educação ambiental e, claro, a produção de cavalos-marinhos em cativeiro como alternativa à exploração das populações naturais.

Palavras-chave: *Hippocampus guttulatus*; cavalo-marinho; produção em cativeiro; reprodução; cultivo de juvenis; conservação.

General Introduction

A live myth

Seahorses (from the Ancient Greek *hippos* meaning "horse" and *kampos* meaning "sea monster") are creatures of myth and fantasy and a symbol of imagination and creative spirit. More than most animals, seahorses inspire wonder. In Ancient Greece and Rome, the seahorse, an animal half fish and half horse rode by Neptune and other sea gods, was considered a sign of strength and power. For the Celts, seahorses were a symbol of courage and forbearance at the sea and, even now, they are a good luck charm for many sailors throughout oceans. In some Asian civilizations, the seahorse was and still is valued as a therapeutic animal that has magical and mystical powers.

The wonder of seahorses goes beyond the myth. In the real life, seahorses still are amazing creatures. Seahorses represent the genus *Hippocampus*, which belong to the family Syngnathidae along with pipefishes, pipehorses and seadragons. Although lacking the greatness of the mythic creatures, these little fish are full of unique and remarkable features. At first sight, seahorses do not look like a fish. Their upright structure, with the head held at a right angle to the body, and the long tubular snout give them an equine profile, while their finless prehensile tail resembles the posterior end of some reptiles. Instead of the typical scales of bony fishes, their body is covered by bony plates arranged in a series of rings, which gives them a tough armored appearance.

Besides their atypical body morphology, seahorses present also an unusual life style. Unlike most fishes, seahorses are poor swimmers and prefer to remain resting, anchored with their prehensile tail to any prospective substrate. Even when they swim, they do not do it like a fish. Their upright swimming highly contrasts with the universally horizontal swim mode of fishes. Rapid undulations of the large dorsal fin provide the main propulsive force, while the smaller ear-like pectoral fins provide steering control. Instead of pursuing preys and escaping from predators, seahorses are masters of camouflage that change color to blend in with their surroundings. Seahorses are ambush predators that mostly depend on patient and stealth to approach their prey. They use their chameleon-like eyes, which can move independently of each other, to detect and their long snout to suck up their planktonic or epibenthic preys. These cryptic fishes are thus preferential inhabitants of sheltered areas and structurally complex habitats such as seagrass beds, coral reefs and mangroves.

Lastly, among the long list of remarkable features of seahorses, is their reproductive strategy. Fidelity is rare among animals but not among seahorses. These fishes generally establish monogamous pair bonds that last through a life or, at least, through a breeding season. Besides faithful husbands, seahorse males are also devoted fathers. Seahorses present the most specialized form of paternal care amongst animals, with the male becoming pregnant and providing extensive care to the developing embryos. Following a long and elaborate courtship that culminates with the pair rising in the water column, the female deposits her eggs inside the male brood pouch. Embryos fully develop within the enclosed pouch environment in close association with the highly vascularized pouch epithelium (Carcupino et al., 2002), in a manner that bears some resemblance to the embryo-placental relationship in mammals. Besides providing protection and a controlled and

safe environment to the embryos, the male pouch is also responsible for gaseous exchange, removal of waste products, osmoregulation and transference of hormones and inorganic compounds during embryonic development (Linton & Soloff, 1964; Boisseau, 1967), playing also an important immunoprotective role (Melamed et al., 2005). Seahorse embryos undergo the most vulnerable developmental stages well protected inside the male pouch and isolated from the uncertainty of the external environment. When juveniles are born, they are already a miniature of adult seahorses, bigger and stronger than the larval stages of most marine fishes.

The great pursuit for seahorses

The peculiarity of seahorses did not pass unnoticed to the world. Their remarkable attributes have been recognized, admired and sought by different cultures and with distinct purposes. Seahorses have long been credited with magic and medicinal value. In Asian civilizations, seahorses have been used for over 600 years in traditional Chinese medicine (TCM) and its derivates. They are believed to increase the energy flows within the body and to have a curative role in a great variety of ailments, such as fatigue, pain, contusions, fractures and strains, open wounds and cuts, throat infections and inflammations, respiratory problems, heart disease and circulatory problems, high cholesterol, kidney and liver disease, incontinence, sexual dysfunction, difficult childbirth, skin afflictions, poor immune system and mental disorders. Although less popular, the use of the medicinal properties of seahorses in Europe is apparently older than in TCM. European records ascend to 342 B.C. and continued until at least the eighteenth century (Vincent, 1996). Although evidence regarding the efficacy of the medicinal properties of seahorses in published scientific literature is scarce (e.g., Qu et al., 1991; Yu et al., 1995; Zhang et al., 2003; Shi et al., 2006), TCM and other traditional medicines are recognized by the World Health Organization as viable health care options.

Seahorses may be jeopardized as much by their unusual beauty as they are by their medicinal properties. Seahorses are also sold around the world as popular aquarium pets and curios. Moreover, there is also who appreciates seahorses as an extravagant delicacy. Seahorses were eaten by ancient Chinese and, currently, they are still sought in some health food restaurants (Vincent, 1996).

Involvement in seahorse trade is global, with at least 32 nations trading seahorses in mid 1990s (Vincent, 1996), a number that has increased to 80 countries in early 2000s (Lourie et al., 2004). Commercial transactions for TCM occur primarily between Asian countries, while the bigger piece of the aquarium trade is reserved for importation into the United States and Europe. Seahorse trade volumes are large, amounting to many millions of seahorses annually. TCM is the largest consumer of seahorses, with estimated values reaching as many as 20 million seahorses traded internationally each year in 1996 (Vincent, 1996), and exceeding the 70 million per year in 2000 (CITES, 2002). A smaller but important international trade in live seahorses for the aquarium market was estimated at several hundred thousand seahorses per year in 1996 (Vincent, 1996), and around 5 million

specimens in early 2000s (Watson & Stokes, 2004). The curios trade occupies also a minor but significant position, monopolizing more than 1 million specimens (Watson & Stokes, 2004).

Although live trade loses in numbers, it may triumph in value. Dried seahorses are a highly-prized ingredient in the traditional medicine, but live seahorses may reach incredibly high prices in the aquarium market. Values vary markedly according with target applications, traded countries, years and seahorse features. Vincent (1996) refers fisher values of 0.02-3.00 and 0.10-1.36 USD and retailer prices of 0.91-17.24 and 1.36-13.00 USD per dried and live seahorses, respectively. However, the prices of live seahorses seem to be highly undervalued. In key aquarium trade areas such as North America and Europe, live seahorses are paid the price of gold. While dried seahorses for curios had a mean value of 0.16 USD per seahorse (Grey et al., 2005), live seahorses were commonly retailed at 9.00-39.00 USD in the USA (Woods, 2007) and 80.00 USD in Canada, with American captive-bred seahorses being sold for up to 170.00 USD by direct selling on the web (Watson & Stokes, 2004).

Demand for seahorses has increased significantly in the last decades and is likely to continue growing. The increased global consumption of seahorses appears to be driven primarily by the economic growth of China, but also by their increasing popularity as aquarium pets worldwide. Seahorses clearly face high levels of exploitation, but demands still invariably exceed supply. Virtually all the seahorses in the medicinal, live aquarium and curios trades were collected from the wild. Natural populations are declining all around the world, and declining rapidly in number and size (Vincent, 1996; Watson & Stokes, 2004). Seahorses once had to be of a certain size and quality before they were accepted for TCM but, now, declining availability of the preferred large, pale and smooth seahorses has been offset by the shift towards prepackaged medicines, which opened space for the previously unexploited juveniles, spiny and dark-colored seahorses.

Along with intensive exploitation and overfishing, seahorses are also being threatened by the widespread degradation of their natural habitats. Seahorses inhabit shallow coastal waters, being generally associated with structurally complex habitats such as seagrass beds, mangroves and coral reefs. Worldwide, these highly productive ecosystems are being degraded and lost as a result of human activities such as dredging, clearing and pollution. Attempts to diminish the impact of global trade on seahorse populations may thus be ineffectual unless protection and management actions are also sought for their vital habitats.

Preserving the myth

Habitat degradation and intensive exploitation are threatening seahorse populations everywhere. The global decline of natural populations has led to the inclusion, in 2002, of seahorse species in the IUCN (International Union for Conservation of Nature) Red List of Threatened Species and in the Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). Of the 37 seahorse species listed in the IUCN Red List, 28 are listed as Data Deficient (i.e.,

there is inadequate information to make a proper assessment of its risk of extinction), 7 as Vulnerable (i.e., it is facing a high risk of extinction in the wild), 1 as Endangered (i.e., it is facing a very high risk of extinction in the wild) and 1 as Least Concern (i.e., it is a widespread and abundant taxa). The listing of most species as Data Deficient, which is not a category of threat, should not however be undervalued and discourage pressing efforts towards their preservation. The lack of information on these species may hide a worrisome and dire reality, and future research may further show that threatened classification is appropriate.

While the IUCN Red List identifies those species most in need of conservation attention without direct implications for exploitation or trade, the CITES Appendix II controls the import and export of species, but only between parties to CITES, which excludes some important countries in seahorse trade. The Appendix II listing requires proper regulatory control but does not prohibit regulated fishing and trade. Export permits or certificates may be granted if the relevant authorities are satisfied that trade will not be detrimental to the survival of the species in the wild. Subsequently to the Appendix II listing, the CITES Animals Committee set a universal minimum permissible size of 10 cm (height) for all seahorses in international trade. The IUCN and CITES listings should not, however, take the place of more specific and strict national laws.

The singular life history characteristics of seahorses make them particularly sensitive to overfishing and disturbance of their natural environment and, thus, an important and pressing focus for global conservation efforts. Low rates of natural adult mortality mean that fishing exerts a relatively substantial selective pressure. Their low mobility and small home ranges increase their vulnerability to harvesting, and reduce their capacity to abandon disturbed areas and colonize new and healthy regions. The low fecundity and lengthy parental care of seahorses limit their reproductive rates and, thus, their capacity to withstand exploitation. Moreover, male pregnancy implies that the survival of the young inside the pouch is reliant upon the survival of the male. Faithful pair bonds mean that social structure is easily disrupted through the loss of a mate with serious consequences for seahorse reproduction. Besides interrupting mating and provoking unusual mate competition, the lost of a mate will require considerable time for lonely seahorses to find a new mate, given the low encounter rates resultant from their sparse distribution, low mobility and reduced mate availability. Moreover, since lengthy and deepen bonds enhance the reproductive performance of seahorse couples, the formation of new pairs will decrease short-term reproductive output.

The publication, in the mid 1990s, of important studies revealing the great dimension of seahorse trade and the related concerns regarding the exploitation and decline of wild populations (e.g., Prein, 1995; Vincent, 1996; Moreau, 1997) has alerted society and the scientific community. Conservational concerns, along with the high value of seahorse traffic, triggered a widespread interest in all and sundry aspects of seahorse biology, and opened the door to seahorse culture.

Breeding in captivity to preserve in the wild

In a time where natural populations become more and more endangered, seahorse conservation turned out to be a primary requirement rather than an option. Strong and diverse efforts must be employed to reduce the pressure on wild stocks and preserve the sustainability of seahorse populations. In addition to habitat preservation and adoption of heavily restrictive fishing procedures, seahorse conservation must pass also through the development of alternative and viable seahorse sources.

In the last decades, captive breeding has been widely used as an important management tool to conserve and enhance endangered and threatened species, being recommended for 36% of the 3314 taxa considered by the Conservation Breeding Specialist Group of the IUCN (Seal et al., 1993) and in 64% of the 314 approved recovery plans for species listed under the Endangered Species Act (Tear et al., 1993). Likewise, captive breeding can be also an essential and viable tool for seahorse conservation. Besides reducing the pressure on wild stocks by assuring, at least to some extent, the commercial demands, captive breeding can be further applied in population supplementation programs when a last-resort recovery strategy is required. Moreover, captive breeding may also contribute to the development of a profitable commercial activity, thus achieving both the conservational and socio-economic goals.

The production of captive seahorses for the traditional medicine may present some potential problems, such as greater susceptibility to disease and altered medicinal properties, but in the aquarium market captive seahorses are without a doubt a better option than wild caught specimens. Seahorses collected from the wild tend to fare poorly in home aquaria, not only because they had experienced the stress and harms from moving out from the wild, but also because they lack the tolerance acquired by captive seahorses to artificial conditions, such as confinement, handling and inert feeding. Moreover, seahorse culture may provide a more stable and foreseeable source of seahorses, in both supply and price, than exploitation of wild populations.

The sedentary life pattern, reduced mobility and general lack of agonistic behaviors in seahorses make them a suitable candidate group to breed and survive under captive conditions. The production of fewer but larger eggs and the release of young at a more advanced developmental stage greatly improve their chances of survival in captivity. Moreover, captive seahorses are also expected to have greater chances of integrate well into the wild, since significant behavioral deficiencies resultant from captivity (especially with respect to foraging, predator avoidance and social behavior) are expected to be least likely in fish with poorer behavioral repertoires, reduced mobility, camouflage as a predator evasion strategy and monogamous pair bonds like seahorses. Even so, seahorse culture did not come up to the high expectations, and proved to be a fighting challenge, at least at the outset.

Long before the western interest in seahorses, the first attempts to rear these fishes in captivity took place in the 1950s in China (Vincent, 1996). In the 1980s, commercial efforts ceased because of combined biological and economic difficulties and, although some attempts continued to be made in

other Asian and occidental countries, seahorse culture barely improved during the second half of the 20th century, remaining experimental rather than becoming commercially viable (reviewed in Koldewey & Martin-Smith, 2010). It was noticeable that numerous challenges (regarding high mortality, disease and nutrition of broodstock and, particularly, offspring) had to be transcended in order to achieve successful and viable seahorse culture protocols. Seahorses proved to require specific husbandry and rearing conditions, to be extremely vulnerable to stress and confinement, and to succumb easily to disease (e.g., gas bubble disease).

With the bloom of research studies and commercial culture attempts in late 1990s and early 2000s, there was, however, a growing understanding of seahorse biology and a great improvement of culture protocols. Seahorse culture has been achieved for several species and, currently, assures part of the commercial demands, particularly to the live aquarium market. The current status of seahorse culture was recently reviewed by Koldewey & Martin-Smith (2010). Since 2004, there have been substantial and rising amounts of captive-bred live seahorses reportedly exported each year, from mean values around 10 thousands in 2004 to 40-50 thousands in subsequent years. In 2005-2006, there were at least 28 seahorse aquaculture operations in 15 countries, most operating at a small scale for less than five years. A total of 13 species are currently commercially cultured with average survival rates to maturity generally greater than 50%. However, there is still much to do in what concerns seahorse culture. Broodstock husbandry and reproduction have been considerably improved but juvenile rearing, more particularly the accomplishment of nutritional requirements, continues to be a major bottleneck in seahorse culture, at least for some species.

Although captive breeding may represent a real option to reduce the pressure on wild stocks, it should not be considered a magic potion that solves the complex problem of seahorse conservation. A multi-faceted approach is likely to offer the best chance of success. Conserving seahorse populations would best be achieved through an integrated combination of biological research, habitat preservation, trade monitoring, environmental education and, without doubt, captive breeding.

Two seahorse species in European waters

The long-snouted (*Hippocampus guttulatus*) and the short-snouted (*H. hippocampus*) seahorses are prominent residents of the European waters. Both species can be found in shallow inshore waters, mainly in seagrass beds and algal stands, all around the eastern Atlantic and the Mediterranean (Lourie et al., 2004). These sympatric seahorses are commonly distinguished by the presence or absence of skin filaments. *Hippocampus guttulatus* generally presents a mane of thick skin filaments, while *H. hippocampus* generally has a smooth appearance. However, skin filaments have proven to be unreliable for identifying these seahorses, since both species can be found with or without skin filaments (Curtis, 2006a). Other morphological traits, such as body size, snout length, trunk shape, coronet form and the number of fin rays, should be rather used for identification (Figure

1). These species differ also in their life style. The quiet *H. guttulatus* prefer to sway passively with the currents while grasping holdfasts in water depths below 12 m, while the more lively *H. hippocampus* can be found in deeper waters (up to 60 m), preferring more open and less complex habitats subjected to greater oceanic influences (Lourie et al., 2004; Curtis & Vincent, 2005; Ribeiro et al., 2006).
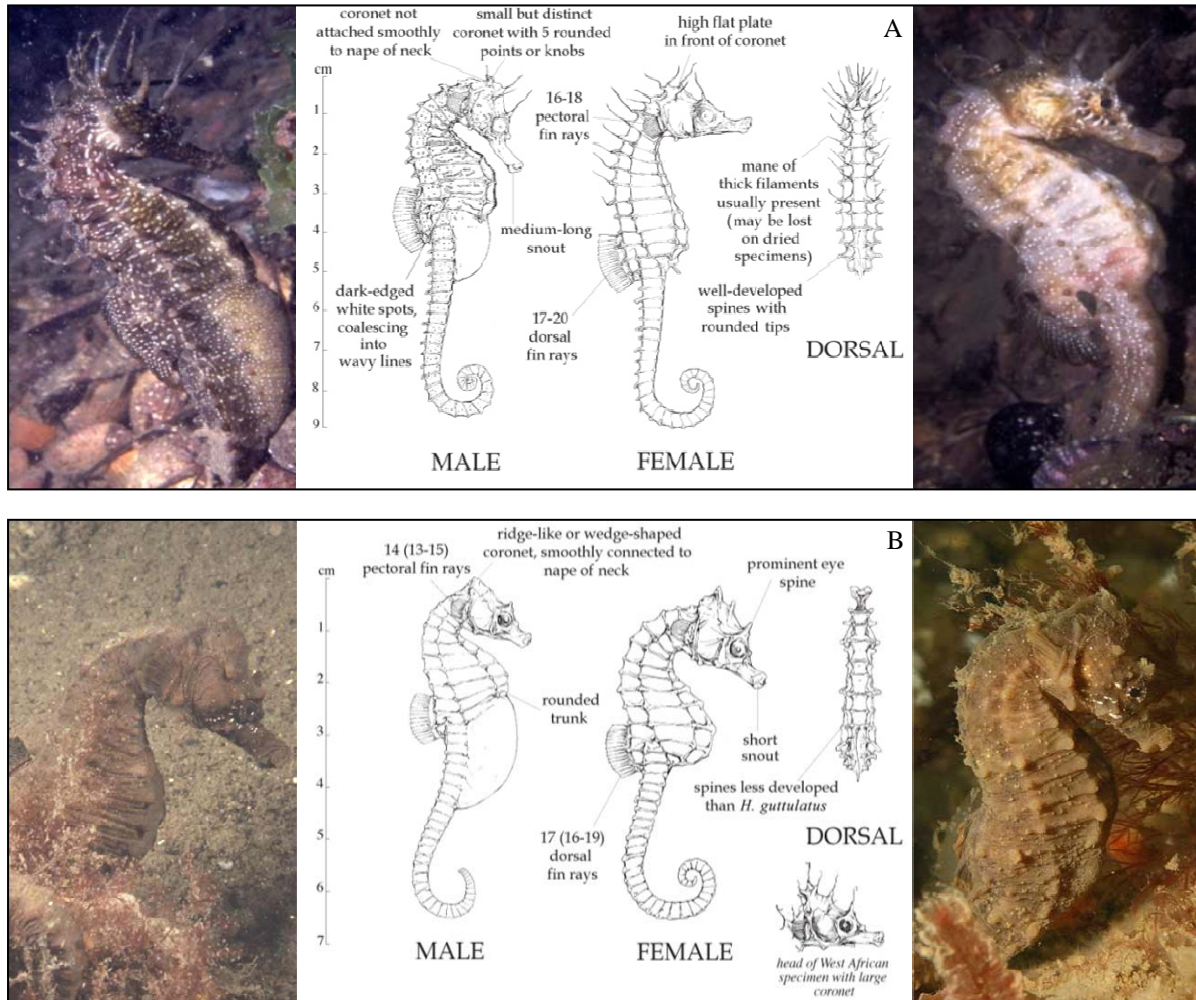
Figure 1 – The two European seahorse species: *Hippocampus guttulatus* (A) and *H. hippocampus* (B) (adapted from Lourie et al. 2004).

Although far below the levels of exploitation in Asian communities, the European species are also caught, both intentionally and incidentally, in several countries (e.g., Italy, France, Spain, Portugal and Croatia), and sold dried for curios and alive for aquarium markets. The volume of this trade is unknown but, without appropriate management, this trade might represent a threat to the species. In Portugal, bycatch and target catches of seahorses to the curios market were in the order of hundreds of kg (Project Seahorse, 2003a,b). In 2004, the CITES Trade Database declared the exportation of 1700 live *H. guttulatus* wild specimens from Portugal to Canada for scientific purpose. With regard to

H. hippocampus, there were no records of exportation of Portuguese specimens, but Portugal imported wild live seahorses from Sri Lanka (6 in 1998, 31 in 1999 and 8 in 2001) and Singapore (8 in 1999, 10 in 2001 and 4 in 2002) for commercial trade, as well as 2 kg of confiscated bodies from Philippines. Since many commercial transactions are not declared, these values are almost certainly underestimated.

Besides harvesting, the European seahorses are also threatened by habitat degradation. Their preferred habitat, seagrass beds, is receding everywhere in the littoral region of the Mediterranean with the increase of various forms of pollution and coastal modification (Vincent, 1996). In Portugal, seahorses, which could be unsurprisingly sawn in their natural environment a few decades ago, can now be found only in some key spots, such as the Ria Formosa lagoon. Compared to *H. guttulatus*, *H. hippocampus* is much less abundant and harder to find (Curtis & Vincent, 2005; Ribeiro et al., 2006).

The two European seahorse species present, therefore, high conservational value. In 1996, they were listed as Vulnerable based on suspected past declines in occupancy, occurrence and habitat, as well as on potential levels of exploitation. However, the reassessment of the species under the new criteria and with greater taxonomic understanding resulted in their listing as Data Deficient in 2003, given the lack of appropriate data on these species and the need for further research. Both species are also listed in the Red Data Book of Portugal, as Undetermined in continental Portugal and Rare in Azores. *Hippocampus guttulatus* is also included in the Red Data Book of France and protected in Slovenia under the 1993 Protection of Threatened Animals Act. Furthermore, the inclusion of the entire genus *Hippocampus* in the CITES Appendix II entails full declaration and monitoring of the trade in live and dead seahorses in the 175 parties to CITES, including Portugal and the other members of the European Union. In Portugal, the capture and retention of seahorse species is forbidden since 2006.

A seahorse oasis in Portugal

In such a globally dire scenario, the Portuguese *H. guttulatus* population is like an oasis in a desert. In early 2000s, this species stood out for presenting one of the most dense seahorse populations in the world, located in the Ria Formosa lagoon. A transect survey covering 5760 m^2 of the lagoon showed that this population (near 2 million individuals) occurred at a mean density of 0.07 ind m^{-2} (range 0 to 0.51 ind m^{-2}: Curtis & Vincent, 2005), reaching 10 ind m^{-2} in small patches on the edges of seagrass beds (reviewed in Foster & Vincent, 2004). *Hippocampus guttulatus* density was one order of magnitude greater than mean densities observed in similar transect surveys of *H. abdominalis* in Australia, *H. capensis* in South Africa and *H. comes* and *H. barbouri* in the Philippines (Curtis & Vincent, 2005).

The Ria Formosa is a shallow coastal lagoon permanently connected to the Atlantic ocean by six inlets. It extends for about 55 km along the south coast of Portugal, with a maximum width of 6 km and an average depth of 2-3 m (Figure 2). The lagoon is bordered by a string of sand dunes that

encloses a complex system of salt marshes, sand and mud flats, subtidal channels and tidal creeks, with a total surface area of approximately 170 km². The intertidal area covers approximately one third of the lagoon, and only 14% of the lagoon surface are permanently submersed (Ribeiro et al., 2008). The system has semi-diurnal tides and high water turnover rates. The freshwater inputs are almost negligible, especially in the summer (Tett et al., 2003). The salinity regime is highly stable (32.1-36.5 psu, except during winter low tide when it falls to 26.8 psu), and temperatures range between 16.4-19.0 ºC in winter and 18.8-28.4 ºC in summer (Newton & Mudge, 2003).
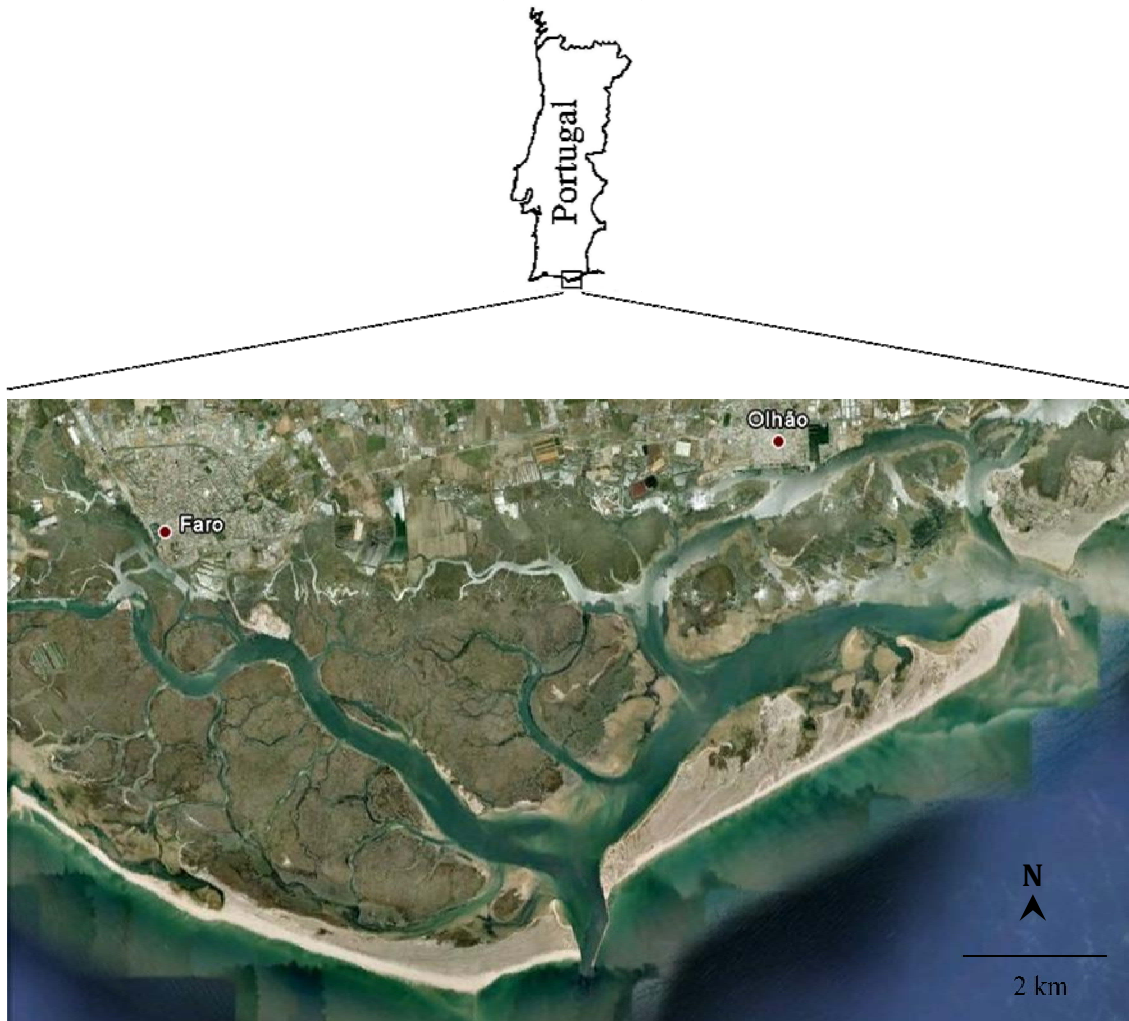
Figure 2 – Satellite image of the western part of the Ria Formosa coastal lagoon
(adapted from Google Earth).

The Ria Formosa lagoon is a highly productive system due to the presence of extensive salt marsh vegetation, high nutrient concentrations, strong insolation and good water exchange. The western part of the lagoon is bounded by a heavily urbanized area and surrounded by agricultural land. Despite the tidal pumping from sediments (Falcão & Vale, 1990) and the exchange with adjacent coastal waters (Falcão and Vale, 2003), nutrients are mainly imported from urban discharges and

agricultural run-off (Newton et al., 2003). Given its high productivity, the Ria Formosa provides a rich environment to a great variety of species, and supports a great variety of socio-economically important industries, namely fishing, aquaculture, salt extraction and tourism.

The great and fast development of human activities in this coastal lagoon is threatening the species and their habitats. Eutrophication has been identified as the major threat to the ecological quality of the Ria Formosa (Newton et al., 2003). The water quality of the lagoon has deteriorated over recent years, reflecting the intense economic development of the surrounding areas and the resultant increase of the levels of bacteria, nutrients and contaminants released by untreated or poorly treated sewage, industrial and agriculture effluents (Bebianno, 1995). Moreover, fisheries, particularly if employing non-selective and ineffective harvest practices, may have also a devastating impact in both populations and habitats. In order to protect the integrity of this important lagoon system, the Ria Formosa was recognized as National Reserve in 1978 and reclassified as Natural Park in 1987. Internationally, it forms part of the European network of protected areas Natura 2000, and it is a protected area of the Ramsar Convention on Wetlands of International Importance.

The fish communities of the Ria Formosa lagoon have evolved throughout the years. A comparative study over a 20-year period showed that, although species composition remained relatively stable between 1980-1986 and 2001-2002, species abundance in number and weight were both significantly greater for the earliest period, mainly due to a sharp decrease in the abundance of Mugilidae (Ribeiro et al., 2008). In contrast, the seahorses *Hippocampus* spp., along with the pipefishes *Syngnathus* spp., significantly increased in number (*Hippocampus* spp.: from 23.67 to 92.00 ind; *Syngnathus* spp.: from 21.63 to 50.38 ind) and weight (*Hippocampus* spp.: from 107.33 to 397.31 g; *Syngnathus* spp.: from 198.00 to 312.81 g), but not the pipefish *Nerophis ophidian* (from 4.63 to 2.00 ind and from 2.58 to 1.07 g). From early 2000s to present, there was however a sudden and tragic reverse and, currently, the sustainability of the formerly prosperous *H. guttulatus* population seems to be jeopardized. Recent observations indicate that, in less than 10 years, this population has experienced 85% reduction in numbers. Potential causes are currently under investigation, and may be related with habitat alteration and fishing.

The inconspicuous long-snouted seahorse

Compared to other seahorse species, *H. guttulatus* has remained quite well camouflaged to researcher eyes throughout time. Until recently, little was known about the seahorses that occur all over the Mediterranean and Atlantic. Following some historical publications (e.g., Gabe, 1957; Durante, 1966; Boisseau, 1967), the interest in *H. guttulatus*, and seahorses in general, fell into oblivion for several decades. Until the time at which this investigation was initiated, there were no more published studies on this species, except a genetic study on five Mediterranean syngnathid species (Vitturi et al., 1998). Even when all eyes were focused on seahorses, this species stayed away

from the spotlight. The bloom of seahorse publications in early 2000s near missed the long-snouted seahorse for some years.

The discovery in 2000 of the prosperous Portuguese population of the Ria Formosa lagoon by the Project Seahorse, an international organization committed to global conservation and focused on seahorses, has triggered the curiosity in this species. From 2005, publications on the long-snouted seahorse started to be gathered round, with almost 30 studies being published in a few years. Besides the articles published (or waiting for publication) resultant from this investigation, many other studies have been published using specimens from the Ria Formosa lagoon, namely on tagging (e.g., Curtis, 2006b), animal preservation (e.g., Nadeau et al., 2008), species identification (e.g., Curtis, 2006a), distribution (e.g., Curtis & Vincent 2005), movement, growth and survival patterns (e.g., Curtis & Vincent, 2006), fecundity (e.g., Curtis, 2007), reproductive behavior (e.g., Naud et al., 2009), broodstock husbandry (e.g., Palma et al., 2008) and evaluation of conservation measures (e.g., Curtis et al., 2007, Curtis & Vincent, 2008). Moreover, studies have also been developed with *H. guttulatus* populations from Spain (e.g., bacterial associations: Balcázar et al., 2010a,b,c,d; genetic: Pardo et al., 2007; López et al., 2010; morphometry: Verdiell-Cubedo et al., 2006; broodstock husbandry: Planas et al., 2008, 2010), Turkish (e.g., morphometry: Gurkan & Taşkavak, 2007), Greece (e.g., natural diet composition: Kitsos et al., 2008) and the French-Belgian border (occurrence: Vandendriessche et al., 2005).

All these studies, along with the present investigation, have notably contributed to a better understanding of *H. guttulatus* biology. The long-snouted seahorse can be found at high local densities (0.04-0.11 ind m^{-2} in transect surveys: Curtis & Vincent, 2005; 0.3–1.5 ind m^{-2} in grid surveys: Curtis & Vincent, 2006). This species presents a patchy distribution, is associated with structurally complex habitats and grasps indiscriminately all prospective holdfast types (Curtis & Vincent, 2005). Adult seahorses maintain small (19.9 ± 12.4 m^2) but strongly overlapping home ranges during multiple reproductive seasons, while benthic juveniles exhibit significantly lower site fidelity than adults. This seahorse species presents an early age at maturity, rapid growth rates, short generation times and relatively low mortalities. Planktonic juveniles measure 1.2 cm at birth, recruit to vegetated habitat at 9.6 cm (3 months old), have mature brood pouches (males only) at 10.9 cm (6 months old), begin maintaining home ranges and reproducing at 12.5-12.9 cm (around 11 months old), and live for 4.3-5.5 years (Curtis & Vincent, 2006). *Hippocampus guttulatus* has a crustacean-based diet dominated by amphipods, anomuran decapods and mysids, but differences in diet composition and feeding activity occur between females, non-brooding and brooding males (Kitsos et al., 2008). This species presents equal sex ratios (Curtis & Vincent, 2006), size-assortative mating and signs of social promiscuity (Naud et al., 2009). Females prepare significantly more clutches than the number of broods produced by males. Males generally brooded 4.2 ± 1.6 broods year^{-1}, while females presented a batch fecundity of 213.9 ± 110.9 eggs and a realized annual fecundity of 903.6 ± 522.4 eggs (Curtis, 2007).

Although *H. guttulatus* may be vulnerable to extended periods of poor recruitment, their life history patterns (such as early age at maturity, rapid growth rates and short generation times) suggest that this species may recover rapidly when direct (e.g., exploitation) and indirect (e.g., bycatch and habitat damage) effects of disturbance cease. Curtis et al. (2007) observed that cession of demersal fishing enhanced habitat structure and complexity and, consequently, *H. guttulatus* abundance. Given their site fidelity to small and overlapping home ranges, the establishment of marine protected areas may be effective for protecting critical spawning biomasses, particularly if established in preferred seagrass beds and macroalgal stands (Curtis & Vincent, 2006). On the other hand, a minimum size limit of 10.0 cm, just above size at maturity (9.7 cm), will not prevent exploited populations from declining. Increasing it to 14.0 cm (the size reached after at least one full reproductive season) or more will lower the risk of decline and extinction with a cost of only 5.6% reduction in long-term catches (Curtis & Vincent 2008).

At the time at which this investigation was initiated, there were no published studies on *H. guttulatus* captive breeding and its viability as both commercial and conservation tools. This investigation represented the first approach into the culture of the long-snouted seahorse. Along with it, other studies have been developed to evaluate the effects of broodstock diet on broodstock condition (e.g., Planas et al., 2010) and egg composition (e.g., Palma et al., 2008; Planas et al., 2008). However, there is still much to do and to discover about seahorses and *H. guttulatus* in particularly. Further research on this species is highly needed to preserve the prosperity of the Portuguese oasis of the Ria Formosa lagoon and the sustainability of natural populations worldwide. It is therefore critical to: 1) determine the disturbing effects that are now threatening the Ria Formosa population; 2) assess the actual state of seahorse populations around Europe; 3) greatly deepen the biological understanding of this species and establish steady knowledge foundations to support conservation actions; 4) foresee the future response of wild populations to expected environmental trends in fishing activities, habitat quality and global warming; 5) and establish successful culture protocols that may be a viable option to exploitation or a last-resort recovery tool for population supplementation programs.

The search for a better understanding

The extraordinary life history of seahorses offers an unusual opportunity to explore our understanding of a great diversity of biological aspects. The present investigation aimed to contribute to a better understanding of *H. guttulatus* biology, to establish a suitable home for the long-snouted seahorse in captivity and to evaluate the potential of captive breeding as a commercial project and a recovery tool for seahorse conservation.

Captive breeding of *H. guttulatus* was studied and optimized at different levels, using different approaches:

− Husbandry and reproduction of wild seahorses in captivity were optimized for a great variety of captive conditions based on social and reproductive behaviors (Chapter 1);

− Reproductive potential of male and female seahorses was evaluated to determine how parental size affects reproduction (Chapters 2 and 3);

− Fatty acid composition of eggs and lipid dynamics during early development were analyzed to estimate the nutritional requirements of both adult seahorses and newborn juveniles (Chapter 4);

− Prey-predator dynamics and the nutritional value of enriched and unenriched *Artemia* nauplii were examined to optimize first feeding and juvenile culture (Chapter 5);

− The disadvantages of captive breeding for the reproductive potential of the species were analyzed based on the number, size, condition and fatty acid composition of wild and captive juveniles (Chapter 6);

− Salinity tolerance and survival capacity of wild and captive juveniles were compared to evaluate the potential of seahorse culture to commercial production and supplementation of wild populations (Chapter 7).

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Seahorse behavior and aquaculture:

how to improve *Hippocampus guttulatus* husbandry and reproduction?

Seahorse behaviour and aquaculture: How to improve *Hippocampus guttulatus* husbandry and reproduction?

Filipa Faleiro [a,*], Luís Narciso [a], Luís Vicente [b]

[a] *Laboratório Marítimo da Guia, Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal*
[b] *Centro de Biologia Ambiental, Departamento de Biologia Animal, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal*

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ABSTRACT

In recent years, aquaculture development has been based on the use of new tools and technologies from different scientific areas. Animal behaviour can also constitute a useful tool for aquaculture and contribute to the improvement of rearing protocols. In this study, a behavioural approach was developed for the long-snouted seahorse (*Hippocampus guttulatus*) culture, in order to improve broodstock husbandry and reproduction. The optimisation of laboratory conditions (such as water column depth, holdfast type and availability, animal density, sex ratio and male/female size) was analysed based on seahorse behaviour. Social and reproductive behaviours were studied in terms of space use preferences, activity levels, distribution patterns, mate competition and selection, courtship, male pregnancy and juvenile birth. In captive conditions, seahorses were diurnally active and more isolated during the night. They were mainly located in the water inlet area, near the bottom, and preferred vertical holdfasts and natural plants. *H. guttulatus* reproduction in captivity was achieved. Seahorse behaviour and reproductive output were affected by the different rearing conditions but not animal welfare. Animal density increased seahorse activity and influenced the number of groups, while holdfast availability affected the group size. Seahorses were generally found in male–female pairs that were not stable over time and tend to select similar size or larger mates. Male competition was rather frequent and was affected by the sex ratio. Courtship occurred during all day and no daily greetings were observed throughout male pregnancy. Water column depth and male competition resulted in mating interruption and juvenile number and size decreased in artificial conditions. Animal behaviour demonstrated to be important for the improvement of seahorse husbandry and reproduction and to constitute a potential tool for aquaculture development.

1. Introduction

The importance of aquaculture has increased in recent decades, not only for the commercial production of aquatic species but also as a tool for species conservation. In order to assure global demands and to improve rearing protocols, aquaculture is based on a multidisciplinary approach that involves the use of new tools and technologies from different scientific areas, such as ecology, physiology, genetics, biochemistry, mathematics, engineering and computer science.

Behavioural studies can also constitute a useful tool for aquaculture and contribute to the improvement of rearing protocols, by allowing the evaluation of animal response to culture conditions and animal welfare assessment. Behavioural alterations can be indicative of environmental disturbances and animal distress (Schreck, 1990; Scherer, 1992; Mench and Mason, 1997) and this can be particularly useful in aquaculture facilities where the intensification of aquaculture procedures increase production but can also create stress conditions that affect animal survival, growth and welfare. Beha-

vioural indicators can be related with different aspects of animal life, such as locomotor activity, social relations, reproduction and feeding (Weinstock, 1997; Braastad, 1998; Lesage et al., 2004), and may include suppression of normal activities, stereotyped and apathetic behaviours, higher aggression frequency, behaviours resulting in injuries to the individual or its conspecifics, inadequate responses to stimuli or responses to inadequate stimuli (Braastad et al., 2006).

Moreover, behavioural experiments in aquaculture can be also very important from a forecast perspective, giving in advance valuable information for the determination of adequate rearing conditions and prevention of welfare related problems. In this context, the establishment of choice experiments can provide excellent cues about the preferences and needs of the animals, based on the assumption that animals tend to select optimal conditions, avoiding situations that affect their survival or reproduction.

Despite all the constraints associated with behavioural studies in artificial conditions, these studies can present several advantages in the evaluation of aquaculture protocols. Firstly, behaviour frequently responds immediately to environmental changes due to the rapid nervous system reaction (Eriksen et al., 2006). Therefore, behavioural shifts are often the first line of defence to stress environments, giving an

early warning about sub-optimal or unfavourable conditions (Oppedal et al., 2006). On the other hand, behavioural observation is non-invasive (Poli et al., 2005) and allows real-time monitoring of animal response to environmental variables (Chandroo, 2000). Moreover, behavioural indicators may show animal reactions to a stressor even when physiological indicators are hard to detect (Schreck, 1990).

Although several studies evidenced the importance of behavioural measures when assessing fish welfare, these methods are not widely used by the aquaculture industry (Chandroo, 2000). In seahorse culture, which has increased in the last decade due to the global decline of natural populations, behavioural research can be particularly advantageous since seahorse husbandry and reproduction in captivity are constrained by their distinctive life history characteristics. These sedentary fishes have a cryptic behaviour and are commonly associated with structurally complex habitats (Flynn and Ritz, 1999). In nature, seahorse distribution is characterised by low densities and by a patchy pattern (Foster and Vincent, 2004), generally associated with the occurrence of male–female pairs (Moreau and Vincent, 2004; Martin-Smith and Vincent, 2005). Seahorses have a long and elaborate courtship that culminates with the pair rising in the water column and males exhibiting specialized parental care, incubating the eggs within a brood pouch (Vincent and Sadler, 1995; Masonjones and Lewis, 1996). Moreover, most species establish monogamous pair bonds that are reinforced through pregnancy by daily greetings (Vincent, 1995a).

Very few studies have been published concerning the long-snouted seahorse (e.g. Curtis and Vincent, 2005; Curtis, 2006; Curtis and Vincent, 2006; Curtis, 2007; Gurkan and Taşkavak, 2007) and none focused seahorse culture. In this study, a behavioural approach was developed for *Hippocampus guttulatus* culture, in order to improve broodstock husbandry and reproduction. Laboratory conditions were optimised based on seahorse social and reproductive behaviours. The specific aims of this study were to: a) characterise daily rhythm, b) determine water flow and holdfast preferences, c) analyse the effect of animal density, sex ratio and holdfast availability on seahorse activity, group structure and fish welfare, d) evaluate sex ratio effect on mate competition, e) characterise mate selection in terms of animal size, f) study courtship behaviour and time occurrence, g) evaluate water depth effect on mating occurrence, h) analyse male and female interactions during male pregnancy, i) determine juvenile birth period, j) compare brood and juvenile size from wild and captive matings and evaluate artificial conditions effect on reproductive output.

2. Materials and methods

2.1. Seahorse collection and general design

H. guttulatus were collected by scuba diving (under Nature Conservation Institute Licence) in Ria Formosa lagoon (36°59'N, 7°51'W), south Portugal, between June and October 2005.

Seahorses were maintained in a recirculated system consisting in three breeding aquaria (180 l, 50×40×90 cm) and four stock aquaria (170 l, 140×35×35 cm). Filtration system consisted in a protein skimmer, biological filtration with bioballs and ultraviolet lamps (11 W). Opaque structures were placed between aquaria to avoid interactions among animals in neighbouring tanks. Artificial plants (similar to *Caulerpa*) were provided for seahorse attachment.

During the experimental period, water parameters were: temperature 24–25 °C, salinity 34–35, pH 8.2–8.3 and constant aeration. Light was provided by two 36 W daylight lamps and photoperiod was set on 14 h light/10 h dark cycles without twilight periods. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg l^{-1} respectively).

Adults were fed *ad libitum* twice a day on live *Artemia* (during the acclimatization week) and on frozen enriched *Artemia* and *Mysis*

(during the experimental period). In order to avoid feeding effects on seahorse behaviour during the experiments, feeding times changed every day according to the specific observation schedule of each day. Aquaria were cleaned every day and 10% water changes were made weekly.

All seahorses were tagged with colour bead necklaces loosely hold around the neck with an elastic cotton string. All individuals were wet weighted and measured through scaled digital photograph. Standard length (SL) was determined as the sum of head (HL), trunk (TrL) and tail (TaL) lengths, according to Lourie (2003). Seahorse size ranged from 9.75 to 23.68 cm SL ($X \pm SE = 17.60 \pm 2.30$ cm).

During the first week, tagging efficiency was monitored based on behavioural observations and no experiments were developed until animals were acclimatized to artificial conditions. A preliminary study was performed in order to establish the ethogram of seahorse behaviour presented in Table 1. During the experimental period, the trials concerning seahorse husbandry were performed in the stock aquaria, while the reproductive experiments were carried out in the breeding aquaria. However, reproductive behaviours exhibited by the seahorses in the stock aquaria were also analysed. In all husbandry and reproductive experiments, results were obtained through direct visual observations.

At the end of the study, necklaces were removed and all seahorses were released on their original habitat without injuries.

2.2. Seahorse husbandry

2.2.1. Daily rhythm

Daily rhythm was studied in two aquaria with 12 holdfasts (artificial plants) and 12 seahorses (six males and six females) each.

Photoperiod was synchronised with the sunrise and sunset rhythms in nature: lights were turned on at sunrise and turned off at sunset. Nine 1-hour periods were established along the day: before (0600–0700 h) and after lights on (0700–0800 h), in the morning (1000–1100 h), at midday (1330–1430 h), in the afternoon (1700–1800 h), before (2000–2100 h) and after lights off (2100–2200 h) and at midnight (0130–0230 h). Behavioural observations during the night were made using red light, after verification that no effects were detected on seahorse behaviour.

In each day period, the behaviour of every seahorse was classified based on the ethogram described in Table 1 and analysed during 2 min, so that seahorse behaviour was characterised during 48 min (24 individuals) in each 1-hour period. The formation of groups was

Table 1
Ethogram of *Hippocampus guttulatus* activity patterns and holdfast attachment

Category	Subcategory	Behaviour description
Activity pattern		
Rest (R)	Stationary (St)	The seahorse does not perform any kind of movement.
	Head movement (Mh)	The seahorse moves slightly the head but maintains the body immobile.
Activity (M)	Slow body movement (Ms)	The seahorse remains in the same place and moves the body slowly without using the fins.
	Fast body movement (Mf)	The seahorse remains in the same place and moves the body rapidly due to the dorsal and/or pectoral fins movement.
	Swimming (Sw)	The seahorse swims actively.
Holdfast attachment		
Unattachment (U)		The seahorse is unattached.
Attachment (A)	Holdfast attachment (Ah)	The seahorse is attached to a holdfast.
	Seahorse attachment (As)	The seahorse is attached to another seahorse.

also analysed for the different day periods. Two or more individuals were considered in the same group if they were apart less than 7 cm and if they were facing each other.

This experiment was repeated using different individuals and, at the end, a total of 96 seahorses were analysed.

2.2.2. Water flow and holdfast preferences

In order to study seahorse distribution along a water flow gradient, three identical areas (in size and holdfast availability) were defined in the aquaria: water inlet (bottom inlet in one edge of the aquarium), central and water outlet (superficial outlet in the opposite edge of the aquarium) areas. Seahorse distribution in each aquarium (six males and six females) was analysed three times a day during 5 days. The proportion of animals present in each area was determined based on a total of 180 seahorses.

Holdfast preferences were analysed independently in terms of substrate type, position and depth:

- Substrate type experiment: six sets of different holdfasts (each set composed by a natural plant of the genus *Caulerpa*, an artificial plant and a green wire structure) with six seahorses in each aquarium;
- Substrate position experiment: 12 green wire structures (with vertical and horizontal branches) with 12 seahorses in each aquarium;
- Substrate depth experiment: 12 holdfasts (artificial plants and green wire structures), extended from the bottom till the water surface and subdivided in three areas (near the bottom, in the water column and near the surface), with 12 seahorses in each aquarium.

In each experiment, the holdfast preference of each seahorse was checked every 30 min during 2 days (8 h a day). These 2-day experiments were repeated using different individuals and, at the end, a total of 48 seahorses (24 males and 24 females) were analysed in each experiment.

2.2.3. Social behaviour

The optimisation of animal density, sex ratio and holdfast (artificial plants) availability was studied based on seahorse social behaviour under different experimental conditions:

- 3M3F-EH trial: three males and three females (12 ind m^{-2}) with exceeding holdfast (six holdfasts with two vertical branches each);
- 6M6F-EH trial: six males and six females (24 ind m^{-2}) with exceeding holdfast (12 holdfasts with two vertical branches each);
- 6M6F-LH trial: six males and six females (24 ind m^{-2}) with limited holdfast (six holdfasts with one vertical branch each);
- 3M9F-EH trial: three males and nine females (24 ind m^{-2}) with exceeding holdfast (12 holdfasts with two vertical branches each);
- 9M3F-EH trial: nine males and three females (24 ind m^{-2}) with exceeding holdfast (12 holdfasts with two vertical branches each).

In each trial, the behaviour of a minimum of six seahorses (three males and three females) was observed during 2 min and classified based on the ethogram described in Table 1. The formation of groups was analysed based on the criteria defined in the daily rhythm study and seahorses present in each group were identified. Both seahorse activity and distribution patterns were analysed for each aquarium three times a day (1230, 1400 and 1530 h) during 5 days. Each 5-day experiment was repeated three times using different individuals.

As a complementary analysis, different welfare indicators were determined for the 3M3F-EH, 6M6F-EH and 6M6F-LH experiments: operculum beating (number of operculum beatings per minute), food uptake (number of *Artemia* ingested during the first 10 min), food waste (wet weight of *Artemia* not ingested), weight variation (difference between seahorse initial and final weights). All welfare indicators (except weight variation) were analysed daily for each 5-day replicate.

2.3. Seahorse reproduction

2.3.1. Mate competition and selection

Sex ratio effect on mate competition was analysed in aquaria with four seahorses and different sex ratios (1:1, 1:3 and 3:1). Males and females of different size (small: 16.65±1.37 cm SL; median: 18.62± 0.68 cm SL; large: 20.75±1.23 cm SL) were selected for each aquarium, in order to study mate selection criteria:

- 1:1 sex ratio trial: a small and a large male with a small and a large female in each aquarium;
- 1:3 sex ratio trial: a median male with a small, a median and a large female in each aquarium;
- 3:1 sex ratio trial: a small, a median and a large male with a median female in each aquarium.

Behaviour observations were made daily in the morning (0800–1200 h) and afternoon (1600–2000 h), during a 4-week period. Seahorses involved in each courtship, behaviour sequences and the individual that started each courtship were described whenever observed.

Three replicates using different individuals were obtained for each sex ratio.

2.3.2. Seahorse courtship and mating

Seahorse courtship and mating were analysed in aquaria with different water column depths: 90 cm in the breeding aquaria and 32 cm in the stock aquaria.

Behaviour observations were made daily in the morning (0800–1200 h) and afternoon (1600–2000 h), during a 4-week period. Courtship behaviours were described and seahorse mating was registered whenever occurred.

2.3.3. Male pregnancy and juvenile birth

Two holdfast sets (each one composed by four vertical branches of artificial plants) were placed respectively in the right and left sides of the breeding aquaria. Seahorse distribution was recorded every hour during the first light hours (0700–1200 h), in the courtship day and during male pregnancy. During pregnancy, daily greetings between the male and the female were registered whenever occurred.

Pregnancy duration, birth period (in the morning, afternoon or night) and brood size were determined for each pregnant male (both for males captured pregnant in the wild and males that mated in captivity). Juvenile birth was described and the occurrence of parental care after birth was analysed.

2.4. Statistical analysis

2.4.1. Seahorse husbandry

To study daily rhythm, the proportion of the different behaviour categories (activity pattern and holdfast attachment) and the group characteristics (number and size of groups) were compared between sex (only for the day periods) and daytime, using the Mann–Whitney, Kruskal–Wallis and Dunn tests (Zar, 1996).

Water flow and holdfast preferences were evaluated using the Chi-square test.

Seahorse distribution pattern in the aquaria was determined through the Index of Dispersion Test (Krebs, 1989).

Group composition and stability were analysed using the Chi-square test only for pairs, since most groups (71.1%) were composed by two seahorses. The gender composition was analysed based on the proportion of male–male, female–female and male–female pairs, while pair stability was analysed based on the frequency of two individuals observed together or separated. Since some individuals were observed together more often than it was expected for a random distribution, the location (in the water inlet, central and water outlet areas) of seahorses that were together in at least 40% of the groups

was analysed in order to evaluate if pair occurrence was related with seahorse distribution in a preferential area of the aquarium.

The influence of seahorse density, sex ratio and holdfast availability in the species social behaviour was analysed by comparing the proportion of the different behaviour categories (activity pattern and holdfast attachment), the group characteristics (number, size and gender composition) and the welfare indicators (operculum beating, food uptake, food waste and weight variation) for the different experimental conditions. All comparisons were made using the Mann–Whitney, Kruskal–Wallis and Dunn tests (Zar, 1996).

Since seahorses in limited holdfast conditions increased group size even with some holdfasts available, the location of groups in a preferential area of the aquarium (water inlet, central and water outlet areas) was analysed using the Chi-square test.

2.4.2. Seahorse reproduction

The influence of sex ratio on mate competition was studied using the Chi-square test, based on the proportion of male initiations and group courtships (courtships between one male and several females or between one female and several males).

Due to the small number of matings observed, mate selection was analysed in the pairs that mated but also in the pairs that were only involved in the courtship. The correlation between male and female lengths and weights was evaluated through the Spearman rank correlation (Zar, 1996). Additionally, the Chi-square test was used to determine if the seahorses selected a similar size mate when in the presence of individuals of different sizes.

Courtship occurrence was compared between morning and afternoon periods. Broodstock distribution in the courtship day and during male pregnancy was analysed based on the number of times that the male and the female were located together in the same side of the aquarium. Juvenile birth was analysed in the morning, afternoon and night periods. All analysis were done using the Chi-square test. Brood size was compared for matings that occurred in nature and in captive conditions, using the t test.

All statistical analysis was performed for a significance level of 0.05, using Statistica 6.0 software (StatSoft Inc. Data).

3. Results

3.1. Seahorse husbandry

3.1.1. Daily rhythm

H. guttulatus were diurnally active, spending more time immobile and attached to the holdfast during the night (Dunn test: $Q \geq 3.50$, $P<0.05$; Fig. 1). No significant differences were found in seahorse behaviour during the day (Dunn test: $Q \leq 3.09$, $P>0.05$). Males were more active than females after lights on (Mann–Whitney U test:
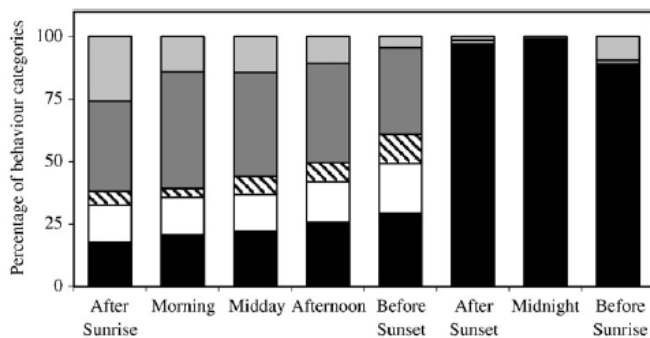

Fig. 1. Changes in activity patterns during the day. Percentage of time spending in rest (■ stationary, ☐ head movement) and in activity (◩ slow body movement, ▨ fast body movement, ▥ swimming).

Table 2
Comparison of seahorse isolation and group characteristics between day and night periods ($X \pm SE$)

Parameter	Day	Night
Isolated seahorses (%)	51.67±13.90	69.44±15.71
Isolated males (%)	40.19±22.17	74.51±17.96
Isolated females (%)	63.15±18.47	66.67±24.75
Number of groups	2.18±0.85	1.56±0.90
Group size	2.40±0.80	2.18±0.47

$U=229.50$, $N_1=N_2=32$, $P<0.05$) and less in the afternoon (Mann–Whitney U test: $U=357.00$, $N_1=N_2=32$, $P<0.05$) and before lights off (Mann–Whitney U test: $U=358.00$, $N_1=N_2=32$, $P<0.05$).

In terms of group characteristics (Table 2), the proportion of isolated seahorses was higher during the night (Mann–Whitney U test: $U=171.50$, $N_1=45$, $N_2=18$, $P<0.05$) due to a higher number of isolated males (Mann–Whitney U test: $U=109.50$, $N_1=45$, $N_2=17$, $P<0.05$). The number of groups was lower during this period (Mann–Whitney U test: $U=263.00$, $N_1=45$, $N_2=18$, $P<0.05$) but no significant differences were found in group size (Mann–Whitney U test: $U=1177.50$, $N_1=99$, $N_2=27$, $P=0.34$).

3.1.2. Water flow and holdfast preferences

Seahorse distribution in the aquaria was influenced by the water flow and was concentrated in the water inlet area (Chi-square test: $\chi^2_2=257.72$, $P<0.05$). Animal occurrence in the water inlet, central and water outlet areas were 50.3%, 26.7% and 23.0% respectively. Comparing both sexes, males selected the water inlet area more frequently than females (Mann–Whitney U test: $U=291837.00$, $N_1=N_2=810$, $P<0.05$).

In terms of holdfast preferences (Table 3), seahorses preferred vertical holdfasts (Chi-square test: $\chi^2_1=12.09$, $P<0.05$) and natural plants (Chi-square test: $\chi^2_2=113.33$, $P<0.05$). They were generally located near the bottom (Chi-square test: $\chi^2_2=2668.04$, $P<0.05$) and males were near the bottom more often than females (Mann–Whitney U test: $U=523981.50$, $N_1=N_2=1076$, $P<0.05$).

3.1.3. Social behaviour

The influence of density, sex ratio and holdfast availability on seahorse activity is represented in Fig. 2. Lower activity levels (higher St % and lower Mf % and Sw %) were observed in lower density conditions (Mann–Whitney U test: $U \leq 3702.00$, $N_1=120$, $N_2=80$, $P<0.05$), with the animals spending more time attached to the holdfast (Mann–Whitney U test: $U=3935.00$, $N_1=120$, $N_2=80$, $P<0.05$). However, no differences were found in time spent attached to other individual between lower and higher density conditions (Mann–Whitney U test: $U=4699.50$, $N_1=120$, $N_2=80$, $P>0.05$). A lower activity (higher St % and lower Mf %) was obtained for a 3:1 sex ratio (Dunn test: $Q \geq 2.48$, $P<0.05$), without significant differences in holdfast attachment periods (Kruskal–Wallis test: $H_2=2.57$, $P=0.28$). Holdfast availability did not affect seahorse activity (Mann–Whitney U test: $U \geq 6303.50$, $N_1=N_2=120$, $P \geq 0.10$) and no agonistic behaviours were observed with limited substrate. In these conditions, seahorses were

Table 3
Seahorse preferences in terms of holdfast type, position and depth ($X \pm SE$)

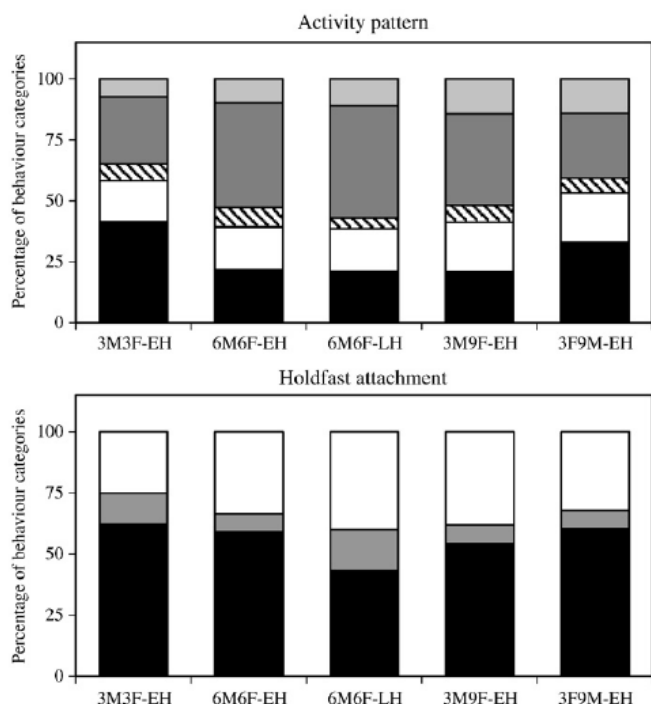| Parameter	Holdfast description	Percentage
Holdfast type	Natural plants	54.29±20.54
	Artificial plants	26.12±16.66
	Green wire structures	19.59±17.56
Holdfast position	Vertical branches	65.08±24.28
	Horizontal branches	34.92±24.28
Holdfast depth	Near the bottom	98.21±4.17
	In the water column	1.30±3.85
	Near the surface	0.49±1.96

Fig. 2. Activity patterns and holdfast attachment in different husbandry conditions: three males and three females with exceeding holdfast (3M3F-EH), six males and six females with exceeding holdfast (6M6F-EH), six males and six females with limited holdfast (6M6F-LH), three males and nine females with exceeding holdfast (3M9F-EH) and nine males and three females with exceeding holdfast (9M3F-EH). Activity patterns – percentage of time spending in rest (■ stationary, □ head movement) and in activity (◪ slow body movement, ▨ fast body movement, ▨ swimming). Holdfast attachment – percentage of time spending attached (■ to a holdfast, ▨ to a seahorse) and unattached (□).

attached to another individual more frequently (Mann–Whitney U test: $U=6019.00$, $N_1=N_2=120$, $P<0.05$), even with some available holdfasts.

Although isolated seahorses were observed in $51.9\pm20.2\%$ ($X\pm SE$) of the time, seahorse distribution in captivity was characterised by an aggregated pattern (Chi-square test: $\chi^2_5=654.73$, $P<0.05$) represented by the formation of pairs (71.1% of the groups). Index of Dispersion, seahorse isolation and group characteristics in the different experiments are represented in Table 4. Seahorse aggregation was higher in limited substrate conditions (characterised by a higher Dispersion Index). Animal density influenced the number of groups (higher for higher density conditions; Mann–Whitney U test: $U=240.00$, $N_1=45$, $N_2=42$, $P<0.05$), while holdfast availability affected the group size (higher for limited substrate conditions; Mann–Whitney U test: $U=3940.50$, $N_1=N_2=98$, $P<0.05$). Consequently, the proportion of iso-

lated seahorses was higher in the low density experiment (Mann–Whitney U test: $U=714.00$, $N_1=N_2=45$, $P<0.05$) and lower in the limited holdfast experiment (Mann–Whitney U test: $U=650.00$, $N_1=45$, $N_2=44$, $P<0.05$). In these conditions, the location of groups was concentrated in the water inlet area (Chi-square test: $\chi^2_2=8.17$, $P<0.05$), even with some available holdfasts in non preferential areas.

In terms of group composition, despite a higher number of male–male pairs observed for a 3:1 sex ratio (Dunn test: $Q=3.77$, $P<0.05$) and female–female pairs for a 1:3 ratio (Dunn test: $Q=4.41$, $P<0.05$), the proportion of male–male, female–female and male–female pairs was the expected for a random distribution (Chi-square test: $\chi^2_2\leq2.72$, $P<0.05$). Although pairs were not stable over time (with the same pair being observed only in $X\pm SE=15.9\pm20.2\%$ of the cases), the Chi-square test demonstrated that pair formation was not totally random, with some pairs observed more often than it was expected (Chi-square test: $\chi^2_2=4.06$, $P<0.05$). However, 86.7% of these pairs were not randomly distributed in the aquaria but located preferentially in the water inlet area (Chi-square test: $\chi^2_2>5.99$, $P<0.05$).

The welfare indicators were not statistically different among the different experimental conditions (Kruskal–Wallis test: operculum beating: $H_2=2.25$, $P=0.32$; food uptake: $H_2=2.90$, $P=0.40$; food waste: $H_2=0.73$, $P=0.69$; weight variation: $H_2=0.45$, $P=0.80$).

3.2. Seahorse reproduction

3.2.1. Mate competition and selection

Seahorse courtship was initiated by the male that approached the female and persisted until she responds. Sex ratio did not influence the gender that initiates the courtship, with the male approaching the female even for a 1:3 proportion.

Mate competition occurred in 29.0% of the courtships, when a seahorse was courted simultaneous by several animals of the opposite sex. In some cases, courtship was responded to only one individual but sometimes it was responded to all seahorses (for one or all together). Male competition was more frequent than female competition (Chi-square test: $\chi^2_1=8.29$, $P<0.05$) and potentially aggressive behaviours were only observed among males ($N=3$) that snapped with the snout toward another male. Courtship between individuals of the same sex was observed for males ($N=2$) and females ($N=2$) respectively during male and female competition. Mate competition was affected by the sex ratio: male competition was only observed in aquaria with equal or higher number of males (Chi-square test: $\chi^2_1=7.50$, $P<0.05$) while the opposite was observed for female competition (Chi-square test: $\chi^2_1=8.00$, $P<0.05$).

In what concerns mate selection, male and female weights were correlated (Spearman rank correlation: $r_s=0.54$, $N=40$, $P<0.05$). When in the presence of different size animals, seahorses selected similar size or larger mates. However, these results were only statistically confirmed for the sex ratio 1:3 (Chi-square test: $\chi^2_2=6.00$, $P<0.05$), probably due to an insufficient number of courtships obtained in these experiments.

Table 4
Index of dispersion, seahorse isolation ($X\pm SE$) and group characteristics ($X\pm SE$) for different husbandry conditions: three males and three females with exceeding holdfast (3M3F-EH), six males and six females with exceeding holdfast (6M6F-EH), six males and six females with limited holdfast (6M6F-LH), three males and nine females with exceeding holdfast (3M9F-EH) and nine males and three females with exceeding holdfast (9M3F-EH)

Parameter	3M3F-EH	6M6F-EH	6M6F-LH	3M9F-EH	9M3F-EH
Index of dispersion	1.381	1.315	1.971	1.229	1.376
Isolated seahorses (%)	64.44±26.61	51.67±13.90	42.23±14.85	48.33±17.27	52.69±18.97
Isolated males (%)	56.30±33.75	40.19±22.17	34.28±16.59	44.81±31.39	51.60±22.46
Isolated females (%)	72.59±27.48	63.15±18.47	50.19±24.97	49.51±19.87	55.93±35.51
Number of groups	0.86±0.64	2.18±0.85	2.23±0.90	2.47±0.93	2.11±1.06
Group size	2.24±0.43	2.40±0.80	2.74±1.08	2.27±0.64	2.43±0.69
MF pairs (%)	61.54±49.61	52.99±43.07	49.14±40.97	50.62±38.77	37.99±38.17
MM pairs (%)	32.69±46.78	24.79±35.38	23.85±32.56	3.10±15.96	58.09±38.35
FF pairs (%)	5.77±21.57	22.22±36.89	27.01±37.11	46.28±38.59	3.92±17.91

3.2.2. Seahorse courtship and mating

Seahorse courtship occurred during all day (48.9% in the morning and 51.1% in the afternoon) with one courtship happening just before the dark period.

H. guttulatus courtship in captivity started with the male approaching the female, brightening and exhibiting an inflated brood pouch. The female was generally attached to the holdfast, exhibiting a prominent abdomen, sometimes with the genital papilla opened and showing the eggs. The male quivered his body toward the female and repeatedly flexed the tail and curved the body, opening the brood pouch. The female started swimming, bending her body forward and backward, and the male followed her. When the female responded to the courtship, the pair rose up together in the water column, swimming in circles in side by side positions. During the successive rises up and down, the male frequently bent the body, flexing the tail and opening the brood pouch. In a latter phase, the pair rose up facing each other, with the female abdomen near the male brood pouch. Finally, in the copulatory rise, the female placed her genital papilla inside the male brood pouch opening and transferred the eggs. After that, male and female became separated.

Six matings occurred during the experimental period: five in the breeding aquaria (90 cm height) and one in the stock aquarium (32 cm height). From the five matings in the breeding aquaria, two occurred for a 1:1 sex ratio, two for 3:1 and one for 1:3.

3.2.3. Male pregnancy and juvenile birth

No daily greetings were observed throughout male pregnancy. In contrast with the courtship day (when the male and the female were observed together in 91.7% of the time, more than the expected for a random distribution; Chi-square test: $\chi^2_1 = 136.98$, $P<0.05$), during pregnancy the male and the female were randomly distributed in the aquaria (Chi-square test: $\chi^2_1 = 2.32$, $P>0.05$) and were only observed together in 28.3% of the time.

Male pregnancy lasted 15 days. Juvenile birth occurred mainly in the night (59.3%) and afternoon (37.0%), with only 3.7% of the births occurring in the early morning (Chi-square test: $\chi^2_2 = 49.23$, $P<0.05$). In 30.6% of the cases, juvenile birth was discontinuous and occasionally occurred in a 3-day period ($N=5$).

During birth, the male frequently bent the body and flexed the tail, forcing the juveniles to leave the brood pouch. These body contractions continued for 1 or 2 h and persisted even after juvenile birth. Immediately after leaving the brood pouch, the juveniles swam directly to the water surface. No parental care was observed after juvenile birth and some males were ready to remate in the day after.

Wild pregnant males produced an average offspring of 426 juveniles (151–696 juveniles) with a total length of 15.53 ± 1.28 mm ($X \pm SE$) and an average weight of 3.69 ± 0.94 mg ($X \pm SE$). In artificial conditions, brood size ($X \pm SE = 255 \pm 125$ juveniles), juvenile length ($X \pm SE = 14.23 \pm 1.07$ mm) and juvenile weight ($X \pm SE = 2.75 \pm 0.63$ mg) were smaller (Paired *t* test: brood size: $t_{24}=2.48$, $P<0.05$; juvenile length: $t_{767}=12.42$, $P<0.05$; juvenile weight: $t_{769}=12.61$, $P<0.05$). However, two smaller broods born in captivity resulted from a mating interrupted by another male and from a mating that occurred in the stock aquarium.

4. Discussion

Seahorse culture present several problems, the first ones related with seahorse husbandry and reproduction. However, as a result of research progress and information availability, seahorse husbandry and reproduction in captivity have been achieved for several species: *Hippocampus abdominalis* (Woods, 2000), *H. capensis* (Lockyear et al., 1997), *H. fuscus* (Vincent, 1995a), *H. reidi* (Silveira, 2000), *H. subelongatus* (Payne and Rippingale, 2000) and *H. zosterae* (Masonjones and Lewis, 1996). This study evidenced that *H. guttulatus* husbandry and reproduction are possible to obtain in captivity. Moreover,

behavioural experiments proved to be important for the improvement of seahorse culture, since helped to determine seahorse preferential conditions and to understand how the species reproductive output can be limited by several rearing conditions.

4.1. Seahorse husbandry

Considering the diversity of cultured species and their specific characteristics, the development of culture protocols should be based on a deep biological knowledge of the target species, including their behavioural patterns. The characterisation of seahorse behaviour in terms of daily rhythm is an important aspect to consider, since light acts as a strong stimulus for physiological and behavioural processes. As most seahorse species (Foster and Vincent 2004), *H. guttulatus* was diurnally active. A higher activity during the day is expected since seahorses are visual predators that depend on light to feed, avoiding predators through camouflage. Differing from previous studies that refer a higher activity in the early morning (Vincent and Sadler, 1995; Masonjones and Lewis, 1996), no differences were found in seahorse activity during the day, with contrasting patterns observed only between day and night periods. Indeed, although the short duration of the twilight periods, it has been suggested that twilight may play a role in fish behaviour and physiology out of proportion to the actual time involved (Helfman, 1986).

In addition to the characterisation of species behaviour, animal preferences are also an important aspect to consider in aquaculture, assuming that animal choices are associated with optimal conditions. Strong water flow conditions and vertical holdfasts, particularly natural plants, were preferential conditions for *H. guttulatus* culture. Conversely, holdfast height was not a limiting factor in seahorse husbandry, since animals were generally positioned near the bottom. Nevertheless, although animal preferences are important, the establishment of a rearing protocol should result from a compromise between animal preference and its feasibility. In this context, artificial plants (which are more practical to keep in artificial conditions) seem to constitute a good alternative to natural plants.

Another important behavioural approach in aquaculture consists in evaluate how artificial conditions influence animal behaviour. A high number of factors throughout the culture period may affect fish behaviour and welfare, including the physicochemical environment (salinity, temperature, water quality and space confinement), biological conditions (animal density, diet, pathogens, parasites and predators) and cleaning and handling procedures. In this study, rearing conditions affected seahorse behaviour in several aspects, such as activity patterns, spatial distribution and group structure.

In what concerns activity patterns, animal density is an important issue to consider in captive conditions, since high stocking densities may affect animal activity and encourage agonistic behaviours that had led to survival and welfare related problems in different fish species (e.g. Feldlite and Milstein, 2000; Anras and Lagardére, 2004; Molnár et al., 2004). Although wild seahorses are generally found in low densities (0.006–0.51 ind m^{-2}) (Foster and Vincent, 2004), the establishment of higher stocking densities in captivity may not limit seahorse survival and welfare due to the general absence of aggressive behaviours in these fishes. Indeed, higher seahorse densities in this study resulted in a greater activity but animal welfare was not affected. In fact, low densities are a recent characteristic of wild populations and a consequence of overexploitation and habitat degradation. Perante et al. (2002) reported densities of 20 ind m^{-2} in coral reefs and 10 ind m^{-2} in *Sargasso* banks in the 60's. Even today, higher densities can be found in some natural microhabitats: 10–15 ind m^{-2} in some seagrass habitats (Vincent, 1996) and 10 ind m^{-2} in *H. guttulatus* population of the Ria Formosa lagoon (Foster and Vincent, 2004).

Another important factor to consider in seahorse culture is holdfast availability, not only because seahorses can become disturbed in limited holdfast conditions (Vincent, 1995b), but also because dense vegetation can difficult feeding and limit courtship behaviours (Giwojna and

Giwojna, 1999). In this study, seahorse activity was not affected by holdfast availability, as it was observed in previous studies. James and Heck (1994) observed that *Hippocampus erectus* spent more time swimming when in the absence of artificial plants. On the other hand, Curtis and Vincent (2005) studied the two seahorse species of the Ria Formosa lagoon and verified that the more active species (*H. hippocampus*) was present in areas characterised by less dense vegetation, while *H. guttulatus* was less active and associated with higher substrate complexity. Moreover, no agonistic behaviours were observed in limited holdfast conditions, neither in the aquaria with higher seahorse density. In fact, the absence of aggressive reactions may be common to all seahorse species. According to Vincent et al. (2005), their planktonic feeding, the establishment of monogamous pair bonds and the embryonic development within the brood pouch reduce the need to protect food, mates, spawning sites or offspring.

Besides seahorse activity, rearing conditions also affected spatial distribution and group structure. In captivity, seahorses showed an aggregated pattern characterised by the formation of groups, usually male–female pairs. These results are in agreement with the patchy distribution observed in natural conditions (Moreau and Vincent, 2004; Martin-Smith and Vincent, 2005), although the differences in spatial scale of wild and captive studies. According to Foster and Vincent (2004), nine of the 12 studies concerning social structure refer that seahorse social groups consist in male–female pairs and that only three species (*H. abdominalis*, *H. breviceps* and *H. guttulatus*) form groups with more than two individuals. Distribution in pairs and the establishment of monogamous pair bonds can be advantageous for species characterised by low density populations, reduced mobility and the use of camouflage to avoid predators, since remove all the costs associated with the search of a new mate and facilitate pair synchronisation (Kvarnemo et al., 2000). Nevertheless, the pairs formed in this study were not stable over time and each seahorse was associated with different individuals. Although some pairs were observed more often than it was expected, this fact may not suggest preferences in pair formation. Firstly, some individuals had a tendency for isolation, what implies that the probability of association with an individual is not identical for all seahorses, as it was assumed in the statistical analysis. Moreover, the higher frequency of some pairs was related with seahorse distribution in a preferential area of the aquarium (the water inlet area) and therefore probably not associated with mate selection.

In artificial conditions, group structure was influenced by seahorse density, sex ratio and holdfast availability. Density affected the number of groups but not the group size, what reinforces the idea that pairs should be the structural basis of social organisation in this species. On the other hand, holdfast availability affected the group size, which increased in limited substrate conditions. However, it is important to refer that some seahorses preferred to increase group size (even with some available holdfasts) instead of maximize substrate occupation. This fact may be explained by the preferential distribution of seahorses in the water inlet area.

4.2. Seahorse reproduction

Animal reproduction depends on a variety of internal factors, such as health and social status, and external conditions, including sex ratio and seasonal cues (Sargent and Gross, 1993). Therefore, several factors must be considered in order to establish an adequate aquaculture environment for broodstock maturation and reproduction. This study demonstrates that *H. guttulatus* reproduction in captivity is possible when adequate rearing conditions are established and that a deep knowledge on different aspects of seahorse reproductive behaviour (such as mate competition and selection, courtship, pregnancy and juvenile birth) is important to optimise seahorse reproduction.

Mate competition in artificial conditions was characterised by males competing more intensely than females: courtship was initiated by the male, courtship behaviours between one female and several

males were rather frequent and agonistic behaviours were only observed among males. Male competition was also observed in all seahorse studies developed so far (Foster and Vincent, 2004), although other syngnathid species can present sex role reversal, characterised by a more intense female competition (Vincent et al., 1992). Although conventional sex roles are not expected for species with high paternal care, seahorses present a stronger male competition due to monogamy constraints that prevent females of mating during male pregnancy and also because males could be prepared for mate faster and remain receptive for longer than females (Vincent, 1994; Masonjones and Lewis, 2000).

Sex ratio is an important factor to consider in sex role assessment, since affects directly the proportion of receptive males and females (operational sex ratio). In this study, sex ratio did not affect the sex that initiate courtship but influenced mate competition, since male competition was only observed for a sex ratio equal or higher than 1 and the opposite was verified for female competition. Seahorse competition for a 1:1 sex ratio can result from the fact that some individuals are unavailable to mate in a certain moment, biasing the operational sex ratio.

In what concerns mate selection, seahorses tend to mate with individuals of similar size (Vincent and Sadler, 1995; Jones et al., 2003; Vincent and Giles, 2003). Size-assortative mating can be advantageous and allow the maximization of pair reproductive potential, since larger females produce more and larger eggs and larger males have larger brood pouches (Vincent, 1990; Teixeira and Musick, 2001; Vincent and Giles, 2003). In this study, mate selection was based on seahorse size. However, the results obtained do not suggest size-assortative mating but evidence a tendency to mate with individuals of similar or larger size.

Similar to other seahorse species (Vincent and Sadler, 1995; Masonjones and Lewis, 1996), *H. guttulatus* courtship was lengthy and characterised by conspicuous behaviours. Although a conspicuous courtship may reduce camouflage and increase predation susceptibility, the risks should be lesser than the benefits to reproduction (e.g. selection of mates with higher fitness and pair synchronisation).

In captive conditions, seahorses performed courtship behaviours during all day and no daily greetings were observed throughout male pregnancy. This is contrary to studies developed so far that report that courtship occurs in the early morning and that daily greetings persist during male pregnancy, reinforcing pair bonds and reproductive synchronisation (Vincent, 1995a; Masonjones and Lewis, 1996). The absence of daily greetings was also reported for a wild population of *Hippocampus breviceps* by Moreau and Vincent (2004). However, it is important to consider that artificial conditions affect animal behaviour and that results obtained in these conditions are not strongly indicative of what happens in natural environments.

Juvenile birth in nature occurs at night (Vincent, 1990; Vincent and Sadler, 1995; Woods, 2000) but in captive conditions juveniles were born during all day. In nature, birth during the night can be advantageous because reduces visual predators risks but not in captive conditions, where the predation pressure is non-existent. Immediately after birth, *H. guttulatus* juveniles swam directly to the water surface, as observed for other seahorse species (Foster and Vincent, 2004). This pelagic behaviour seems associated with the air bladder inflation and may give juveniles access to zooplankton preys as well as act as a dispersal mechanism (Woods, 2000).

Juvenile number and size vary greatly between species, since 34 juveniles with 2.0 mm average length in *Hippocampus bargibanti* up to higher values in the larger species: 2000 juveniles in *H. ingens* and 16.2 mm average length in *H. abdominalis* (Foster and Vincent, 2004). Comparatively with other species, although *H. guttulatus* produced a small brood size (151–696 juveniles), newborn length was rather higher (15.53 mm average length). While some seahorse species invest in the production of a higher number of juveniles, *H. guttulatus* looses the advantageous of a large brood but benefits with the birth of larger juveniles that potentially have a higher competitive ability.

Seahorse mating in captivity resulted in a decrease of juvenile number and size. The smaller brood size was a consequence of mating interruption due to reduce aquarium depth and male competition, while the decrease in juvenile size should be related mainly with broodstock nutrition and welfare. Suboptimal conditions may limit female investment in egg production and male contribution during embryonic development and, consequently, offspring condition.

5. Conclusion

The behavioural experiments performed in this study demonstrated to be important for the improvement of seahorse culture, since rearing conditions modified seahorse behaviour and influenced the species reproductive output. Seahorses were preferably distributed in male–female pairs and tend to select similar size or larger mates. Higher densities and limited holdfast availability affected seahorse behaviour and distribution but it appears that animal welfare was not affected. Water column depth and male competition resulted in mating interruption and the consequent brood size decrease; therefore, after pair formation, their isolation seems to be advantageous in order to improve *H. guttulatus* reproductive success in captivity. However, more important than the specific results obtained in this study is the evidence that animal behaviour can constitute an important and useful tool for aquaculture improvement, which can be applied to all species, in different ways and using different approaches. Nevertheless, it is necessary to consider not only the behavioural studies benefits but also their restrictions and it is important to combine multiple biological indicators, in order to successfully improve culture protocols.

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The bigger the better:

an assessment of reproductive potential in seahorses

The bigger the better:

an assessment of reproductive potential in seahorses

Filipa Faleiro* and Luís Narciso

Universidade de Lisboa, Faculdade de Ciências, Centro de Oceanografia, Laboratório Marítimo da Guia,
Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal
* Corresponding author (e-mail: filipafaleiro@gmail.com)

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Conventionally, the mother usually plays the key role in reproduction. Seahorses offer a rare opportunity to undertake a more comprehensive assessment of reproductive success, given the important role of the male pouch in embryonic development. This study evaluates the effects of both male and female size on the reproductive output of the long-snouted seahorse, *Hippocampus guttulatus*. Relationships within and between parents and newborn seahorses were analyzed regarding male and female size, egg size, pouch volume, embryo density, juvenile number and size. Results clearly demonstrated that, with regard to parental quality, the bigger the better. Seahorses selected similar-size or larger mates. Larger females produced bigger eggs with larger yolk reserves. Larger males had larger brood pouches. Brood size and mass were similar among small and large males, but embryo density was lower inside the pouch of larger males. Juveniles brooded at higher densities had smaller size and greater size dispersion than those brooded at lower densities. Both male and female parents proved to play a decisive role in the reproductive output of *H. guttulatus*. Along with female and egg size, the capacity of the male pouch also influences the size of newborns by changing the embryo density inside the pouch.

Additional keywords: Hippocampus guttulatus, reproductive potential, egg size, embryo density, newborn juvenile.

INTRODUCTION

Conventionally, the mother usually plays the key role in reproduction. Females are the main responsible for embryo nourishment, either through the yolk reserves of the eggs or by direct supply of nutrients, and generally invest more in reproduction than males. Amongst fishes, however, the father seems to be a more devoted parent than most males in the animal kingdom. Although females still produce the larger gamete and are the main responsible for embryo nutrition, parental care in fishes is normally provided by the male. The strong paternal care in some species raises the question of whether females still remain the key determinant of reproductive success in fish or if there is a high place for males.

Seahorses exhibit the most specialized form of paternal care amongst animals. The female deposits her eggs inside the male brood pouch, and embryos fully develop within the enclosed pouch environment. The pouch epithelium is penetrated by an abundant network of blood vessels and the embryos, which become embedded within depressions (Carcupino *et al.*, 2002), develop in close association with the brood pouch epithelium in a manner that bears some resemblance to the embryo-placental relationship in mammals. The male is responsible for gaseous exchange, removal of waste products and osmoregulation during embryonic development (Linton and Soloff, 1964), playing also an important immunoprotective role (Melamed *et al.*, 2005). Seahorse males are thus expected to have a more determinant role in reproductive success than most fish parents.

Studies on parental energy investment proved that pregnancy has high energetic costs for male pipefish (e.g.,

Berglund *et al.*, 1986a; Svensson, 1988). In seahorses, the reproductive costs of male pregnancy are expected to be greater than in pipefish, given their extremely elaborated form of paternal care. However, the paternal contribution during embryonic development is not always perceptible in the relationship between male and newborn characteristics. Some studies found that the number and size of juveniles were not related to male size, and that female size was the key determinant of juvenile characteristics in some syngnathids (e.g., Berglund *et al.*, 1986b; Vincent and Giles, 2003; Woods, 2007). In contrast, other studies observed that male and juvenile characteristics were positively related in some seahorses (e.g., Boisseau, 1967; Teixeira and Musick, 2001; Woods, 2007) and pipefish (e.g., Berglund *et al.*, 1986b; Ahnesjö, 1992, Gasparini and Teixeira, 1999; Watanabe and Watanabe, 2002), even though this relationship had been frequently interpreted as a indirect consequence of larger males mating with larger females.

Seahorse embryos present an essentially lecithotrophic nutrient pattern, depending primarily upon their maternally-derived yolk reserves. Syngnathid embryos have proven to be self-sustaining from a nutritional perspective and to fully develop outside the brood pouch (Azzarello, 1991), but nutrient supplementation by the father (e.g., reviewed in Stölting and Wilson, 2007) is expected to enhance the nutritional condition of the embryos and, consequently, their growth and development. The size of young is thus expected to reflect both the initial size of eggs and the embryonic growth rate, which is mainly related to the paternal supply of essential environmental conditions and important elements during embryonic development. The role of syngnathid fathers in reproductive output becomes more obvious when conditions inside the pouch encourage embryo competition.

Higher densities of competing embryos may affect the efficacy of the embryo-pouch interactions and, consequently, juvenile size at birth, as well as their future growth and development (Ahnesjö, 1992; Watanabe and Watanabe, 2002; Dzyuba *et al.*, 2006).

Seahorses provide an unusual opportunity to study the relative roles of the two sexes in reproductive success, given their high maternal investment in gametogenesis and their extreme form of paternal care during embryonic development. Furthermore, the reproductive output is a better indicator of reproductive success for seahorses than for other fishes, since survival of juveniles seahorses is expected to be considerably higher than that of fish larvae, given the smaller number but greater size and advanced development stage of seahorses at birth in relation to other fish, including species with paternal care. Nevertheless, the reproductive biology of seahorses involves such a complex combination of male and female influences that may hamper the separation of maternal and paternal contributions. Factors like mate selection, the number and size of eggs, brood pouch volume, male condition and embryo density inside the pouch, besides other external variables, interact all in such an intricate way that makes harder to assign responsibilities in the reproductive output of seahorses.

In this study we examine the effects of both male and female size on the reproductive output of the long-snouted seahorse, *Hippocampus guttulatus*, and explore the role of size in mate selection. Egg size was correlated with female size, and juvenile characteristics were related to parent male size, pouch volume and embryo density.

MATERIALS AND METHODS

Experimental methodology

H. guttulatus were collected by scuba diving (under official permission) in Ria Formosa lagoon (36°59'N, 7°51'W), southern Portugal, between June and October 2009, and maintained under laboratory conditions. Seahorses were tagged with color bead necklaces loosely hold around the neck with an elastic cotton string, weighted alive in seawater and measured through scaled digital photograph, according to Lourie (2003). Standard length (SL) was determined as the sum of head (HL), trunk (TrL) and tail (TaL) lengths. Brood pouch dimensions (PL – length; PW – width; PD – depth) were determined for each male, after juvenile birth.

Seahorses were maintained in recirculated rearing systems. Pregnant males and some females were individually placed in 27 L incubation aquariums with independent juvenile collectors to study reproductive output. Females and non-pregnant males were kept in 180 L reproduction aquariums (50x40x90 cm) and in 170 L stock aquariums (140x35x35 cm) to study mate selection. Artificial plants were provided for seahorse attachment. Husbandry conditions were similar to those observed in their natural environment: photoperiod 14L:10D, temperature 24-25 ºC, salinity 34-35 psu, and pH 8.2-8.3. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg L^{-1}, respectively). Adults were fed *ad libitum* twice a day on live adult *Artemia* during the acclimatization week and on frozen enriched adult *Artemia* and *Mysis* in the following weeks. The aquariums were cleaned every day and 10% water changes were made weekly.

Mate selection was studied in reproduction aquariums with four seahorses at different sex ratios (2:2, 1:3 and 3:1). Males and females of different size (small: 16.65±1.37 cm SL; median: 18.62±0.68 cm SL; large: 20.75±1.23 cm SL) were methodically selected for each aquarium: a small and a large males with a small and a large females in the 1:1 trial; a median male with a small, a median and a large females in the 1:3 trial; a small, a median and a large males with a median female in the 3:1 trial. Three replicates using different individuals were obtained for each sex ratio. Behavior observations were made daily in the morning and afternoon, during a 4-week period. Seahorses involved in each courtship or mating were recorded.

The reproductive potential of seahorse males and females was evaluated in the incubation aquariums, based on the characteristics of the juveniles released by the males and of the unfertilized eggs that were dropped by the females in the bottom of the tanks, respectively. Thirty eggs from each female (n=9) were measured through micrometric analysis under light stereomicroscope. Four measures were taken for each egg: length and width of eggs (EL and EW, respectively) and yolk-sac reserves (YL and YW, respectively). Thirty newborn juveniles from each brood (n=20) were wet weighed in a high-precision balance and measured according to Lourie (2003).

At the end of the study, necklaces were removed, and adult seahorses and survivor juveniles were released back to their original habitat without injuries.

Statistical analysis

Sexual dimorphism regarding the weight (W), length (SL, HL, TrL and TaL) and body proportions (HP, TrP and TaP for head, trunk and tail proportions, respectively) of male and female seahorses was evaluated through the Mann-Whitney *U* test.

Mate selection was analyzed for pairs that mated (n=5) but also for pairs involved in courtships that did not result in egg transference (n=39), given the reduced number of mating occurrences in captivity. The relationship between male and female lengths and weights was evaluated through correlation analysis (Spearman correlation coefficient). The Chi-square test was used to determine if seahorses selected a similar size mate when in the presence of individuals of different sizes.

The volume of eggs and yolk-sac reserves was estimated based on length (L) and width (W) measures, using the volume formula of a prolate spheroid: $V = 1/6(\pi LWW)$. The yolk percentage in eggs (YP) was determined based on the volume of eggs and yolk reserves. The relationship between female length (SL) and the size of eggs (EL, EW and EV) and yolk-sac reserves (YL, YW, YV and YP) was evaluated through the Spearman correlation coefficient.

The volume of the male brood pouch was estimated based on length (L), width (W) and depth (D) measures, using the volume formula of a half cone: $V = 1/6(\pi LWD)$. The relationship between male length (SL) and the size and volume of the brood pouch (PL, PW, PD and PV) was evaluated through correlation analysis (Pearson correlation coefficient).

Juvenile size was examined in terms of weight (W), length (SL, HL, TrL and TaL) and body proportions (HP, TrP and TaP). Broods were analyzed regarding brood size (n), total brood weight (nW), the nSL parameter (determined through the multiplication of juvenile number and average length), embryo density (D=n/PV) and coefficients of variation (CV=standard deviation/mean) in juvenile size (CV_W, CV_{SL}, CV_{HL}, CV_{TrL} and CV_{TaL}). Juvenile and brood characteristics were correlated with male size and pouch volume (Spearman or Pearson correlation coefficients). Coefficients of variation were related to brood size, embryo density and juvenile size (Pearson correlation coefficient), while juvenile size was correlated with brood size and embryo density (Spearman correlation coefficient).

Seahorses were classified into two distinct length groups: small (SL between 11.0-18.0 mm in males and 13.0-18.0 mm in females) and large (SL between 18.0-23.0 mm in males and 18.0-21.0 mm in females). The size of eggs (EL, EW and EV) and yolk-sac reserves (YL, YW, YV and YP) were compared for small and large females through the Mann-Whitney U test. Brood pouch dimensions (PL, PW, PD and PV) and brood characteristics (n, nW, nSL, D, CV_W and CV_{SL}) were compared between small and large males using the t test, while newborn characteristics (W, SL, HL, TrL, TaL, HP, TrP and TaP) were compared using the Mann-Whitney U test.

All statistical analyses were performed for a significance level of 0.05.

RESULTS

H. guttulatus did not present sexual dimorphism in relation to weight or standard length. Males ranged from 4.47 to 24.88 g (average weight 14.55±5.43 g) and from 9.75 to 22.39 cm (average SL 17.65±2.51 cm), while females varied from 3.29 to 22.14 g (average weight 13.47±3.76 g) and from 11.22 to 23.68 cm (average SL 17.54±2.05 cm). Differences between sexes were only found in trunk and tail measures. Compared to males, females presented higher TrL (males: 4.79±0.77 cm; females: 5.29±0.82%; U=3170.0; p=0.000) and TrP (males: 27.14±2.24 cm; females: 30.13±2.59%; U=1892.5; p=0.000) and lower TaL (males: 10.30±1.67 cm; females: 9.65±1.24%; U=3473.0; p=0.001) and TaP (males: 58.21±3.04 cm; females: 54.96±1.66%; U=986.5; p=0.000).

Mate selection and mating were, in some way, size-assortative. Male and female weights were weakly correlated (Fig. 1; R^2=0.145, p=0.011), but not their total length (R^2=0.059, p=0.113). In the presence of different size animals, seahorses always selected similar size or larger mates, even though differences were only statistically significant for the 1:3 sex ratio (χ^2=6.00, p<0.05), probably due to the reduced number of courtship occurrences.

The relationship between female size and egg size is described in Table 1 and Fig. 2. The size of seahorse eggs was positively correlated with female size, with larger females producing bigger eggs than smaller females. Larger eggs had larger yolk reserves, even though the yolk percentage in the egg was significantly smaller in larger rather than smaller eggs. Egg enlargement was particularly marked in width (EW and YW) rather than length measures (EL and YL).
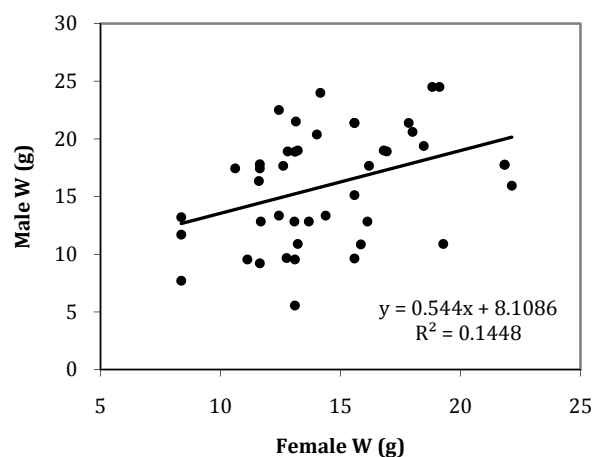
Fig. 1 – Relationship between male and female weights in courtship pairs.

Table 1 – Variation in the size of eggs (EL – egg length; EW – egg width; EV – egg volume) and yolk reserves (YL – yolk length; YW – yolk width; YV – yolk volume; YP – yolk proportion) with female size (W – weight; SL – standard length).

	Comparison of small and large females				Correlation with W		Correlation with SL	
	Small	Large	U	p	R^2	p	R^2	p
EL (mm)	2.30±0.39	2.45±0.55	6102.0	0.100	0.214	0.001	0.149	0.021
EW (mm)	1.33±0.16	1.57±0.28	3361.0	0.000	0.173	0.008	0.362	0.000
EV (µL)	2.21±0.77	3.43±1.72	4034.0	0.000	0.202	0.002	0.314	0.000
YL (mm)	1.54±0.36	1.61±0.51	5098.5	0.800	0.137	0.049	-0.048	0.493
YW (mm)	1.20±0.16	1.34±0.22	3160.5	0.000	0.167	0.016	0.197	0.005
YV (µL)	1.22±0.52	1.64±0.85	3792.0	0.001	0.169	0.015	0.110	0.117
YP (%)	53.39±15.59	43.98±15.31	3502.0	0.000	-0.134	0.056	-0.325	0.000
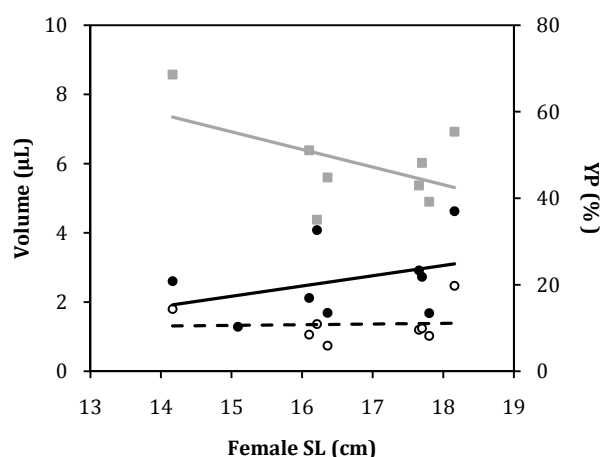
Fig. 2 – Relationship between egg characteristics (● EV – egg volume; ○ YV – yolk volume; ■ YP – yolk proportion) and female size (SL – standard length).

Larger males had larger brood pouches (Table 2, Fig. 3). The relationship between male size and the characteristics of broods and newborns is described in Table 3. With the exception of embryo density, brood characteristics were not correlated with male size or brood pouch volume. The broods of larger males presented similar size, mass and dispersion in juvenile weight and length, but lower embryo density than broods from smaller males. Except for juvenile condition, the characteristics of newborn juveniles were positively correlated with male size, with larger males producing bigger and heavier juveniles with smaller head proportions (Fig. 4).

Table 2 – Variation in pouch size (PL – pouch length; PW – pouch width; PD – pouch depth; PV – pouch volume) with male size (W – weight; SL – standard length).

	Comparison of small and large males				Correlation with W		Correlation with SL	
	Small	Large	t	p	R^2	p	R^2	p
PL (cm)	2.31±0.45	2.95±0.51	-5.44	0.000	0.31	0.000	0.40	0.000
PW (cm)	0.94±0.07	1.35±0.18	-12.67	0.000	0.81	0.000	0.89	0.000
PD (cm)	0.76±0.22	1.09±0.23	-5.86	0.000	0.49	0.000	0.49	0.000
PV (mL)	0.90±0.37	2.31±0.85	-8.90	0.000	0.66	0.000	0.70	0.000

Fig. 3 – Relationship between pouch volume (PV) and male size (SL – standard length).

Besides the relationship with male size, brood and juvenile characteristics were also related with each other (Table 4). Dispersion in juvenile length (but not weight) was negatively correlated with juvenile length but not with brood size or embryo density. Juvenile size was negatively correlated with the density of embryos inside the brood pouch, which was negatively correlated with male size (Fig. 5).

DISCUSSION

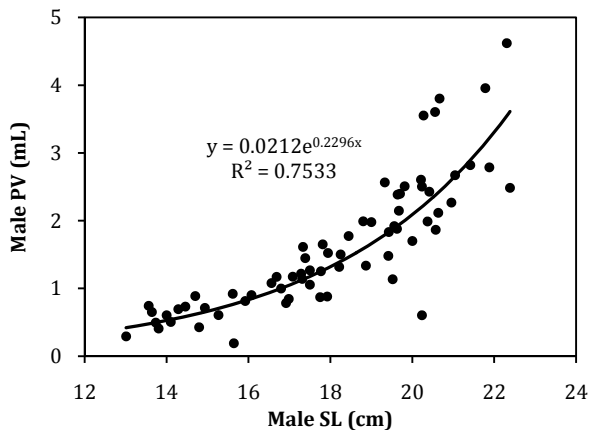In most animals, mate choice is not arbitrary but rather an important decision to make. Animals have to choose the best mate and the best parent in order to maximize the reproductive success of the species. There are a great variety of appealing traits among species but, in most cases, there is an intuitive belief that the bigger the better. Larger body size may confer some obvious fitness benefits, such as decreased susceptibility to predators, access to a greater range of food items, greater competitive ability, increased resistance to unsuitable conditions or disease, and greater reproductive output.

Since seahorses are normally faithful and devoted partners that make a choice for a life, or at least for a breeding season, the selection of a suitable mate gains particularly relevance in these fish. Seahorses have shown to prefer similar-size or larger mates (e.g., Vincent and Giles, 2003; reviewed in Foster and Vincent, 2004; Woods, 2007). In this study, *H. guttulatus* also showed a similar trend, even though the inclusion of pairs involved in courtships that didn't result in mate might have introduced some entropy, thus preventing the acquisition of stronger correlations and greater statistical significance.

Given the lack of sexual dimorphism in seahorse length and weight, size-assortative mating can be advantageous and optimize the reproductive potential of seahorse couples. Males seek large females with more and larger eggs, while females seek large males with greater pouch capacities and able to hold all their eggs. Even though males and females may have an instinct of choosing larger mates, it may be advantageous for both sexes to mutually choose a well-matched mate in order to increase the fitness of the species. Seahorse males that prefer to mate with larger females may take a chance on incubating a smaller brood due to the larger size of eggs. The question remains open whether more or bigger eggs are the best strategy for seahorses or animals in general.

In what concerns the reproductive output of *H. guttulatus* females, the bigger was indeed the better. Larger females produced bigger eggs with larger yolk reserves, a common trend among syngnathids (e.g., Berglund *et al.*, 1986b; Gasparini and Teixeira, 1999; Teixeira and Musick, 2001; Watanabe and Watanabe, 2002). The positive relationship between female length and egg size may result from larger females having greater body reserves for reproduction. However, even though larger eggs had larger yolk reserves, their yolk percentage was lower than in smaller eggs, indicating that the increase in egg volume reflects mainly an increase in egg albumen rather than in egg yolk. The production of large eggs with considerable yolk supply may be viewed as a kind of extended maternal care for developing embryos, which confer a survival advantageous to juveniles even though they are totally independent from the mother. Moreover, besides producing bigger eggs, larger females may also produce more eggs. Ovarian length was found to be roughly proportional to trunk length in *H. guttulatus* (Boisseau, 1967) and, thus, larger females are expected to present higher fecundity, as observed in other syngnathids (e.g., Berglund *et al.*, 1986b; Vincent, 1990; Teixeira and Musick, 2001).

More and larger eggs are expected to produce more and larger offspring. Interspecific studies have demonstrated that egg size is positively correlated with larval size at hatching in teleost fish (Bagarinao and Chua, 1986) and with juvenile size at birth in seahorses (reviewed in Foster and Vincent, 2004). Likewise, intraspecific differences in egg size are also likely to result in differences in length at birth, with larger eggs (that have larger yolk reserves and protein-rich albumen contents) producing larger seahorses.

Table 3 – Variation in brood (n – brood size; nW – total brood mass; nSL – nSL parameter; D – embryo density; CV_W – coefficient of variation in weight; CV_{SL} – coefficient of variation in standard length) and juvenile characteristics (W – weight; SL – standard length; HL – head length; TrL – trunk length; TaL – tail length; HP – head proportion; TrP – trunk proportion; TaP – tail proportion) with male size (W – weight; SL – standard length) and pouch volume (PV).

| Brood	Comparison of small and large males				Correlation with W		Correlation with SL		Correlation with PV	
	Small	Large	t	p	R^2	p	R^2	p	R^2	p
n (ind)	427.33±85.64	425.00±144.95	0.04	0.971	0.002	0.864	0.039	0.446	0.093	0.234
nW (mg)	1486.97±403.83	1595.92±622.32	-0.39	0.700	0.073	0.294	0.160	0.112	0.199	0.072
nSL (mm)	6420.62±1382.20	6704.27±2350.11	-0.27	0.788	0.024	0.549	0.093	0.235	0.152	0.122
D (ind mL^{-1})	382.39±88.70	176.91±68.31	5.20	0.000	0.509	0.001	0.484	0.002	0.474	0.002
CV_W	0.08±0.02	0.09±0.04	-0.88	0.392	0.059	0.350	0.030	0.508	0.002	0.862
CV_{SL}	0.04±0.01	0.04±0.01	-0.04	0.969	0.048	0.396	0.013	0.660	0.003	0.849
Juvenile	**Small**	**Large**	**U**	**p**	**R^2**	**p**	**R^2**	**p**	**R^2**	**p**
W (mg)	3.35±0.72	4.12±1.01	24803.5	0.000	0.349	0.000	0.296	0.000	0.248	0.000
SL (mm)	15.01±1.05	16.19±1.24	20604.0	0.000	0.436	0.000	0.410	0.000	0.373	0.000
HL (mm)	3.28±0.29	3.36±0.25	36054.0	0.001	0.067	0.104	0.012	0.762	0.209	0.000
TrL (mm)	4.26±0.40	4.70±0.57	23516.5	0.000	0.406	0.000	0.388	0.000	0.320	0.000
TaL (mm)	7.47±0.56	8.13±0.62	18698.0	0.000	0.479	0.000	0.464	0.000	0.397	0.000
HP (%)	21.89±1.62	20.81±1.65	27992.0	0.000	-0.342	0.000	-0.370	0.000	-0.157	0.000
TrP (%)	28.35±1.13	28.96±1.71	35844.0	0.001	0.192	0.000	0.194	0.000	0.063	0.162
TaP (%)	49.76±1.49	50.23±1.02	35451.0	0.000	0.165	0.000	0.207	0.000	0.097	0.030
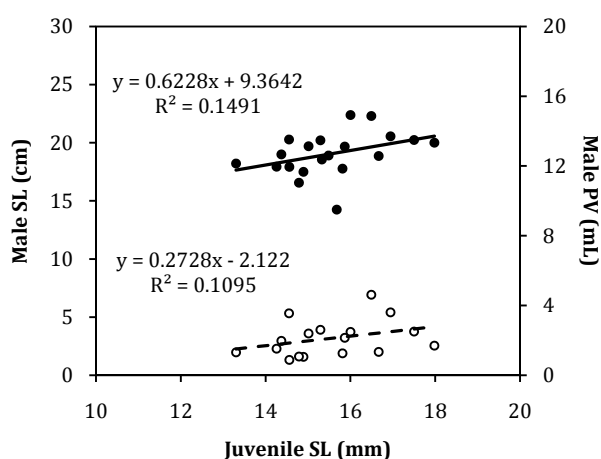
Fig. 4 – Relationship between juvenile size (SL – standard length) and male characteristics (● SL - standard length; ○ PV – pouch volume).

The fact that larger *H. guttulatus* males had larger brood pouches is not surprising. A positive relationship between male and pouch size has been the rule among syngnathids (e.g., Watanabe and Watanabe, 2002; Woods, 2007). Body proportions are expected to hold on relatively stable with increasing overall size, thus resulting in larger males presenting larger brood pouches. All rules have, however, exceptions. Some tiny seahorses may present incredible large pouches (e.g., Woods, 2000) or the other way around.

Larger males with larger pouches have a higher carrying capacity and, thus, greater reproductive potential. A positive correlation between brood size and body size can be found across seahorse species (reviewed in Foster and Vincent, 2004), but within species this relationship seems to be less straightforward. Although some studies have found a positive, even though not always strong, relationship between male and brood size (e.g., Boisseau, 1967; Teixeira and Musick, 2001; Woods, 2007), others found no significant relation with male size (Vincent and Giles, 2003; Woods,

2007). Instead, they found that females rather than males were the main determinant in the number of young released by the male.

A strong relationship between female and brood size may be expected for some syngnathids like monogamous pipefish, which do not possess a close brood pouch that greatly restricts their carrying capacity, but in seahorses (and polygamous pipefish as well) male size is expected to be the key determinant of brood size. Seahorse females generally produce more eggs than the male can handle (e.g., Vincent, 1990; Vincent, 1994), including the long-snouted seahorse *H. guttulatus* (Movchan, 1988), or at least as many as they can hold (e.g., Teixeira and Musick, 2001). A significant maternal effect on brood size would be expected only if the female produce fewer eggs than the male is able to carry. This may occur if the species presents a particularly large pouch (as *H. abdominalis* seems to present: Vincent, 1990), or if small males mate with large females. Even though, it is always possible to find some exceptions: Woods (2000) noted that the largest brood was carried by one of the smaller males, which had a particularly deep abdomen with a remarkably large pouch.

In this study, there were no differences in the brood size of small and large males, probably because larger males mated with larger females and received bigger eggs, thus leaving less space available inside the pouch for extra eggs. However, when male size and pouch volume were related to brood mass (nW) and the nSL parameter, it was still not possible to find strong correlations or significant differences between small and large males. There are too many factors influencing the number of embryos inside the male pouch. Besides the number and size of the oocytes and the capacity of the male pouch, other factors such as mate competition and pair bond duration may also be determinant. Faleiro *et al*. (2008) observed that mate competition, particularly between males, was responsible for disturbing mating and interrupting the transference of eggs, thus resulting in reduced brood size. Moreover, well-known partners with

longer pair bonds are expected to present better reproductive performance than newly-formed couples. Vincent (1994) found that, if a male and female had been together for more than seven days prior to mating, the male subsequently produced a significantly larger brood than if they had only been together for less than seven days. Monogamous seahorses are known to perform daily greetings in order to reinforce pair bonding and reproductive synchronization (Vincent, 1995). Faleiro *et al*. (2008) observed that the only captive *H. guttulatus* pair able to mate in a water column depth of just 30 cm was a pair that had mated previously in suitable water column depth conditions. We believe that, if we could control for such factors, a significant relationship between male or pouch size and brood mass would be easily detected.

While the carrying capacity of the male pouch can be expected to be the main determinant of brood size in seahorses, the size of newborns is mainly related to female and egg size. Although the male pouch has an important role in embryos nourishment, namely in the transference of inorganic compounds such as calcium, but also in the production of the hormone prolactin that promotes an enzyme which initiates the breakdown of the egg chorion into a fluid of nutritional value to the embryos (Linton and Soloff, 1964), the maternally-derived yolk reserves of the eggs seem to remain the only or, at least, the main source of nutrients during embryonic development. Larger eggs, which have larger yolk reserves, will thus produce larger juveniles. Positive relationships between juvenile size and male characteristics are, at least in part, an indirect consequence of larger males mating with larger females that produce larger eggs.

Table 4 – Variation in intra-brood size dispersion (CV_W – coefficient of variation in weight; CV_{SL} – coefficient of variation in standard length; CV_{HL} – coefficient of variation in head length; CV_{TrL} – coefficient of variation in trunk length; CV_{TaL} – coefficient of variation in tail length) and juvenile characteristics (W – weight; SL – standard length; HL – head length; TrL – trunk length; TaL – tail length; HP – head proportion; TrP – trunk proportion; TaP – tail proportion) with brood size (n), embryo density (D) and juvenile size (W – weight; SL – standard length).

	Correlation with n		Correlation with D		Correlation with W		Correlation with SL	
	R^2	p	R^2	p	R^2	p	R^2	p
CV_W	0.035	0.469	0.002	0.866	0.092	0.237	0.091	0.240
CV_{SL}	0.002	0.863	0.003	0.827	0.252	0.040	0.317	0.019
CV_{HL}	0.003	0.844	0.004	0.817	0.545	0.001	0.577	0.000
CV_{TrL}	0.043	0.425	0.012	0.670	0.333	0.015	0.341	0.014
CV_{TaL}	0.072	0.299	0.031	0.501	0.201	0.071	0.272	0.032

	R^2	p	R^2	p
W	0.155	0.000	-0.236	0.000
SL	0.172	0.000	-0.341	0.000
HL	0.434	0.000	-0.029	0.520
TrL	0.063	0.124	-0.338	0.000
TaL	0.155	0.000	-0.370	0.000
HP	0.262	0.000	0.317	0.000
TrP	-0.283	0.000	-0.230	0.000
TaP	-0.117	0.005	-0.108	0.015

Nevertheless, the seahorse father may also have a decisive role in the size of newborns. Syngnathid embryos proved to be self-sustaining from a nutritional perspective, being able to complete their development outside the male pouch (Azzarello, 1991), but nutrient supplementation by the father (e.g., reviewed in Stölting and Wilson, 2007) may enhance the nutritional condition of the embryos and, consequently, their growth and development. Moreover, the male pouch is responsible for gas exchange, waste products removal and osmorregulation (Linton and Soloff, 1964; Boisseau, 1967), and seems also to have an important immunoprotective function (Melamed *et al*., 2005). The size of young is thus expected to reflect both the initial size of eggs and the embryonic growth rate inside the pouch.
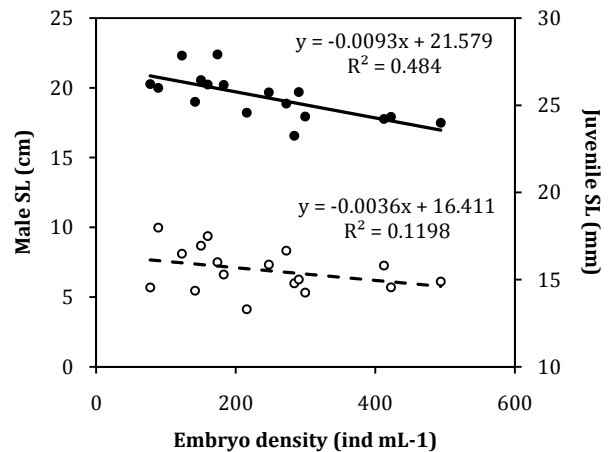
Fig. 5 – Relationship between embryo density and the size of males (● SL – standard length) and juveniles (○SL – standard length).

In this study, the size of newborns was positively correlated to male size and negatively related to the density of embryos inside the pouch, thus revealing a direct paternal effect. Similar brood sizes among small and large males resulted in a higher density of embryos inside the smaller pouch of the smaller males and, consequently, in reduced juvenile size. Embryos that developed at higher densities gave rise to smaller juveniles with greater head proportions and increased intra-brood size dispersion. Higher embryo density may not only restrict the room for growth but also increase intra-pouch competition between offspring. Dzyuba *et al*. (2006) found that embryos experience different degrees of paternal physiological support according with the attachment site in the pouch: embryos located dorsally in the deep pits are immersed in their own individual microenvironment, whereas those located ventrally and merely attached to the wall in shallow depressions may receive less optimal support. At lower densities, *H. guttulatus* embryos probably had the opportunity to attach to the most functionally advantageous sites, thus receiving a greater level of physiological support and further presenting enhanced growth. In contrast, embryos brooded at higher densities probably had to occupy all available sites, which resulted in some resource or supply limitation for the embryos attached to the poorest quality sites and, consequently, in reduced growth and greater size dispersion.

Superior juveniles (not always longer but generally heavier) have been produced when fewer newborns are present in the pouch of pipefish (e.g., Ahnesjö, 1992;

Watanabe and Watanabe, 2002) and seahorses (e.g., Dzyuba *et al.*, 2006; Woods, 2007). However, some studies found no relationship between juvenile size and brood size or embryo density in *H. whitei* (Vincent and Giles, 2003) and *H. abdominalis* (Woods, 2007). Woods (2007) speculated that, in *H. abdominalis*, brood sizes were not large enough in relation to pouch volume for resource or space competition to be a significant factor amongst the developing juveniles, given the proportionately larger pouch of this species with extra septa (3 to 5 instead of the normal one) that provide larger internal volume for brooding (Vincent, 1990).

The effects of parental size on the reproductive success of seahorses go far beyond the size of newborns and further affect the survival capacity and growth rate of the juveniles. The higher growth rates of newborns from larger males and smaller broods (e.g., Ahnesjö, 1992; Dzyuba *et al.*, 2006; Ortega-Salas and Reyes-Bustamante, 2006; Lin *et al.*, 2008) highlight the importance of the prenatal conditions experienced by the embryos in the pouch upon their future development and survival. Studies with *H. guttulatus* found that conditions during gametogenesis and embryonic development affect the reproductive output of the species, with smaller and thinner juveniles presenting poorer ability to tolerate salinity fluctuations and reduced survivorship (unpubl. data). The smaller size of the newborns from smaller *H. guttulatus* parents may, therefore, restrict the survival capacity of the juveniles and the reproductive success of the species.

In conclusion, both parents proved to play a decisive role in the reproductive output of *H. guttulatus*. Along with female size and initial egg size, the capacity of the male pouch seems also to influence the size of newborns by changing the density of embryos inside the pouch. With regard to parental quality, the bigger the better. Bigger seahorses, both males and females, have greater reproductive potential. The diversity and the weak power of the relationships between parental and juvenile characteristics among studies reflect the complexity of factors and interactions involved in the reproductive output of these fish. Besides mate selection, the number and size of the oocytes, the capacity of the male pouch and embryo density, other factors such as mate competition (e.g., Faleiro *et al.*, 2008), parents age (e.g., Dzyuba *et al.*, 2006), pair bond duration (e.g., Vincent, 1994), the number of prior broods (e.g., Vincent and Giles, 2003), temperature (e.g., Lin *et al.*, 2006) and stress (unpubl. data) may all affect the reproductive success of seahorses. To control all these factors and separate maternal from paternal contributions is not an easy task but, if means can be developed to do it, seahorses may offer a rare opportunity among fishes to undertake a more comprehensive assessment of reproductive success.

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Pregnant and responsible for offspring size:

a male story

Pregnant and responsible for offspring size:

a male story

Filipa Faleiro & Luís Narciso

Universidade de Lisboa, Faculdade de Ciências, Centro de Oceanografia, Laboratório Marítimo da Guia,
Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal

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Conventionally, the mother usually plays the key role in reproduction[1], but what about the most dedicated father amongst animals? In seahorses, it is the male that becomes pregnant, incubating the embryos inside the brood pouch in a manner that bears some resemblance to the embryo-placental relationship in mammals. The female still is the main responsible for embryo nourishment, but the male plays an important role in gaseous exchange, removal of waste products, osmoregulation and immunoprotection[2]. Male pregnancy raises the question of whether the female still is the key determinant of reproductive success or if she shares some responsibility with the male. Here we give direct evidence that the size of newborn seahorses is, in part, paternally determined. We found that the size of juveniles from the same parents was negatively related to brood size and embryo density, thus evidencing a trade-off between the number and the size of embryos inside the pouch. Males were responsible for a variation of 30% and 15% in juvenile weight and length, respectively, between different-size consecutive broods. Our results demonstrate that the male seahorse may affect offspring size even without being responsible for embryo nourishment. For the first time, males have proven to be much more than just a sperm donor and to directly contribute to the size of the offspring.

Conventionally, the mother usually plays the key role in reproduction. While males produce the numerous but tiny and unprovisioned sperm, females are responsible for the big, highly provisioned and energetically costly eggs. Females typically invest more in the offspring than males and are, therefore, the main determinant of offspring quality. In fishes, the size of the fingerlings is strongly correlated with the size of eggs[1], but what happens when males invest more in the offspring?

Seahorses are the most dedicated fathers in the animal kingdom. In these fishes, it is the male that becomes pregnant. Embryos fully develop within the enclosed environment of the male brood pouch, in close association with the highly vascularized pouch epithelium in a manner that bears some resemblance to the embryo-placental relationship in mammals. Although females still produce the large and provisioned gamete and are the main responsible for embryo nourishment, males are responsible for gaseous exchange, removal of waste products and osmoregulation during embryonic development, playing also an important immunoprotective role[2]. Male pregnancy raises the question of whether females still are the key determinant of reproductive success in seahorses or if males share some of the responsibility.

Studies with syngnathids have shown that, even though embryos are self-sustaining from a nutritional perspective and fully develop outside the male pouch[3], pregnancy has high energetic costs for males[4,5]. Recent studies have evaluated parental effects on the reproductive output of seahorses[6-9] and pipefish[10-13], but without dissociating the paternal contribution from the maternal one. The complex reproductive biology of seahorses makes hard to separate male and female roles and to assign responsibilities for reproductive output.

In this study, we establish a controlled breeding experiment that dissolves the maternal contribution and assesses the role of the father in the reproductive success of seahorses. We followed five long-snouted seahorse, *Hippocampus guttulatus*, couples in captivity over two breeding events. Breeding conditions were kept constant, except for water column depth. A suitable water depth for reproduction was set for the first breeding event but, in the second event, seahorses were forced to reproduce in a restrictively low depth, in order to obtain two different-size consecutive broods from the same couples. Since the transference of oocytes from the female to the male pouch occurs at the same time the pair rises up in the water column and is interrupted when the pair reaches the water surface, reduced water column depth was responsible for limiting the transference to a smaller number of eggs (t=8.8, p=0.000). Females were able to transfer 333±54 eggs in the first mating but, in the second mating, they only managed to deposit 113±13 eggs inside the male pouch. As a result, embryos from the smaller broods were incubated at lower densities than those from the larger broods (larger broods: D=425.6±62.4 juv mL^{-1}; smaller broods: D=145.8±28.3 juv mL^{-1}; t=9.1, p=0.000).

Brood size and embryo density had a significant effect on the size of the offspring (Figure 1). Newborn juveniles from the same parents were bigger and heavier when incubated in smaller and lower-density broods (U<1401.0, p<0.001). Juveniles incubated at higher densities had higher head and trunk proportions and smaller tail proportion (U<9776.0, p<0.050), which may indicate poorer nutritional condition, as nutritional restrictions are expected to be more evident in

the tail which comprise mainly musculature. Differences in juvenile size were the sole responsibility of the male, since the size and nutritional content of eggs from the same females are expected to be identical under constant breeding conditions.
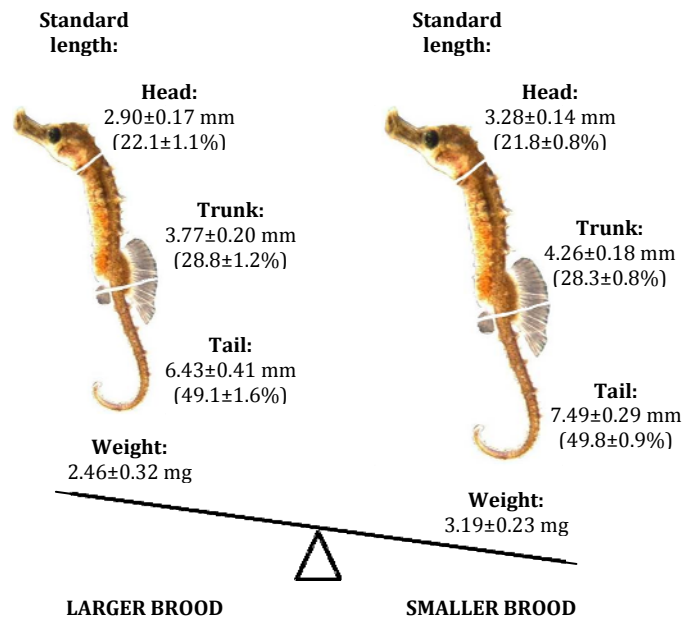

Standard length:

Head:
2.90±0.17 mm
(22.1±1.1%)

Trunk:
3.77±0.20 mm
(28.8±1.2%)

Tail:
6.43±0.41 mm
(49.1±1.6%)

Weight:
2.46±0.32 mg

LARGER BROOD

Standard length:

Head:
3.28±0.14 mm
(21.8±0.8%)

Trunk:
4.26±0.18 mm
(28.3±0.8%)

Tail:
7.49±0.29 mm
(49.8±0.9%)

Weight:
3.19±0.23 mg

SMALLER BROOD

Figure 1 – Comparative size (weight, length and body proportions) of newborn juveniles from different-size consecutive broods produced by the same seahorse couples.

Results demonstrate that the size of newborn seahorses is, in part, paternally determined. Males incubating smaller broods were able to increase juvenile weight and length by 30% and 15%, respectively. The trade-off between the number and size of embryos inside the pouch clearly indicates a limited carrying capacity of the male. Since seahorse embryos may experience different degrees of paternal physiological support according with the attachment site in the pouch, intra-pouch competition may result in reduced juvenile size[8]. At lower densities, seahorse embryos may have the opportunity to attach to the most functionally advantageous sites, thus receiving a greater level of physiological support and further presenting enhanced growth. However, embryos brooded at higher densities have to occupy all available sites, which may result in some resource or supply limitation for the embryos attached to the poorest quality sites and, consequently, in reduced growth and greater size dispersion. Compared to the larger broods, the smaller broods had lower intra-brood size dispersion in both weight (larger broods: $CV_W=0.13$; smaller broods: $CV_W=0.07$; t=14.8, p=0.000) and length (larger broods: $CV_{SL}=0.05$; smaller broods: $CV_{SL}=0.03$; t=18.3, p=0.000).

For the first time, a male has proven to be much more than just a sperm donor and to directly contribute to the size of the offspring, even without being responsible for embryo nourishment. Paternal supply of essential environmental conditions and important elements during embryonic development affect juvenile size and may further influence their future development and survival. The seahorse father can incubate a greater number of eggs or can give birth to bigger juveniles, but cannot achieve both at the same time.

Larger juveniles have greater survival capacities and better chances of reaching adulthood, but increased quantity of offspring may offset their greater vulnerability by improving the frequency probabilities. Seahorses have therefore a hard decision to make. More or bigger? Both is "seahorsely" impossible!

Methods Summary

H. guttulatus were collected by scuba diving (under official permission) in Ria Formosa lagoon (36°59'N, 7°51'W), southern Portugal, in June 2009. Seahorses were maintained under laboratory conditions in recirculated rearing systems. After mate selection and pair formation, five couples were isolated in 180 L reproduction aquariums with suitable water column depths (50x40x90 cm). Following mating, pregnancy and juvenile birth, the pairs were transferred to 170 L stock aquariums with restrictive water depths (140x35x35 cm).

Husbandry conditions were similar to those observed in their natural environment: photoperiod 14L:10D, temperature 24-25 ºC, salinity 34-35 psu, and pH 8.2-8.3. Artificial plants were provided for seahorse attachment. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg L^{-1}, respectively). Adults were fed *ad libitum* twice a day on live adult *Artemia* during the acclimatization week and on frozen enriched adult *Artemia* and *Mysis* in the following weeks. The aquariums were cleaned every day and 10% water changes were made weekly.

Adult seahorses, as well as 30 newborn juveniles from each brood, were weighed and measured. Standard length was determined as the sum of head, trunk and tail lengths. Brood pouch dimensions (L – length; W – width; D – depth) were determined for each male at a non-brooding condition. The volume of the male pouches was estimated using the volume formula of a half cone: $V=1/6(\pi LWD)$. Embryo density (D=n/V) and coefficients of variation (CV=standard deviation/mean) in juvenile weight and standard length were estimated for each brood. Brood characteristics (brood size, embryo density and size dispersion) were compared between first (n=5) and second broods (n=5) using the *t* test. Juvenile size (weight, length measures and body proportions) were compared between first (n=150) and second broods (n=150) using the Mann-Whitney *U* test. All comparisons were made for a significance level of 0.05.

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Lipid dynamics during early development of *Hippocampus guttulatus* seahorses: searching for clues on fatty acid requirements

Lipid dynamics during early development of *Hippocampus guttulatus* seahorses: Searching for clues on fatty acid requirements

Filipa Faleiro *, Luís Narciso

Centro de Oceanografia, Laboratório Marítimo da Guia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal

A B S T R A C T

The biochemical composition of eggs and the nutrient dynamics during early ontogeny can provide better understanding on the nutritional requirements of both broodstock and first-feeding fish, thus constituting a valuable tool for improvement of aquaculture procedures. This study constitutes the first record of lipid composition and fatty acid dynamics during early development of seahorses. Lipid content and fatty acid profile were determined for *Hippocampus guttulatus* eggs and newborn juveniles, and their patterns of consumption were analyzed during embryonic development. *H. guttulatus* produced small broods (426 ± 128 juveniles) but invested in the production of large eggs (2.44 ± 0.37 mm) that resulted in large fingerlings (15.53 ± 1.28 mm), which may have a high survival capacity. The total lipid content of seahorse eggs (17.6% DW) was within the typical range of marine fish eggs. Eggs were dominated by PUFA (43.9%), mainly n−3 HUFA (17.4% of DHA and 13.2% of EPA), and showed a lower MUFA content (22.5%). Extremely low AA levels (0.6%) and particularly high EPA concentrations resulted in a low DHA:EPA ratio (1.3) and high EPA:AA (22.3) and n−3:n−6 (12.2) ratios. Fatty acid consumption during embryonic development was considerably high (67.8%), reflecting the high requirements of seahorse embryos. Fatty acids were depleted at different rates, with PUFA being preferentially consumed (75.2%), while SFA were selectively retained (59.2%). PUFA constituted the major source of metabolic energy and the fatty acids 16:0, EPA and DHA were the main fatty acids to fulfill the energetic demands of seahorse embryos. Essential fatty acids (AA, EPA and DHA) were not preferentially retained, with EPA being catabolized at a particularly high rate (88.0%). These patterns of embryonic consumption resulted in newborn juveniles with an exceptionally low lipid content (5.0% DW), a predominance of SFA (41.0%) and extremely low EPA levels (5.0%). Fatty acid composition of *H. guttulatus* eggs and juveniles provided important clues to determine seahorse requirements, so that suitable feeding protocols could be developed. The use of broodstock and juvenile diets that reflect the lipid profile of eggs will allow the accomplishment of nutritional requirements and improvement of seahorse production.

1. Introduction

The annual recruitment of marine fish stocks constitutes a key element to preserve the sustainability of wild populations, and is affected by several factors, including the reproductive success of the broodstock and several environmental conditions. The quantity and quality of progeny can have significant effects in fish recruitment, since low fecundity or unhealthy progeny may lead to subsequent low survival, mainly if occurring at periods of unfavorable environmental conditions. Although most attention has been addressed to fecundity as a measure of reproductive success, the qualitative aspects of the reproductive output are currently developing a rising interest. Different measures have been proposed to assess egg, embryo or larvae quality, including egg size (e.g., Keckeis et al., 2000), histological criteria (e.g., Bromage et al., 1994), hormone levels (e.g., Lam, 2007), genetic analysis (e.g., Brooks et al., 1997), enzyme activity (e.g., Clarke et al., 1992) and biochemical composition (e.g., Washburn et al., 1990; Fernández-Palacios et al., 1995).

The nutritional status of eggs, embryos and larvae can be highly related with their chance of survival and enhanced growth. Since fish embryos and yolk-sac larvae obtain nutrients from the endogenous reserves of the yolk, eggs must contain all the nutrients necessary to support both homeostasis and development until the end of the lecithotrophic phase, prior to exogenous feeding. Moreover, the nutritional profile of larvae will influence the amount of metabolically available energy at hatching, which will determine how long larvae

will be able to survive in limited feeding conditions, until adequate food becomes available.

Within the nutritional profile of eggs, lipid and fatty acid composition is of major importance, not only because lipid reserves are the most important energy source during embryonic development of most marine organisms, but also because they provide important structural components of biomembranes and precursors of an important group of hormones known collectively as eicosanoids (Sargent et al., 2002). Indeed, lipid levels and fatty acid composition have been correlated with reproductive parameters of many fish species, including egg quality, spawning, fertilization and hatching rates and larval viability (e.g., Rainuzzo et al., 1997; Yanes-Roca et al., 2009).

The nutritional composition and dynamics during early ontogeny can provide better understanding on the nutritional requirements of both broodstock and first-feeding fish. Such an understanding can be of major importance to enhance progeny quality, constituting a valuable tool for aquaculture improvement, particularly in a time when the production of marine organisms holds a key role in species conservation and commercialization. Worldwide, the growing eminence of marine aquaculture has created a high global demand for healthy animals, which will rely on the ability to achieve good reproductive rates and high larval and juvenile performances. However, the lack of knowledge on specific nutritional requirements has raised major difficulties in culturing marine organisms, with nutrition constituting one major challenge in marine larviculture.

Information on the nutritional profile of eggs and newly-hatched larvae will allow estimation of nutritional requirements and improvement of diets at two different levels. The first one relates to parental nutrition. Since the biochemical composition of eggs is directly derived from the parental reserves built up in the period preceding gametogenesis (Sargent, 1995), the nutritional state of broodstock can greatly influence the biochemical composition of eggs. Indeed, several studies confirmed that qualitatively and quantitatively inadequate diets, especially in terms of lipid content and fatty acid composition, adversely affect eggs and larvae production (e.g., Rainuzzo et al., 1997). The second level relates to progeny nutrition. Following the complete absorption of the yolk-sac reserves, larval feeding is exclusively dependent on exogenous sources. Therefore, diets should fulfill all the nutritional requirements of larvae, in order to achieve high survival and growth rates.

In the last decade, seahorse culture has developed a rising interest worldwide. The intensification of seahorse exploitation for traditional medicines, aquarium trade and curios has led to the global decline of natural populations (Vincent, 1996) and to the inclusion in 2002 of seahorse species in the IUCN Red List and in the CITES Appendix II. In order to assure global demands and to protect the sustainability of natural populations, seahorse culture shows a great potential not only as a commercial project but also as a tool for species conservation. Nevertheless, the development of suitable protocols for seahorse culture has revealed several problems, namely on broodstock husbandry, reproduction and juvenile rearing. Although rearing protocols have been improved and seahorse culture has been achieved for several species, optimization of juvenile rearing, more particularly the accomplishment of nutritional requirements, continue to be a major bottleneck in seahorse culture.

Despite the importance of assessing seahorse lipid requirements, no information is available on the biochemical profile of seahorse eggs and juveniles. This study presents the first data on fatty acid composition and dynamics during early development of a seahorse species. Lipid content and fatty acid profile of eggs and newborn juveniles of the long-snouted seahorse, *Hippocampus guttulatus*, were determined and their patterns of consumption were analyzed during embryonic development, in order to estimate lipid requirements and develop suitable feeding protocols for long-snouted seahorse culture.

2. Materials and methods

2.1. Experimental methodology

Long-snouted seahorses were collected by scuba diving (under official permission) in Ria Formosa lagoon (36°59′N, 7°51′W), south Portugal, in June 2007. Females and pregnant males were kept in a recirculated system with distinct incubation tanks. Husbandry conditions were: salinity 34–35 PSU, water temperature 24–25 °C, pH 8.2–8.3, photoperiod 14 L:10 D and constant aeration. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg L^{-1}, respectively). Seahorses were fed daily on live adult *Artemia*.

Unfertilized eggs that were dropped by the females were removed from the bottom of the tanks. Thirty eggs from each female ($n = 28$) were measured (to the nearest 0.05 mm) through micrometric analysis under light stereomicroscope. Four measures were taken for each egg: length and width of eggs (EL and EW, respectively) and yolk-sac reserves (YL and YW, respectively). Thirty newborn juveniles from each brood ($n = 20$) were wet weighed in a high-precision balance (to the nearest 0.01 mg) and measured (to the nearest 0.05 mm) according to Lourie (2003). Standard length (SL) was determined as the sum of head (HL), trunk (TrL-straight line) and tail (TaL) lengths. Because some juveniles were born at a premature state, their morphological characteristics were described. Eggs from three different females and newborns from three different males were frozen individually for fatty acid analysis.

Frozen samples of seahorse eggs and juveniles were freeze-dried for fatty acid analysis. The wet weight of frozen samples and the dry weight of freeze-dried samples were measured in a high-precision balance (to the nearest 0.01 mg) to determine the water content of eggs and newborn juveniles. Lipids were extracted based on the Bligh and Dyer (1959) method and esterified to fatty acid methyl esters according to Metcalfe and Schmitz (1961). The fatty acid methyl esters were injected into a capillary column Omega Wax 320 WCOT (30 m fused silica, 0.32 internal diameter) installed in a Varian Star 3400CX gas–liquid chromatograph (GLC). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. Oven temperature was 180 °C for 7 min and then increased to 200 °C (with a temperature gradient of 4 °C min^{-1}) over a period of 71 min. Both the split injector (100:1) and the FID detector were set at 250 °C. GLC data acquisition and handling were performed using a Varian integrator 4290 connected to the GLC. Peak quantification was carried out with a Star Chromatography workstation installed in an IBM PS/1, and the fatty acid C19:0 (nonadecanoic acid methyl ester) was used as internal standard. Peak identification was performed using well-characterized cod liver oil chromatograms as a reference.

At the end of the experiment, seahorses and survivor juveniles were released on their original habitat without injuries.

2.2. Statistical analysis

The volume of eggs and yolk-sac reserves was estimated based on length (L) and width (W) measures, using the volume formula of an ovoid: $V = 1/6(\pi LWW)$. The relationship between the volume of eggs and yolk-sac reserves was studied through regression analysis.

Water content, TL content and fatty acid composition (in absolute and relative values) were compared between eggs and newborn juveniles through t test. All fatty acids were analyzed but special emphasis was given to the essential fatty acids AA, EPA and DHA. Comparisons were also made for TFA content and fatty acid classes (BFA, SFA, MUFA, PUFA, HUFA, n−3 and n−6 fatty acids), as well as for the ratios of n−3:n−6, EPA:AA and DHA:EPA. Consumption of lipids and fatty acids during embryonic development were estimated by comparing the composition of eggs and newborn juveniles.

All statistical analyses were performed for a significance level of 0.05, using Statistica 8.0 software.

3. Results

3.1. Size of broods, eggs and newborn juveniles

Unfertilized eggs of the long-snouted seahorse were demersal, pear-shaped and transparent, containing one orange yolk-sac (Fig. 1A). Eggs (n = 840) presented an average length of 2.44 ± 0.37 mm, width of 1.50 ± 0.25 mm and volume of 3.02 ± 1.40 μL. Yolk-sac length and width were 1.55 ± 0.39 mm and 1.28 ± 0.21 mm, respectively, and the average volume was 1.41 ± 0.67 μL. Yolk-sac volume was positively correlated with egg volume ($R^2 = 0.34$, p = 0.000, n = 840), indicating that larger eggs have larger yolk-sac reserves (Fig. 2).

Embryonic development within the male brood pouch is illustrated in Fig. 1. After female deposition of unfertilized eggs inside the male pouch, the male releases sperm into the pouch, fertilizing the eggs. The embryos develop within the male pouch, nourished by their individual yolk-sacs. Just before hatching, the embryos resemble an adult seahorse and it is already possible to distinguish the head with detailed eyes and a snout albeit short, the body trunk with the yolk-sac and the long tail. After egg eclosion, seahorse growth continues inside the brood pouch, with the development of internal organs and fins. The snout length increases gradually and the yolk reserves are slowly reabsorbed. At birth, the yolk-sac has been completely reabsorbed and newborn juveniles are a structurally perfect replica of adults.

H. guttulatus brood size (n = 20) was 426 ± 128 juveniles, with newborns (n = 600) having 15.53 ± 1.28 mm in SL and presenting an average weight of 3.69 ± 0.94 mg.

3.2. Lipid content, water content and fatty acid profile of eggs and newborn juveniles

The TL content showed a significant decrease during embryonic development ($t = 2.99$, p = 0.040, n = 3), with eggs presenting a higher lipid content (17.60 ± 5.39% DW) than newborn juveniles (5.00 ± 2.56% DW). No differences were found ($t = 1.91$, p = 0.129, n = 3) in the water content of eggs (92.06 ± 1.02%) and juveniles (89.22 ± 1.83%).
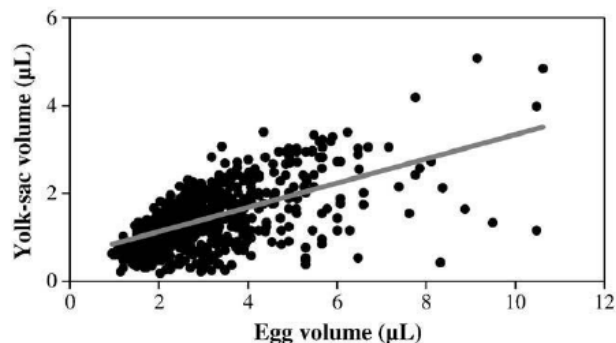
Fig. 2. Positive correlation between volume of *H. guttulatus* eggs and yolk-sac reserves.

The fatty acid profile of eggs and newborn juveniles is described in Table 1. A significant decrease was observed in TFA content during embryonic development (67.76%), from 99.36 ± 17.72 μg mg^{-1} DW in eggs to 32.04 ± 3.09 μg mg^{-1} DW in newborns. Indeed, compared to eggs, newborn seahorses had lower amounts of the majority of the fatty acids (including the essential fatty acids AA, EPA and DHA), which resulted in lower quantities of TFA, BFA, SFA, MUFA, PUFA, HUFA, n−3 and n−6 fatty acids. A slight increase in fatty acid concentrations was only observed for the SFA 13:0 and 20:0, although differences between eggs and newborns were not significant.

Consumption rates varied among fatty acids, from extremely low levels (1.65 and 2.38% for the fatty acids 20:1n−5 and 20:0, respectively) to total consumption (100%). Nevertheless, consumption rates of most fatty acids were greater than 65%, with higher rates being generally observed for fatty acids present in lower amounts. PUFA were preferentially used during development (75.19%), while SFA were catabolized at a lower rate (59.20%). Consequently, the fatty acid profile of eggs was dominated by PUFA (43.88 ± 0.75%), while newborns were rich in SFA (41.01 ± 1.83%). Both groups had very low quantities of BFA and showed a considerably higher proportion of n−3 fatty acids when compared to n−6 fatty acids.

In terms of absolute amounts, although PUFA continue to be the major group catabolized (32.87 μg mg^{-1} DW), a higher decrease
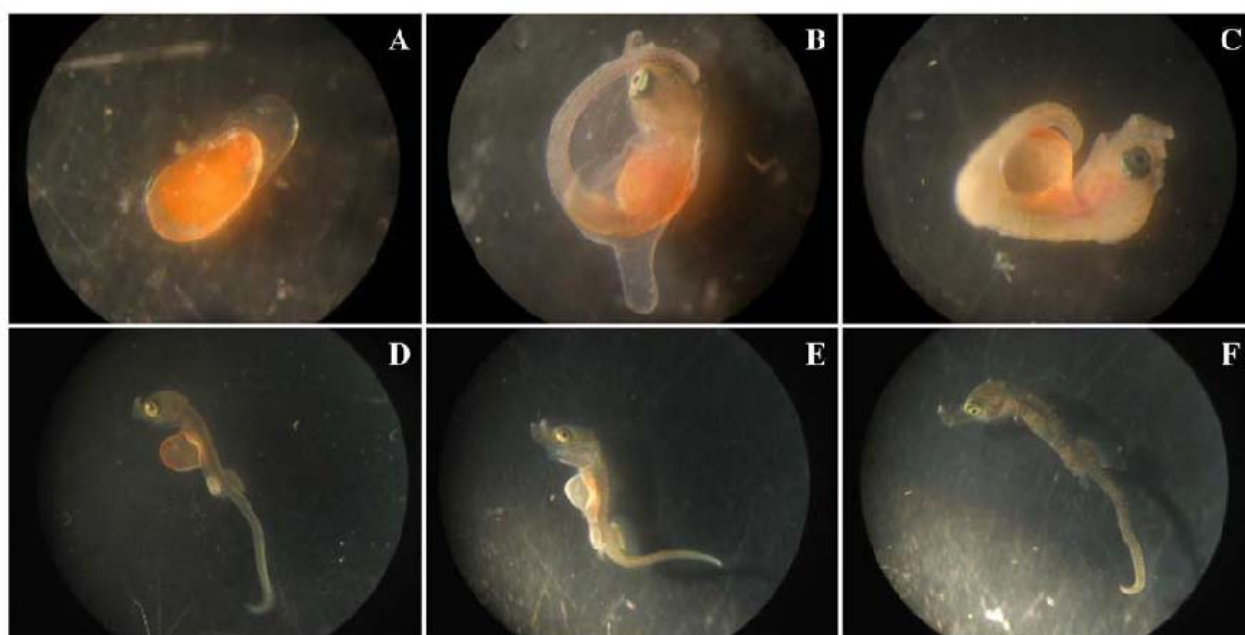
Fig. 1. Embryonic development of *H. guttulatus* (A—unfertilized egg; B—embryo inside the egg; C,D,E—yolk-sac juveniles after egg eclosion; F—newborn juvenile).

Table 1

Absolute ($\mu g\ mg^{-1}$ DW) and relative (% TFA) fatty acid compositions during early development of *H. guttulatus* (nd—not detected). Mean values ± standard errors of triplicate samples for eggs (E) and newborn seahorses (NB). Significant differences ($p<0.05$) between eggs and newborns are marked in bold.

|	Absolute profile ($\mu g\ mg^{-1}$ DW)			Relative profile (% TFA)			Embryonic consumption	
	E	NB	p	E	NB	p	$\mu g\ mg^{-1}$ DW	%
Iso 15:0	0.20 ± 0.14	0.07 ± 0.00	0.258	0.18 ± 0.13	0.21 ± 0.01	0.787	0.13	66.77
Ant 15:0	0.06 ± 0.04	0.00 ± 0.00	0.124	0.05 ± 0.04	0.00 ± 0.00	0.117	0.06	100.00
Iso 16:0	0.22 ± 0.06	0.05 ± 0.01	**0.019**	0.22 ± 0.05	0.15 ± 0.02	0.139	0.17	78.18
Iso 17:0	0.65 ± 0.15	0.18 ± 0.03	**0.015**	0.65 ± 0.06	0.57 ± 0.12	0.494	0.47	72.02
BFA	1.13 ± 0.40	0.30 ± 0.04	**0.044**	1.10 ± 0.27	0.93 ± 0.15	0.482	0.83	73.76
13:0	0.00 ± 0.00	0.08 ± 0.11	0.374	0.00 ± 0.00	0.27 ± 0.38	0.374	−0.08	
14:0	2.21 ± 0.44	0.61 ± 0.08	**0.008**	2.21 ± 0.05	1.90 ± 0.13	**0.033**	1.60	72.41
15:0	0.88 ± 0.11	0.21 ± 0.04	**0.001**	0.90 ± 0.07	0.64 ± 0.07	**0.023**	0.67	76.37
16:0	19.86 ± 3.24	6.75 ± 0.31	**0.005**	20.04 ± 0.37	21.17 ± 1.17	0.263	13.11	66.02
17:0	1.03 ± 0.17	0.47 ± 0.08	**0.015**	1.04 ± 0.01	1.50 ± 0.37	0.151	0.56	54.42
18:0	7.52 ± 0.82	4.37 ± 0.36	**0.008**	7.70 ± 0.97	13.65 ± 0.35	**0.001**	3.15	41.88
20:0	0.49 ± 0.15	0.47 ± 0.17	0.953	0.50 ± 0.16	1.48 ± 0.54	0.070	0.01	2.38
22:0	0.11 ± 0.01	0.13 ± 0.04	0.470	0.11 ± 0.01	0.40 ± 0.09	**0.011**	−0.02	−23.38
SFA	32.09 ± 4.60	13.09 ± 0.77	**0.005**	32.50 ± 1.19	41.01 ± 1.83	**0.005**	19.00	59.20
14:1n−5	0.06 ± 0.08	0.00 ± 0.00	0.374	0.08 ± 0.11	0.00 ± 0.00	0.374	0.06	100.00
16:1n−7	5.50 ± 1.11	1.09 ± 0.11	**0.005**	5.52 ± 0.37	3.41 ± 0.05	**0.001**	4.41	80.17
16:1n−5	0.05 ± 0.06	0.00 ± 0.00	0.374	0.04 ± 0.05	0.00 ± 0.00	0.374	0.05	100.00
17:1n−8	0.38 ± 0.10	0.08 ± 0.03	**0.013**	0.38 ± 0.04	0.23 ± 0.07	0.063	0.31	80.00
18:1n−9	9.84 ± 1.98	3.85 ± 0.34	**0.015**	9.86 ± 0.30	12.03 ± 0.09	**0.001**	5.99	60.87
18:1n−7	4.91 ± 0.79	1.50 ± 0.21	**0.004**	4.96 ± 0.09	4.68 ± 0.25	0.211	3.40	69.35
18:1n−5	0.12 ± 0.03	0.05 ± 0.04	0.087	0.12 ± 0.01	0.15 ± 0.13	0.753	0.08	62.41
19:1n−8	0.05 ± 0.04	0.01 ± 0.01	0.206	0.05 ± 0.03	0.03 ± 0.05	0.712	0.04	81.82
19:1n−10	0.04 ± 0.03	0.03 ± 0.02	0.642	0.04 ± 0.03	0.09 ± 0.07	0.397	0.01	32.30
20:1n−9	0.66 ± 0.13	0.44 ± 0.17	0.221	0.67 ± 0.07	1.33 ± 0.39	0.079	0.22	33.77
20:1n−7	0.43 ± 0.03	0.50 ± 0.26	0.689	0.44 ± 0.05	1.52 ± 0.63	0.074	−0.08	−18.52
20:1n−5	0.07 ± 0.10	0.07 ± 0.10	0.990	0.06 ± 0.08	0.24 ± 0.34	0.507	0.00	1.65
22:1n−11	0.17 ± 0.14	0.09 ± 0.07	0.506	0.16 ± 0.14	0.27 ± 0.20	0.551	0.08	46.27
22:1n−9	0.14 ± 0.11	0.09 ± 0.07	0.647	0.15 ± 0.14	0.31 ± 0.24	0.454	0.04	32.56
MUFA	22.43 ± 4.27	7.81 ± 1.03	**0.010**	22.52 ± 0.50	24.29 ± 0.82	0.060	14.62	65.20
16:4n−3	0.08 ± 0.05	0.00 ± 0.00	0.118	0.07 ± 0.05	0.00 ± 0.00	0.117	0.08	100.00
18:2n−6	1.95 ± 0.42	0.53 ± 0.06	**0.010**	1.95 ± 0.07	1.66 ± 0.07	**0.015**	1.42	72.63
18:3n−3	1.44 ± 0.62	0.13 ± 0.04	**0.042**	1.38 ± 0.42	0.41 ± 0.16	**0.037**	1.31	91.02
18:4n−3	0.76 ± 0.23	0.04 ± 0.03	**0.012**	0.75 ± 0.16	0.11 ± 0.08	**0.008**	0.72	95.14
20:3n−6	0.23 ± 0.12	0.06 ± 0.04	0.132	0.24 ± 0.13	0.18 ± 0.13	0.635	0.17	73.98
20:4n−6	0.60 ± 0.15	0.16 ± 0.03	**0.017**	0.60 ± 0.10	0.50 ± 0.05	0.268	0.44	73.39
20:3n−3	0.18 ± 0.03	0.09 ± 0.02	**0.019**	0.18 ± 0.02	0.27 ± 0.05	0.085	0.09	51.81
20:4n−3	3.08 ± 0.45	1.49 ± 0.23	**0.012**	3.14 ± 0.34	4.63 ± 0.30	**0.009**	1.59	51.63
20:5n−3	13.19 ± 2.90	1.59 ± 0.22	**0.005**	13.17 ± 0.91	5.02 ± 0.96	**0.001**	11.60	87.96
21:5n−3	0.34 ± 0.07	0.00 ± 0.00	**0.002**	0.34 ± 0.03	0.00 ± 0.00	**0.000**	0.34	100.00
22:4n−6	0.50 ± 0.10	0.18 ± 0.04	**0.012**	0.52 ± 0.14	0.57 ± 0.06	0.703	0.32	63.65
22:5n−3	4.08 ± 0.57	0.94 ± 0.19	**0.002**	4.13 ± 0.16	2.90 ± 0.31	**0.008**	3.14	76.99
22:6n−3	17.29 ± 3.49	5.64 ± 0.79	**0.008**	17.40 ± 1.27	17.52 ± 0.74	0.912	11.66	67.41
PUFA	43.72 ± 8.50	10.85 ± 1.36	**0.005**	43.88 ± 0.75	33.77 ± 1.15	**0.000**	32.87	75.19
HUFA	39.28 ± 7.17	3.05 ± 0.45	**0.004**	39.52 ± 0.52	9.47 ± 0.49	**0.000**	29.35	74.31
n−3	40.43 ± 8.00	9.91 ± 1.20	**0.005**	40.56 ± 0.85	30.86 ± 1.06	**0.001**	30.52	75.49
n−6	3.28 ± 0.51	0.94 ± 0.16	**0.004**	3.32 ± 0.11	2.90 ± 0.21	0.067	2.35	71.49
TFA	99.36 ± 17.72	32.04 ± 3.09	**0.006**				67.32	67.76
n−3:n−6	12.24 ± 0.66	10.67 ± 0.72	0.085	12.24 ± 0.66	10.67 ± 0.72	0.085		
DHA:EPA	1.33 ± 0.18	3.63 ± 0.75	**0.013**	1.33 ± 0.18	3.63 ± 0.75	**0.013**		
EPA:AA	22.27 ± 2.32	10.45 ± 3.09	**0.012**	22.27 ± 2.32	10.45 ± 3.09	**0.012**		

was observed in SFA (19.00 $\mu g\ mg^{-1}$ DW) rather than in MUFA (14.62 $\mu g\ mg^{-1}$ DW). Among fatty acids, 16:0, DHA and EPA were the main fatty acids to fulfill the energetic demands of seahorse embryos, with a decrease of 13.11, 11.66 and 11.60 $\mu g\ mg^{-1}$ DW, respectively. While the fatty acid profile of eggs was dominated by the fatty acids 16:0, DHA and EPA, and in lesser quantities by 18:1n−9, 18:0, 16:1n−7, 18:1n−7 and 22:5n−3, newborn seahorses were mainly rich in 16:0, DHA, 18:0 and 18:1n−9, having also significant amounts of EPA, 18:1n−7 and 20:4n−3. In what concerns essential fatty acids, higher AA, EPA and DHA concentrations were present in eggs, but differences in their proportions were only significant for EPA. Moreover, newborns presented a lower EPA:AA ratio and a higher DHA:EPA ratio than eggs.

4. Discussion

4.1. Parental investment in egg size and number

The reproductive success of a species is dependent on their investment in offspring production. While some species invest in a large number of small eggs, others prefer to produce a small brood with larger eggs. This seems to be the case of most teleosts fish with parental care (Goodwin et al., 2002), including seahorses. Both reproductive strategies can be successful in contributing with descendents to future generations and, generally, the reproductive success of a species arises from a compromise between female fecundity and egg quality.

Seahorse eggs are distinct from the spherical eggs of most teleosts by their pear-shape which, according to Carcupino et al. (2002), can increase the egg surface for gas exchange between the embryos and the male. Moreover, seahorses have in general relatively larger eggs than other marine teleosts (Foster and Vincent, 2004). Such an extremely high investment in reproduction is not surprising given that syngnathids, particularly the seahorses, present the most specialized form of parental care among fish. Their larger eggs result in relatively larger fingerlings, including those of other marine teleosts with parental care (Foster and Vincent, 2004). The large size of newborn seahorses results not only from the large size of eggs, but also from the lengthy embryonic development inside the brood pouch. Birth of seahorses at a more advanced stage may result in increased survival, due to a better ability to resist unfavorable environmental conditions, survive food deprivation and compete for resources. Nonetheless, a trade-off between offspring number and size occurs, with seahorse brood size being lower than the fecundity of other teleosts of similar size, even among marine teleosts with parental care (Foster and Vincent, 2004).

Egg, newborn and brood size vary considerably among seahorse species, from 0.9 to 2.0 mm in egg diameter, 2.0 to 16.2 mm in newborn length and 34 to 2000 in maximum brood size (Foster and Vincent, 2004). Within seahorse species, H. guttulatus seems to show an exceptionally high investment in brood quality instead of quantity, producing a relatively low number of juveniles (426 ± 128) but investing in larger eggs (2.41 ± 0.33 mm in EL) that result in larger fingerlings (15.53 ± 1.28 mm in SL). Larger eggs have larger yolk reserves and are expected to have a greater nutritional content, as observed in other marine organisms (e.g. Keckeis et al., 2000).

Besides the size of eggs and yolk reserves, their nutritional composition is also important to assess the reproductive success of a species. In fish and many other organisms, the yolk reserves of eggs are the only source of nutrients and essential compounds during embryonic development and must therefore meet all the nutritional requirements and metabolic needs during this endogenous feeding stage. In seahorses, which present the most specialized form of paternal care with the male becoming pregnant and incubating the eggs within his brood pouch, the male pouch has important respiratory, osmoregulatory and excretory functions, and play an important role in embryos nourishment (Linton and Soloff, 1964). Nevertheless, although paternally-derived inorganic compounds such as calcium are transferred to the embryos (Linton and Soloff, 1964), the yolk reserves seem to be the only source of nutrients during the development of seahorse embryos. The quantity and quality of egg reserves are therefore important parameters that will impact quality and survival of newborn seahorses.

4.2. Lipid and fatty acid profile of eggs

Although protein is the main component of eggs in marine organisms (Kamler, 1992), lipids play a central role in the embryonic metabolism, since they constitute a major source of metabolic energy and also of essential fatty acids for the formation of cell and tissue membranes (Sargent et al., 2002). Indeed, hatching and fertilization rates and early survival of fish larvae have been correlated with the fatty acid profile of eggs (e.g. Fernández-Palacios et al., 1995; Pickova et al., 1997; Bruce et al., 1999; Mazorra, 2000; Castell et al., 2001), particularly with increased levels of n−3 and n−6 HUFA and higher DHA:EPA ratios.

The lipid content and fatty acid composition of eggs vary considerably among species. Table 2 summarizes the main aspects of lipid and fatty acid composition of eggs of several marine fish species, and information on marine crustaceans is also presented, given their similarities with seahorse eggs in their lengthy development and large yolk reserves. Some data had to be converted to standard units to allow comparison among species.

Among the species listed in Table 2, fish eggs have in general lipid levels between 12 and 20% DW, although some exceptions can present values higher than 30% DW. Similar levels (10–17% DW) have also been mentioned as the typical lipid content of marine teleosts eggs by Craik and Harvey (1987) and Wiegand (1996). The TL content of H. guttulatus eggs (17.6% DW) is close to the upper limit of the typical range of fish roe and is comparable to the levels of decapods eggs, but the TFA content is lower. Since high-lipid levels can be essential in providing abundant energy for rapid embryonic development or flotation of pelagic eggs and early larvae (Eldridge et al., 1983), the demersal trait of seahorse eggs and their lengthy development within the brood pouch may therefore not justify a high-lipid content on a dry-weight basis. Nevertheless, given the larger size of seahorse eggs, the quantity of TL and TFA in each egg will be greatly higher than in other fish. Considering that seahorse development is dependent upon the yolk reserves until the juvenile stage is reach, while most fish hatch at the larval stage, such a high egg content will be essential to fulfill the high-lipid requirements expected for seahorse embryos.

Fish lipids are known to present high PUFA contents, but the fatty acid class composition of eggs may vary among species. Like most fish species listed in Table 2, H. guttulatus eggs were dominated by PUFA, although some species produce eggs with similar levels of SFA, MUFA and PUFA. Differences among species may reflect distinct habitat conditions and early life histories. For instance, while PUFA have important structural and physiological roles, SFA and MUFA are readily catabolized to generate metabolic energy (Sargent et al., 1999a) and may, therefore, be abundant in species with higher energetic demands. Moreover, fatty acid profiles are thought to reflect habitat temperature, and a higher degree of unsaturation seems to constitute an adaptation to cold water environments (e.g. Wiegand et al., 1991; Los and Murata, 2004). Since the MUFA:SFA ratio seems to be the main determinant of fluidity in membrane phosphoglycerides (Sargent et al., 2002), the preponderance of SFA rather than MUFA in H. guttulatus eggs may not be surprising in a species characteristic from temperate environments.

Seahorse eggs were dominated by the fatty acids 16:0 (20.0%), DHA (17.4%) and EPA (13.2%), and to a lesser extent by 18:1n−9, 18:0, 16:1n−7 and 18:1n−7. These fatty acids are typically the most quantitatively important fatty acids present in fish and decapods eggs, although there are slight differences in their order of abundance. This is not surprising given that 16:0 and 18:1n−9, together with EPA and DHA, are the main fatty acids of the phosphoglycerides that constitute cell membranes in fish in general and, since they are largely consumed during fish growth, they are also major sources of metabolic energy (Sargent et al., 2002).

Marine fish eggs, including the long-snouted seahorse eggs, are characterized by a high n−3 HUFA content, but also by a prevalence of DHA rather than EPA. Indeed, the DHA:EPA ratio in fish roe is generally higher than that of body triacylglycerols (Sargent et al., 2002), which is consistent with selective catabolism of EPA relative to DHA in fatty acid oxidative processes producing energy for gametogenesis. Nevertheless, H. guttulatus eggs had a lower DHA:EPA ratio (1.3) than other fish, due to an exceptionally high EPA proportion. Such a low ratio seems to be rather a trait of decapods eggs.

In contrast to n−3 HUFA, the importance of n−6 HUFA has been largely neglected and only recently has been shown (e.g., Bell and Sargent, 2003). Although a minor component of fish cell membranes, AA is particularly concentrated in fish eggs and newly-hatched larvae, and proved to be an essential fatty acid for normal growth and development of marine fish. Although AA is required in very small amounts, its concentration is likely to be species-dependent and influenced by environmental conditions, the life cycle and the life style of the species. Fish species that experience stressful conditions (at least at specific stages of the life cycle) have higher demands for AA, while species that occupy more constant and less turbulent environments need lesser AA levels (Sargent et al., 1999b). H. guttulatus eggs had a very low AA

Table 2
Total lipid content and fatty acid composition of eggs of marine fish and crustaceans (NL—neutral lipids; PL—polar lipids; Y—yolk; OG—oil globule).

	TL (% DW)	TFA (μg mg DW)	BFA (%)	SFA (%)	MUFA (%)	PUFA (%)	HUFA (%)	n−3 (%)	n−6 (%)	AA (%)	EPA (%)	DHA (%)	n−3: n−6	EPA: AA	DHA: EPA	Reference
Marine fish																
Hippocampus guttulatus	17.6	99.4	1.1	32.5	22.5	43.9	39.5	40.6	3.3	0.6	13.2	17.4	12.2	22.3	1.3	Present study
Centropomus undecimalis				34.5	31.5	34.9		23.8	9.5	3.7	2.4	13.7	2.5	0.6	5.8	Yanes-Roca et al. (2009)
Clupea harengus	14.6															Tocher et al. (1985)
Clupea harengus (NL)				30.6	33.5	32.9		30.8	2.1	0.6	9.7	17.1	14.7	16.2	1.8	Tocher et al. (1985)
Clupea harengus (PL)				32.4	15.0	48.7		47.1	1.6	1.0	13.7	31.4	29.4	13.7	2.3	Tocher et al. (1985)
Coryphaena hippurus	31.5															Ostrowski and Divakaran (1991)
Dentex dentex	24.4	168.4		27.4	24.8	47.8		43.0	4.8	1.5	5.3	25.7	9.0	3.6	4.8	Mourente et al. (1999)
Dicentrarchus labrax	30.7			22.3	39.1	38.5										Devauchelle and Coves (1988)
Gadus morhua (NL)				18.0	43.0	37.0		35.0	2.0	1.0	12.0	19.0	17.5	12.0	1.6	Fraser et al. (1988)
Gadus morhua (PL)				26.0	19.0	53.0		50.0	3.0	3.0	15.0	33.0	16.7	5.0	2.2	Fraser et al. (1988)
Hippoglossus hippoglossus	12.0			33.6	31.2	30.0		24.5	6.0	4.9	7.3	15.1	4.1	1.5	2.1	Falk-Petersen et al. (1989)
Morone saxatilis (Y)				28.6	26.9	36.5		31.6	4.9	3.3	8.6	19.9	6.4	2.6	2.3	Eldridge et al. (1983)
Morone saxatilis (OG)				8.5	58.6	25.1		21.4	3.7	1.3	6.4	10.0	5.8	4.9	1.6	Eldridge et al. (1983)
Pagrus major				29.3	31.5	38.3		29.8	8.5	1.0	7.7	17.8	3.5	7.7	2.3	Izquierdo et al. (1989)
Sarda sarda	19.4	142.0		29.0	26.1	44.9	35.8	39.0	5.9	1.1	8.4	23.0	6.6	7.6	2.7	Ortega and Mourente (2009)
Sebastes maliger	31.0	37.1		19.6	29.3	51.1		46.5	4.7	3.1	17.5	24.7	9.9	5.7	1.4	Sewall and Rodgveller (2009)
Solea senegalensis	16.3	103.1		30.6	20.7	48.8		43.5	5.3	1.8	5.0	29.8	8.3	2.8	5.9	Mourente and Vasquez (1996)
Sparus aurata				26.9	28.0	29.1				0.8	2.3	14.8	2.0	2.9	6.3	Naz (2009)
Thunnus thynnus	19.5	170.6		26.7	24.3	49.0	39.8	42.9	6.0	1.5	7.5	27.8	7.2	4.9	3.7	Ortega and Mourente (2009)
Marine crustaceans																
Alpheus saxidomus	19.4			37.5	25.6	36.3	25.3	24.5	7.9	5.1	16.5	2.4	3.1	3.2	0.1	Wehrtmann and Graeve (1998)
Chlorotocus crassicornis		122.9		37.2	31.9	29.9					7.2	12.3			1.7	Rosa et al. (2007)
Homarus gammarus	26.2	285.7	1.1	24.4	43.4	31.0	29.4	25.5	5.5	3.6	13.0	10.5	4.6	3.6	0.8	Rosa et al. (2005)
Lysmata seticaudata	17.1	140.7	1.8	30.5	29.5	38.3		29.0	9.3	4.5	11.5	8.5	3.1	2.6	0.7	Calado et al. (2005)
Macropipus tuberculatus		127.4		26.2	38.6	34.5					10.1	13.5			1.3	Rosa et al. (2007)
Nephrops norvegicus	15.2	285.4	1.1	24.7	44.3	29.9	27.1	25.4	4.5	2.0	7.6	14.8	5.6	3.8	1.9	Rosa et al. (2003)
Palaemon elegans	19.5	205.2		25.2	28.2	45.8	39.4	39.1	6.7	2.6	26.6	5.8		10.2	0.2	Morais et al. (2002)
Palaemon serratus	17.7	203.9		21.4	33.1	43.6	28.7	23.9	19.7	3.5	12.6	7.7	1.2	3.6	0.6	Morais et al. (2002)
Palaemonetes schmitti	21.5			36.0	23.9	40.1	33.9	31.9	4.9	3.1	17.8	11.6	6.5	5.7	0.7	Wehrtmann and Graeve (1998)
Plesionika martia martia	17.5	191.7		23.0	42.1	34.0	29.8	28.0	6.0	2.5	11.8	12.8	4.7	4.8	1.1	Morais et al. (2002)
Plesionika narval		151.8		32.2	40.7	26.4					5.4	7.1			1.3	Rosa et al. (2007)
Polybius henslowii		155.7		25.9	27.1	46.2					13.4	22.1			1.6	Rosa et al. (2007)

content (0.6%) and a extremely high EPA:AA ratio (22.3) when compared to other species (0.6–4.9% and 0.6–7.7, respectively). This low AA content would not be expected for a species that inhabit highly unstable environments, with great temperature and salinity fluctuations. Indeed, a direct relationship between the AA content of fish eggs and stressful conditions is not observed in some species present in Table 2. Other factors such as the competitive relationship between AA and EPA in eicosanoid production must have therefore a more decisive role.

Requirements for n−3 HUFA are quantitatively more significant than for n−6 HUFA. The dominance of fatty acids from the n−3 series is especially evident in *H. guttulatus* eggs, which present a n−3:n−6 ratio (12.2) considerable higher than most fish and decapods species (2.0–9.9). The optimal balance between these two series is extremely important due to competitive interactions in fatty acid biosynthesis. Excessive n−3 fatty acids can result in overly high EPA:AA ratios and, consequently, decreased eicosanoid actions, immunosuppressive effects and higher mortality (Gatlin, 2002). On the other hand, excessive n−6 PUFA can change the physiological state to a pro-inflammatory state, with many human diseases being associated with increase n−6 and decrease n−3 fatty acids intake (Bergé and Barnathan, 2005).

4.3. Lipid and fatty acid consumption during embryonic development

In the same way that the fatty acid profile of eggs varies among species, their patterns of consumption during development are also species-dependent. Decrease in TL and TFA contents and consumption of fatty acid classes and essential fatty acids during development were determined for several fish and decapods species and are presented in Table 3.

During embryogenesis, all nutrients required for growth, cellular and organ differentiation and metabolism have to be originated from the yolk reserves, until the onset of exogenous feeding. Since lipids are a major energetic source, the lipid content of eggs has been typically found to decrease during fish and crustacean development due to embryonic consumption. *H. guttulatus* was not an exception and embryonic consumption greatly decreased the lipid content from 17.6% DW in eggs to 5.0% DW in newborn seahorses. Moreover, their extremely high rate of TFA consumption (67.8%) was not observed in other fish and decapods species (23.4–59.8%, except for *Homarus gammarus*), thus reflecting the high requirements of seahorse development.

As lipids are broken down for use during development, the resulting fatty acids may be conserved as structural components of new tissues or metabolic compounds, modified into new fatty acids or consumed as energy sources. In some marine fish, fatty acids appear to be utilized in a non-selective way (in order of their abundance), while in other species some fatty acids are preferentially retained. In what concerns seahorse embryos, some fatty acids were selectively conserved, while the higher loss percentages were observed for the fatty acids present in lower amounts. Nevertheless, in terms of absolute amounts, 16:0, DHA and EPA were the main fatty acids to fulfill the energetic demands of seahorse embryos. Although they are the main fatty acids of the phosphoglycerides that constitute cell membranes in fish in general (Sargent et al., 2002), they seem also to have an important energetic role.

All fatty acids (SFA, MUFA and PUFA) can be metabolized for provision of energy during development of marine eggs but selective retention of HUFA has been quite consistently observed during development of marine fish, with MUFA being preferentially used for energetic purposes. In contrast, PUFA (including HUFA) were not selectively retained during *H. guttulatus* embryogenesis and constituted

Table 3
Decrease in total lipid content and embryonic consumption of fatty acids during development of marine fish and crustaceans, until the full consumption of the yolk-sac reserves.

	TL (Δ% DW)	TFA (%)	BFA (%)	SFA (%)	MUFA (%)	PUFA (%)	HUFA (%)	n−3 (%)	n−6 (%)	AA (%)	EPA (%)	DHA (%)	Reference
Marine fish													
Hippocampus guttulatus	12.6	67.8	73.7	59.1	65.1	75.3	74.4	75.6	71.4	73.3	87.9	67.8	Present study
Clupea harengus	−9.1												Tocher et al. (1985)
Coryphaena hippurus	5.7												Ostrowski and Divakaran (1991)
Dentex dentex	9.7	59.8		50.5	77.9	55.7		56.1	51.8	22.8	78.9	38.4	Mourente et al. (1999)
Gadus morhua	−10.6												Fraser et al. (1988)
Sarda sarda	1.5	23.9		27.3	31.9	17.0	15.6	19.4	1.5	−43.5	22.5	17.3	Ortega and Mourente (2009)
Sebastes maliger	5.3	54.5		54.2	53.2	55.4		56.1	49.3	39.5	60.8	52.5	Sewall and Rodgveller (2009)
Solea senegalensis	1.0	23.5		20.4	45.8	16.1		17.3	5.6	−24.6	40.0	9.2	Mourente and Vasquez (1996)
Marine crustaceans													
Chlorotocus crassicornis		38.4	31.3	27.4	47.7	42.9		48.2	25.6	83.3	75.3	50.3	Rosa et al. (2007)
Homarus gammarus	16.2	76.5	83.2	76.3	82.9	67.5	68.3	67.2	69.0	72.9	66.3	68.1	Rosa et al. (2005)
Lysmata seticaudata	10.8	44.6	46.8	45.2	51.0	39.1		39.0	39.3	27.0	27.0	37.2	Calado et al. (2005)
Macropipus tuberculatus		31.8	42.9	33.9	35.4	25.7		26.9	19.7	−43.8	28.1	16.9	Rosa et al. (2007)
Nephrops norvegicus	11.5	51.0	61.9	40.2	55.7	52.7	54.0	54.8	40.9	29.2	27.5	67.2	Rosa et al. (2003)
Palaemon elegans	11.4	29.0	27.8	26.5	33.0	28.0	27.8	28.0	27.7	40.0	28.9	22.9	Morais et al. (2002)
Palaemon serratus	13.6	57.2	63.2	50.6	68.3	52.0	45.0	41.5	64.3	87.5	30.4	51.9	Morais et al. (2002)
Plesionika martia martia	10.3	23.4	36.8	15.2	26.6	24.6	21.5	21.8	36.5	28.6	16.4	21.6	Morais et al. (2002)
Plesionika narval		48.0	61.5	48.3	50.8	42.9		45.5	38.5	28.0	47.0	42.1	Rosa et al. (2007)
Polybius henslowii		41.4	20.0	41.4	44.5	39.9		40.3	36.6	43.8	34.1	40.4	Rosa et al. (2007)

the major source of metabolic energy. Indeed, PUFA were consumed at a higher rate (75.2%) than the average rate of TFA consumption (67.8%), while SFA and MUFA were catabolized at a lower rate (59.2 and 65.2%, respectively).

Considering that AA, EPA and DHA are essential fatty acids for marine fish and are known to positively affect egg and larval quality, a preferential retention of these fatty acids would be expected during fish embryogenesis. Indeed, conservation of AA and DHA seems to be a common trait of fish development, although EPA is generally consumed at a higher rate than TFA. In contrast, n−3 and n−6 HUFA were not preferentially retained during *H. guttulatus* embryogenesis, being

used at rates equal to or higher than the average rate of TFA consumption. EPA and DHA have important structural and physiological roles, but are also expected to be a major energetic source in n−3 HUFA-rich marine eggs. Indeed, catabolism of EPA and DHA for energy has been reported during the early life history of many marine fish (Tocher, 2003).

4.4. Lipid and fatty acid profile of juveniles

Lipid composition of early development stages differs markedly among species (Table 4), as a consequence of specific needs during

Table 4
Total lipid content and fatty acid composition of early development stages of marine fish and crustaceans after consumption of the endogenous reserves (NL—neutral lipids; PL—polar lipids).

	TL (% DW)	TFA (μg mg DW)	BFA (%)	SFA (%)	MUFA (%)	PUFA (%)	HUFA (%)	n−3 (%)	n−6 (%)	AA (%)	EPA (%)	DHA (%)	n−3: n−6	EPA: AA	DHA: EPA	Reference
Marine fish																
Hippocampus guttulatus	5.0	32.0	0.9	41.0	24.3	33.8	9.5	30.9	2.9	0.5	5.0	17.5	10.7	10.4	3.6	Present study
Clupea harengus	23.7															Tocher et al. (1985)
Clupea harengus (NL)				30.7	37.4	28.8		27.1	1.7	0.4	6.8	15.6	15.9	17.0	2.3	Tocher et al. (1985)
Clupea harengus (PL)				31.3	15.8	50.6		49.0	1.6	1.0	13.9	32.5	30.6	13.9	2.3	Tocher et al. (1985)
Coryphaena hippurus	25.8															Ostrowski and Divakaran (1991)
Dentex dentex	14.7	67.7		33.7	13.7	52.7		46.9	5.8	2.9	2.8	39.4				Mourente et al. (1999)
Gadus morhua	20.5															Fraser et al. (1988)
Gadus morhua (NL)				15.0	27.0	56.0		52.0	4.0	3.0	13.0	35.0	13.0	4.3	2.7	Fraser et al. (1988)
Gadus morhua (PL)				27.0	18.0	53.0		49.0	4.0	4.0	14.0	32.0	12.3	3.5	2.3	Fraser et al. (1988)
Hippoglossus hippoglossus				25.5	28.0	44.6		40.4	5.8	4.4	11.0	25.4	7.0	2.5	2.3	Falk-Petersen et al. (1989)
Pagrus major				40.6	21.8	36.6		28.9	7.7	1.6	7.2	18.7	3.8	4.5	2.6	Izquierdo et al. (1989)
Sarda sarda	17.9	108.1		27.7	23.3	48.9	39.7	41.3	7.6	2.1	8.6	25.0	5.4	4.1	2.9	Ortega and Mourente (2009)
Sebastes maliger	25.7	16.9		19.7	30.1	50.1		44.9	5.3	4.1	15.0	25.8	8.5	3.7	1.7	Sewall and Rodgveller (2009)
Solea senegalensis	15.3	78.8		31.4	14.4	52.9		46.5	6.4	2.9	3.9	35.0				Mourente and Vasquez (1996)
Marine crustaceans																
Alpheus saxidomus				44.9	24.2	38.7	28.8	27.1	8.3	5.8	18.1	3.5	3.3	3.1	0.2	Wehrtmann and Graeve (1998)
Chlorotocus crassicornis		75.7		43.9	27.1	27.8					2.9	9.9			3.5	Rosa et al. (2007)
Homarus gammarus	10.0	67.0	0.8	24.7	31.6	43.0	39.8	35.7	7.3	4.2	18.7	14.3	4.9	4.5	0.8	Rosa et al. (2005)
Lysmata seticaudata	6.2	77.9	1.7	30.1	26.1	42.1	0.0	31.9	10.2	5.9	15.2	9.7	3.1	2.6	0.6	Calado et al. (2005)
Macropipus tuberculatus		86.9		25.3	36.5	37.5					10.6	16.5			1.6	Rosa et al. (2003)
Nephrops norvegicus	3.6	139.7	0.9	30.2	40.1	28.9	25.4	23.4	5.5	2.9	11.3	9.9	4.3	3.9	0.9	Rosa et al. (2003)
Palaemon elegans	8.1	145.7		26.1	26.6	46.4	40.0	39.6	6.8	2.6	26.6	6.2	5.9	10.1	0.2	Morais et al. (2002)
Palaemon serratus	4.1	87.2		24.8	24.6	49.0	36.8	32.7	16.4	3.2	20.5	8.6	2.0	6.5	0.4	Morais et al. (2002)
Palaemonetes schmitti				38.6	21.3	40.1	35.7	33.3	4.2	2.8	17.7	13.7	7.9	6.3	0.8	Wehrtmann and Graeve (1998)
Plesionika martia martia	7.2	146.8		25.4	40.3	33.5	30.6	28.5	5.0	2.1	12.9	13.1	5.8	6.0	1.0	Morais et al. (2002)
Plesionika narval		79.0		32.0	38.5	29.0					5.6	7.9			1.4	Rosa et al. (2007)
Polybius henslowii		91.3		25.9	25.7	47.4					15.0	22.5			1.5	Rosa et al. (2007)

embryonic development, at larvae hatching and after the onset of exogenous feeding. Some fish hatch as yolk-sac larvae and are therefore dependent on endogenous reserves, overcoming potential food restrictions in the environment and ensuring larvae development to less vulnerable stages. At the onset of exogenous feeding, species that inhabit less rich and more unstable environments are expected to produce larvae with higher lipid content, in order to survive unfavorable environmental conditions.

H. guttulatus newborns presented exceptionally low TL and TFA contents (5.0% DW and 32.0 µg mg^{-1} DW, respectively) when compared to seahorse eggs (17.6% DW and 99.4 µg mg^{-1} DW, respectively), due to the high consumption rates observed during embryonic development. Such low contents were not observed in other fish (TL: 14.7–25.8% DW; TFA: 67.7–108.1 µg mg^{-1} DW, except for *Sebastes maliger*), although similar TL values were common among decapods (3.6–10.0% DW). This may result from the extension of the lecithotrophic phase until the juvenile stage in seahorses, instead of the highly vulnerable larval stages of marine fish in general. Moreover, since this species inhabits shallow inshore waters with high food availability, newborn seahorses may not require as much lipid reserves as species that inhabit less rich environments.

Differences between species are also evident in the proportion of fatty acid groups. Most marine fish produce PUFA-rich eggs that will result in PUFA-rich larvae. Even some species that produce eggs with similar proportions of SFA, MUFA and PUFA may originate larvae with a fatty acid profile dominated by PUFA. In contrast, *H. guttulatus* newborns were rich in SFA due to a preferential consumption of MUFA and, particularly, PUFA during development. The high consumption of the essential n−3 and n−6 HUFA resulted in juveniles with extremely low levels of AA, EPA and DHA. While *H. guttulatus* eggs were characterized by EPA levels higher than those present in other marine fish eggs, the fatty acid profile of newborn juveniles was much less rich in EPA (5.0%) and DHA (17.5%) than the larvae profile of other fish at the starting of exogenous feeding (EPA: 7.2–15.0%, except for *Dentex dentex* and *Solea senegalensis*; DHA: 18.7–39.4%).

4.5. Lipid and fatty acid dynamics as a tool for aquaculture improvement

The establishment of a suitable protocol for seahorse culture that will allow achievement of good reproductive output and maintenance of high growth and survival rates in captivity is largely dependent on a good understanding of their nutritional requirements. The production of high-quality eggs and juveniles is mainly determined by the nutritional composition of eggs and, thus, by parental investment. In *H. guttulatus*, larger eggs have larger yolk reserves and are expected to have a higher nutritional content, producing larger and higher-quality juveniles that will have a higher survival capacity. Given the intraspecific differences in the reproductive output of the species, the selection of populations and individuals with higher reproductive potential (for instance, larger-size individuals and populations with high-fitness genes) will be essential to improve egg quality.

Moreover, the nutritional and physiological state of the broodstock may also affect parental investment in gamete production. Thus, a nutritionally suitable diet that fulfills all the nutritional requirements of males and, particularly, females is essential to produce high-quality gametes. One possible way of assessing the nutritional requirements for egg production is by determining the nutritional composition of eggs from wild stocks and manipulating the diets in order to reproduce a similar profile in eggs from captive fish. Considering that the extremely high-lipid requirements of seahorse embryos are accomplished through the production of larger eggs, instead of eggs with an extremely high-lipid content (on a dry-weight basis), the use of increasingly high-lipid diets will not be necessary to meet seahorse

requirements, thus avoiding the severe consequences of excessive lipid levels. An optimal diet for *H. guttulatus* reproduction should reflect the PUFA-rich profile of eggs. Extremely high levels of n−3 HUFA must be provided, while low AA concentrations should be sufficient to fulfill the n−6 HUFA requirements.

Independent of the high quality of eggs and newborn juveniles, the optimization of progeny nutrition is vital to achieve successfully rearing protocols, because diets must fulfill all the nutritional requirements of young seahorses at the start of exogenous feeding. Considering the exceptionally low lipid level of newborn seahorses, the importance of the nutritional quality of the first feed seems to be particularly important for rearing *H. guttulatus* juveniles. Given their long embryonic development, it seems likely that seahorses born at a suboptimal nutritional state, and juvenile diets must therefore reproduce the richer profile of the eggs. Indeed, eggs can be regarded as a valuable "black box", since they enclose all the information necessary to understand what happens before and after egg production, allowing us to go back to the past and detect unsuitable conditions during gametogenesis, and leading us into the future by foreseeing the performance of fingerlings during development, including their survival and growth rates, stress tolerance and malformation incidence.

5. Conclusion

Nutritional studies can provide important clues for the improvement of culture protocols via the manipulation of broodstock, larval and juvenile diets. Moreover, considering that egg viability and larval or juvenile fitness are dependent on their nutritional quality, biochemical composition of eggs, larvae or juveniles can be used as an important diagnostic tool to predict egg viability and larvae or juvenile rearing success. These lines of research can be particularly useful, and even essential, for overcoming important challenges in seahorse culture. Seahorses have currently an important conservational value and aquaculture potential, and information on the size and fatty acid composition of eggs and newborn juveniles and lipid dynamics during embryonic development can enable significant advances in broodstock management and juvenile production.

This study presents the first data on lipid composition and fatty acid dynamics during early development of seahorse eggs and juveniles, giving useful information on seahorse lipid requirements. Despite the special life features common to all seahorses, species-specific differences should not be disregarded and nutritional requirements should be assessed for each species. Moreover, regardless of the structural and metabolic importance of fatty acids during early development of *H. guttulatus*, lipid classes, proteins and free amino acids are also important aspects to consider. Not all classes of lipids are equivalent with respect to metabolic availability (MacFarlane and Norton, 1999) and studies on the dynamics of distinct lipid classes may provide greater knowledge on the amount of energy available to sustain growth and survival. Moreover, recent findings have shown that a pool of free amino acids is available as an additional endogenous resource in marine fish eggs (Fyhn, 1989; Rønnestad et al., 1994). Thus, further studies on nutritional requirements of seahorse species will allow improving rearing protocols and juvenile production, in order to face the global decline of natural populations.

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Prey-predator dynamics in seahorses:

deciphering fatty acid clues

Prey-predator dynamics in seahorses:

deciphering fatty acid clues

Filipa Faleiro[1,*] and Luís Narciso[1]

[1] Laboratório Marítimo da Guia, Centro de Oceanografia, Faculdade de Ciências da Universidade de Lisboa, Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal
* Corresponding author (Tel.: +351 214 869 211; fax: +351 214 869 720; e-mail:filipafaleiro@gmail.com)

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Understanding the flow of fatty acids between trophic levels can provide important clues on prey-predator dynamics and nutritional requirements of the species. This study investigates the fatty acid flow between enrichment emulsions, *Artemia* nauplii and *Hippocampus guttulatus* juveniles, and evaluates the nutritional value of enriched and unenriched *Artemia* for newborn seahorses. The fatty acid profile of *Artemia* and seahorses generally reflected the dietary composition, but fatty acids were not linearly transferred between trophic levels. The incorporation of dietary fatty acids showed to be a more complex process involving dietary composition, predator metabolism and nutritional requirements. *Artemia* composition resulted from a dynamic balance between what was assimilated and metabolized by the nauplii during enrichment. Prey fatty acids were incorporated in seahorses but HUFA, particularly DHA, were selectively retained to fulfill their high requirements. *Hippocampus guttulatus* newborns were not successfully reared on *Artemia* nauplii, not even on enriched *Artemia*, with low survival rates (15.0-26.7%) being observed in all feeding treatments. The high MUFA content and low DHA level of *Artemia* did not fulfill the high SFA and PUFA requirements of newborn juveniles, particularly their great DHA demands. Higher survivorship was obtained with enriched *Artemia* but no differences were detected in seahorse growth.

Keywords: Hippocampus guttulatus, juvenile seahorses, *Artemia* enrichment, fatty acid profile, survival, growth.

Abbreviations: AA – arachidonic acid (20:4n-6); BFA – branched fatty acids; DHA – docosahexaenoic acid (22:6n-3); DW – dry weight; EPA – eicosapentaenoic acid (20:5n-3); HUFA – highly unsaturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; SFA – saturated fatty acids; TFA – total fatty acids.

1. Introduction

Marine fish larvae have major and specific nutritional requirements that must be fully met by the provision of high quality live prey. At the start of the exogenous feeding, after depletion of the yolk reserves, diets should fulfill all the nutritional requirements of the fingerlings. Lipid requirements are particularly important, because lipids represent the most important energy source during embryonic development of marine fish, and are also a source of essential fatty acids for the synthesis of new cellular lipid (Sargent *et al.* 2002), being highly required during early development.

The development of high quality diets for marine fish larviculture is largely dependent on a good knowledge of the nutritional requirements of the fingerlings and on a clear understanding of the trophic dynamics between enrichment emulsions, zooplanktonic prey and predator fish. Live prey has an important role in accomplishing the high energetic requirements of fish larvae, but also in delivering essential fatty acids to the fingerlings, since these fatty acids cannot be synthesized by fish and must therefore be provided in the diet. Although the nutritional profile of the higher trophic levels is largely dependent on the composition of the lower levels, the flow of nutrients between trophic levels is greatly complex.

The lack of knowledge on larval nutrition has raised major difficulties in rearing marine fish larvae. The two most commonly used live prey, the rotifer *Brachionus* spp. and nauplii of the brine shrimp *Artemia* sp., have a poor nutritional composition, particularly in what concerns the essential fatty acids n-3 HUFA. Since enhanced larval survival and growth have been correlated with higher levels of HUFA, particularly EPA and DHA (e.g., Watanabe *et al.* 1989; Watanabe 1993; Salhi *et al.* 1994), enrichment products and protocols have been largely developed to overcome the nutritional deficiencies of these prey items.

Development of suitable protocols for seahorse culture has raised a growing interest worldwide, due to the global decline of natural populations and inclusion, in 2002, of seahorse species in the IUCN Red List and in the CITES Appendix II. Seahorse culture has a major role in reducing the pressure on wild stocks, assuring global demands and protecting the sustainability of natural populations, thus accomplishing both conservational and commercial goals. Although several progresses have been recently achieved, optimization of juvenile feeding still remains a major bottleneck in seahorse culture, at least for some species. The nutritional quality of the first feed seems to be particularly

important for rearing juvenile seahorses, given their exceptionally low lipid content at birth, as a result of their long embryonic development and high lipid requirements of seahorse embryos (Faleiro & Narciso 2010).

Artemia nauplii have been widely used to feed juvenile seahorses. Although low survivorship has been attained with newly-hatched nauplii (*Hippocampus* sp: Chang & Southgate 2001; *H. reidi*: Olivotto *et al.* 2008), several species have been successfully reared on enriched *Artemia* (*Hippocampus* sp.: Chang & Southgate 2001; *H. abdominalis*: Woods 2000a; Martinez-Cardenas & Purser 2007; *H. barbouri*: Payne 2003; *H. erectus*: Lin *et al.* 2008; *H. kuda*: Hilomen-Garcia *et al.* 2003). Nonetheless, enriched *Artemia* proved to be an unsuitable monodiet for some juveniles (*H. abdominalis*: Woods 2000b; *H. erectus*: Gardner 2003; *H. subelongatus*: Payne & Rippingale 2000), and alternative prey such as copepods showed to be a better feed for juvenile seahorses (*H. barbouri*: Wilson & Vincent 2000; *H. erectus*: Gardner 2003; *H. fuscus*: Wilson & Vincent 2000; *H. kuda*: Lin *et al.* 2006; Wilson & Vincent 2000; *H. reidi*: Olivotto *et al.* 2008; *H. subelongatus*: Payne & Rippingale 2000).

This study represents the first approach into the culture of the long-snouted seahorse *Hippocampus guttulatus* and evaluates the aquaculture potential of this species. The main aim of this study was to optimize the first feeding of the long-snouted seahorse, not only by evaluating the nutritional value of *Artemia* nauplii to newborn juveniles, but also by presenting a more clear understanding on the trophic dynamics between enrichment products, *Artemia* nauplii and juvenile seahorses, so that improvement of prey quality and seahorse feeding become a less obscure issue and an easier goal to achieve. The nutritional quality of newly-hatched and enriched *Artemia* was compared and their effect on juvenile survival, growth and fatty acid composition was analyzed during the first month of seahorse development.

2. Materials and methods

2.1. Experimental methodology

Pregnant seahorse males were collected by scuba diving (under official permission) in Ria Formosa lagoon (36°59'N, 7°51'W), south Portugal, in June 2007. Pregnant males were kept in a recirculated system and fed daily on live adult *Artemia*. Husbandry conditions were similar to those observed in their natural environment: salinity 34-35 g L^{-1}, water temperature 24-25 ºC, pH 8.2-8.3, photoperiod 14 L:10 D and constant aeration. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg L^{-1}, respectively).

Newborn juveniles were reared in a recirculated system composed of 10-L cylindrico-conical fiberglass tanks with an upward water flow. Inert plastic netting was provided for juvenile attachment. Seahorse density in tanks was 2 juveniles L^{-1} and culture conditions were the same used in the husbandry system.

Four feeding treatments were established in order to analyze the effect of *Artemia* enrichment on juvenile culture: newly-hatched nauplii (NHA) and *Artemia* enriched with Algamac-2000 (AEA), Culture HUFA (CEA) and Protein HUFA (PEA). Proximate analysis and fatty acid composition of the three

enrichment emulsions are described in Table 1. Three replicate tanks were setup for each treatment.

Table 1 – Proximate analysis and n-3 HUFA composition of the three enrichment emulsions used for *Artemia* nauplii supplementation

		Algamac 2000	Culture HUFA	Protein HUFA
Proximate Analysis	Protein (%)	20.2	36.0	42.0
	Fat (%)	38.1	17.0	18.0
	Carbohydrate (%)	17.1	35.0	28.0
	Ash (%)	20.4	3.0	3.0
	Moisture (%)	4.2	9.0	9.0
Fatty Acid Profile	EPA (%)	0.5	0.6	3.5
	DHA (%)	27.0	2.5	5.0

Artemia franciscana (Great Salt Lake) was cultured based on the Van Stappen (1996) protocol. *Artemia* enrichment was conducted for 24 hours, at a maximum density of 30 nauplii mL^{-1}, using the recommended concentrations for each product (Algamac-2000: 200 mg L^{-1}; Culture HUFA: 125 mg L^{-1}; Protein HUFA: 125 mg L^{-1}). Seahorses were fed to excess (3 nauplii mL^{-1}) and *Artemia* nauplii were replaced every day. Three *Artemia* samples from each treatment were frozen for fatty acid analysis.

Seahorse survival was analyzed daily during four weeks and juvenile growth was assessed at the end of the experiment. With this purpose, 30 newborns and 28-day old juveniles from each treatment were wet-weighed in a high-precision balance (to the nearest 0.01 mg) and measured by micrometric analysis under light stereomicroscope (to the nearest 0.05 mm). Standard length (SL) was determined as the sum of head (HL), trunk (TrL-straight line) and tail (TaL) lengths, according to Lourie (2003). Triplicate samples of newborn seahorses and 28-day old juveniles from each treatment were frozen for fatty acid analysis.

Frozen samples of *Artemia* nauplii and seahorse juveniles were freeze-dried for fatty acid analysis. Lipids were extracted based on the Bligh & Dyer (1959) method and esterified to fatty acid methyl esters according to Metcalfe & Schmitz (1961). The fatty acid methyl esters were injected into a capillary column Omega Wax 320 WCOT (30 m fused silica, 0.32 internal diameter) installed in a Varian Star 3400CX gas-liquid chromatograph (GLC). Helium was used as a carrier gas at a flow rate of 1 mL min-1. Oven temperature was 180 ºC for 7 min and then increased to 200 ºC (with a temperature gradient of 4 ºC min-1) over a period of 71 min. Both the split injector (100:1) and the FID detector were set at 250 ºC. GLC data acquisition and handling were performed using a Varian integrator 4290 connected to the GLC. Peak quantification was carried out with a Star Chromatography workstation, and the fatty acid C19:0 (nonadecanoic acid methyl ester) was used as internal standard. Peak identification was performed using well-characterized cod liver oil chromatograms as a reference.

At the end of the experiment, the males and survivor juveniles were released on their original habitat without injuries.

2.2. Statistical analysis

Fatty acid composition (both absolute and relative profiles) was analyzed for newborn seahorses, *Artemia* nauplii and 28-day old juveniles from the different feeding treatments. All fatty acids were considered but special emphasis was given to the essential fatty acids AA, EPA and DHA. Comparisons were also made for TFA content and fatty acid classes (BFA, SFA, MUFA, PUFA, HUFA, n-3 and n-6 fatty acids), as well as for the ratios n-3:n-6, EPA:AA and DHA:EPA.

Changes in the fatty acid profile between trophic levels were analyzed at two distinct levels: from the enrichment emulsions to the *Artemia* nauplii, by comparing the profile of the enriched nauplii (AEA, CEA and PEA) through Anova and Tukey's *a posteriori* test, and from the *Artemia* nauplii to the juvenile seahorses, by comparing their profile for each feeding treatment using the *t* test or its nonparametric analogue, the Mann-Whitney U test. A regression analysis was used to examine if juvenile composition (namely, fatty acid classes and essential fatty acids) was influenced by the profile of the diets.

In order to determine the best feeding for juvenile seahorses, prey and juvenile quality were compared between the different treatments through Anova and Tukey's *a posteriori* test. Prey quality was further evaluated by comparing the profile of each prey item with the profile of seahorse eggs (described by Faleiro & Narciso 2010), using the *t* test or the Mann-Whitney U test. Since the nutritional profile of eggs is expected to reflect the nutritional requirements of young juveniles, this comparison will allow the detection of potential gaps in the nutritional composition of each prey item. Juvenile quality was evaluated by comparing the composition of 28-day old juveniles with the profile of newborn seahorses using the *t* test or the Mann-Whitney U test. Additionally, daily survival, final growth (standard length and weight increase) and body proportions (HP – head, TrP – trunk and TaP – tail proportions) were compared between feeding treatments using Anova and Tukey's *a posteriori* test. Since no differences were found between treatments, enriched treatments were analyzed together and compared with the unenriched treatment using the *t* test or the Mann-Whitney U test.

All statistical analyses were performed for a significance level of 0.05.

3. Results

3.1. Fatty acid profile of Artemia nauplii, seahorse eggs and juveniles

Fatty acid composition of eggs, newborn seahorses, prey and juveniles from the different feeding treatments is presented in Tables 2 (absolute profile) and 3 (relative profile), and statistical differences between them are described in Table 4. The fatty acid profile varied greatly between eggs, newborn seahorses, *Artemia* nauplii and 28-day old juveniles. Eggs and newly-hatched *Artemia* presented the higher values of TFA (99.4±17.7 and 91.9±3.6 µg mg^{-1} DW, respectively), while newborns (32.0±3.1 µg mg^{-1} DW) and 28-day old juveniles (from 20.6±4.4 to 33.0±9.3 µg mg^{-1} DW) had the lower amounts. Enriched *Artemia* showed intermediate quantities, with TFA values varying between 53.4±2.8 and 75.3±5.9 µg mg^{-1} DW.

Except for eggs (rich in PUFA – 43.9%) and newborns (rich in SFA – 41.0%), fatty acid profiles were dominated by MUFA (between 39.8 and 51.4%). All groups presented low quantities of BFA (from 0.3 to 1.7%) and HUFA (between 3.4 and 10.5%, except for eggs), and showed a considerable higher proportion of n-3 fatty acids (from 17.1 to 40.6%) when compared to n-6 fatty acids (from 2.9 to 7.8%).

The fatty acid profile of eggs was dominated by the fatty acids 16:0, DHA and EPA, and in lesser quantity by 18:1n-9, 18:0, 16:1n-7, 18:1n-7 and 22:5n-3. Newborn seahorses were mainly rich in 16:0, DHA, 18:0 and 18:1n-9, having also significant quantities of EPA, 18:1n-7 and 20:4n-3. *Artemia* nauplii were characterized by the dominance of 16:0, 16:1n-7, 18:1n-9, 18:1n-7 and DHA, while 28-day old juveniles were rich in 16:0, 16:1n-7, 18:0, 18:1n-9 and 18:1n-7.

3.1.1. From enrichment products to Artemia nauplii

Artemia nauplii differed significantly among treatments. Compared to enriched *Artemia*, newly-hatched nauplii had higher fatty acid concentrations, particularly of BFA, SFA, MUFA and fatty acids from the n-3 series, which resulted in a higher TFA content. No differences were found in the essential fatty acids AA and DHA, but a higher EPA concentration was present in the NHA nauplii. In terms of fatty acid proportions, enriched nauplii had a lower MUFA content and higher SFA and PUFA levels, although differences were only significant for the SFA proportion. Significant differences were also detected in the essential fatty acid EPA, with higher proportions being observed in newly-hatched nauplii.

Besides differences between enriched and unenriched *Artemia*, some differences were also found among enriched treatments. The higher lipid content and DHA percentage of Algamac-2000 resulted in nauplii with a significantly higher TFA concentration and greater proportions of PUFA, HUFA and DHA. In contrast, the higher EPA content of Protein HUFA was not reflected in the composition of PEA nauplii, with higher EPA proportions being observed in AEA nauplii. AEA nauplii had a higher EPA:AA ratio, while CEA nauplii presented a lower n-3:n-6 proportion.

Regardless the dietary treatment, all diets had lower TFA contents than eggs. Compared to eggs, the fatty acid composition of *Artemia* nauplii (both absolute and relative profiles) was characterized by lower levels of SFA, PUFA, HUFA and n-3 fatty acids, but higher MUFA contents. While the fatty acid profile of eggs was dominated by PUFA, *Artemia* was rich in MUFA and presented a low PUFA content. In what concerns essential fatty acids, *Artemia* nauplii had lower amounts of AA, EPA and DHA, lower DHA:EPA ratios and higher EPA:AA ratios than eggs. Nevertheless, differences in the fatty acid composition between prey and newborn juveniles were particularly marked in NHA nauplii and less accentuated in AEA nauplii.

Table 2 – Fatty acid composition (absolute profile in µg mg^{-1} DW) of eggs, newborn seahorses, *Artemia* nauplii and 28-day old juveniles from the different feeding treatments

			NHA treatment		AEA treatment		CEA treatment		PEA treatment	
	Eggs	Newborns	Artemia	Juveniles	Artemia	Juveniles	Artemia	Juveniles	Artemia	Juveniles
Iso 15:0	0.18±0.14	0.07±0.00	0.63±0.02	0.21±0.08	0.37±0.08	0.07±0.05	0.25±0.01	0.04±0.05	0.19±0.13	0.08±0.07
Ant 15:0	0.05±0.04	nd	0.22±0.01	nd	0.12±0.01	nd	0.08±0.01	nd	0.12±0.01	nd
Iso 16:0	0.22±0.06	0.05±0.01	0.32±0.01	nd	0.20±0.02	nd	0.14±0.01	nd	0.17±0.01	0.03±0.05
Ant 16:0	nd	nd	nd	nd	0.03±0.04	nd	0.01±0.02	nd	0.03±0.05	0.04±0.06
Iso 17:0	0.64±0.16	0.18±0.03	0.42±0.02	nd	0.32±0.03	0.03±0.04	0.26±0.01	0.02±0.03	0.30±0.03	nd
BFA	**1.09±0.40**	**0.30±0.04**	**1.60±0.04**	**0.21±0.08**	**1.04±0.10**	**0.09±0.08**	**0.75±0.03**	**0.06±0.08**	**0.81±0.16**	**0.15±0.16**
12:0	nd	nd	0.01±0.01	nd	nd	nd	nd	nd	nd	nd
13:0	nd	0.09±0.11	0.08±0.00	nd	0.01±0.02	nd	nd	nd	0.02±0.02	nd
14:0	2.20±0.45	0.61±0.08	1.48±0.02	1.13±0.42	2.05±0.85	0.70±0.02	0.86±0.08	0.71±0.09	1.52±0.12	0.74±0.07
15:0	0.89±0.11	0.21±0.04	0.70±0.02	0.23±0.08	0.46±0.04	0.13±0.01	0.34±0.02	0.13±0.09	0.37±0.04	0.05±0.08
16:0	19.91±3.31	6.78±0.31	12.07±0.38	4.73±1.53	11.56±1.18	3.40±0.41	6.57±0.09	2.78±0.22	7.65±0.53	3.20±0.34
17:0	1.03±0.17	0.48±0.08	2.09±0.08	0.65±0.27	1.28±0.15	0.37±0.08	0.93±0.03	0.43±0.16	1.02±0.07	0.61±0.14
18:0	7.65±0.83	4.37±0.36	3.98±0.16	3.47±0.90	4.70±0.52	2.82±0.48	3.72±0.12	2.58±0.21	3.85±0.24	3.08±0.45
20:0	0.50±0.15	0.47±0.17	0.30±0.06	0.32±0.18	0.28±0.12	0.24±0.05	0.22±0.11	0.08±0.10	0.27±0.06	0.15±0.13
22:0	0.11±0.01	0.13±0.04	0.24±0.02	0.18±0.11	0.26±0.06	0.20±0.04	0.26±0.01	0.21±0.04	0.25±0.02	0.23±0.04
SFA	**32.29±4.71**	**13.14±0.77**	**20.95±0.64**	**10.71±3.00**	**20.60±1.72**	**7.86±1.01**	**12.91±0.34**	**6.91±0.57**	**14.95±1.02**	**8.04±1.14**
14:1n-5	0.08±0.08	nd	0.27±0.02	nd	0.15±0.02	nd	0.06±0.04	nd	0.13±0.15	nd
16:1n-7	5.48±1.13	1.09±0.11	18.26±0.72	3.44±1.45	11.24±0.75	1.91±0.35	7.19±0.27	1.45±0.43	8.28±0.90	1.67±0.49
16:1n-5	0.04±0.06	nd	0.15±0.01	nd	0.07±0.01	nd	0.02±0.02	nd	0.02±0.03	0.03±0.06
17:1n-8	0.38±0.10	0.07±0.03	0.60±0.03	nd	0.26±0.18	0.03±0.05	0.27±0.01	0.04±0.06	0.31±0.03	0.03±0.05
18:1n-9	9.80±2.02	3.85±0.34	14.36±0.64	4.96±1.70	9.91±1.44	3.33±0.54	9.04±0.19	3.20±0.54	9.83±0.77	3.58±0.54
18:1n-7	4.93±0.80	1.50±0.21	11.18±0.39	3.43±1.20	9.83±0.71	2.62±0.55	7.46±0.13	2.17±0.54	7.60±0.67	2.47±0.67
18:1n-5	0.12±0.03	0.05±0.04	0.08±0.01	nd	0.04±0.03	nd	0.02±0.02	nd	0.02±0.03	0.34±0.57
19:1n-8	0.05±0.04	0.01±0.01	0.58±0.07	nd	0.18±0.10	nd	0.18±0.02	nd	0.18±0.02	nd
19:1n-10	0.04±0.03	0.03±0.02	0.09±0.03	nd	0.11±0.06	nd	0.06±0.01	nd	0.02±0.03	nd
20:1n-9	0.66±0.13	0.43±0.17	0.78±0.06	2.90±3.30	0.67±0.07	0.65±0.23	0.68±0.19	0.81±0.54	1.04±0.19	0.63±0.38
20:1n-7	0.43±0.03	0.49±0.26	0.56±0.17	0.14±0.12	0.28±0.06	0.25±0.07	0.24±0.03	0.31±0.31	0.32±0.14	0.26±0.29
20:1n-5	0.06±0.10	0.08±0.10	0.04±0.05	nd	0.08±0.06	nd	0.06±0.08	nd	0.10±0.15	nd
22:1n-11	0.16±0.14	0.09±0.07	0.07±0.00	0.44±0.37	0.14±0.06	0.15±0.10	0.17±0.07	nd	0.42±0.13	0.27±0.17
22:1n-9	0.15±0.11	0.10±0.07	0.21±0.01	0.16±0.14	0.20±0.05	0.08±0.13	0.20±0.04	0.34±0.09	0.14±0.10	0.09±0.15
MUFA	**22.38±4.34**	**7.78±1.03**	**47.21±1.70**	**15.47±5.43**	**33.16±2.98**	**9.02±1.25**	**25.64±0.56**	**8.33±2.44**	**28.40±2.43**	**9.36±2.39**
16:4n-3	0.07±0.05	nd	nd	0.13±0.21	0.07±0.06	nd	0.02±0.02	0.05±0.07	0.02±0.03	nd
18:2n-6	1.94±0.42	0.53±0.06	3.72±0.20	0.98±0.25	2.40±0.41	0.73±0.18	3.97±0.19	0.99±0.17	2.62±0.23	0.80±0.09
18:3n-3	1.37±0.62	0.13±0.04	2.39±0.10	0.34±0.10	1.53±0.29	0.18±0.06	1.33±0.10	0.13±0.12	1.20±0.12	0.15±0.12
18:4n-3	0.74±0.23	0.03±0.03	0.80±0.04	nd	0.36±0.04	nd	0.23±0.05	nd	0.33±0.04	nd
20:2n-6	nd	nd	0.03±0.04	nd	nd	nd	nd	nd	nd	nd
20:3n-6	0.24±0.12	0.06±0.04	0.05±0.03	nd	nd	nd	nd	nd	nd	nd
20:4n-6	0.60±0.15	0.16±0.03	0.10±0.01	0.01±0.00	0.08±0.01	0.01±0.00	0.17±0.09	0.32±0.05	0.19±0.05	0.34±0.07
20:3n-3	0.18±0.03	0.09±0.02	0.20±0.03	nd	0.22±0.05	nd	0.14±0.05	nd	0.16±0.06	nd
20:4n-3	3.12±0.46	1.48±0.23	2.67±0.19	1.72±0.25	2.62±0.27	1.20±0.27	1.51±0.28	1.22±0.10	1.77±0.34	1.33±0.14
20:5n-3	13.09±2.94	1.61±0.22	12.11±0.61	2.31±0.34	8.88±0.60	1.37±0.25	6.01±0.62	1.38±0.24	6.66±0.78	1.66±0.32
21:5n-3	0.33±0.07	nd	nd	0.22±0.19	nd	nd	nd	nd	nd	nd
22:4n-6	0.52±0.09	0.18±0.04	nd	nd	1.10±0.95	0.40±0.06	0.02±0.03	nd	nd	nd
22:5n-6	nd	nd	nd	nd	nd	nd	nd	0.14±0.26	nd	0.18±0.31
22:5n-3	4.11±0.59	0.93±0.19	0.02±0.02	0.17±0.28	0.10±0.15	0.28±0.08	0.06±0.09	0.38±0.06	0.13±0.09	0.40±0.02
22:6n-3	17.29±3.33	5.61±0.79	0.08±0.06	0.77±0.41	3.18±3.07	1.53±0.28	0.67±0.54	0.69±0.07	1.49±0.70	1.08±0.43
PUFA	**43.60±8.33**	**10.82±1.36**	**22.16±1.23**	**6.65±1.09**	**20.53±4.45**	**5.70±1.16**	**14.12±2.05**	**5.30±1.36**	**14.58±1.99**	**5.94±1.27**
HUFA	**39.48±7.08**	**10.12±0.15**	**15.22±0.03**	**5.20±0.87**	**16.17±0.39**	**4.79±0.22**	**8.57±0.20**	**4.12±0.91**	**10.39±0.19**	**4.99±1.53**
n-3	40.30±7.82	9.89±1.20	18.27±0.95	5.66±0.90	16.96±3.78	4.57±0.92	9.96±1.73	3.85±0.54	11.76±2.00	4.61±0.64
n-6	3.30±0.52	0.93±0.16	3.89±0.28	0.99±0.25	3.57±0.67	1.13±0.23	4.16±0.32	1.46±0.87	2.81±0.28	1.33±0.87
TFA	99.36±17.72	32.04±3.09	91.92±3.58	33.03±9.30	75.33±5.89	22.67±3.41	53.42±2.79	20.60±4.35	58.74±4.43	23.50±4.81
n-3:n-6	12.24±0.66	10.67±0.72	4.70±0.09	5.77±0.87	4.73±0.15	4.03±0.09	2.39±0.22	3.02±1.07	4.24±0.85	4.31±1.75
DHA:EPA	1.33±0.18	3.63±0.75	0.01±0.01	0.35±0.16	0.36±0.32	1.12±0.00	0.11±0.07	0.50±0.09	0.22±0.08	0.68±0.30
EPA:AA	22.27±2.32	10.45±3.09	127.46±11.50	232.89±39.81	445.57±56.38	150.65±10.10	42.44±14.09	51.10±6.27	38.14±12.54	115.25±18.04

Table 3 – Fatty acid composition (relative profile in % TFA) of eggs, newborn seahorses, *Artemia* nauplii and 28-day old juveniles from the different feeding treatments

	Eggs	Newborns	NHA treatment		AEA treatment		CEA treatment		PEA treatment	
			Artemia	Juveniles	Artemia	Juveniles	Artemia	Juveniles	Artemia	Juveniles
Iso 15:0	0.18±0.13	0.21±0.01	0.69±0.00	0.64±0.10	0.49±0.11	0.30±0.22	0.46±0.03	0.17±0.24	0.32±0.23	0.36±0.26
Ant 15:0	0.05±0.04	nd	0.24±0.00	nd	0.16±0.00	nd	0.16±0.02	nd	0.20±0.01	nd
Iso 16:0	0.22±0.05	0.15±0.02	0.35±0.03	nd	0.26±0.03	nd	0.27±0.01	nd	0.30±0.01	0.14±0.20
Ant 16:0	nd	nd	nd	nd	0.04±0.05	nd	0.02±0.03	nd	0.06±0.08	0.16±0.22
Iso 17:0	0.65±0.06	0.57±0.12	0.46±0.00	nd	0.42±0.04	0.12±0.17	0.49±0.00	0.11±0.16	0.51±0.02	nd
BFA	1.10±0.27	0.93±0.15	1.74±0.03	0.64±0.10	1.37±0.12	0.42±0.32	1.40±0.03	0.28±0.40	1.39±0.28	0.65±0.56
12:0	nd	nd	0.01±0.01	nd	nd	nd	nd	nd	nd	nd
13:0	nd	0.27±0.38	0.08±0.00	nd	0.02±0.03	nd	nd	nd	0.03±0.04	nd
14:0	2.21±0.05	1.90±0.13	1.60±0.05	3.41±0.39	2.72±0.96	3.10±0.41	1.61±0.07	3.42±0.27	2.59±0.02	3.14±0.63
15:0	0.90±0.07	0.64±0.07	0.76±0.00	0.70±0.07	0.61±0.06	0.56±0.04	0.63±0.03	0.65±0.57	0.63±0.02	0.19±0.27
16:0	20.04±0.37	21.17±1.17	13.13±0.10	14.31±1.72	15.34±0.56	15.01±0.98	12.31±0.56	13.48±1.98	13.03±0.65	13.60±1.57
17:0	1.04±0.01	1.50±0.37	2.28±0.01	1.96±0.43	1.70±0.22	1.65±0.14	1.74±0.13	2.06±0.51	1.74±0.07	2.59±0.10
18:0	7.70±0.97	13.65±0.35	4.33±0.02	10.51±1.11	6.24±0.83	12.43±0.30	6.97±0.13	12.53±1.62	6.56±0.10	13.11±1.48
20:0	0.50±0.16	1.48±0.54	0.33±0.08	0.98±0.85	0.37±0.14	1.07±0.22	0.42±0.17	0.37±0.53	0.47±0.07	0.65±0.51
22:0	0.11±0.01	0.40±0.09	0.26±0.01	0.55±0.40	0.34±0.09	0.87±0.08	0.49±0.04	1.00±0.06	0.42±0.01	0.96±0.06
SFA	32.50±1.19	41.01±1.83	22.79±0.20	32.42±2.95	27.35±0.25	34.68±1.02	24.16±0.65	33.52±4.36	25.45±0.70	34.24±3.04
14:1n-5	0.08±0.11	nd	0.29±0.01	nd	0.20±0.02	nd	0.12±0.09	nd	0.22±0.23	nd
16:1n-7	5.52±0.37	3.41±0.05	19.86±0.10	10.42±2.05	14.92±0.86	8.41±0.83	13.47±1.07	7.05±0.58	14.10±1.27	7.11±0.74
16:1n-5	0.04±0.05	nd	0.16±0.01	nd	0.09±0.00	nd	0.03±0.04	nd	0.03±0.05	0.15±0.21
17:1n-8	0.38±0.04	0.23±0.07	0.65±0.01	nd	0.35±0.25	0.15±0.21	0.50±0.04	0.21±0.29	0.53±0.01	0.12±0.16
18:1n-9	9.86±0.30	12.03±0.09	15.62±0.09	15.01±2.02	13.15±2.22	14.67±0.43	16.91±0.54	15.52±0.64	16.73±0.50	15.22±0.92
18:1n-7	4.96±0.09	4.68±0.25	12.16±0.07	10.39±1.50	13.05±1.00	11.54±0.85	13.97±0.64	10.56±0.42	12.93±0.31	10.51±0.85
18:1n-5	0.12±0.01	0.15±0.13	0.09±0.01	nd	0.05±0.03	nd	0.03±0.04	nd	0.04±0.05	1.45±2.05
19:1n-8	0.05±0.03	0.03±0.05	0.63±0.05	nd	0.25±0.14	nd	0.33±0.04	nd	0.30±0.02	nd
19:1n-10	0.04±0.03	0.09±0.07	0.10±0.03	nd	0.15±0.07	nd	0.12±0.02	nd	0.03±0.04	nd
20:1n-9	0.67±0.07	1.33±0.39	0.85±0.09	8.77±8.48	0.89±0.06	2.87±1.50	1.28±0.28	3.93±1.63	1.78±0.28	2.67±1.41
20:1n-7	0.44±0.05	1.52±0.63	0.61±0.21	0.43±0.60	0.37±0.08	1.12±0.47	0.44±0.03	1.53±1.18	0.54±0.21	1.12±1.15
20:1n-5	0.06±0.08	0.24±0.34	0.04±0.06	nd	0.10±0.08	nd	0.10±0.15	nd	0.17±0.24	nd
22:1n-11	0.16±0.14	0.27±0.20	0.07±0.00	1.32±0.94	0.19±0.07	0.65±0.47	0.32±0.11	nd	0.71±0.27	1.13±0.80
22:1n-9	0.15±0.14	0.31±0.24	0.22±0.02	0.49±0.69	0.27±0.07	0.36±0.51	0.37±0.08	1.66±0.11	0.24±0.18	0.37±0.53
MUFA	22.52±0.50	24.29±0.82	51.37±0.22	46.83±4.67	44.02±4.44	39.78±0.94	48.00±1.75	40.46±3.12	48.35±1.97	39.85±2.32
16:4n-3	0.07±0.05	nd	nd	0.39±0.55	0.10±0.08	nd	0.03±0.04	0.26±0.37	0.04±0.05	nd
18:2n-6	1.95±0.07	1.66±0.07	4.05±0.07	2.97±0.31	3.19±0.60	3.23±0.38	7.44±0.07	4.81±0.59	4.46±0.22	3.41±0.54
18:3n-3	1.38±0.42	0.41±0.16	2.60±0.03	1.02±0.12	2.03±0.41	0.79±0.15	2.49±0.07	0.65±0.47	2.05±0.06	0.62±0.45
18:4n-3	0.75±0.16	0.11±0.08	0.87±0.02	nd	0.47±0.03	nd	0.44±0.06	nd	0.57±0.03	nd
20:2n-6	nd	nd	0.03±0.04	nd	nd	nd	nd	nd	nd	nd
20:3n-6	0.24±0.13	0.18±0.13	0.05±0.04	nd	nd	nd	nd	nd	nd	nd
20:4n-6	0.60±0.10	0.50±0.05	0.10±0.01	0.03±0.00	0.10±0.02	0.04±0.00	0.32±0.15	1.57±0.31	0.32±0.08	1.45±0.33
20:3n-3	0.18±0.02	0.27±0.05	0.22±0.03	nd	0.30±0.05	nd	0.25±0.07	nd	0.27±0.10	nd
20:4n-3	3.14±0.34	4.63±0.30	2.90±0.12	5.20±1.18	3.47±0.14	5.30±0.39	2.82±0.37	5.90±0.82	3.01±0.50	5.64±0.69
20:5n-3	13.17±0.91	5.02±0.96	13.18±0.15	6.99±1.19	11.78±0.16	6.03±0.40	11.25±0.57	6.69±0.28	11.33±0.83	7.06±0.72
21:5n-3	0.34±0.03	nd	nd	0.67±0.95	nd	nd	nd	nd	nd	nd
22:4n-6	0.52±0.14	0.57±0.06	nd	nd	1.46±1.16	1.76±0.05	0.04±0.05	nd	nd	nd
22:5n-6	nd	nd	nd	nd	nd	nd	nd	0.69±0.97	nd	0.78±1.11
22:5n-3	4.13±0.16	2.90±0.31	0.02±0.02	0.52±0.74	0.14±0.19	1.23±0.17	0.11±0.15	1.85±0.22	0.22±0.15	1.71±0.33
22:6n-3	17.40±1.27	17.52±0.74	0.09±0.07	2.33±0.82	4.22±3.76	6.77±0.45	1.26±0.92	3.33±0.64	2.54±1.10	4.60±1.74
PUFA	43.88±0.75	33.77±1.15	24.11±0.43	20.12±2.97	27.26±4.31	25.14±1.73	26.44±2.42	25.75±1.25	24.81±2.39	25.26±1.42
HUFA	39.52±0.52	9.47±0.49	3.44±0.14	6.43±2.01	5.61±1.53	8.32±0.55	3.80±0.86	10.51±1.75	4.00±0.83	9.95±3.26
n-3	40.56±0.85	30.86±1.06	19.87±0.28	17.14±2.82	22.51±3.70	20.14±1.33	18.65±2.23	18.68±1.51	20.03±2.68	19.62±1.81
n-6	3.32±0.11	2.90±0.21	4.23±0.14	2.98±0.31	4.74±0.61	5.00±0.41	7.79±0.21	7.07±2.48	4.79±0.30	5.64±2.66
n-3:n-6	12.24±0.66	10.67±0.72	4.70±0.09	5.77±0.87	4.73±0.15	4.03±0.09	2.39±0.22	3.02±1.07	4.24±0.85	4.31±1.75
DHA:EPA	1.33±0.18	3.63±0.75	0.01±0.01	0.35±0.16	0.36±0.32	1.12±0.00	0.11±0.07	0.50±0.09	0.22±0.08	0.68±0.30
EPA:AA	22.27±2.32	10.45±3.09	127.46±11.50	232.89±39.81	445.57±56.38	150.65±10.10	42.44±14.09	51.10±6.27	38.14±12.54	115.25±18.04

Table 4 – Statistical comparisons between the fatty acid profile (in terms of essential fatty acids and fatty acid classes) of eggs (E), newborn seahorses (N), *Artemia* nauplii (NA – NHA treatment, AA – AEA treatment, CA – CEA treatment, PA – PEA treatment) and 28-day old juveniles (NJ – NHA treatment, AJ – AEA treatment, CJ – CEA treatment, PJ – PEA treatment) from the different feeding treatments ("=" non significantly different; ">" significantly higher in the first group; "<" significantly lower in the first group)

		E/NA	E/AA	E/CA	E/PA	NA/AA/CA/PA	NA/NJ	AA/AJ	CA/CJ	PA/PJ	N/NJ	N/AJ	N/CJ	N/PJ	NJ/AJ/CJ/PJ
Absolute profile	20:4n-6	>	>	>	>	=	<	=	=	=	<	<	=	=	=
	20:5n-3	=	=	>	>	NA>AA>CA,PA	<	<	<	<	=	=	=	=	=
	22:6n-3	>	>	>	>	=	=	=	=	=	<	<	<	<	AJ>NJ,CJ,PJ
	TFA	=	=	>	>	NA>AA>CA,PA	<	<	<	<	=	<	<	=	=
	BFA	=	=	=	=	NA>AA,CA,PA	<	<	<	<	=	<	<	=	=
	SFA	>	>	>	>	NA,AA>CA,PA	<	<	<	<	=	<	<	<	=
	MUFA	<	<	=	=	NA>AA>CA,PA	<	<	<	<	=	=	=	=	=
	PUFA	>	>	>	>	=	<	<	<	<	<	<	<	<	=
	HUFA	>	>	>	>	=	<	<	<	<	<	<	<	<	=
	n-3	>	>	>	>	NA>CA,PA	<	<	<	<	<	<	<	<	=
	n-6	=	=	=	=	=	<	<	<	=	=	=	=	=	=
Relative profile	20:4n-6	>	>	>	>	=	<	=	=	=	<	<	=	=	=
	20:5n-3	=	=	=	=	NA>CA,PA	<	<	<	<	=	=	=	=	=
	22:6n-3	>	>	>	>	=	>	=	=	=	<	<	<	<	AJ>NJ,CJ
	BFA	<	=	=	=	=	<	<	<	=	=	=	=	=	=
	SFA	>	>	>	>	AA>CA,PA>NA	>	>	>	>	<	<	=	=	=
	MUFA	<	<	<	<	=	=	=	<	<	>	>	>	>	=
	PUFA	>	>	>	>	=	=	=	=	=	<	<	<	<	=
	HUFA	>	>	>	>	=	=	=	>	=	=	=	=	=	=
	n-3	>	>	>	>	=	=	=	=	=	<	<	<	<	=
	n-6	<	<	<	<	CA>NA,AA,PA	<	=	=	=	=	>	=	=	=
Fatty acid ratios	n-3:n-6	>	>	>	>	NA,AA,PA>CA	=	<	=	=	<	<	<	<	=
	DHA:EPA	>	>	>	>	=	>	>	>	=	<	<	<	<	AJ>NJ,CJ
	EPA:AA	<	<	<	<	AA>NA>CA,PA	<	>	=	<	<	<	<	<	NJ>AJ,PJ>CJ

3.1.2. From Artemia nauplii to juvenile seahorses

The composition of *H. guttulatus* juveniles was significantly influenced by the profile of the diets ($R^2=0.59$). Among treatments, this influence was extremely marked in AEA ($R^2=1.00$) and NHA ($R^2=0.84$) trials and less obvious in CEA ($R^2=0.50$) and PEA ($R^2=0.33$) treatments. In terms of fatty acid classes (Fig. 1), a significant correlation between prey and juvenile profiles was only detected for SFA ($R^2=0.93$), while HUFA levels presented the less obvious relationship. In what concerns essential fatty acids (Fig. 2), a positive correlation was only observed for AA ($R^2=0.99$) and DHA ($R^2=0.99$), and also for the DHA:EPA ratio ($R^2=0.96$).
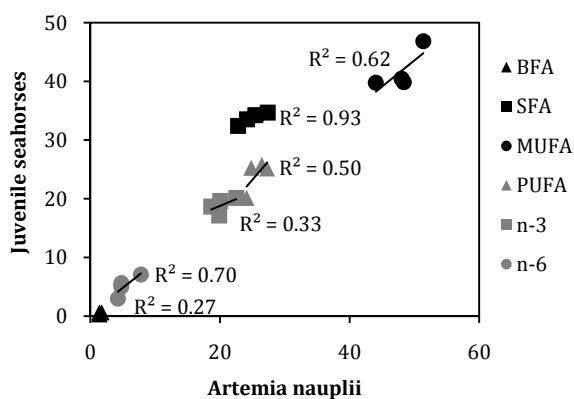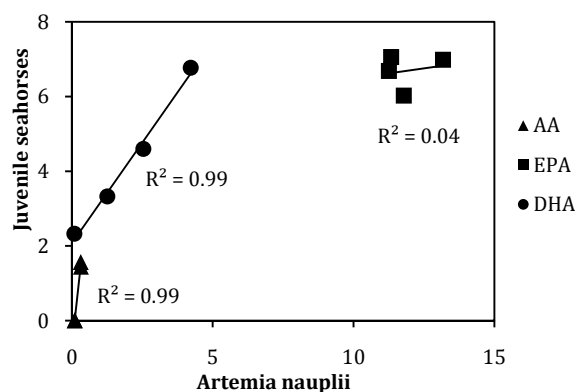
Fig. 2 – Correlation between the relative profile (%) of *Artemia* nauplii and 28-day old juveniles in what concerns essential fatty acids

The fatty acid composition of 28-day old juveniles somehow reflected the profile of the live feeds. SFA-rich newborn seahorses fed diets with a high MUFA content produced juveniles with high SFA levels but richer in MUFA. Whenever the proportion of the fatty acid classes was higher in prey than in newborn seahorses, the proportion increased during juvenile development, decreasing when prey values were lower than in newborns. The significant differences found in *Artemia* composition between treatments were generally reflected in 28-day old juveniles but were not sharp enough to be detected in the statistical analysis. Nevertheless, the transference of fatty acids from *Artemia* nauplii to juvenile seahorses varied with the diet. Although PEA nauplii had

Fig. 1 – Correlation between the relative profile (%) of *Artemia* nauplii and 28-day old juveniles in what concerns fatty acid classes

lower amounts of TFA, BFA, SFA, MUFA and PUFA than AEA nauplii, PEA juveniles presented higher concentrations than juveniles of the AEA treatment.

Compared to *Artemia* nauplii, 28-day old juveniles had a lower TFA content, due to lower concentrations of BFA, SFA, MUFA, PUFA, HUFA, n-3 and n-6 (except in the PEA treatment) fatty acids. Higher concentrations were only observed for the fatty acid 22:5n-3 in juveniles fed CEA and PEA. No differences were found in the essential fatty acids AA (except in the NHA treatment, with juveniles having lower amounts than *Artemia* nauplii) and DHA, but a lower EPA concentration was observed in 28-day old juveniles, resulting in a higher DHA:EPA ratio (except in the PEA treatment).

In what concerns fatty acid proportions, differences between prey and juvenile profiles were not so consistent among treatments. Lower juvenile proportions were found for BFA (except in the PEA treatment), for MUFA in the CEA and PEA treatments and for n-6 fatty acids in the NHA treatment, while higher values were observed for SFA in all treatments and for HUFA in the CEA treatment. Nevertheless, although differences were not significant, all juveniles presented a higher percentage of HUFA than the respective diets, even when PUFA proportions were lower than dietary levels. In terms of essential fatty acids, juveniles fed AA-rich prey (CEA and PEA nauplii) presented higher AA contents than the respective diets, while juveniles fed the less rich diets (NHA and AEA nauplii) showed insignificant AA levels, although differences were only significant for the NHA treatment. DHA proportions of 28-day old juveniles were higher than dietary levels (although statistical differences were only detected in the NHA treatment), but EPA proportions were lower, although juveniles fed nauplii with a lower EPA proportion presented values comparable to those observed in juveniles fed the EPA-rich diet (NHA nauplii).

Although the composition of *Artemia* nauplii was significantly different between treatments, very few differences were found in 28-day old juveniles among treatments. Seahorses fed CEA presented a higher 18:2n-6 proportion, while juveniles fed AEA nauplii had higher DHA levels (either in absolute and relative amounts) and, consequently, a higher DHA:EPA ratio. Significant differences were also detected in the EPA:AA ratio, with lower values in CEA juveniles and greater ratios in NHA seahorses.

Compared to newborn seahorses, 28-day old juveniles had a lower TFA content (except in the NHA and AEA treatments), due to lower levels of BFA (only in juveniles fed on AEA and CEA nauplii), SFA (except in the NHA treatment), PUFA, HUFA and n-3 fatty acids. No differences were found in the essential fatty acid EPA, although lower AA (only in juveniles fed on NHA and AEA nauplii) and DHA levels were detected in 28-day old seahorses, resulting in higher EPA:AA ratios (only in the NHA and AEA treatments) and lower DHA:EPA proportions. In terms of fatty acid proportions, older juveniles had higher MUFA contents and lower SFA (only significant for the NHA and AEA treatments) and PUFA proportions. The essential fatty acid EPA remained quite stable but older juveniles presented lower AA (except in the CEA and PEA treatments) and DHA proportions than newborn seahorses. Nevertheless, the differences between newborn and 28-day old juveniles were more evident in the NHA treatment and less accentuated in AEA juveniles.

3.2. Survival and growth of juvenile seahorses

Survival curves for the different feeding treatments are presented in Fig. 3. Juvenile survival was near 100.0% in the first five days, showing a pronounced decline after that. No differences were found in juvenile survival between dietary treatments. However, when comparing the unenriched with the enriched treatments, juveniles fed enriched *Artemia* showed a higher final survival (25.0±4.1%) than juveniles fed newly-hatched nauplii (15.0±10.8%).
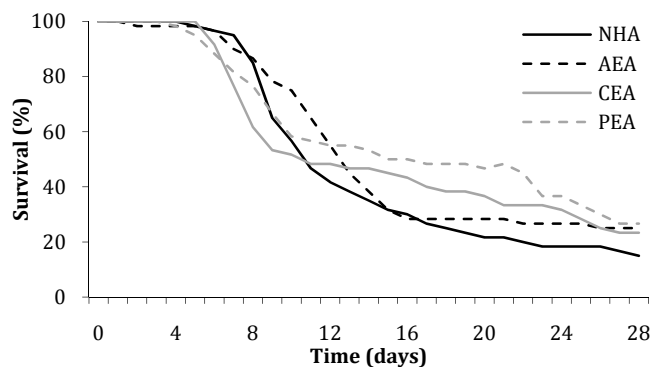

Fig. 3 – Survival curves of juveniles from the different feeding treatments

Newborn juveniles presented an average weight of 4.52±0.37 mg and standard length of 16.80±0.60 mm (HL: 3.31±0.15 mm; TrL: 5.02±0.25 mm; TaL: 8.46±0.34 mm). Body proportions were 21.5±0.7%, 28.7±0.9% and 49.9±1.0% for head, trunk and tail, respectively. After four weeks of culture, juveniles showed an average increase of 1021.4±425.0% in weight and 100.5±25.9% in standard length. Size of 28-day old juveniles from the different feeding treatments is presented in Fig. 4. Although a higher mean weight was observed in juveniles fed AEA nauplii, no significant differences were found in juvenile weight, length and body proportions among feeding treatments, neither between the unenriched and enriched treatments.
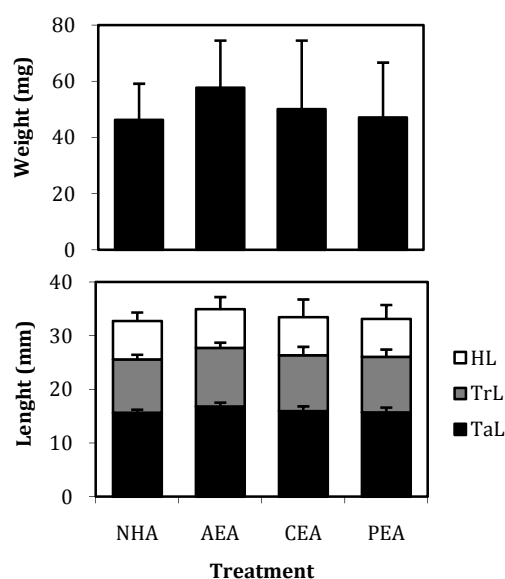

Fig. 4 – Weight and length of 28-day old juveniles from the different feeding treatments

4. Discussion

4.1. "Following" fatty acids through trophic levels

4.1.1. From enrichment products to Artemia nauplii

Suspension feeders like *Artemia* filter suspended matter and food particles from the water. The enrichment process, which consists in rearing the nauplii in a water emulsion with supplementing oils, results in nauplii ingesting the enriching preparation and, at least partially, assimilating and metabolizing it. After enrichment with n-3 HUFA-rich oils, *Artemia* nauplii are expected to have a higher percentage of HUFA and, consequently, a low percentage of SFA and MUFA. In this study, enriched *Artemia* presented in general higher PUFA and lower MUFA proportions, but had a significantly higher SFA proportion. This, together with the low TFA content of the enriched nauplii, indicates that the enrichment process can be improved.

The lipid composition of the emulsions was somehow reflected in the TFA content and n-3 HUFA profile of the enriched *Artemia*. The higher lipid content and DHA proportion of Algamac-2000 resulted in nauplii with a higher TFA concentration and greater proportions of PUFA, HUFA and DHA. In contrast, the higher EPA proportion of Protein HUFA was not reflected in PEA nauplii. Although PEA nauplii might have assimilated a higher EPA content, the high DHA proportion assimilated by AEA nauplii was likely converted to EPA during enrichment, resulting in a higher EPA proportion. Consequently, the DHA:EPA ratio decreased considerably from the enrichment emulsions to the enriched nauplii, a trend commonly observed during *Artemia* supplementation (e.g., Narciso *et al.* 1999).

The fatty acid profile of enriched *Artemia* seems therefore to result from a dynamic balance between what is assimilated and what is metabolized by the nauplii during enrichment. Since *Artemia* metabolism may overshadow the assimilation process, it was not possible to conclude that fatty acids were proportionally transferred from oils to enriched nauplii. Nevertheless, given the highly distinct patterns observed among studies (for instance, EPA and DHA proportions were found to reflect the dietary levels by Chang & Southgate (2001) and Estévez *et al.* (1999), while Pousão-Ferreira *et al.* (1997) found no relationship between the n-3 HUFA composition of the emulsions and the enriched nauplii), it is likely that fatty acids are not assimilated exactly by their order of abundance, and that their incorporation may vary with the enrichment product. Among other factors, the lipid content and the lipid class composition of the emulsions may affect fatty acid assimilation: increased lipid levels can depress the fatty acid synthesis through enzyme inhibition (Sargent *et al.* 1989), while high neutral lipid levels may reduce lipid digestion and fatty acid absorption (Morais *et al.* 2005).

4.1.2. From Artemia nauplii to juvenile seahorses

Marine fish have a limited ability to biosynthesize *de novo* fatty acids (Sargent *et al.* 2002), particularly at early development stages where biological functions may not be fully developed. Although the dietary composition is expected to have a strong effect on the fatty acid profile of the fingerlings, the flow of fatty acids from live prey to fish seems to be a non linear process, given all the different patterns of fatty acid assimilation observed among studies. In some cases the fry profile matched the profile of the diet (e.g., Chang & Southgate 2001) but, in general, this relationship was not so clear and dietary levels were only reflected in some fish fatty acids (e.g., Pousão- Ferreira *et al.* 1997; Morais *et al.* 2004).

The fatty acid composition of *H. guttulatus* juveniles somehow reflected the profile of the diets. SFA-rich newborns were fed MUFA-rich diets and, in a 28-day period, produced juveniles with high SFA levels but richer in MUFA. Whenever newborn juveniles had a lower fatty acid class proportion than the respective prey, their proportion increased through development, decreasing when prey presented lower values. However, the dietary effect was not as clear as the one observed in an unknown seahorse species (Chang & Southgate 2001). The significant differences found in the composition of *Artemia* nauplii between treatments were broadly reflected in 28-day old juveniles but were not sharp enough to be detected in the statistical analysis, which is not surprising given the decline in prey quality after nauplii hatching and throughout the feeding period.

Fatty acid assimilation was shown to vary with the diet: the flow of fatty acids from prey to juvenile seahorses was more linear in NHA and, particularly, AEA treatments, and less in CEA and PEA treatments. Indeed, PEA juveniles presented the highest fatty acid concentrations among enriched treatments despite their reduced dietary concentrations, indicating that incorporation of dietary fatty acids was improved in these juveniles. The reason why may rely, among other factors, on the total lipid content or lipid class composition of the diet.

The flow of fatty acids during juvenile feeding also varied with the fatty acid in question. While SFA were linearly transferred from prey to juvenile seahorses, the flow of MUFA and PUFA was less straightforward, particularly in what concerns HUFA. Indeed, it is interesting to notice that, although *H. guttulatus* juveniles had lower PUFA proportions than the respective diets, they presented a higher percentage of HUFA. Conservation of dietary HUFA is not surprising since n-3 and n-6 HUFA are essential fatty acids for marine fish, being particularly important during early development where the synthesis of new cellular lipid is higher.

Signs of n-3 and n-6 HUFA conservation have been commonly observed among marine fish species. Fingerlings generally present higher DHA, EPA and AA proportions than the respective diets (e.g., Pousão-Ferreira *et al.* 1997; Chang & Southgate 2001; Morais *et al.* 2004), particularly when dietary levels are low as in newly-hatched *Artemia*. Selective retention of n-3 and n-6 HUFA was also observed in *H. guttulatus* juveniles. Indeed, juveniles with small or trace amounts of DHA and EPA in the diet still have these essential fatty acids in considerable amounts. Nevertheless, the increase in DHA:EPA ratios indicates that DHA has a higher conservative pattern than EPA, evidencing their greater importance in fish development. Given that DHA is generally present at about twice the level of EPA in fish phospholipids, being particularly abundant in neural tissues (Sargent *et al.* 2002), fingerlings (including *H. guttulatus* juveniles) have higher requirements for DHA than EPA.

The importance of overcoming dietary deficiencies and increasing DHA levels is particularly clear in NHA juveniles. Since most ingested NHA nauplii are likely to lack DHA (because their extremely low DHA content, less than 0.1 µg mg^{-1} DW, is expected to rapidly disappear due to the retroconversion of DHA into EPA), the DHA present in NHA juveniles (0.8 µg mg^{-1} DW) is not derived from dietary retention but from yolk retention during seahorse development, although a limited capacity to biosynthesize DHA could also be consider, as reported in other fish (e.g., Linares & Henderson 1991). Moreover, it is interesting to notice that juveniles fed DHA-rich diets had lower DHA concentrations (AEA: 1.5 µg mg^{-1} DW; PEA: 1.1 µg mg^{-1} DW) than the respective diets (AEA: 3.2 µg mg^{-1} DW; PEA: 1.5 µg mg^{-1} DW), while nauplii and juveniles from the CEA treatment presented similar contents (ca. 0.7 µg mg^{-1} DW), very close to the DHA amount retained by the NHA juveniles. This may indicate that, although the optimal DHA concentration for *H. guttulatus* juveniles is expected to be around 17.3 µg mg^{-1} DW (Faleiro & Narciso 2010), a concentration of 0.7-0.8 µg mg^{-1} DW is likely to have important biological implications, constituting the least amount tolerated by young seahorses, although higher DHA levels are expected to greatly improve seahorse survival and growth rates.

4.2. Optimizing juvenile feeding for seahorse culture

4.2.1. Prey quality

Prey quality can be evaluated by comparing the fatty acid profile of live prey with the profile of seahorse eggs, since their fatty acid composition is expected to be indicative of the nutritional requirements of juvenile seahorses, at least during early development (Faleiro & Narciso 2010). Nevertheless, although dietary proportions should reflect the relative profile of the eggs, the absolute amounts have to be higher in prey so that equivalent amounts can be incorporated into juvenile body tissues.

H. guttulatus juveniles born at a highly nutritionally-depleted state and have therefore high nutritional requirements (Faleiro & Narciso 2010). Compared to seahorse eggs, *Artemia* nauplii had lower fatty acid contents, particularly the CEA and PEA nauplii.

Nevertheless, low fatty acid contents can be overcome with increased nauplii ingestion. Although the mechanisms that regulate ingestion rates are not well defined, it is likely that prey intake is regulated by the fatty acid amounts that are ingested rather than the number of nauplii. However, at the same time that higher ingestion rates can balance lower dietary contents, they can also reduce the assimilation efficiency due to shortened retention in the gut.

When the nutritional value of a diet is evaluated, fatty acid proportions have therefore a more important role than the TFA content. Unbalanced proportions will result in suboptimal levels of the less representative fatty acids, while dominant fatty acids will occur in excessive concentrations. Given the competitive interactions that occur between fatty acids, unbalanced proportions will affect fish welfare. Suboptimal DHA:EPA ratios will lead to EPA substituting DHA in tissue phospholipids (Mourente *et al.* 1991) and, ultimately, to high fish mortalities (e.g., Watanabe 1993),

while excessive EPA:AA ratios will inhibit AA synthesis and AA-derived eicosanoid production and, consequently, reduce immunosuppressive effects (Sargent *et al.* 1999; Sargent *et al.* 2002) and, eventually, fish survival (e.g., Erdal *et al.* 1991; Li *et al.* 1994).

Artemia nauplii proved to be a nutritionally poor prey for young seahorses. Their high MUFA content and relatively low PUFA levels are not fitting to fulfill the great PUFA and SFA requirements of newborn juveniles (ca. 43.9% and 32.5%, respectively). Moreover, the essential fatty acid levels in *Artemia* nauplii were generally above the great EPA (ca. 13.2% or 13.1 µg mg^{-1} DW) and DHA (ca. 17.4% or 17.3 µg mg^{-1} DW) amounts and the low AA levels (ca. 0.6% or 0.6 µg mg^{-1} DW) required by young seahorses.

Nevertheless, enrichment proved to be an essential tool to enhance the composition of *Artemia* nauplii. Unenriched *Artemia* presented the less suitable fatty acid profile, while AEA nauplii had the highest SFA and PUFA contents and the greatest DHA proportion. Indeed, Algamac-2000 has shown to be highly efficient in improving the quality of *Artemia* nauplii (e.g., Brown *et al.* 2000; Woods 2003; Calado *et al.* 2005), due to their extremely high DHA content. Achieving high DHA levels and DHA:EPA ratios has been the main challenge in prey optimization. Although enhanced enrichment conditions are expected to improve the fatty acid incorporation and the nutritional value of *Artemia* nauplii for the long-snouted seahorse, high quality *Artemia* will be difficult to achieve even with optimal enrichment conditions, given the poor nutritional quality of the nauplii and their inherent ability to convert DHA into EPA.

Moreover, it is now known that prey quality cannot be optimized without at the same time consider the relative proportions of phospholipids and triacylglycerols, as well as the global macronutrient composition of the diet. Marine fish have a limited ability to biosynthesize phospholipids *de novo*, having therefore a partial dietary requirement for intact phospholipids (Sargent *et al.* 2002). Since enrichment emulsions are rich in triacylglycerols that have lower n-3 PUFA content (Sargent *et al.* 1989), *Artemia* enrichment often results in excessive lipid contents and suboptimal phospholipids levels. On the other hand, deficiency or excess of essential amino acids will limit protein synthesis and fish growth (Rønnestad *et al.* 1999). Besides their poor fatty acid composition, *Artemia* nauplii also proved to be nutritionally incomplete in the essential amino acids histidine, methionine, phenylalanine and threonine (Hoff & Snell 1987).

4.2.2. Juvenile quality

The fatty acid composition of *H. guttulatus* juveniles changed considerably from birth to 28-days old. The distinct composition of newborns and 28-day old juveniles resulted almost certainly from dietary restrictions rather than changes in seahorse requirements during the first month of development. The poorer composition of *Artemia* nauplii was responsible for decreasing the fatty acid concentrations of the newborns (except for MUFA), which are expected to be in a nutritionally-depleted state. Indeed, the low lipid content of enriched *Artemia* was responsible for decreasing the TFA content from 32.0 µg mg^{-1} DW in newborns to 20.6-23.5 µg mg^{-1} DW in 28-day old juveniles; even the richer

lipid content of newly-hatched nauplii was insufficient to increase the depleted levels of newborn seahorses.

Changes were also observed in the relative composition of *H. guttulatus* juveniles, from SFA-rich newborns to MUFA-rich 28-day old juveniles. Moreover, the unbalanced n-3 HUFA profile of the *Artemia* nauplii led to increased EPA proportions, decreased DHA levels and, consequently, lower DHA:EPA ratios in the older juveniles. Suboptimal concentrations were particularly evident for the essential fatty acid DHA. Juvenile concentrations (0.7-1.5 µg mg⁻¹ DW) and proportions (0.7-1.5%) were far below the levels of newborn seahorses (5.6 µg mg⁻¹ DW and 17.5%, respectively), indicating that dietary DHA must exceed considerably the levels present in the diets (0.1-3.2 µg mg⁻¹ DW and 0.1-4.2%, respectively). Indeed, juvenile concentrations were far below the minimum level supporting optimal survival of *Hippocampus* sp. fry (6.4 µg mg⁻¹ DW), even though growth greatly increased when juvenile levels were above 7.6 µg mg⁻¹ DW (Chang & Southgate 2001).

The fatty acid profile of 28-day old juveniles was suboptimized in all feeding treatments, but higher-quality juveniles were obtained in the AEA treatment. AEA juveniles had a superior fatty acid class (lower MUFA and higher SFA and PUFA proportions) and n-3 HUFA (greater DHA concentrations and DHA:EPA ratios) composition, while NHA juveniles presented the worst fatty acid profile. Given the important biochemical, cellular and physiological functions of n-3 HUFA, suboptimal fatty acid concentrations will have major costs to fish development and welfare.

4.2.3. Juvenile survival and growth

Although seahorses have been successfully reared (*Hippocampus* sp.: Chang & Southgate 2001; *H. abdominalis*: Woods 2000a; Martinez-Cardenas & Purser 2007; *H. barbouri*: Payne 2003; Wilson & Vincent 2000; *H. fuscus*: Wilson & Vincent 2000; *H. kuda*: Wilson & Vincent 2000; Job *et al.* 2002; Lin *et al.* 2006; *H. whitei*: Wong & Benzie 2003), some species appear to be particularly sensitive to rearing conditions (*H. reidi*: Forteath 1996; Gardner 2003; Olivotto *et al.* 2008), which seems also to be the case of *H. guttulatus* juveniles. Survival rates of newborns varied between 15.0 and 26.7% during the first month and were far below the rates observed for most seahorses. Distinct survival rates among species may arise from specific nutritional demands and their capacity to tolerate culture conditions.

The top illumination and the upward water flow in the tanks may not be the most suitable conditions for seahorse culture, since may lead to *Artemia* congregating near the water surface and juveniles ingesting air when preying on it. Indeed, gas bubbles were detected inside the digestive tract of some juveniles, which may cause buoyancy problems and restrict feeding. Nevertheless, the insignificant mortality recorded during the first five days and the sudden decline observed in juvenile survival after that indicate that the feeding regime was the major cause of low survivorship. Newborn seahorses depend entirely on exogenous feeding sources to fulfill their nutritional requirements but, in starvation conditions, the nutrient reserves in their body tissues will allocate enough energy for metabolism and survival during a limited period. Exhaustion of the maternally-derived nutrient reserves of *H. guttulatus* newborns near day 5 is in conformity with the 5.3 days average survival of *H. guttulatus* juveniles under starvation conditions (unpublished data) and with the 5-day period of full survival observed in other species (*H. barbouri*: Payne 2003; *H. kuda*: Sheng *et al.* 2007; *H. subelongatus*: Payne & Rippingale 2000; *H. trimaculatus*: Sheng *et al.* 2007).

Starvation during feeding may arise from problems in prey ingestion and digestion, but also from unsuitable dietary compositions. Since prey ingestion was confirmed with the presence of *Artemia* nauplii in seahorse gut, prey digestion seems therefore a more feasible cause for the low survivorship of *H. guttulatus* juveniles. Because *Artemia* is not a natural prey for seahorses, juveniles may be unable of digesting them. Moreover, the high content of triacylglycerols (lipids less easy to digest than phospholipids) in *Artemia* nauplii (Navarro *et al.* 1999) may strengthen their poor digestion. Indeed, *H. subelongatus* (Payne & Rippingale 2000) and *H. abdominalis* (Woods 2003) juveniles proved to be unable to digest *Artemia*, with nauplii passing intact through the digestive tract and, occasionally, remaining alive.

Although juveniles may have some problems in digesting *Artemia*, the poor nutritional quality of the nauplii seems also responsible for juvenile mortality, given the different survival rates observed in juveniles fed newly-hatched and enriched nauplii. Low survivorship has been obtained for seahorses fed newly-hatched *Artemia* (*Hippocampus* sp: Chang & Southgate 2001; *H. reidi*: Olivotto *et al.* 2008) but, despite some exceptions (*H. erectus*: Gardner 2003; *H. subelongatus*: Payne & Rippingale 2000), juveniles have been successfully reared on enriched nauplii (*Hippocampus* sp.: Chang & Southgate 2001; *H. abdominalis*: Woods 2000a; Martinez-Cardenas & Purser 2007; *H. barbouri*: Payne 2003; *H. erectus*: Lin *et al.* 2008; *H. kuda*: Hilomen-Garcia *et al.* 2003).

H. guttulatus newborns were not successfully reared on *Artemia* nauplii, not even on enriched *Artemia*. Although both enriched and unenriched nauplii had unsuitable fatty acid compositions for newborn seahorses, enrichment was responsible for improving nauplii composition (increased DHA contents and DHA:EPA ratios) and rising juvenile survival from 15.0 to 25.0%. A positive effect of dietary n-3 HUFA, particularly DHA, has been commonly observed on survival (e.g., Watanabe *et al.* 1989; Watanabe 1993; Salhi *et al.* 1994; Southgate & Kavanagh 1999) and growth (e.g., Watanabe *et al.* 1989; Izquierdo *et al.* 1992; Mourente *et al.* 1993; Watanabe 1993) of marine fingerlings, including juvenile seahorses (e.g., Chang & Southgate 2001; Payne 2003; Wong & Benzie 2003). Moreover, even though differences in seahorse growth were not significant between treatments, the higher mean weight of juveniles fed AEA nauplii (the most DHA-rich prey) may become more obvious later in seahorse development.

Nevertheless, the role of dietary n-3 HUFA on the improvement of fish survival and growth is not totally clear. *Solea senegalensis* larvae fed unenriched *Artemia* (deficient in essential fatty acids) showed a better growth than larvae fed enriched nauplii (higher HUFA content and DHA:EPA ratio) (Morais *et al.* 2004). Moreover, high dietary HUFA levels did not promote higher survival or growth rates in pipefish juveniles (Payne *et al.* 1998). In what concerns

seahorses, growth rates of 6-month old *H. abdominalis* juveniles were optimized with both the lowest and the highest DHA:EPA ratios (Woods 2003). Indeed, as far as the requirement of essential fatty acids for somatic growth is accomplished, an excess of n-3 HUFA may cause negative effects in fish (Corraze 2001).

5. Conclusion

Although the fatty acid profile of *Artemia* nauplii and *H. guttulatus* juveniles generally reflected the dietary composition, fatty acids were not linearly transferred from lower to higher trophic levels. The incorporation of dietary fatty acids proved to be a more complex process that varies with the diet and with the fatty acid in question. The characteristics of the diet seem to regulate the extent to which fatty acids are assimilated, while their incorporation appears to result from a trade-off between dietary input and nutritional requirements. At the zooplankton level, the composition of *Artemia* nauplii seemed to result from a balance between what was assimilated and metabolized by the nauplii during the enrichment process, with fatty acid assimilation varying with the enrichment emulsion. At the next trophic level, incorporation of dietary fatty acids in seahorse tissues varied among fatty acids and dietary treatments, depending on the capacity to conserve and synthesize each fatty acid, so that nutritional requirements could be met when dietary levels are suboptimized.

H. guttulatus newborns were not successfully reared on *Artemia* nauplii, not even on enriched *Artemia*. Even though enrichment enhanced the composition of newly-hatched nauplii, the MUFA-rich profile of *Artemia* was unsuitable to fulfill the high PUFA and SFA seahorse requirements. Although juvenile survivorship was far behind the desired rates, results help to clarify some aspects of seahorse feeding and give important clues to optimize prey quality and fulfill seahorse requirements, opening space for further investigation. Survival and growth rates of the long-snouted seahorse are expected to increase with optimization of the rearing system (e.g., light and water flow conditions), *Artemia* enrichment (e.g., cysts quality, emulsion composition, supplementation level and duration) and feeding procedures (e.g., photoperiod and feeding frequency). Nevertheless, given the indigestibility, poor composition and metabolism of the *Artemia* nauplii, further studies should also evaluate the potential of alternative prey items such as copepods, the main component of the diet of wild seahorses (Tipton & Bell 1988) and a suitable prey to rear juvenile seahorses due to their greater digestibility and superior nutritional composition (e.g., Payne & Rippingale 2000; Wilson & Vincent 2000; Gardner 2003; Lin *et al.* 2006; Olivotto *et al.* 2008).

Acknowledgments

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The disadvantages of mating outside home:

implications of captive breeding for seahorse reproduction

The disadvantages of mating outside home:

implications of captive breeding for seahorse reproduction

F. Faleiro[1,*] and L. Narciso[1]

[1] Universidade de Lisboa, Faculdade de Ciências, Centro de Oceanografia, Laboratório Marítimo da Guia,
Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal
[*] Corresponding author (e-mail: filipafaleiro@gmail.com; phone: +351214869211; fax: +351214869720)

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Mating outside home may have a negative impact on the reproductive success of the species. This study evaluates the effect of captive breeding on some quantitative and qualitative aspects of the reproductive success of the long-snouted seahorse, *Hippocampus guttulatus*. Wild and captive broods were compared in terms of juvenile number, size, condition and fatty acid profile at birth. Reproductive success of *H. guttulatus* was highly affected by the captive environment. Juveniles from captive broods were fewer in number (254.6±134.8 juveniles), smaller (2.75±0.63 mg of weight and 14.23±1.07 mm in SL), generally thinner and with lower fatty acid contents (per juvenile) than those from wild broods (425.7±124.5 juveniles, 3.69±0.94 mg and 15.53±1.28 mm, respectively), although their fatty acid composition (µg mg^{-1} DW or %TFA) was not significantly affected. In captivity, reproductive quality took priority over the quantity, as seahorses preferred to counter energetic restrictions by reducing the amount of energy reserves in eggs and juveniles in order to maintain a suitable composition. This study reveals some negative impacts that captive breeding can have on the reproductive success of seahorses, and gives important clues to improve the quantity and quality of the offspring and, consequently, the success of commercial production and reintroduction programs in the wild.

Keywords: captive breeding, fatty acid, *Hippocampus guttulatus*, reproductive success, seahorse, wild.

Introduction

In captive environments, husbandry conditions are very distinct from those experienced by fish in the wild, and may therefore have a significant effect on several aspects of fish life, including their behavior (e.g., Mathews et al. 2005; Pearsons et al. 2007; Blanchet et al. 2008; Kydd and Brown 2009), morphology (e.g., Blanchet et al. 2008), physiology (e.g., reviewed in Portz et al. 2006; Antilla et al. 2008), survival (e.g., Srivastava and Brown 1991; Kostow 2004) and reproduction (e.g., Srivastava and Brown 1991; Harrell and Woods 1995; Palacios et al. 2007; Faleiro et al. 2008; Ozaki et al. 2008; Seaborn et al. 2009). The reproductive success of fish can be affected by the captive environment in many different ways, including the size of eggs and fingerlings, fecundity, fertility, fry survival and nutritional composition. Even though some species have proven to be well adapted to captivity by presenting a similar reproductive performance in both wild and captive environments (e.g., Harrell and Woods 1995; Martínez-Chavez et al. 2003; Kjesbu 2006), in most cases the quantity and quality of eggs and fingerlings are negatively affected by the captive conditions (e.g., Srivastava and Brown 1991; Harrell and Woods 1995; Palacios et al. 2007; Ozaki et al. 2008; Seaborn et al. 2009).

Breeding seahorses in captivity has raised a great interest worldwide. Habitat degradation and intensive exploitation for traditional medicines, aquarium trade and curios are threatening seahorse populations everywhere. The great extent and economic importance of seahorse exploitation, along with the global decline of natural populations, have raised the need for an alternative source of seahorses, which replaces harvesting of natural populations and assures, at least to some extent, the commercial demands. Besides reducing the pressure on wild stocks, captive breeding can be further applied in population supplementation programs if a last-resort recovery strategy becomes necessary.

Seahorses have been successfully bred in captivity (e.g., Vincent 1995; Masonjones and Lewis 1996; Lockyear et al. 1997; Silveira 2000; Woods 2000; Payne and Rippingale 2000; Faleiro et al. 2008). However, the success of captive breeding is far beyond the feat of breeding in captive conditions, being largely dependent on the quantity and quality of the offspring produced. Unsuitable or suboptimal husbandry conditions may reduce the reproductive success of cultured fish and jeopardize the commercial feasibility of seahorse culture and the success of supplementation programs in the wild.

This study intends to evaluate how the captive environment affects the reproductive success of seahorses. Understanding how male and female seahorses respond to the generally restrictive conditions in captivity will help to improve the captive environment and, consequently, the reproductive output of captive seahorses. The effects of captive breeding on the quantity and quality of the offspring were studied for the long-snouted seahorse, *Hippocampus guttulatus*. This species was known to present one of the most dense seahorse populations in the world, located in the Ria Formosa lagoon, southern Portugal (Curtis and Vincent 2005). However, the current sustainability of this large population seems to be jeopardized. Degradation of seagrass

habitats along with directed and by-catch fisheries represent a significant long-term threat to this species (OSPAR, 2005).

Materials and methods

Experimental methodology

Hippocampus guttulatus were collected by scuba diving (under official permission) in Ria Formosa lagoon (36°59'N, 7°51'W), south Portugal, between June and October 2009. Pregnant males were placed in a recirculated incubation system composed of 27 L aquariums with independent juvenile collectors. Females and non-pregnant males were kept in a recirculated reproduction system composed of 180 L aquariums (50x40x90 cm) with 2 males and 2 females each. Artificial plants were provided for seahorse attachment. Husbandry conditions were similar to those observed in their natural environment: photoperiod 14L:10D, temperature 24-25 ºC, salinity 34-35 psu, and pH 8.2-8.3. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg L^{-1}, respectively). Adults were fed *ad libitum* twice a day on live adult *Artemia* during the acclimatization week and on frozen enriched adult *Artemia* and *Mysis* in the following weeks. Proximate analysis of the frozen diets is described in Table 1. The aquariums were cleaned every day and 10% water changes were made weekly.

Table 1 – Proximate analysis of frozen diets of adult seahorses in captivity

	Artemia	*Mysis*
Crude protein (%)	5.0	10.2
Crude fat (%)	1.0	1.1
Crude fiber (%)	0.9	0.4
Moisture (%)	92.0	87.7
Ash (%)	1.1	0.6

After birth, seahorse broods were studied in terms of juvenile number, size, condition and fatty acid composition. Males captured pregnant and respective broods (n=20) were classified as "wild", while males that mated in captivity and respective broods (n=7) were designated as "captive". In the "wild" treatment, gamete production and mating took place exclusively under natural conditions, but incubation by pregnant males occurred partially in captivity. Time in captivity of wild pregnant males prior to juvenile birth was about 10 days. Captive males were on average 14 and 29 days in captivity prior to mating and juvenile birth, respectively. If we consider only the males whose broods were examined in the fatty acid analysis, wild males were on average 14 days in captivity prior to juvenile birth, while captive males were about 9 and 24 days in captivity prior to mating and juvenile birth, respectively.

Brood size was determined for wild (n=20) and captive (n=7) broods. Males (after juvenile parturition) and 30 newborns from each brood were weighed alive (to the nearest 0.01 and 0.00001 g, respectively) in seawater. Males were measured through scaled digital photograph, while newborn juveniles were measured through micrometric analysis in a stereomicroscope. Standard length (SL) was determined as the sum of head (HL), trunk (TrL - straight line) and tail (TaL) lengths, according to Lourie (2003).

Newborn samples (about 100-200 newborns each) from six different broods (three wild and three captive) were frozen independently and freeze-dried for fatty acid analysis. Lipids were extracted based on the Bligh and Dyer (1959) method and esterified to fatty acid methyl esters according to Metcalfe and Schmitz (1961). The fatty acid methyl esters were injected into a capillary column Omega Wax 320 WCOT (30 m fused silica, 0.32 internal diameter) installed in a Varian Star 3400CX gas-liquid chromatograph (GLC). Helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. Oven temperature was 180 ºC for 7 min and then increased to 200 ºC (with a temperature gradient of 4 ºC min^{-1}) over a period of 71 min. Both the split injector (100:1) and the FID detector were set at 250 ºC. GLC data acquisition and handling were performed using a Varian integrator 4290 connected to the GLC. Peak quantification was carried out with a Star Chromatography workstation, and the fatty acid C19:0 (nonadecanoic acid methyl ester) was used as internal standard. Peak identification was performed using well-characterized cod liver oil chromatograms as a reference.

At the end of the study, adult seahorses and survivor juveniles were released back to their original habitat without injuries.

Statistical analysis

Wild and captive broods were compared with regard to juvenile number, size, condition and fatty acid composition, as well as to total parental investment per brood. Male weight (W) and length (SL) were compared for males that bred in natural and artificial conditions. All comparisons were performed for a significance level of 0.05, using the *t* test or the Mann-Whitney U test when the parametric assumptions were not accomplished.

Juvenile size was analyzed in terms of weight, length (SL, HL, TrL and TaL) and body proportions (HP, TrP and TaP for head, trunk and tail proportions, respectively). The condition (K) of the juveniles was evaluated through Fulton's condition factor: K = 100 W / L^3, where W = weight (g) and L = total length (cm). Wild and captive juveniles were distributed into length classes (1 mm of amplitude) and their average weight was compared within each class.

The fatty acid composition of wild and captive juveniles was analyzed in terms of absolute and relative profiles. All fatty acids were considered but special emphasis was given to the essential fatty acids AA (arachidonic acid, 20:4n-6), EPA (eicosapentaenoic acid, 20:5n-3) and DHA (docosahexaenoic acid, 22:6n-3). Comparisons were also made for TL (total lipids) and TFA (total fatty acids) contents and fatty acid classes (BFA - branched fatty acids, SFA - saturated fatty acids, MUFA - monounsaturated fatty acids, PUFA - polyunsaturated fatty acids, HUFA - highly unsaturated fatty acids, n-3 and n-6 fatty acids), as well as for the ratios n-3:n-6, EPA:AA and DHA:EPA.

Total parental investment per brood was estimated based on the total brood weight (nW), the nSL parameter (determined as the multiplication of juvenile number and average length) and the indicators of TL and TFA contents per brood (nWTL and nWTFA, respectively).

Results

Juvenile number, size and condition

The number, size and condition of wild and captive juveniles are compared in Table 2. Breeding in captivity resulted in smaller broods than reproduction in natural conditions. While captive broods ranged in size from 113 to 416 juveniles, the size of wild broods varied between 151 and 696 juveniles. Wild newborn juveniles were heavier and bigger than captive juveniles (Figure 1), and presented a smaller HP. No significant differences were detected in the condition factor of wild and captive juveniles (Table 2), but the length-class distribution analysis (Table 3) showed that wild juveniles were significantly heavier than captive juveniles for the length classes between 13.0 and 16.0 mm.

Table 2 – Comparison of wild and captive newborn juveniles regarding the number (n), weight (W), length (SL, HL, TrL and TaL), body proportions (HP, TrP and TaP) and condition factor (K) of the juveniles

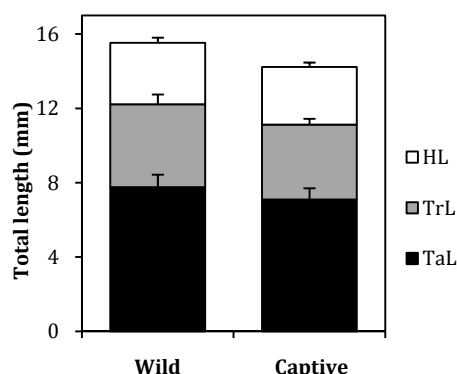|	**Wild**	**Captive**	**p**
n (juv)	425.7±124.5	254.6±134.8	0.006
W (mg)	3.69±0.94	2.75±0.63	0.000
SL (mm)	15.53±1.28	14.23±1.07	0.000
HL (mm)	3.32±0.28	3.10±0.24	0.000
TrL (mm)	4.46±0.53	4.02±0.32	0.000
TaL (mm)	7.76±0.67	7.10±0.61	0.000
HP (%)	21.41±1.72	21.84±0.90	0.019
TrP (%)	28.62±1.44	28.30±1.07	0.243
TaP (%)	49.97±1.32	49.86±1.24	0.322
K	0.00097	0.00095	0.321

Figure 1 – Length measures (HL – head length, TrL – trunk length, TaL – tail lenght) of wild and captive juvenile seahorses

Table 3 – Comparison of the average weight of wild (W_W) and captive juveniles (W_C) for each length class (SL)

SL (mm)	W_W (mg)		W_C (mg)		p
11-12	1.81±0.00	(n=1)	2.16±0.07	(n=2)	0.144
12-13	2.20±0.71	(n=9)	2.38±0.26	(n=17)	0.362
13-14	2.60±0.49	(n=50)	2.40±0.29	(n=64)	0.009
14-15	3.08±0.34	(n=136)	2.66±0.47	(n=51)	0.000
15-16	3.49±0.38	(n=202)	3.30±0.52	(n=37)	0.008
16-17	4.15±0.54	(n=97)	4.26±0.39	(n=8)	0.585
17-18	5.13±0.54	(n=75)	4.33±0.00	(n=1)	0.148
18-19	5.89±0.26	(n=18)			
19-20	6.34±0.00	(n=1)			

Juvenile fatty acid composition

Wild and captive newborns had a similar TL content (wild juveniles: 4.4±2.2% DW; captive juveniles: 3.9±1.9% DW; p=0.824) and fatty acid composition (Table 4). Both the TFA content (wild juveniles: 32.0±3.1 µg mg⁻¹ DW; captive juveniles: 33.8±2.9 µg mg⁻¹ DW) and the fatty acid class composition were identical, with both wild and captive newborns being dominated by SFA (41.0±1.8 and 38.6±1.7%, respectively), followed by PUFA (33.8±1.2 and 34.8±2.3%, respectively) and MUFA (24.3±0.8 and 26.0±3.7%, respectively). The concentrations and proportions were equal for most fatty acids, including the essential fatty acids AA, EPA and DHA. However, given the smaller size of captive juveniles, the total fatty acid contents per juvenile (µg juv⁻¹) are lesser in captive than in wild juveniles, even though their contents per dry weight (µg mg⁻¹ DW) are similar.

Total parental investment per brood

Total parental investment per brood of wild and captive seahorses is described in Table 5. All the indicators used in this study (i.e., the brood weight and the parameters nSL, nWTL and nWTFA) were significantly greater in wild broods, indicating a lower reproductive investment of seahorses kept in artificial conditions. No differences were found in the size of wild and captive pregnant males that could have induced discrepancies in their reproductive performance (weight: W_W=17.8±4.2 g, W_C=15.1±4.3 g, t=1.13, p=0.271; length: SL_W=19.0±1.6 cm, SL_C=18.0±1.6 cm, t=1.15, p=0.263).

Discussion

The disadvantages of mating outside home

In captivity, fish have in general less energy available for reproduction than in the natural environment, due to poorer dietary composition but also because captive fish have to spend part of their energy budget facing suboptimal husbandry conditions. *Hippocampus guttulatus* was not an exception. Total parental investment in captivity was far below the levels observed for wild seahorses. Captive conditions were responsible for decreasing the number, size, total fatty acid contents per juvenile (µg juv⁻¹) and the condition of newborn seahorses, although their fatty acid composition (µg mg⁻¹ DW or %TFA) was not affected.

Differences between wild and captive broods were almost certainly an effect of space confinement, broodstock diet and the level of stress to which they were exposed, since the photoperiod and the physicochemical parameters of the water largely reproduced the natural conditions. Broodstock confinement can directly affect seahorse courtship through space restriction and mate competition, while broodstock diet directly influences the amount of energy available for reproduction and, consequently, the nutritional composition of the gametes. Moreover, both factors can have also an indirect effect on reproduction by reducing the reproductive output through stress-induction. When fish are subject to stressful conditions, less energy is available for gamete production because the energetic reserves are reallocated from secondary or non-essential physiological processes

(e.g., growth and reproduction) towards primary or essential processes (e.g., metabolism and immune function). For that reason, stress has had a negative impact on several aspects of fish reproduction (e.g., Campbell et al. 1994; Wilson et al. 1995; Kjesbu 2006).

How seahorse females respond to restrictions in captivity?

When the energetic reserves available for reproduction are restricted, female fish may reduce the reproductive investment either by producing less, smaller and/or poorer-quality eggs. Although direct evidence on egg characteristics of *H. guttulatus* females was not available due to logistical and conservational constraints, newborn juveniles are expected to reflect egg characteristics, since the maternally-derived yolk reserves of the eggs are the only or, at least, the main source of nutrients during embryonic development. The smaller size of captive juveniles thus indicates that captive females produced smaller eggs that have smaller yolk reserves.

Table 4 – Comparison of the fatty acid composition (absolute profile in µg mg^{-1} DW and relative profile in %TFA) of wild and captive juveniles (nd – not detected)

	Absolute Profile			Relative Profile		
	Wild	Captive	p	Wild	Captive	p
Iso 15:0	0.07±0.00	0.09±0.06	0.663	0.21±0.01	0.24±0.18	0.663
Ant 15:0	nd	0.02±0.03	0.374	nd	0.06±0.09	0.374
Iso 16:0	0.05±0.01	0.02±0.02	0.160	0.15±0.02	0.05±0.07	0.122
Iso 17:0	0.18±0.03	0.09±0.07	0.170	0.57±0.12	0.29±0.22	0.191
BFA	**0.30±0.04**	**0.22±0.12**	**0.445**	**0.93±0.15**	**0.64±0.35**	**0.348**
13:0	0.08±0.11	nd	0.374	0.27±0.38	nd	0.374
14:0	0.61±0.08	0.56±0.05	0.475	1.90±0.13	1.64±0.01	0.081
15:0	0.21±0.04	0.19±0.05	0.660	0.64±0.07	0.55±0.09	0.311
16:0	6.75±0.31	6.74±0.70	0.990	21.17±1.17	19.92±0.70	0.264
17:0	0.47±0.08	0.57±0.18	0.528	1.50±0.37	1.69±0.56	0.701
18:0	4.37±0.36	4.45±0.28	0.821	13.65±0.35	13.17±0.38	0.255
20:0	0.47±0.17	0.41±0.08	0.675	1.48±0.54	1.25±0.34	0.636
22:0	0.13±0.04	0.12±0.01	0.675	0.40±0.09	0.35±0.05	0.494
SFA	**13.09±0.77**	**13.03±1.11**	**0.952**	**41.01±1.83**	**38.57±1.66**	**0.234**
16:1n-7	1.09±0.11	1.03±0.02	0.470	3.41±0.05	3.07±0.35	0.250
17:1n-8	0.08±0.03	0.05±0.04	0.574	0.23±0.07	0.17±0.12	0.538
18:1n-9	3.85±0.34	4.58±0.81	0.306	12.03±0.09	13.50±1.82	0.081
18:1n-7	1.50±0.21	1.57±0.29	0.804	4.68±0.25	4.62±0.60	0.903
18:1n-5	0.05±0.04	0.08±0.06	0.498	0.15±0.13	0.23±0.17	0.626
19:1n-8	0.01±0.01	nd	0.374	0.03±0.05	nd	0.374
19:1n-10	0.03±0.02	0.04±0.03	0.748	0.09±0.07	0.12±0.08	0.721
20:1n-9	0.44±0.17	0.70±0.46	0.486	1.33±0.39	2.04±1.28	0.494
20:1n-7	0.50±0.26	0.52±0.09	0.928	1.52±0.63	1.53±0.15	0.970
20:1n-5	0.07±0.10	nd	0.374	0.24±0.34	nd	0.374
22:1n-11	0.09±0.07	0.23±0.05	0.080	0.27±0.20	0.67±0.08	0.058
22:1n-9	0.09±0.07	nd	0.134	0.31±0.24	nd	0.135
MUFA	**7.81±1.03**	**8.81±1.63**	**0.500**	**24.29±0.82**	**25.95±3.65**	**0.562**
16:4n-3	nd	0.03±0.04	0.374	nd	0.09±0.13	0.374
18:2n-6	0.53±0.06	1.13±0.86	0.436	1.66±0.07	3.29±2.43	1.000
18:3n-3	0.13±0.04	0.28±0.26	0.466	0.41±0.16	0.81±0.73	0.498
18:4n-3	0.04±0.03	0.03±0.04	0.894	0.11±0.08	0.09±0.13	0.867
20:3n-6	0.06±0.04	0.06±0.02	0.873	0.18±0.13	0.19±0.06	0.895
20:4n-6	0.16±0.03	0.15±0.02	0.707	0.50±0.05	0.45±0.07	0.471
20:3n-3	0.09±0.02	0.07±0.02	0.442	0.27±0.05	0.20±0.04	0.216
20:4n-3	1.49±0.23	1.99±0.33	0.159	4.63±0.30	5.84±0.52	0.046
20:5n-3	1.59±0.22	1.36±0.33	0.457	5.02±0.96	4.05±1.03	0.386
22:4n-6	0.18±0.04	0.20±0.09	0.762	0.57±0.06	0.60±0.22	0.853
22:5n-3	0.94±0.19	0.94±0.10	0.972	2.90±0.31	2.84±0.55	0.896
22:6n-3	5.64±0.79	5.51±1.45	0.919	17.52±0.74	16.38±4.25	0.728
PUFA	**10.85±1.36**	**11.76±1.14**	**0.504**	**33.77±1.15**	**34.84±2.32**	**0.591**
HUFA	**10.00±1.28**	**10.16±1.99**	**0.935**	**31.14±1.49**	**30.16±1.48**	**0.249**
n-3	**9.91±1.20**	**10.21±1.58**	**0.839**	**30.86±1.06**	**30.31±4.46**	**0.872**
n-6	**0.94±0.16**	**1.55±0.78**	**0.336**	**2.90±0.21**	**4.53±2.15**	**0.383**
TFA	**32.04±3.09**	**33.83±2.92**	**0.584**			
n-3:n-6	**10.67±0.72**	**8.47±3.76**	**0.460**	**10.67±0.72**	**8.47±3.76**	**0.461**
DHA:EPA	**3.63±0.75**	**4.04±0.15**	**0.494**	**3.63±0.75**	**4.04±0.15**	**0.493**
EPA:AA	**10.45±3.09**	**8.95±1.26**	**0.556**	**10.45±3.09**	**8.95±1.26**	**0.559**

Table 5 – Total parental investment per brood of wild and captive seahorses, with respect to the indicators nW (g), nSL (m), nWTL (g . % DW) and nWTFA (g . µg mg^{-1} DW)

	Wild broods	**Captive broods**	**p**
nW	1.6±0.5	0.7±0.2	0.003
nSL	6.6±2.0	3.9±1.6	0.007
nWTL	6822.9±2370.6	2707.3±829.0	0.001
nWTFA	212067.4±64749.8	130689.6±54411.1	0.013

Besides decreasing the size of eggs, captive females may have also produced a smaller number of eggs, given the smaller size of captive broods. However, seahorse fecundity is hard to assess, particularly in the wild, and cannot be directly inferred from brood size. Since the quantity of oocytes produced by the female generally exceeds the capacity of the male brood pouch (Vincent 1994), reduced fecundity is not expected to affect brood size. Indeed, smaller broods in captivity were a direct consequence of male competition and mating interruption caused by confinement, reduced water column depth and high stocking densities. Although the aquariums used in this study were specifically made for seahorse reproduction, their great vertical height (90 cm) still restricted *H. guttulatus* courtship. Compared to other species (e.g., Sobolewski 1997), *H. guttulatus* seems to require particularly high water column depths, i.e., greater than five times the seahorse height.

With regard to egg quality, the results indicate that captive females invested less in the nutritional content of eggs. Even though wild and captive juveniles had a similar fatty acid composition per dry weight (µg mg^{-1} DW), total fatty acid contents per juvenile (µg juv^{-1}) were smaller in captivity. The matching composition of captive and wild juveniles is highly unexpected given the differences between the natural and artificial diets. Wild seahorses eat a varied diet, dominated by amphipods, decapods, mysids and algae (e.g., Teixeira and Musick 2001; Woods 2002; Kitsos et al. 2008); in captivity, seahorse diets are much less varied, generally composed by *Artemia* and mysids. Since dietary fatty acids are stored in the adipose tissue and utilized as metabolic energy or incorporated into new lipids during oogenesis, the poorer nutritional composition of artificial diets in relation to natural food generally leads to decreased egg quality in captive fish (e.g., Srivastava and Brown 1991; Harrell and Woods 1995; reviewed in Brooks et al. 1997; Ozaki et al. 2008; Seaborn et al. 2009). Planas et al. (2008) found that seahorse eggs showed a progressive decrease in the content of essential fatty acids with time in captivity, even though female growth and condition were successfully attained.

The lack of differences in the fatty acid profile of wild and captive *H. guttulatus* juveniles may have resulted from a female ability to retain certain dietary fatty acids so that gametogenesis requirements can be accomplished as a trade-off with their growth and body composition. The fatty acid composition of fish eggs is generally more conserved and relatively less influenced by the diet than other tissue compositions, reflecting the importance of specific compositions in the gametes (Sargent et al. 2002). Moreover, seahorse embryos may have also the ability to manage yolk consumption in order to reach a suitable fatty acid composition in detriment of their growth.

The lower fatty acid content (per juvenile) and greater head proportion of captive juveniles are indicative of poorer nutritional condition, as nutritional restrictions are expected to be less evident in seahorse head, and more in the trunk and tail which comprise mainly musculature. Indeed, captive newborns were smaller but also generally thinner than wild juveniles, even though both juveniles had a similar condition factor. The condition factors generally applied to fish may not, however, be the most suitable to evaluate seahorse condition, since the presence of an exoskeleton that contributes considerably to the total weight of the animal can easily camouflage the loss of weight in the soft body parts.

How seahorse males respond to restrictions in captivity?

Females are not the only responsible for reproductive success. The overall state of the male can directly influence the quality of the sperm and, consequently, the fertilization success (reviewed in Kamler 2005). In seahorses, the nutritional and physiological wellbeing of the pregnant male is also expected to directly affect embryo development and offspring size. Seahorse embryos fully develop within the enclosed environment of the male brood pouch, in close association with the highly vascularized pouch epithelium in a manner that bears some resemblance to the embryo-placental relationship in mammals (Stölting and Wilson 2007). The male is responsible for gaseous exchange, removal of waste products and osmoregulation (Linton and Soloff 1964), playing also an important immunoprotective role (Melamed et al. 2005). Even though syngnathid embryos are self-sustaining from a nutritional perspective (Azzarello 1991), the male still has an important role in embryo nourishment (Stölting and Wilson 2007).

The nutritional content of eggs is expected to be the main determinant of newborn size, but recent studies have shown that paternal supply of essential elements and environmental conditions during embryonic development may also be important. Bigger juveniles have been produced when fewer newborns are present in the male pouch due to reduced intra-pouch competition (e.g., Dzyuba et al., 2006; Woods, 2007). Studies with *H. guttulatus* also showed that the size of newborns is, in part, paternally determined (F. Faleiro, unpublished data). Therefore, suboptimal conditions during male pregnancy in captivity may also have accounted for the reduced reproductive success of captive *H. guttulatus*.

Wild vs. captive breeding – A first denouement!

Reproductive investment and breeding success of *H. guttulatus* decreased considerably in captivity. Modification of the natural conditions during gametogenesis, mating and embryonic development may have affected seahorse reproduction in many different ways (Figure 2). Although the precise mechanisms that affected *H. guttulatus* reproduction in captivity remain to some extent uncertain, the captive environment was responsible for noticeably decreasing the number, size, total fatty content (per juvenile) and the condition of newborn seahorses, but not their composition (µg mg^{-1} DW or %TFA). When subject to restrictive energetic conditions, the long-snouted seahorse seems therefore to choose the quality over the quantity, by

reducing the amount of energy reserves in eggs and juveniles in order to maintain a suitable fatty acid composition.

Given the present results, it is clear that there is still much to do in order to improve the captive breeding of the long-snouted seahorse. Structurally complex habitats, higher water column depths, stronger water flow conditions, lower densities, balanced sex ratios, similar-size mates and pair isolation are essential to improve the welfare and reproductive success of *H. guttulatus* in captivity (Faleiro et al. 2008). Moreover, the use of broodstock diets tailor-made for seahorses and with enhanced nutritional composition (not only the lipid and fatty acid profiles, but also the global macronutrient composition) will improve egg and juvenile quality, along with increased feeding frequency.

The smaller size and poorer condition of captive juveniles may affect their survival capacity and further jeopardize the success of commercial production and reintroduction programs in the wild. Long-term captive breeding will improve the rearing success and the survival capacity of captive seahorses, which will become more and more adapted to the captive environment and less to the natural one. Domestication will be advantageous for commercial production but not for reintroduction programs in the wild, because significant genetic and phenotypic changes may compromise fitness in the wild (reviewed in Lynch & O'Hely 2001). Limit captive breeding programs to short-term situations will decrease animal domestication and increase their reestablishment potential in the wild.
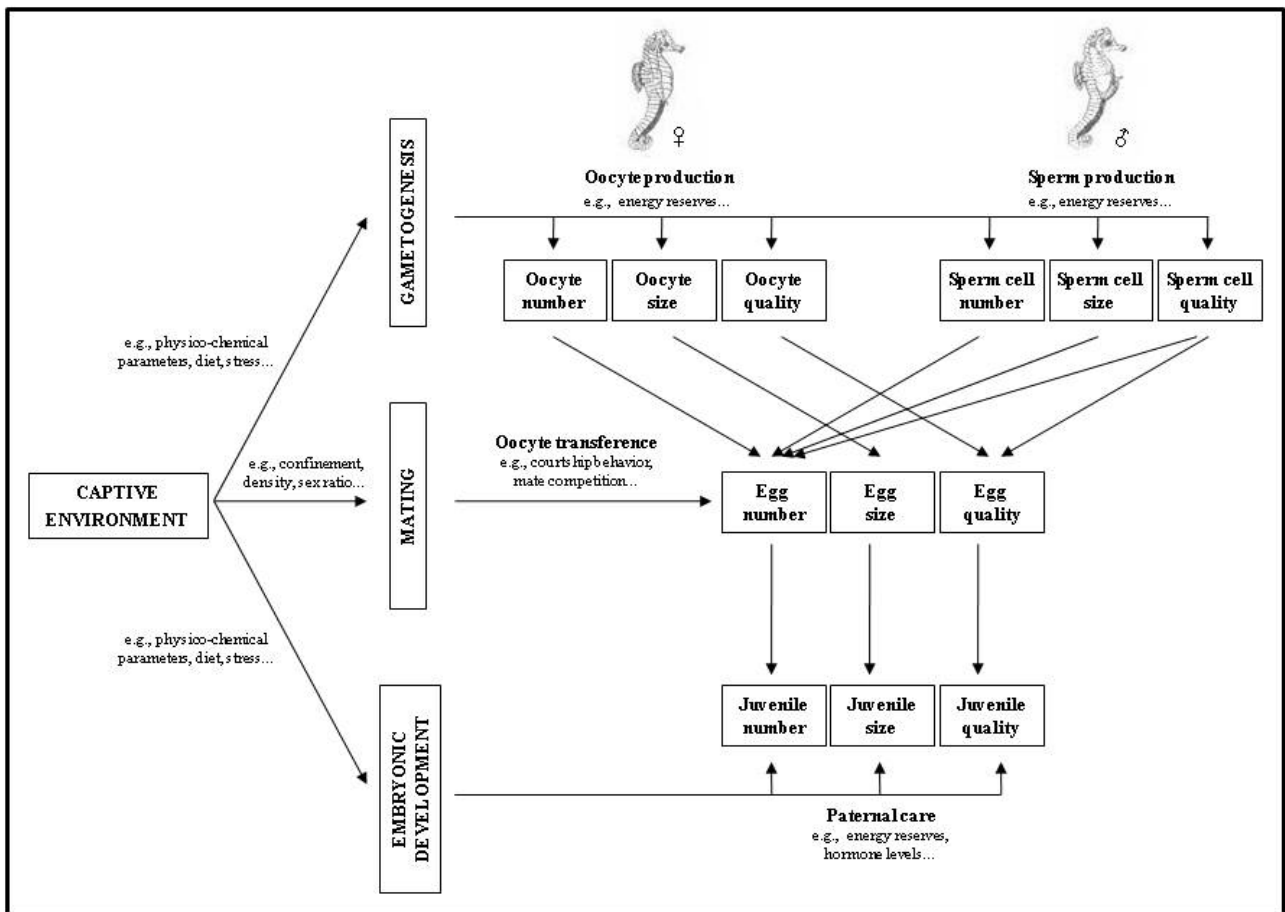

Figure 2 – Network scheme illustrating the overall consequences of captive breeding to the reproductive output of both male and female seahorses

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The bitter victory of wild against captive seahorse juveniles

The bitter victory of wild against captive seahorse juveniles

Filipa Faleiro[1,*], Armando J. Almeida[1], Pedro Ré[1] and Luís Narciso[1]

[1] Universidade de Lisboa, Faculdade de Ciências, Centro de Oceanografia, Laboratório Marítimo da Guia,
Avenida Nossa Senhora do Cabo 939, 2750-374 Cascais, Portugal
[*] Corresponding author (e-mail: filipafaleiro@gmail.com; phone: +351214869211; fax: +351214869720)

The success of supplementation programs in the wild is highly dependent on the quality of the offspring produced. Captive breeding showed to decrease the reproductive performance of the long-snouted seahorse, but its effect on the survival capacity of the juveniles remains unknown. This study intends to evaluate if and how the captive environment affects the survival capacity of *Hippocampus guttulatus* juveniles through a salinity stress test. Optimal salinity, tolerance range and average survival were compared for wild and captive newborn juveniles. *Hippocampus guttulatus* proved to be an efficient osmoregulator that tolerates a wide salinity range at early development stages. Wild juveniles tolerated well salinities from 5 to 55 psu and presented optimal performance at 5 psu, but captive seahorses had a narrower tolerance range (15-45 psu) and enhanced survival at 20 psu. The lower salinity tolerance and survival capacity of the captive seahorses is discussed in relation to their smaller size, lower physiological condition and reduced nutritional reserves for osmoregulation and stress handling. This study demonstrates that captive seahorses are competitively inferior to wild seahorses, which may have consequences for seahorse conservation. The greater vulnerability of captive seahorses may decrease their chances of survival in the natural environment and jeopardize the success of supplementation programs in the wild.

Keywords: Hippocampus guttulatus, seahorse juveniles, wild, captive, salinity stress tolerance, survival.

Introduction

Captive breeding is an important management tool for species conservation, and represents the last chance of survival for many species facing imminent extinction in the wild. Besides reducing the pressure on wild stocks by assuring, at least to some extent, the commercial demands, captive breeding can be further applied in population supplementation programs when a last-resort recovery strategy is required.

Much controversy has arisen regarding whether this technique can efficiently boost the population size and establish a self-sustaining natural population. Some studies have shown that captive breeding can efficiently enhance the population size without obvious short-term fitness costs (e.g., Araki et al. 2007; Caroffino et al. 2008) but, in general, supplementation programs have proven to be incapable of maintaining self-sustainable natural populations, particularly in a long-term perspective (e.g., reviewed in Einum and Fleming 2001; reviewed in Fleming and Peterson 2001).

Successive failures of reintroductions in the wild are related to the innumerous limitations that captive breeding can present in species recovery (reviewed in Snyder et al. 1996), but also with an excessive and inaccurate use of captive breeding as a recovery technique. Besides problems concerning the high costs (both financial and physical resources) and the biological unsuitability of the species to successfully breed and survive under captive conditions, supplementation of wild populations also entails risks such as disease introductions, increased competition for resources, as well as genetic, morphological and behavioral changes that often decrease the fitness of the supplemented populations.

The success of supplementation programs is therefore largely dependent on the quality of the offspring produced, and how captive organisms will integrate into natural populations. Larger and higher-quality fingerlings are expected to have a survival advantage in the wild, due to their reduced vulnerability and greater capacity to face ecological challenges. Smaller fingerlings are more vulnerable to predation and able to eat a lower variety of prey items. Their reduced body reserves allow them to survive food deprivation for shortened periods and restrict the energetic reserves available for basal metabolism, particularly if facing challenging environmental conditions. Although some exceptions (e.g., Kjesbu 2006; Shimada et al. 2007), captive breeding has been responsible for decreasing the reproductive performance of fish (e.g., Srivastava and Brown 1991; Harrell and Woods 1995; Palacios et al. 2007; Ozaki et al. 2008; Seaborn et al. 2009), with repercussions to the survival capacity of the fingerlings (e.g., Srivastava and Brown 1991; Kostow 2004). Optimization of breeding conditions in captivity is therefore essential to produce high quality fingerlings and increase their chances of survival in the wild.

Seahorses have raised a growing interest worldwide. Habitat degradation and intensive exploitation for traditional medicines, aquarium trade and curios have led to the global decline of natural populations and to the inclusion in 2002 of seahorse species in the IUCN Red List and in the CITES Appendix II. The singular life history characteristics of

seahorses (e.g., low mobility, sparse distribution, small home range, monogamy and lengthy parental care) make them particularly sensitive to overfishing and perturbations of their natural environment and, thus, an important and pressing focus for global conservation efforts. Given the need to assure commercial demands, reduce the pressure on wild stocks and protect the sustainability of the natural populations, captive breeding has been attained for several seahorse species (e.g., Vincent 1995; Masonjones and Lewis 1996; Lockyear et al. 1997; Payne and Rippingale 2000; Silveira 2000; Faleiro et al. 2008; Woods 2000).

The long-snouted seahorse, *H. guttulatus*, is classified by the IUCN as 'Data-Deficient', but degradation of seagrass habitats along with directed and by-catch fisheries in several countries are a significant long-term threat to this species (OSPAR 2005). *Hippocampus guttulatus* was known to present one of the most dense seahorse populations in the world, localized in the Ria Formosa lagoon, southern Portugal (Curtis and Vincent 2005), but habitat degradation and fisheries seem to jeopardize the current sustainability of this large population. Recent observations indicate that, in less than 10 years, this population has experienced about 85% reduction in numbers. Captive breeding may thus be an important tool for *H. guttulatus* conservation. The negative effects of captive breeding on the reproductive performance were also observed for this species (e.g., decreased number, size, total fatty acid content and condition of newborn seahorses: unpublished data), but the repercussions on the survival capacity of the juveniles remain unknown. The aim of this study is to evaluate if captive breeding affects the survival capacity of *H. guttulatus* newborns and how it may jeopardize the success of reintroduction programs in the wild. The survival capacity of wild and captive juveniles was evaluated through a salinity stress test and analyzed in terms of optimal salinity, tolerance range and average survival.

Methods

Experimental methodology

H. guttulatus were collected by scuba diving (under official permission) in Ria Formosa lagoon (36°59'N, 7°51'W), southern Portugal, between June and October 2007. Seahorses were maintained in a recirculated rearing system: pregnant males were placed in stock aquariums (170 L, 140x35x35 cm), while females and non-pregnant males were kept in reproduction aquariums (180 L, 50x40x90 cm). Artificial plants were provided for seahorse attachment. Husbandry conditions were similar to those observed in their natural environment: photoperiod 14L:10D, temperature 24-25 ºC, salinity 34-35 psu and pH 8.2-8.3. Ammonia, nitrites and nitrates concentrations were maintained below detection levels (0.1, 0.3 and 10.0 mg L^{-1} respectively). Adults were fed twice a day on live adult *Artemia* (during the acclimatization week) and on frozen adult *Artemia* and *Mysis* (during the experimental period). The aquariums were cleaned every day and 10% water changes were made weekly.

After juvenile birth, two distinct groups of juveniles were considered: "wild" juveniles, from males captured pregnant, and "captive" juveniles, which resulted from mating in captivity. The tolerance of wild and captive juveniles to

stressful conditions was evaluated through a salinity stress test. Groups of 10 juveniles from the same brood were placed in 2-L tanks full of water with different salinities: 5, 15, 25, 35, 45, 55, 65 and 75 psu. Triplicates using juveniles from six different broods (three wild and three captive) were obtained for each salinity (n=30). Survival was checked every hour during the first 16 hours and every 8 hour after that, during a maximum of 10 days. No food was provided during the experimental period.

At the end of the study, adult seahorses and survivor juveniles were released back to their original habitat without injuries.

Statistical analysis

A survival analysis was used to compare the tolerance of newborn juveniles to the different salinity conditions. The Cox's F-Test was used to compare survival of wild and captive juveniles for each salinity, while the overall tolerance of each group was analyzed for all salinities by a multiple-sample test (an extension of Gehan's generalized Wilcoxon test, Peto and Peto's generalized Wilcoxon test and the log-rank test).

The average survival (number of hours) was compared between wild and captive juveniles for each salinity using the Mann-Whitney U test, and between salinities using analysis of variance and the Tukey's HSD *a posteriori* test.

All statistical analyses were performed for a significance level of 0.05.

Results

The tolerance of wild and captive juveniles to the different salinities is summarized in Table 1. Survival of newborns (both wild and captive juveniles) varied with salinity, either in terms of average survival (Fig. 1) or survival curve (Fig. 2). Stress tolerance of wild newborns was enhanced at 5 psu and declined as salinity increased. In contrast, captive juveniles were not able to survive for a long period at 5 psu, presenting maximum tolerance near 20 psu.

Table 1 – Comparison of the survival time of wild and captive juveniles exposed to different salinities

		Survival (hours)		Average Survival		Survival Curve	
	Salinity	Wild	Captive	Z	p	F	p
	All	102.1±78.1	43.1±40.3	-7.3	0.000		
	5	232.0±17.8	6.0±1.9	-6.6	0.000	11.7	0.000
	15	155.5±46.3	100.5±7.4	-5.5	0.000	4.2	0.000
	25	143.2±29.8	82.7±21.3	-5.8	0.000	4.5	0.000
	35	128.3±22.5	77.3±16.0	-6.2	0.000	4.7	0.000
	45	91.7±20.9	59.7±20.3	-5.5	0.000	4.1	0.000
	55	60.0±30.6	10.0±3.0	-6.2	0.000	4.8	0.000
	65	3.4±0.9	5.1±1.0	5.2	0.000	3.3	0.000
	75	2.6±0.6	3.0±0.3	2.4	0.019	1.5	0.076
Average Survival	**F**	276.5	329.3				
	p	0.000	0.000				
Survival Curve	**χ²**	212.1	210.7				
	p	0.000	0.000				

■ Wild

$y = 9E\text{-}05x^4 - 0.0147x^3 + 0.7984x^2 - 18.885x + 307.49$ ($R^2 = 0.954$)

◆ Captive

$y = 2E\text{-}06x^5 - 0.0005x^4 + 0.0391x^3 - 1.6074x^2 + 30.273x - 108.42$ ($R^2 = 0.927$)

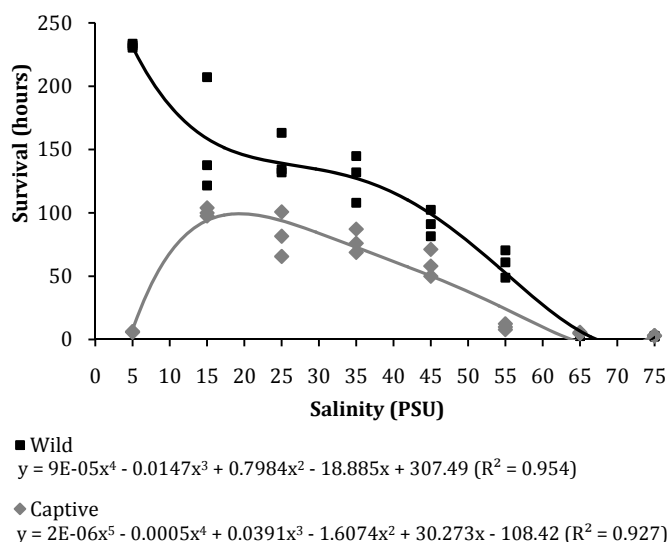**Fig. 1 –** Average survival of wild and captive juveniles throughout a salinity gradient from 5 to 75 psu
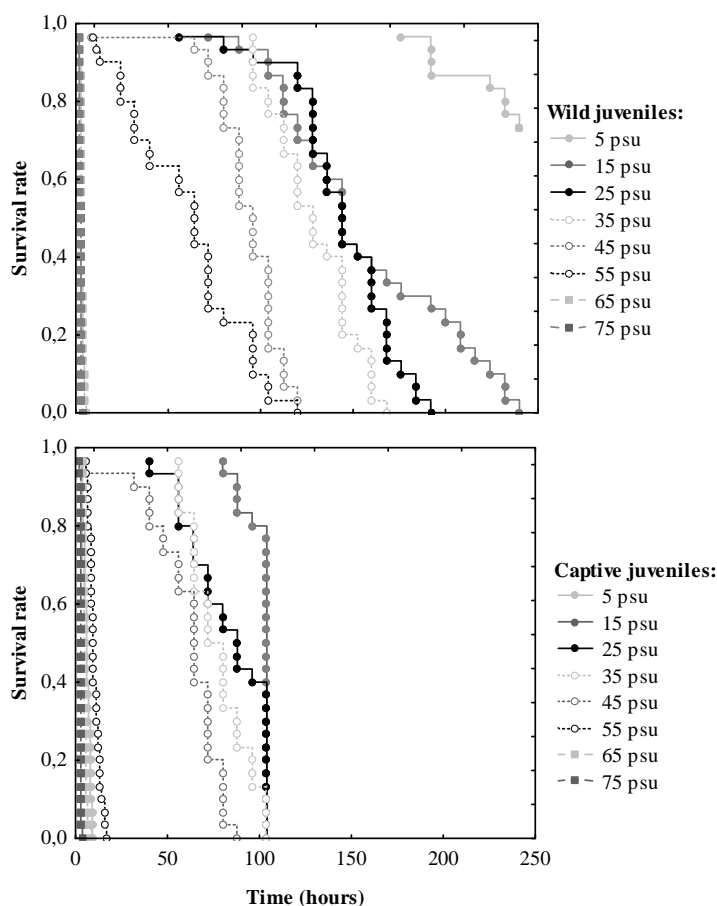

Fig. 2 – Comparison of the survival curves of wild and captive juveniles between different salinities

Compared to captive juveniles, wild newborns presented a higher survival capacity: their average survival was significantly higher at all salinities (Fig. 1), and the survival curves were significantly different at all salinities excluding

75 psu (Fig. 3). Although the survival curve of captive juveniles was dislocated down in the graph (Fig. 1), the curves of wild and captive juveniles showed a divergent pattern at the lower limit of the salinity range (5 psu), but converged near the upper limit (65 and 75 psu). The salinity tolerance range of wild juveniles was therefore wider than that of captive juveniles: while wild newborns survived for a relatively long period between salinities 5 and 55 psu, the salinity range of captive newborns shrank to 15 and 45 psu.

Discussion

Captive conditions are very distinct from those experienced by fish in the wild and have, therefore, a significant effect on the quantity and quality of the offspring produced. In seahorses, artificial conditions were responsible for decreasing the number, size, total fatty acid content (per juvenile) and condition of *H. guttulatus* newborns (unpublished data). The nutritional state and physiological condition of the fingerlings can have a strong impact on their survival capacity and growth rate. Decreased fry survival has been commonly observed in captive fingerlings when compared to wild specimens (e.g., Srivastava and Brown 1991; Kostow 2004), in part related to their smaller size and poorer nutritional composition. Likewise, the captive and smaller *H. guttulatus* juveniles presented lower survivorship than wild seahorses.

The captive environment affected not only the survival capacity of the newborns but also their osmoregulatory skills. In general, *H. guttulatus* proved to be an efficient osmoregulator that tolerates a wide salinity range at early developmental stages, which can be particularly advantageous for estuarine organisms with cryptic behavior and extremely reduced mobility like the long-snouted seahorse, because they need other mechanisms rather than behavioral osmoregulation (i.e., fish motion to seek a medium isotonic with their body fluids) to face unsuitable salinities. Even though seahorse embryos are unable to osmoregulate properly, requiring the male to provide an optimum osmotic environment (Linton and Soloff 1964), newborn seahorses already have functional osmoregulatory organs, such as gills and kidneys, and are able to self-osmoregulate.

The higher salinity tolerance of the wild and larger *H. guttulatus* juveniles is not surprising, as osmoregulatory capacity was shown to improve with size (e.g., reviewed in Varsamos et al. 2005). Even so, the tolerance to salinity stress is expected to reflect a more general physiological condition of the organism to tolerate adverse environmental conditions, rather than just their osmoregulatory capacity. Besides having more developed gills, larger juveniles are also expected to have larger nutritional reserves that can be used directly for osmoregulation or to withstand stress. Moreover, the nutritional condition (lipids in particularly) can further affect the osmoregulatory mechanisms by influencing gill area and ramification, the fatty acid composition of membrane phospholipids in gills and, consequently, the permeability to water and ions and the activity of the Na^+/K^+-ATPase pump (Palacios et al. 2004).
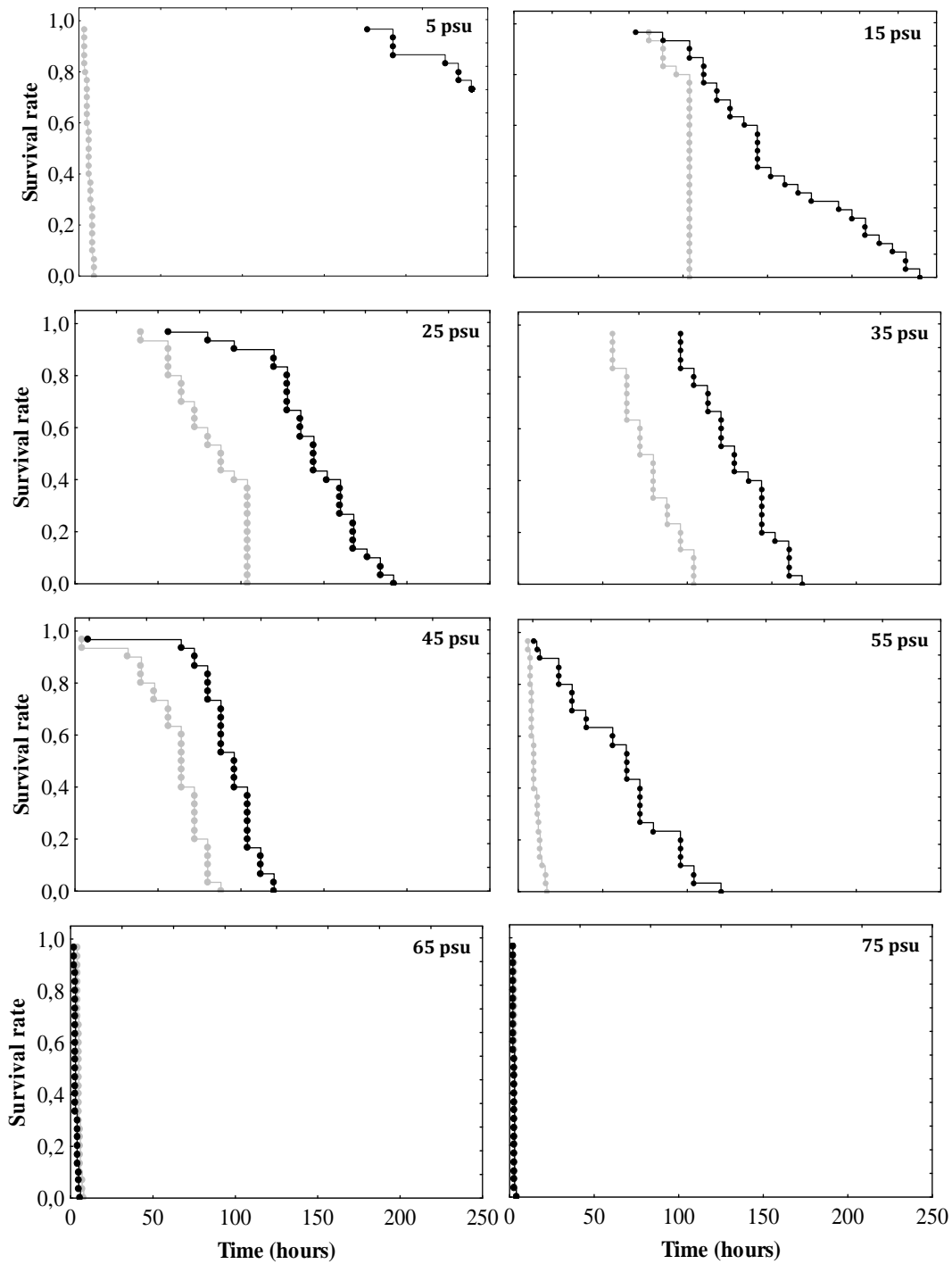
Fig. 3 – Comparison of the survival curves between wild (●) and captive (●) juveniles kept at different salinities

One interesting aspect of the salinity tolerance of *H. guttulatus* juveniles was that, besides differing in their average survival and tolerance range, wild and captive juveniles also differed in their optimal salinity. Stress tolerance of captive newborns was enhanced near 20 psu, while wild juveniles presented maximum tolerance at 5 psu. Since changes in the optimal salinity (which is likely to be genetically determined) are highly unexpected, the high mortality of captive juveniles at 5 psu is probably a consequence of their reduced ability to tolerate such a great

osmotic shock (from 35 to 5 psu), rather than incapacity to osmoregulate at such a low salinity.

This assumption is corroborated by the shrinkage of the tolerance range from 5-55 psu in wild juveniles to 15-45 psu in captive seahorses. The highest survival of wild juveniles at 5 psu shows that the lower tolerance limit of this species is below this salinity. The divergent pattern in the survival curves of wild and captive seahorses at 5 psu probably resulted from their distinct capacity to tolerate great osmotic shocks. On the other hand, the convergence of both curves at 65 psu suggests that the mortality of captive juveniles at this

salinity did not result from the great osmotic shock (from 35 to 65 psu), but rather from an inability to survive at such an extremely high salinity, thus establishing an upper limit in the physiologically acceptable salinity range of this species. *Hippocampus guttulatus* juveniles thus showed a higher tolerance to lower salinities than to higher concentrations. Wild and captive juveniles tolerated well a hypoosmotic shock of 30 and 20 psu, but were only able to tolerate a hyperosmotic shock of 20 and 10 psu, respectively.

The great hyper-regulation capacity of the long-snouted seahorse at low salinities is somehow surprising given the aglomerular seahorse kidney (reviewed in Hickman and Trump 1969). Freshwater and euryhaline species have in general kidneys with high glomeruli density, because glomeruli have a critical role in excreting excess water and fewer ions through dilute urine in freshwater adaptation (reviewed in Beyenbach 2004). However, the functional inferiority of the aglomerular seahorse kidney remains unproved. The kidney is essential in eliminating less abundant divalent ions (reviewed in McCormick 2001), but it plays a minor osmoregulatory role than gills (Sangiao-Alvarellos et al. 2003).

Both the low optimal salinity of *H. guttulatus* juveniles and their higher tolerance to hypoosmotic shocks are related to their low internal osmolarity. Irrespective of the salinity of the external environment, teleost fish maintain their plasma osmotic concentration about one-third that of seawater (e.g., Sampaio and Bianchini 2002; reviewed in Varsamos et al. 2005; Prodocimo et al. 2008), including pipefishes (e.g., Partridge et al. 2007). Comparable values are also expected for seahorses. Although optimal salinity is expected to reflect plasma osmolarity, because the energy costs of osmoregulation are predictable lowest in isotonic environments, other factors must be also considered. Osmoregulation involves a complex network of physiological reactions that occurred in the plasma and in a great variety of organs (Sangiao-Alvarellos et al. 2003). For instance, reduced salinity has shown to enhance protein and lipid digestion (e.g., Ringø 1991), to increase nitrogen assimilation (e.g., Usher et al. 1990) and to improve carbohydrate and lipid storage (e.g., Woo and Chung 1995).

Moreover, acclimation to different environmental salinities involves both stress and osmoregulation components (Morgan 1997; Sangiao-Alvarellos et al. 2003). Cortisol (the primary stress-response hormone) plays an important role in seawater adaptation of teleost fish, while prolactin (a key endocrine hormone involved in seahorse pregnancy) is responsible for preventing the loss of ions and the uptake of water in freshwater adaptation (reviewed in Varsamos et al. 2005). Since the maternal-origin hormones from the yolk are important to the development of the embryos which lack a functional endocrine system (Tanaka et al. 1995), but also because male hormones may pass through the placental barrier to the embryos, stressful conditions during broodstock husbandry and reproduction in captivity may thus affect hormonal regulation and the osmoregulatory capacity of the offspring. Prolactin production during seahorse pregnancy can enhance hyper-regulation at low salinities and substantiate the greater tolerance of *H. guttulatus* juveniles to low salinities, while enhanced production of cortisol as a response to stressful conditions in captivity may improve the hypo-regulatory capacity of the

captive juveniles and explain their higher optimal salinity relatively to wild juveniles.

Little is known regarding the salinity tolerance of seahorses. Hilomen-Garcia et al. (2003) observed that growth and survival of 9-week old *Hippocampus kuda* juveniles born and reared in captivity tended to peak in diluted seawater salinities of 15 and 20 psu, with signs of osmoregulatory decompensation at 10 and 50 psu setting the salinity tolerance limits for these juveniles. These results cannot, however, be directly compared to those obtained for *H. guttulatus* newborns. Besides species-specific differences, discrepancies in seahorse age (*H. guttulatus*: newborn juveniles; *H. kuda*: 9-week old juveniles), water temperature (*H. guttulatus*: 24-25 ºC; *H. kuda*: 27-28 ºC) and, particularly, feeding and rearing conditions (*H. guttulatus*: subject to starvation and osmotic shock; *H. kuda*: fed and reared at different salinities) may explain the differences in optimal salinity and tolerance range between the species.

Survival to salinity stress tests and survival under normal rearing conditions are generally viewed as the two sides of the same coin. Since salinity is one of the key factors that affect fish survival and metabolism, salinity stress tests have been widely used as a predictor of subsequent quality (e.g., Aquacop et al. 1991; Rees et al. 1994; Álvarez et al. 2004; Racotta et al. 2004). This relationship is based on the assumption that a higher physiological condition allows a higher tolerance to salinity stress. Healthier or better quality fish are expected to present higher survivorship than unhealthy or poorer quality fish later in development, because any characteristics that can be useful during the salinity stress test are expected to remain advantageous during rearing, even if an abrupt change in salinity is not involved. Álvarez et al. (2004) provided direct evidence for an association between salinity stress resistance and later performance, but this relationship is not so straightforward. Factors like starvation and osmotic shock can affect salinity tolerance and survival, whereat direct associations between these two sides of the same coin should be carefully made.

Conclusion

Captive breeding had a strong impact on the reproductive success of *H. guttulatus*. The lower size, condition and fatty acid content of captive newborns (unpublished data) resulted in poorer ability to tolerate stressful conditions and reduced survivorship. The inferiority of captive seahorses should not, however, efface the potential of captive breeding as a tool for *H. guttulatus* conservation. Improvement of breeding conditions in captivity (see Faleiro et al., 2008) will easily enhance the quality of seahorse eggs and juveniles and, consequently, their integration into natural populations. Seahorses are a suitable candidate group to breed and survive under captive conditions, given their sedentary life pattern, reduced mobility and general lack of agonistic behaviors. Moreover, captive seahorses are expected to have better chances to integrate well into the wild, since significant behavioral deficiencies resultant from captivity (especially with respect to foraging, predator avoidance and social behavior) are expected to be least likely in fish with poorer behavioral repertoires, reduced mobility, camouflage as a predator evasion strategy and monogamous pair bonds like seahorses.

Despite being a promising tool for seahorse conservation, supplementation of wild populations should be carefully applied as a conservational strategy, bearing in mind all the potential limitations of captive breeding in endangered species recovery. Reintroductions in the wild should be a last-resort recovery strategy rather than a prophylactic or long-term solution. Moreover, captive breeding should not divert attention from dealing with the real problems (such as habitat loss and overexploitation) and, when adopted as a recovery technique, should be always integrated with simultaneous efforts to maintain or restore natural populations and habitats.

Aknowledgments

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General Discussion and Final Considerations

Giving back to nature

Biodiversity is one of the most precious gifts from nature, but also the most devalued. Over the years, man has given back to nature with progress and knowledge, but also with pollution, climate change, devastation, overexploitation and extinction, and rarely with its preservation. Faced with such an overwhelmingly drastic scenario, only recently man started to try avoiding repeat the mistakes of the past and to change direction towards the preservation of life. In the last decades, major efforts have been developed to preserve the ecological balance of the natural environment and its sustainability. Conservational actions have been simultaneously developed in different directions, from habitat preservation to harvesting restriction and, more recently, to captive breeding.

Even though cultivation of species is a very ancient practice that was instinctively developed to facilitate human access to food, the dire situation of the living resources in the last decades has triggered a sudden interest in species culture, firstly as a profitable solution to accomplish human demands, but more recently as a prominent tool for species conservation. There are more than 18 000 species currently listed as threatened, and numbers are expected to continue increasing at a galloping rate. For some species it is too late. For others captive breeding may be the only hope. Hopefully, at least for some, it may represent a new future, a more optimistic one.

A new home for the long-snouted seahorse

Breeding the long-snouted seahorse in captivity proved to be a challenging but promising goal to achieve. Even though this seahorse (in particularly the little and fragile juveniles) had shown to be more problematic than other seahorse species, it seems likely that a suitable home in captivity can be established for this species in a near future. Some challenges have been discovered during this investigation, some have been overhauled, but many remain to solve or improve.

Wild *H. guttulatus* lived healthy and reproduced well in their new home. Taylor-made rearing systems, suitable environmental conditions and good water quality were essential to successfully breed this species. High water flow conditions, greater holdfast availability and lower stocking densities were preferential conditions for seahorse husbandry. Although effects on animal welfare were not detected, high densities and scarce holdfasts were responsible for increasing seahorse activity and the number and size of groups, which may create a stressful environment and further affect the long standing of this species in captivity. Regarding reproduction, high water column depth, low density, balanced sex ratio and similar-size mates were important to decrease mate competition and enhance the reproductive performance of the long-snouted seahorse. After mate selection, isolation of seahorse pairs, which was the basis of social structure of captive *H. guttulatus*, may thus be advantageous.

All seahorses were dedicated parents, but some of them proved to be better parents than others or, at least, to have better conditions to raise their broods. Although females are the main responsible

for embryo nourishment, seahorse males proved to have a key role in offspring size. With respect to parental size, the bigger the better. Larger females produced bigger eggs with larger yolk reserves and, thus, greater nutritional content. Larger males had larger brood pouches and, consequently, higher carrying capacities. As the saying goes, like father like son. Juveniles from larger parents were bigger and heavier and, hence, expected to have a survival advantage in relation to broods from smaller parents. Given the importance of parental size to the reproductive performance of *H. guttulatus*, the selection of large males and females with greater reproductive potential will increase its chances of success in captivity.

Besides choosing the best parents, it is also important to provide them a proper environment. Good nutrition is essential to fulfill the high nutritional demands of reproduction and to achieve good reproductive performance. Even though adult seahorses were easily adapted to frozen diets, the dietary composition may have affected their reproductive performance. Adult seahorses, particularly the females, require high levels of dietary PUFA, mainly of the essential fatty acids DHA and EPA, in order to produce high quality gametes and juveniles.

Seahorses have the advantage of being born at a more advanced and less vulnerable developmental stage than the larval stages of marine fishes. *Hippocampus guttulatus*, in particularly, seems to invest heavily in the production of a relatively small number of exceptionally large eggs and juveniles. Even though the long-snouted seahorse could, therefore, be expected to be particularly resistant at birth, newborn juveniles showed to be highly vulnerable to captive conditions and to present low survival rates in the first month of life. Given their long and demanding embryonic development, juvenile seahorses were born at an exceptionally nutritionally depleted state. The nutritional quality of the first feed, which must fulfill all the nutritional requirements of young seahorses at the start of the exogenous feeding, is therefore particularly important for rearing *H. guttulatus* juveniles. The fatty acid composition of seahorse eggs, which is expected to reflect the nutritional requirements of newborn juveniles, revealed that newborns have high PUFA requirements, particularly of the n-3 HUFA DHA and EPA, but lower MUFA requisites.

Artemia nauplii were not a suitable prey for *H. guttulatus* juveniles, not even the enriched metanauplii. The high MUFA content and low DHA level of this prey item did not fulfill the high PUFA requirements of newborn seahorses. Supplementation with n-3 HUFA rich oils enhanced the composition of newly-hatched nauplii but, even though superior enrichment is expected to improve the nutritional value of *Artemia*, the MUFA-rich profile of this prey and its inherent catabolism of DHA into EPA impose major restrictions and hamper the accomplishment of high PUFA levels, particularly of DHA. The potential of alternative preys such as copepods, the main dietary component of wild juveniles and a superior prey to rear juvenile seahorses, should therefore be evaluated. The development of high-quality diets for juvenile seahorses that reflect the rich profile of the eggs (not only the lipid and fatty acid profiles, but also the global macronutrient composition) is essential to accomplish their high nutritional requirements and successfully culture newborn seahorses. Moreover, optimization of the rearing system (e.g., light and water flow conditions) and culture

procedures (e.g., feeding frequency and photoperiod) will also contribute to the rearing success of *H. guttulatus* juveniles.

The bit victory of wild against captive seahorses

Mating outside home had a strong impact on the reproductive performance of the long-snouted seahorse. Captive conditions were responsible for decreasing the size, condition and nutritional reserves of captive newborns and, consequently, their ability to tolerate stressful conditions. The lower salinity tolerance and survival capacity of the captive seahorses strongly suggest that they will have lesser chances of surviving in both the captive and natural environments. The quality of the offspring may thus jeopardize the economic viability of commercial production and the success of supplementation programs in the wild. In order to improve the cost-benefit relation and to achieve competitive prices that face those of wild-caught seahorses, captive juveniles must be in good shape and present high survival rates. Moreover, larger and higher-quality juveniles will have a survival advantage in the natural environment, due to their reduced vulnerability and greater capacity to withstand ecological challenges (such as environmental changes, food deprivation and predation), thus improving the success of reintroductions in the wild.

Although not greatly encouraging, the poor reproductive performance of captive seahorses, along with the low survival rates in the first month of life, should not efface the potential of captive breeding as a tool for *H. guttulatus* conservation. This study was a first approach into the culture of the long-snouted seahorse, still leaving a long road ahead. Results helped to clarify some aspects of seahorse biology and gave important clues to optimize seahorse captive breeding, opening space for further investigation. Further research will certainly allow deciphering the mysteries behind *H. guttulatus* culture, closing the life cycle of the species in captivity and developing a suitable culture protocol for the long-snouted seahorse.

A handful of opportunities for *H. guttulatus* captive breeding

In a time where natural populations become more and more endangered and global demands continue increasing, captive breeding needs to be part of the solution. Wild seahorses must be left alone to recover their natural balance, and captive seahorses must replace them in the race for global demands supply. There is no doubt that captive seahorses do present an opportunity to meet, at least in part, the commercial supply, but also any future increases in global demand. The question that arises is whether this opportunity will be a feasible, a desirable and a profitable one?

With time and dedication and also investment, *H. guttulatus* captive breeding may rapidly go from a challenging mission to a steady accomplishment. Establishing a suitable environment and successfully hosting successive generations in captivity may not be such a far-away reality for the

long-snouted seahorse, at least at a small commercial scale. Upgrading seahorse culture to large commercial production is, however, a different reality and should not be underestimated.

Hippocampus guttulatus is currently sought for the aquarium and curios markets, thus constituting a highly desirable live product. Since the production of ornamental fish is a rapidly growing sector of the aquaculture industry, there is increasing pressure within the aquarium trade to develop reliable and sustainable culture procedures for many fish species. Given their unusual beauty and peculiar life style, seahorses are thus expected to become an increasingly desired aquarium pet. The relatively large size and hairy look of *H. guttulatus* makes it a particularly attractive species to the live aquarium market. In this specific market, captive seahorses will be, without a doubt, a better option than their wild rivals. Cultured seahorses are undeniably better and easier companions to keep in home aquariums, at least for those looking for a tough and long-lived companion. Captive *H. guttulatus* may also be a valuable source of dried seahorses for the curios market. Nevertheless, instead of encouraging profit from the beauty of dead seahorses, we should rather inspire people to value the splendor of live seahorses.

Although a desirable species for the aquarium and curios markets, the long-snouted seahorse lacks the preferential attributes for the TCM, which is the most important and threatening seahorse market. Moreover, the TCM has kept the door closed for captive specimens because of their doubtful medicinal properties. The chances of captive *H. guttulatus* are thus extremely low in this specific market. However, given the current decline of wild stocks, the TCM community has indicated a willingness to accept culture animals, and many TCM traders are now looking to aquaculture to meet their demands (Koldewey & Martin-Smith, 2010). Studies that evaluate the medicinal properties of captive and wild seahorses, as well as of preferred and non-preferred species, may help to open space for captive *H. guttulatus* in the TCM market, or not. Studies on the medicinal properties of seahorses may be a double-edged sword. The scientific validation of the healing power of seahorses, which seems to be the most likely scenario, may increase even more the great pursuit for seahorses, particularly in western communities.

Given the high market prices of seahorses, particularly in the live aquarium trade, *H. guttulatus* captive breeding can become a gold mine. With time and knowledge, the long-snouted seahorse culture is likely to become a profitable commercial project, thus providing a new opportunity for economic investment and development. Even though the biggest challenge to viability of seahorse aquaculture in developed countries seems to be the cost of production relative to sale price (Koldewey & Martin-Smith, 2010), competitive prices may not be an issue for the European and North American markets. Besides the extravagant prices achieved in these markets, most costumers are certainly willing to pay a premium for the higher quality and life expectancy of captive-bred seahorses. Moreover, who knows if the long-snouted seahorse can gather a group of fans ready to pay extra money for their extremely attractive skin filaments? On the contrary, the prices of cultured seahorses for the curios and TCM markets may not be competitive with the low prices of wild-caught seahorses, particularly for a species with low TCM value like the long-snouted seahorse. Until the

costs of production become economically competitive with fishing prices, expansion of seahorse culture to the dried market may be unfeasible.

The potential of *H. guttulatus* culture goes, however, far beyond their commercialization. Captive breeding may also play an important and direct role in species conservation, by supplying seahorses for population supplementation programs when a last-resort recovery strategy becomes crucial. Reintroductions in the wild have become a prominent and regular measure for species conservation. Even though captive juveniles proved to perform worse than wild juveniles at the end of this investigation, enhanced culture protocols will allow improving juvenile quality and their chances of survival in the wild. Moreover, captive seahorses are expected to have greater chances of integrate well into the wild than other fishes. Behavioral deficiencies resultant from captivity, especially with respect to foraging, predator avoidance and social behavior, are expected to have less severe consequences in fish with poorer behavioral repertoires, reduced mobility, camouflage as a predator evasion strategy and monogamous pair bonds like seahorses.

It is however important to keep in mind that the supply of captive seahorses for commercial markets and for population supplementation programs are two entirely distinct goals. High-quality juveniles for the live aquarium trade will be attained with long-term captive breeding, since captive generations will become more and more adapted to artificial conditions and, consequently, improve their performance in captivity. On the other hand, long-standing captive generations will almost certainly perform poorly in natural conditions. With time in captivity, animals undergo significant genetic and phenotypic changes that will compromise fitness in the wild (reviewed in Lynch & O'Hely, 2001). While long-term captive breeding improves the rearing success and the survival capacity of seahorses to commercial markets, limit captive breeding programs to short-term situations will decrease animal domestication and increase their reestablishment potential in the wild.

A new future for the long-snouted seahorse

Guaranteeing a future for seahorses is not a matter of what can we do, but of what should we do? Supplementation of wild populations, whilst a valuable tool for species preservation, should be carefully applied as a conservational strategy, bearing in mind all the potential limitations that captive breeding can present in endangered species recovery (reviewed in Snyder et al., 1996). Besides problems regarding the high costs (both financial and physical resources) and the biological unsuitability of the species to successfully breed and survive under captive conditions, supplementation of wild populations also entails risks such as disease introductions, increased competition for resources, as well as genetic, morphological and behavioral changes that often decrease the fitness of the supplemented populations. Reintroductions in the wild should, therefore, be a last-resort recovery strategy rather than a prophylactic or long-term solution.

Even though captive breeding may represent the last chance of survival for many species facing imminent extinction in the wild, this is not the case of *H. guttulatus*, at least for now. Long before even considering the possibility of supplying natural populations with captive seahorses, other conservational actions should be evaluated and implemented. Harvesting and habitat degradation are the main threats to *H. guttulatus* populations, and captive breeding should not divert attention from dealing with the real problems. Reintroductions will never succeed without dissolving the threats that menace seahorse populations in the wild, and should be always integrated with simultaneous efforts to maintain or restore natural populations and habitats.

Unsustainable harvesting cannot persist if we want to preserve natural populations and ensure a future for seahorses. In Portugal, the capture of seahorses is entirely forbidden since 2006. Other populations may not require such a harsh measure, but precautionary measures must be implemented in order to avert crisis management later. There is a great diversity of fishery management tools that can be potentially adopted. The question is to determine the best and most successful combination.

Output controls, which directly limit the amount of fish that can be taken from the water, may not be the most efficient option. Measures such as size limits or sex-selective fishing to protect pregnant males may not be fully efficient if seahorses are returned to the water dead, with physical injuries or after experienced deleterious effects such as habitat damage, home range disruption and pair detachment. Output controls will be also nearly inefficient in managing bycatch seahorses. Moreover, the minimum permissible height of 10 cm recommended by CITES may not be biologically appropriate for some species, including the long-snouted seahorse, which begin reproducing at a standard length of 12.5-12.9 cm (Curtis & Vincent, 2006). A proper minimum size limit must be considerably above the height at first reproduction, to allow seahorses to have the opportunity to reproduce at least once, thus reducing the problem of recruitment overfishing.

In contrast, input controls, which limit directly the amount of fishing effort and indirectly the amount of fish caught, may be a better choice than output controls. Temporal closures during the high reproductive season may be helpful but may cause discontinuities in seahorse supply and fisheries subsistence. On the other hand, the establishment of marine protected areas offers a valuable measure for conservation in general, protecting the target and bycatch species, as well as the whole ecosystem. Indeed, seahorse conservation will be ineffectual unless protection and management actions are also sought for their vital habitats. Given the effects of holdfast availability on the distribution and social structure of *H. guttulatus* in captivity, degradation of structurally complex habitats will certainly affect several important aspects of seahorse life, including reproductive performance.

Conservation actions for the long-snouted seahorse cannot be evaluated without taking into consideration its effects on neighboring species, including the even more uncommon short-snouted seahorse, *H. hippocampus*. These sympatric seahorses respond differently to disturbances that modify habitat structure and complexity. Reduced fishing pressure and increased habitat cover

favored higher densities of *H. guttulatus* but lower abundances of *H. hippocampus* (Curtis et al., 2007). Given the differences in habitat preference among benthic marine fishes, simultaneous attempts to manage populations of sympatric species may require complementary strategies that support the persistence of diverse habitat types.

Additionally to habitat preservation and fishing restriction, captive breeding has also an important role in *H. guttulatus* conservation. Even though supplementation of natural populations is not, at least for now, a critical measure for the long-snouted seahorse, captive breeding is essential to replace harvesting of natural populations and provide an alternative source of seahorses for commercial trade. However, the cure must not be worse than the ailment. Aquaculture has long been known for its harmful impacts on the natural environment. To avoid adding to conservation concern, it is important that seahorse culture learns from examples of detrimental aquaculture and avoids ecological risks such as effluent discharges, escapes, introduction of exotic species and dependence of wild-caught broodstock and prey. Many of these impacts can be minimized or mitigated through careful planning. Sustainable sourcing of broodstock and live prey, genetic management, closed rearing systems and culture of native species may strongly reduce the environmental impact of aquaculture practices.

Seahorse culture may have also strong social implications. Seahorses are the main annual income of many fishers in several countries, and are also economically important to a great variety of people along the trade routes. Captive breeding cannot replace and take out the subsistence of these communities, so other forms of income generation must be identified and promoted. Any aquaculture project that does not involve fishers will have, therefore, few benefits to seahorse conservation. Even though social implications may be less severe in European countries, commercial seahorse culture may still provide economic opportunities for European fishers. Moreover, management options for seahorse conservation will not succeed without the understanding and acceptance of fisheries communities and other interested groups. No one has greater interest in preserving the sustainability of the natural resources than the people who live at the expense of their exploitation. It is a manner of reaching the consciousness of fishermen and come to a comprehensive agreement between all concerned groups.

Captive breeding provides a handful of opportunities for seahorse conservation and economic development, but should not be considered a magic potion that solves all prospective problems. Conserving seahorse populations would have best chances of success with an integrated combination of biological research, national and international conservation measures and trade monitoring, comprehensive environmental education, modification of seahorse supply and habitat protection. Increasing awareness of the threats to seahorse populations may motivate fishers to respect management actions, consumers to choose cultured over wild-caught animals, and each single person to give its own personal contribution. General public interest in seahorses and their conservation can be nurtured through a great variety of popularization actions, including magazine articles, television broadcasts, exhibitions in public aquaria and local awareness raising measures.

Combined efforts will help to preserve seahorse populations and the biodiversity of life on earth. There is no better way of giving back to nature.

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