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Work Authorship Declaration

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

I declare to be the author of this work, which is unique and unprecedented. Authors and works consulted are properly cited in the text and are included in the listing of references.

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Resumo

Os cavalos-marinhos (género *Hippocampus*) são peixes teleósteos pertencentes à família Syngnathidae, juntamente com as marinhas e dragões-marinhos. Os membros da família Syngnathidae destacam-se de outros peixes ósseos por exibirem cuidados especializados uni parentais que são assegurados pelos machos. Estes mantêm os embriões numa bolsa incubadora, e fornecem todos os cuidados parentais pós-fertilização. O elevado grau de especialização no cuidado parental faz de Syngnathidae a família com maior diversidade de espécies dentro da ordem Gasterosteiformes, na qual está incluída.

A criação de cavalos-marinhos em cativeiro tornou-se essencial para a conservação das populações selvagens, uma vez que a elevada pressão antropogénica atualmente exercida, aliada à sua biologia peculiar, tem levado a um risco acrescido de extinção das populações naturais. Os cavalos-marinhos são caracterizados pela sua baixa fertilidade, cuidado parental duradouro, aparente estratégia monogâmica, baixa mobilidade, ocupação de pequenas áreas e uma dispersão esparsa ao longo da costa (Foster & Vincent, 2004). Todas estas características conferem a estas espécies um elevado grau de vulnerabilidade a perturbações antropogénicas. Problemas como a destruição de habitats, sobre-pesca, captura para aquariofilia e medicina tradicional (principalmente a Chinesa) e como souvenirs (Vincent, 1996) ocorrem muitas vezes de forma interligada contribuindo para a diminuição das populações destas espécies e devem invariavelmente ser tidos em consideração ao realizar esforços globais para sua conservação.

O presente estudo permitiu caracterizar o comportamento reprodutivo do cavalo-marinho-de-focinho-curto, *Hippocampus hippocampus*, uma das duas espécies europeias de cavalos-marinhos que ocorrem na Ria Formosa, sul de Portugal. A informação recolhida permitiu ainda detalhar o comportamento de corte, o efeito das proporções de sexos e o potencial reprodutivo de ambos os sexos de *H. hipocampus*. O comportamento reprodutivo foi estudado em termos de atividade da fêmea, interação de "novos" machos com a fêmea, medição do "time-out" reprodutivo de cada um dos sexos na presença do sexo oposto e observação do comportamento após a postura inicial do macho. Para superar as dificuldades associada à recolha de informação sobre o comportamento de corte desta espécie no meio selvagem, o estudo foi realizado com exemplares adultos de *H. hippocampus*, pertencentes a um grupo de adultos reprodutivos, mantidos em cativeiro

na Estação Experimental de Aquicultura do Ramalhete localizada na Ria Formosa. O período experimental teve uma duração de 3 meses, com início em maio coincidente com a época reprodutiva em ambiente natural. Antes do início da experiência, ambos os sexos foram mantidos num mesmo tanque, em contacto visual, mas separados através por uma rede plástica, de forma a manter o isolamento reprodutivo até o início das observações. Os indivíduos foram transferidos para um tanque de observação após começarem a apresentar comportamentos típicos de corte.

Os resultados aqui apresentados mostraram semelhanças e diferenças comparativamente ao descrito para outras espécies de cavalo-marinho. O período de corte de *H. hippocampus* é caracterizado por comportamentos conspícuos realizados principalmente no período da manhã, sendo bastante menos frequentes no período da tarde. Como observado para o *H. whitei* (Vincent & Sadler, 1995), estes comportamentos mantiveram-se durante a gravidez do macho, apesar de ocorrerem com menor frequência. Até ao acasalamento, o *H. hippocampus* necessita de um longo período de corte composto por várias interações diárias entre os indivíduos predispostos. Em contraste, *H. fuscus* (Vincent, 1994a) necessita de apenas duas manhãs de comportamento de corte, acasalando no terceiro dia consecutivo. No rácio sexual 1♀:1♂, através do qual se definiu o comportamento padrão da espécie, observou-se uma inversão dos papeis sexuais convencionais, com a fêmea a assumir a iniciativa na maioria dos comportamentos de corte observados. Nos demais rácios sexuais 1♀:3♂ e 3♀:1♂, a proporção de machos e de fêmeas influenciou os papeis de cada sexo durante o período de corte. Assim, no rácio 3♀:1♂ observou-se novamente uma inversão dos papeis convencionais, sendo a fêmea o sexo mais ativo durante a corte. Por outro lado, no rácio 1♀:3♂ foram observados papéis sexuais tradicionais, onde o macho compete pela fêmea.

O comportamento de corte segue um padrão ritualizado, que se inicia com a abordagem dos indivíduos do sexo oposto e onde ocorre uma rápida mudança da cor original que no *H. hippocampus* passa de verde-escuro/castanho-escuro, para um amarelo suave/creme, mantendo uma faixa escura no dorso. Em seguida, os indivíduos entrelaçam as caudas, dando início a um comportamento sincronizado, que se assemelha a uma dança. Na maioria das vezes, foi a fêmea que interrompeu este comportamento, afastando-se do macho. Após o início do período de corte, os indivíduos realizam comportamentos específicos que demonstram a sua predisposição para o acasalamento, sendo estes o “Bombeamento” (*Pumping*) realizado pelo macho e o “Apontar” (*Pointing*) por parte da fêmea. De acordo com Vincent (1994b), são as fêmeas que impõem os longos períodos de corte, pois o comportamento de “Bombeamento” é exibido antes do “Apontar”. Os resultados aqui apresentados demonstram uma correlação negativa entre o número de indivíduos e o tempo despendido em comportamentos de corte, sugerindo que a competição pelo acasalamento pode acelerar o processo reprodutivo.

Os partos revelaram-se processos descontínuos, que, apesar de terem início durante a noite, por vezes prolongavam-se durante o dia ou mesmo vários dias consecutivos. O número de juvenis por gestação foi relativamente pequeno comparado ao observado em estudos anteriores para esta espécie (Boisseau, 1967; Otero-Ferrer *et al.*, 2010; Cabo, 1979; Foster & Vincent, 2004). Uma vez que o alimento vivo utilizado na alimentação dos indivíduos em estudo foi capturado diretamente da Ria, a sua qualidade não era controlável, podendo desempenhar um papel importante na explicação das posturas de menores dimensões.

Embora estudos anteriores tenham classificado a estratégica reprodutiva de *Hippocampus* spp. como sendo estritamente monogâmica, o mesmo não foi observado para este estudo. Após o término de cada parto, observou-se que os machos recebiam sempre ovos de diferentes fêmeas. Isso deve-se ao fato de as fêmeas apresentarem menor potencial reprodutivo do que os machos, fazendo com que o vínculo do casal termine após o ciclo reprodutivo.

A intensidade e a variação da competição para o acasalamento podem ser explicadas através da teoria da razão sexual operacional (OSR), pois, conforme observado, prevê que o nível de competição entre indivíduos do mesmo sexo aumente com a escassez de potenciais parceiros. Nas situações em que um dos sexos predomina sobre o outro, o sexo em maior número compete mais intensamente pelo sexo oposto devido à existência de potenciais competidores e à limitação de potenciais parceiros reprodutivos. Assim, machos e fêmeas podem demostrar tanto escolha como competição por um parceiro, dependendo do rácio sexual operacional de uma determinada população em um determinado momento.

A informação obtida neste estudo permitiu uma melhor compreensão sobre o comportamento reprodutivo do *H. hippocampus*, que até à data era assumido como sendo idêntico ao descrito para outras espécies de cavalo-marinho. Assim, com base nesse conhecimento, protocolos adequados podem ser desenvolvidos para o cultivo e manutenção dessa espécie em cativeiro, servindo também como ferramenta de gestão e conservação em seu ambiente natural.

Palavras-chave: *Hippocampus hippocampus*; comportamento de corte; seleção sexual; proporção de sexos; estratégia reprodutiva

Abstract

Seahorses are teleost fish of the Syngnathidae family where members stand out among other bony fish by exhibiting specialized uniparental male care, with males bearing the embryos in a brood pouch and providing all post-fertilization parental care. This study aimed to detail and increase the knowledge of the reproductive and courtship behavior of the short-snouted seahorse, *Hippocampus hippocampus*, the effect of sex ratio, and the reproductive potential of both sexes. Reproductive behavior was studied in terms of female activity, the interaction of "new" males with the female, measurement of the reproductive "time-out" of each sex in the presence of the opposite sex, and observation of behavior after the male's initial posture. In this study, adult *H. hippocampus* from a captive broodstock kept in the Experimental Station of Aquaculture of Ramalhete located in Ria Formosa were used. The trial period started in May and lasted approximately 3 months. Prior to the start of the experiment, fish were kept in two tanks, with sexes separated by a plastic net to maintain reproductive isolation, at the same time the reproductive stimuli were maintained through visual contact. Later, selected animals were placed in the observation tanks at the ratios of 1 female ♀ + 1 male ♂, 1 female ♀ + 3 males ♂, and 3 females ♀ + 1 male ♂. *H. hippocampus* revealed both conventional and reversed sex roles, with courtship being mostly initiated by females in 1♀:1♂ (60.7%) and 3♀:1♂ (60%) sex ratios, and by males in 1♀:3♂ (59.1%) sex ratio. With no limitation of available partners, individuals tend to choose different reproductive partners between consecutive mattings. Thus, *H. hippocampus* revealed a non-monogamous mating strategy, which led to differences in the reproductive potential of both sexes.

Keywords: *Hippocampus hippocampus*; courtship behavior; sexual selection; sex ratios; reproductive strategy.

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List of Abbreviations, Acronyms and Symbols

CITES - Convention on International Trade in Endangered Species

GB – Gigabyte

IUCN - International Union for Conservation of Nature

MMC- Multimedia Card

OSR - Operational Sex Ratio

Ø - Diameter

General Introduction

Seahorses (*Hippocampus* genus) are included in the family Syngnathidae, one of the largest families of teleost fish included in the order Gasterosteiformes. This family comprises 55 genera from which, along with seahorses, pipefish and sea dragons are also distinguished (Kuiter, 2000). Members of this family stand among other bony fish by exhibiting specialized uniparental male care, where males, rather than females, carry and care for the brood. Postfertilization male parental care ranges from simple attachment of eggs to the skin in the *Entelurus* and *Nerophis* genera, to protection and osmoregulation in the highly specialized brood pouches of *Hippocampus* and *Syngnathus* (Dawson, 1985). The high degree of specialization in parental care makes Syngnathidae, the family with the highest species diversity in the order Gasterosteiformes, with approximately 230 described species (Dawson, 1985 in Wilson *et al.*, 2001).

Currently, at least 46 species of seahorses are known, although there are still doubts about taxonomic identification (Lourie *et al.*, 1999b; Kuiter, 2001, 2009; Koldewey & Martin-Smith, 2010). Identifying seahorse species can be challenging due to limited morphological variation between species, the ability of seahorses to camouflage themselves, which creates variation within species, poor type descriptions, and independent designation of the same name for different species (Lourie *et al.*, 1999a). This problem could be addressed by applying genetic techniques that would likely change the number of known species, confirming or describing more seahorse species (*e.g.*, Teske *et al*., 2005; Lourie, 2006).

Seahorses possess a significant number of synapomorphic morphological features that distinguish them from other Syngnathids. These characters support the monophyly of seahorses, all of which are included in the *Hippocampus* genus (Rafinesque, 1810) (Žalohar *et al*., 2009). Some of these features, such as an elaborately ornamented body with the ability to develop skin filaments, upright posture, prehensile tail, long tubular snout, and the ability to change color, made them able to live camouflaged in algae and corals while ambushing copepods, mysids, and other zooplankton (Foster & Vincent, 2004; Koldewey & Martin-Smith, 2010). Short-term color changes can also occur during courtship and other intra-species interactions (Lourie *et al.*, 2004). Additional distinguishing features are the presence of a sealed brood pouch along the midline of the body in males, the absence of a caudal fin, and the characteristic elevated dorsal fin (Fritzsche, 1980; Teske *et al*., 2004, 2007).

Seahorse species are globally distributed in tropical and temperate waters, between latitudes 50° South and 50° North, being most abundant in the Indo-Pacific region (Lourie *et al.,* 2004). Inhabiting shallow coastal regions up to 30 meters deep, such as estuaries and lagoons, most of the seahorse species live in association with seagrasses and mangroves, in addition to macroalgae, sponges and corals (Foster & Vincent, 2004). However, there are reports of species occurring in deeper waters from 40 to 100 meters deep (*e.g*., *H. bargibanti* to 60 m; *H. minotaur* to 100 m) (Vincent, 1996; Lourie *et al.,* 1999a). Being poor swimmers, seahorses rarely venture into open water to chase mobile prey, instead, they are mainly found attached by the prehensile tail to corals, algae, and roots, among other substrates, and in sandy or muddy bottoms (Foster & Vincent, 2004). Seahorses are carnivores and ambush predators, remaining immobile until the approach of the prey that is captured through the projection of the snout and rapid suction of water. Adults feed mainly on small crustaceans, caridean shrimps, mysids, and nematodes, while larvae and juveniles feed on zooplankton, mainly copepods and copepodite (Kendrick & Hyndes, 2005; Castro *et al.,* 2008).

Global *Hippocampus* conservation faces significant problems including over-fishing, habitat destruction, capture for the ornamental fish market, traditional medicine (mainly Chinese) and souvenirs (Vincent, 1996). Although many seahorse species are directly exploited for international trade, such as the species *H. comes* (Cantor, 1850) (Perante *et al*., 1998), most are accidentally caught mainly by non-selective fishing nets such as trawls (Vincent, 1996; Baum *et al.*, 2003). This significant exposure to anthropogenic pressures has led to the current listing of 14 species belonging to the *Hippocampus* genus as “Vulnerable” or “Threatened” on the IUCN Red List of Endangered Species (International Union for Conservation of Nature, 2017). To date, 17 species are still considered “Data Deficient”, which means that there is still not enough information to be able to assess, directly or indirectly, their extinction risk based on their distribution and/or population status (Planas *et al*., 2008). The entire *Hippocampus* genus is also listed in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna) (Project Seahorse, 2004), allowing greater control over its exports, in search of sustainability. All CITES members must demonstrate that the international trade in seahorses, does not threaten wild populations. However, this trade remains complex, requiring a lot of effort and research to get more informed data (Foster, 2016). As an example, it is estimated that more than 20 million animals are traded each year, and only 6 million are reported to CITES (Foster *et al*., 2016).

The short-snouted seahorse *H. hippocampus* (Linnaeus, 1758) is a moderate-sized species of seahorse distributed along the coastlines of the north-east Atlantic Ocean, from the British Isles, throughout the Mediterranean Sea and the Wadden Sea southward to the Gulf of Guinea, including Azores, Madeira, and the Canary Islands (Lourie *et al.,* 1999b, 2004; Pérez-Ruzafa *et al.,* 2004; Curtis & Vincent, 2005; Pinnegar *et al.,* 2008; Valladares *et al*., 2014). According to the IUCN (2009), it is listed as "Data Deficient" on a global level, although it has recently been re-evaluated as a "Near Threatened" in the Mediterranean Sea (Pollom, 2014, 2017). It is also included in the Canary Islands Catalog of Endangered Species, where it was classified as "Vulnerable" (Government of the Canary Islands, D. 151/2001, 23 July). *H. hippocampus* is one of the seahorse species found in Ria Formosa, a coastal lagoon located in the south of Portugal, where it co-occurs sympatrically with its congener *H. guttulatus* (Culver, 1829). These species are generally found in shallow waters in sheltered bays, estuaries, and lagoon systems, where each occupies different ecological niches within the same geographic distribution (Curtis & Vincent, 2005; Woodall, 2009). *H. hippocampus* is associated with more open habitats having the ability to explore less complex habitats, such as areas of sparse vegetation (Curtis & Vincent, 2005). On the contrary, *H. glutulatus* is associated with more complex habitats with a distribution positively correlated with the percentage of vegetation cover and immobile benthic invertebrates (Curtis & Vincent, 2005). Despite differences in habitat complexity preferences, both species preferentially grasp holdfasts with their prehensile tails (Curtis & Vincent, 2005), apparently to maintain stability and crypsis. Both species are morphologically very similar. However, *H. hippocampus* has a shorter snout, a more rounded trunk shape, smaller body size, fewer fin rays, a pattern of non-coalescing spots on its body and a different head shape with a taller crown attached to the nape of the neck (Lourie *et al.*, 1999b; Curtis *et al.,* 2017).

In the wild, adults of *H. hippocampus* show seasonality in their reproductive activity that runs from Spring (April) to Autumn (October) (Foster & Vincent, 2004), when water temperatures are warmer (in the order of 22-23ºC) and the light regime is higher (Otero Ferrer *et al*., 2012). The male releases his brood after a gestation period of between 2 and 4 weeks (Boisseau, 1967), depending on the water temperature. During early life, the juveniles of *H. hippocampus* go through a pelagic phase that can last around 2-3 weeks (Wilson & Vincent, 2000; Damerval *et al*. 2003) after which there is a shift to the permanent benthic phase with the use of the prehensile tail to settle. Sexual maturity is then reached at around 16-18 weeks for males and 18 for females (Damerval *et al*., 2003).

Syngnathids exhibit a wide variety of mating patterns (Vincent *et al*., 1992; Jones & Avise 2001; Wilson *et al.,* 2003), with some species exhibiting strict monogamy, through the formation of long-term sexually faithful pairs (Kvarnemo *et al*., 2000; Vincent *et al.,* 2004). The monogamous mating strategy, rare in most taxa, is well documented in several *Hippocampus* spp (*H. fuscus*, Vincent 1994a, b, 1995; *H. whitei*, Vincent *et al*., 1995, 2004; *H. zosterae*, Masonjones & Lewis, 1996; *H. angustus,* Jones *et al.,* 1998; *H. subelongatus*, Kvarnemo *et al*., 2000). Even though females have some potential to exceed their mate's reproductive rate, they will wait until he gives birth before mating again, even if additional males are present (Vincent, 1994b). However, for some species, polygamy was observed (Woods, 2000; Kvarnemo *et al*., 2000; Foster & Vincent, 2004; Silveira, 2009, 2005; Wilson & Martin-Smith, 2007; Naud *et al*. 2009). The functional costs of finding new partners, the low density of potential mates, the ability to synchronize breeding activity and the improvement in reproduction success in consecutive mattings with the same partner should all favor monogamous relationships in seahorses (Vincent, 1990). Studies have shown that ritualized daily greetings, which usually occur early in the morning, play an important role in maintaining pair bonds and synchronizing female egg hydration with male parturition (Vincent, 1994b, 1995). Therefore, in the period before the mating season, seahorses show a long and elaborate courtship behavior, which, culminates in the pair's ascent in the water column, at the moment of mating, commonly defined as the copula (Vincent & Sadler, 1995; Masonjones & Lewis, 1996).

Sexual selection theory predicts that the relative parental investment per offspring is a primary predictor of the intensity of sexual selection and patterns of courtship behavior within species (Campbell, 1972; Williams, 1975; Thornhill & Gwynne, 1986). In most species, female investment in offspring normally exceeds that of males (reviewed by Clutton-Brock, 1991) and, as the theory correctly predicts, traditional sex roles occur during courtship, where males play a more active role in competition for access to females, these being the most selective in choosing a partner (Trivers, 1972; Williams, 1975; Gwynne, 1991). Male pregnancy leads to the deduction that female seahorses compete more intensely for access to a reproductive partner than males since they limit the reproduction of females. However, behavioral studies of several seahorse species suggest that, despite high levels of paternal investment, males are the predominant competitors for mates (reviewed in Vincent *et al.*, 1992; Vincent, 1994b; Masonjones & Lewis, 1996; Moore, unpublished results). In the selection of partners, females take into account the size of males as a guarantee of good performance, with reproductive success and the display of good parental care being greatly secured by larger males (Masonjones & Lewis, 2000). These traditional sex roles were observed in *H. fuscus* (Vincent *et al.*, 1992) and *H. zosterae* (Masonjones & Lewis, 2000), thus showing that extreme male care is not necessarily associated with courtship-role reversal, as previously assumed (Trivers, 1985).

Seahorses, including *H. hippocampus*, exhibit the most specialized forms of mating and male parental care among teleost species (Foster & Vincent, 2004). This makes seahorses a suitable model for testing the prediction that relative parental investment determines sex roles during courtship and for studying the evolution of parental care (Masonjones & Lewis, 1996; Wilson *et al.*, 2001). Daily greetings initiate copulation, which in turn ends with the female transferring her eggs to the male although sometimes the female can lay her eggs outside the male's hatching bag (Woods, 2003; Lin *et al.*, 2008; Planas *et al.*, 2008). The role of females during reproduction is restricted to the transfer of eggs in the male's hatching pouch at the time of copulation, from which it will not provide any additional care to the embryos. All the additional care provided to the embryos is carried out by the male who protects, aerates, osmoregulate and nourish them throughout their embryonic development, after which they are released as independent juveniles (Leiner, 1934, 1936; Linton & Soloff 1964; Boisseau, 1967; Haresign & Shumway, 1981).

The particularity of the male's parental care and the role of each gender during reproduction has led to several studies on the reproductive behavior of numerous *Hippocampus* species. Of the two European species, *H. guttulatus* and *H. hippocampus*, the first has been the focus of most studies related to its ecology (Curtis & Vincent, 2005, 2006; Curtis, 2007; Curtis *et al.*, 2007; Curtis & Vincent, 2008; Faleiro & Vincent, 2008; Planas *et al.*, 2008, 2009; Naud *et al.*, 2009; Fialho, 2009; Caldwell & Vincent, 2012, 2013; Correia *et al.*, 2015; Woodall *et al.*, 2015) Few studies have been published on the ecology of *H. hippocampus*, in part due to the species lower abundance (Curtis & Vincent, 2005; Curtis, 2007; Woodall *et al.*, 2011; Caldwell & Vincent, 2012; Correia *et al.*, 2015), low catchability (Curtis *et al.*, 2007), challenges associated with species differentiation (Lourie *et al.*, 1999b; Curtis, 2006) and technical difficulties in culture and breeding in captivity (Fenner, 1998; Hargrove, 1998). The study carried out on the life history and ecology of *H. hippocampus* in the Ria Formosa (Curtis *et al.,* 2017), corresponds to the only reference found on the study topic. This article summarized information on the life history, ecology and behavior of *H. hippocampus* based on data obtained over 5 years, including a capture-recapture study, visual surveys obtained from diving, and an experimental study of seine fishing.

The production of sounds is used by several groups of animals as a way of transmitting information (Bradbury & Vehrencamp, 2011). Fish stand out from other vertebrates for their great diversity of mechanisms associated with the production of sounds (Ladich & Fine 2006; Bass & Ladich 2008), probably making them the most diverse group to produce sounds (Ladich, 2004). Most of the sounds produced by fish are in the form of pulsating signals, varying in frequency and duration (Bass & Ladich, 2008). Sounds can contain relevant biological information and are used during a variety of situations such as disturbances, territorial defense, feeding, mate attraction, courtship, and spawning (for a review, see Ladich & Myrberg, 2006; Myrberg *et al.*, 2006; Kasumyan, 2009; Luczkovich *et al.,* 2011).

During sexual selection, it is common for males to produce acoustic signals to attract receptive females and convey information about location, species discrimination, sexual motivation, and sexual identity (Bradbury & Vehrencamp, 2011). The sounds produced by fish during courtship can also lead to sexual synchronization between the sexes and encourage and/or announce spawning (Fish, 1953; Myrberg *et al.*, 2006; Lobel *et al.*, 2010). According to Amorim *et al*., (2013), acoustic signals also provide information about male size and fitness, indicators that are taken into account by the female during mate selection. This was observed in the *Pomacentridae* family, with females showing a preference for lower frequency sounds, as these were indicators that the male had a larger size. (Myrberg *et al.*, 1986).

Sound production in seahorses (*Hippocampus* spp.) is generated by a skull stridulatory mechanism produced by the friction between the bones of the skull (Fish & Mowbray, 1970), resulting in clicking sounds (Colson *et al*., 1998). Seahorses have been recorded emitting clicking sounds in the course of a few behaviors, being more frequent during feeding (*e.g.,* Gill 1905; James & Heck, 1994; Berget & Wainright, 1997; Felício *et al*., 2006; Anderson, 2009; Chakraborty *et al*., 2014; Oliveira *et al*., 2014; Lim *et al.,* 2015), but also in stressful situations such as the introduction of these fish into a new environment (Fish, 1953) and during courtship behavior (Anderson, 2009; Furtado, 2013; Oliveira *et al.,* 2014).

Studying the behavior of seahorses, especially regarding courtship and mating, can be an advantageous tool since captive culture and breeding are determined by their distinctive life history characteristics (Flynn & Ritz, 1999). Although behavioral observations can induce stressful conditions that affect animal survival, growth, and welfare (Faleiro *et al*., 2008), they are considered non-invasive (Poli *et al*., 2005), and allow monitoring of animals' responses to environmental variables in real-time (Chandroo, 2000). Therefore, they represent a beneficial contribution to the improvement of seahorse culture protocols (Faleiro *et al*., 2008) by determining suitable breeding conditions and preventing welfare-related problems. It is also noteworthy that direct observations of the mating competition and sexual selection differentials in nature, when combined with behavioral observations under experimental conditions, may offer a better means of determining the importance of intrasexual competition and choice of sex partner in reproductive interactions. Studies aimed at understanding the natural biology of threatened and unknown species are also essential for a better understanding of population structure and to provide data for conservation projects, where behavior plays an adaptive role in the survival of an organism, leading to evolutionary success (Krebs *et al.*, 1996).

Captive seahorse breeding has become essential for the conservation of wild populations, at a time when anthropogenic pressure combined with their peculiar biology has led to an increased risk of extinction of the natural populations. Characteristics such as low fertility, lasting parental care, apparent fidelity to the partner, low mobility, small living areas, and sparse distribution (Foster & Vincent, 2004), makes seahorses vulnerable to human pressure. Combined with the protection of ecosystems, fisheries management and trade control, captive seahorse breeding is essential to ensure, to some extent, commercial demands without creating pressure on wild stocks and, consequently, reducing the risk of extinction. On the other hand, captive breeding can also be applied in population supplementation programs, if a recovery strategy is needed and as a valuable source of information about the reproductive biology of seahorses.

Some aspects related to the reproductive biology of *H. hippocampus,* such as its reproductive cycle and the pattern of courtship behavior remains poorly known, assuming that they are identical to those described to date for other seahorse species. The present work aims to describe and characterize the reproductive and courtship behavior of the short-snouted seahorse, *H. hippocampus,* and to evaluate the effect of sex ratio during it. It was also considered important to characterize and temporally delimit the stages that constitute the reproductive cycle of this species, analyze their reproductive strategy, and define the reproductive potential of each of the sexes.

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Characterization of the reproductive behavior of the short-snouted seahorse (*Hippocampus hippocampus*)

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Keywords: *Hippocampus hippocampus*; courtship behavior; sexual selection; sex ratios; reproductive strategy.

Abstract

Seahorses are teleost fish of the Syngnathidae family where members stand out among other bony fish by exhibiting specialized uniparental male care, with males bearing the embryos in a brood pouch and providing all post-fertilization parental care. This study aimed to detail and increase the knowledge of the reproductive and courtship behavior of the short-snouted seahorse, *Hippocampus hippocampus*, the effect of sex ratio, and the reproductive potential of both sexes. Reproductive behavior was studied in terms of female activity, the interaction of "new" males with the female, measurement of the reproductive "time-out" of each sex in the presence of the opposite sex, and observation of behavior after the male's initial posture. In this study, adult *H. hippocampus* from a captive broodstock kept in the Experimental Station of Aquaculture of Ramalhete located in Ria Formosa were used. The trial period started in May and lasted approximately 3 months. Prior to the start of the experiment, fish were kept in two tanks, with sexes separated by a plastic net to maintain reproductive isolation, at the same time the reproductive stimuli were maintained through visual contact. Later, selected animals were placed in the observation tanks at the ratios of 1 female ♀ + 1 male ♂, 1 female ♀ + 3 males ♂, and 3 females ♀ + 1 male ♂. *H. hippocampus* revealed both conventional and reversed sex roles, with courtship being mostly initiated by females in 1♀:1♂ (60.7%) and 3♀:1♂ (60%) sex ratios, and by males in 1♀:3♂ (59.1%) sex ratio. With no limitation of available partners, individuals tend to choose different reproductive partners between consecutive mattings. Thus, *H. hippocampus* revealed a non-monogamous mating strategy, which led to differences in the reproductive potential of both sexes.

Keywords: *Hippocampus hippocampus*; courtship behavior; sexual selection; sex ratios; reproductive strategy.

1. Introduction

Captive seahorse breeding has become essential for the conservation of wild populations, at a time when anthropogenic pressure combined with their peculiar biology has led to an increased risk of extinction of the natural populations. Characteristics such as low fertility, lasting parental care, apparent fidelity to the partner, low mobility, small living areas, and sparse distribution (Foster & Vincent, 2004), makes seahorses vulnerable to human pressure. Global conservation efforts have considered problems such as fishing, habitat destruction, collection of seahorses for home aquariums, traditional medicine (mainly Chinese) and souvenirs (Vincent, 1996). Although many species of seahorses are exploited directly for international trade, such as *Hippocampus comes* (Cantor, 1850) (Perante *et al.*, 1998), most are caught accidentally (Vincent, 1996) mainly by non-selective fishing nets such as trawls (Vincent, 1996; Baum *et al.*, 2003). Seahorse aquaculture is therefore seen as a possible solution to the current risk of extinction of wild seahorse stocks (Oliver, 2003), not only for commercial production but also as a tool for species conservation.

Combined with the protection of ecosystems, fisheries management and trade control, captive seahorse breeding is essential to ensure, to some extent, commercial demands without creating pressure on wild stocks and, consequently, reducing the risk of extinction. On the other hand, captive breeding can also be applied in population supplementation programs, if a recovery strategy is needed and as a valuable source of information about the reproductive biology of seahorses. It is also noteworthy that direct observations of the mating competition and sexual selection differentials in nature, when combined with behavioral observations under experimental conditions, may offer a better means of determining the importance of intrasexual competition and choice of sex partner in reproductive interactions.

The particularity of the male's parental care and the role of each gender during reproduction has led to several studies on the reproductive behavior of numerous *Hippocampus* species. Of the two European species, *H. guttulatus* and *H. hippocampus*, the first has been the focus of most studies related to its ecology (Curtis & Vincent, 2005, 2006; Curtis, 2007; Curtis *et al.*, 2007; Curtis & Vincent, 2008; Faleiro *et al.*, 2008; Planas *et al.* 2008, 2009; Naud *et al.*, 2009; Fialho, 2009; Woodall *et al.*, 2011, 2015; Caldwell & Vincent, 2012, 2013; Correia *et al.*, 2015). Few studies have been published on the ecology of *H. hippocampus*, in part due to the species lower abundance (Curtis & Vincent, 2005, 2007; Caldwell & Vincent, 2012; Correia *et al.,* 2015), low catchability (Curtis *et al.,* 2007), challenges associated with species differentiation (Lourie *et al.,* 1999; Curtis, 2006) and technical difficulties in culture and breeding in captivity (Fenner, 1998; Hargrove, 1998).

During the courtship period, fish produce acoustic signals containing relevant biological information, which can increase spawning and sexual synchronization between the sexes (Fish, 1953; Myrberg *et al.,* 2006; Lobel *et al*., 2010). Sound production in seahorses (*Hippocampus* spp.) is generated by a skull stridulatory mechanism produced by the friction between the bones of the skull (Fish & Mowbray, 1970), resulting in clicking sounds (Colson *et al.,* 1998). Seahorses have been recorded emitting clicking sounds in the course of a few behaviors, being more frequent during feeding (*e.g.,* Gill 1905; James & Heck, 1994; Berget & Wainright, 1997; Felício *et al*., 2006; Anderson, 2009; Chakraborty *et al*., 2014; Oliveira *et al*., 2014; Lim *et al.,* 2015), but also in stressful situations such as the introduction of these fish into a new environment (Fish, 1953) and during courtship behavior (Anderson, 2009; Furtado, 2015; Oliveira *et al.*, 2014).

Studying the behavior of seahorses, especially regarding courtship and mating, can be an advantageous tool since captive culture and breeding are determined by their distinctive life history characteristics (Flynn & Ritz, 1999). Therefore, they represent a beneficial contribution to the improvement of seahorse culture protocols (Faleiro *et al*., 2008) by determining suitable breeding conditions and preventing welfare-related problems. Studies aimed at understanding the natural biology of threatened and unknown species are also essential for a better understanding of population structure and to provide data for conservation projects, where behavior plays an adaptive role in the survival of an organism, leading to evolutionary success (Krebs *et al.*, 1996).

The present study investigates the reproductive behavior of the ‘Data-Deficient’ (IUCN, 2004) seahorse *H. hippocampus*. Like other seahorses, it exhibits the most specialized forms of male mating and parental care among teleost species (Foster & Vincent, 2004). Daily greetings initiate copulation, which in turn ends with the female transferring her eggs to the male although sometimes the female can lay her eggs outside the male's hatching bag (Woods, 2003; Lin *et al.,* 2008). The role of females during reproduction is restricted to the transfer of eggs in the male's hatching pouch at the time of copulation, from which it will not provide any additional care to the embryos. All the additional care provided to the embryos is carried out by the male who protects, aerates, osmoregulate and nourish them throughout their embryonic development (Vincent & Sadler, 1995), after which they are released as independent juveniles (Boisseau, 1967).

Some aspects related to the reproductive biology of *H. hippocampus,* such as its reproductive cycle and the pattern of courtship behavior remains poorly known, assuming that they are identical to those described to date for other seahorse species. The present work aims to describe and characterize the reproductive and courtship behavior of the short-snouted seahorse, *H. hippocampus*, and to evaluate the effect of sex ratio during it. It was also considered important to characterize and temporally delimit the stages that constitute the reproductive cycle of this species, analyze their reproductive strategy, and define the reproductive potential of each of the sexes.

2. Materials and Methods

2.1. Seahorse Maintenance and Handling

The experimental work took place at the Ramalhete Aquaculture Experimental Station, belonging to the University of Algarve, where adult short-snouted seahorse, *H. hippocampus*, were kept in captivity. The trial period lasted for 3 months, from May to August.

Prior to the start of the experiments, a group of *H. hippocampus* were individually selected from the initial available broodstock for a preliminary period of reproductive isolation. 80 individuals (40 males and 40 females) were assigned in to two 250 liters tanks assembled in a semi-open system and with adequate aeration (40 animals (20 males and 20 females) per tank). A plastic net (1cm mesh Ø) was used to divide the tanks and keep the sexes apart, to avoid physical interaction, but allow visual and chemical contact, ensuring that the animals are predisposed to mate. The purpose of this step was to predispose the seahorses for reproduction, at the same time it was assured that no reproductive activity took place and none of the males would show signs of pregnancy at the start of the experiment.

To avoid male selection, by the females, based on size, similar size individuals were selected for each set. The measurements of the animals were carried out according to Pritchard (1993), through the sum of two main measurements: that of the head, through the distance between the tip of the snout and the coronet (tip of the head) and the height, through the distance between the coronet and the tip of the tail. This method was chosen over the more complex method by Lourie *et al.* (1999), in order to minimize the stress during sampling (Palma *et al.*, 2012). In order to observe the standard breeding behavior of this species as well as any potential effect of the sex ratio in that same behavior, animals were placed in the observation tanks at the ratios of 1 female ♀ + 1 male ♂, 1 female ♀ + 3 males ♂, and 3 females ♀ + 1 male ♂. Observed males and females had an average length of respectively, 13.8±1.6 (cm) and 12.9±1.2 (cm). The initial selection of individuals was based on the predisposition to mate, with the display of characteristic courtship behaviors, such as the attempt to approach the opposite sex, with the fixation in the net that reproductively isolated them, and the dilatation of the abdomen in the case of females (sign indicative of ovarian development).

The selected pairs of *H. hippocampus* were placed in 150 liter glass tanks, assembled in the same rearing system of the broodstock thus maintaining the same husbandry conditions and environmental parameters (inc. temperature, dissolved oxygen, and light intensity). Artificial structures made of nautical rope (0.3 cm Ø) were provided as holdfasts for the animals under observation. The water inflow to the tanks was done through polystyrene tubes (2cm in diameter) placed at the surface to minimize disturbance, and the water level was kept close to the edge in order to maximize the height of the water column. During the experiment, the water temperature varied between 20 and 23ºC in a similar trend to the observed in the wild during the same period. Tanks were illuminated from above with 2 ×36W fluorescent tubes, with an intensity of 600 ± 25 lux at the water surface and a photoperiod controlled by a timer. Photoperiod was adjusted to match the natural photoperiod during the observation period (14 hours light – 10 hours dark) and was regulated so the light period would start when the observer was in the premises. The back and lateral tank walls were covered with a black sticker to visually isolate the fish placed in each of the observation tanks. Over the front wall of the tanks, a wood frame was assembled allowing a 30 cm gap between the tank wall and a black curtain that visually separated the animals under observation from the observer. The video cameras (SONY® FDR-X1000V) were set in place in the inside part of the frame.

During the experimental period, fish were fed with a variable mix of live mysids (*Mesopodopsis slabberi* and *Leptomysis* sp.) captured in the surrounding ponds belonging to the station in a percentage corresponding to ≈ 5% body weight day^{-1}. The animals were fed and the tanks were siphoned daily, always after the observation period to not interfere with the observations. Water temperature and salinity were recorded daily, and the remaining water quality parameters (ammonia, nitrates and nitrites) were recorded twice a week and kept stable throughout the experiment.

2.2. Observation of Courtship Behavior

To visually isolate each of the observation tanks, the back and lateral tank walls were covered with a black sticker. In the front wall, a wood frame covered with a black curtain was assembled allowing a 30 cm gap between the tank wall and the curtain to visually separated the animals under observation from the observer. This setup allowed isolating tanks from each other, so that there was no visual contact between the animals in the different observation tanks, at the same time it was also isolated from the observer and any potential interferences that could occur in the observation room. To minimize contact with the animals, external video cameras were used and set in place in the inside part of the wood frame.

The *H. hippocampus* courtship behavior was observed with the help of HD video cameras (SONY® FDR-X1000V) placed in each tank as described above, to record the frequency and duration of the different behaviors displayed by the individuals. In the set containing one female and one male (1♀: 1♂), as there could only be direct interaction between the sexes, the objective was to observe and define the standard behavior of courtship between a couple of individuals with sexual predisposition. In the remaining two sets, each comprising four individuals with a ratio of one female to three males (1♀: 3♂), and three females to one male (3♀: 1♂) the objective was to verify whether the sex ratio of the ready to mate individuals influenced the behavior pattern compared to the ones observed in the sex ratio of one female to one male (1♀: 1♂).

During the observation period, individuals were replaced by others in two different situations: the replacement of an individual with little or no interest in the opposite sex and the replacement of all individuals in a tank when suspected pregnancy occured. Throughout the experiment, a total of 39 viable individuals (which displayed reproductive behavior) were used, 13 individuals (6♀;7♂) in the proportion (1♀:1♂), 11 individuals (5♀;6♂) in the proportion (1♀:3♂) and 15 individuals (10♀;5♂) in the proportion (3♀:1♂).

According to Vincent (1994a) and Masonjones & Lewis (1996) regardless of the species, seahorse courtships mostly occur in the morning, thus to confirm this assumption, for the first week of observations, seahorses were observed starting in the morning period and continued through the afternoon period. However, as just a residual number of courtship behavior interactions were observed in the afternoon, the observation period was set to the morning period. Animals in each tank were continuously observed for 3 hours after the tank lights were turned on, and a detailed ethological record of the courtship behavior for each of the sex ratios, using video equipment was recorded.

To attain individual recognitions of the animals, that later in the observation period would allow to identify which animal was engaged in the courtship, individual recognition was done according to the method described by Correia *et al.*, (2021). In short, as the *H. hippocampus* individual recognition is possible due to the different shape of the tip of the coronet, the animals that became engaged in the initial courtship behaviors were individually identified so later, it could be determine if they extend their participation to the follow-up behaviors.

2.3. Categorization and Description of Behavior

To categorize the *H. hippocampus* courtship behavior, an ethogram (Table 2.1) adapted from Fialho (2009) including different behavioral categories was used.

Table (2.1): Ethogram of the *H. hippocampus* behavioral courtship pattern

Courtship Behavior	Male	Female
Which sex first approaches to initiate courtship behavior?		
Which sex changes color first?		
Which sex first weaves the tail into the partner's tail?		
Which sex initiates “Pairing”?		
“Pump” by the male, Yes or No?		
Which of the sexes first returns to its original coloration?		
Which sex abandons courtship behavior?		
How many males interact with the female?		
Was there direct competition between males?		
How long did the courtship behavior last?		
How long was the interval between courtship behaviors?		

As previously mentioned, the categorization of the standard courtship behavior of *H. hippocampus* was observed in the 1♀: 1♂ sex ratio. Thus, it was intended to define:

- which sex has a more active role in courtship, taking the initiative for mating, through the behaviors of "Approach", "Alteration of the original color" and " Tail Interlacing ";

- if either or both sexes exhibit some type of characteristic behavior during each courtship behavior, such as “Pairing”, and if sex specific behaviors such “Pointing” only displayed by females, and “Pumping”, only displayed by males were observed;

- which sex first "returns to the initial color" (dark brown / dark green) and "Walks away", abandoning the courtship behavior.

The total courtship period during which, all described behaviors occurred was calculated based on the records of the duration of each courtship behavior. The time interval between two consecutive courtship behaviors was also recorded during the observation time. For the 1♀: 3♂ sex ratio, three additional traits were added and recorded, “Number of intervening males” in each behavior, the existence of “Direct competition” between males and “Only interaction between males”.

2.4. Description of Standard Behaviors

To facilitate its identification during observation, the following table (Table 2.2) adapted from Fialho (2009) containing the description of the standard behaviors was used.

Table (2.2): Description of the standard behavior of males (M) and females (F) *H. hippocampus*

Standard Behaviors	Description
"Approach” (M/F)	One of the individuals approaches the partner of the opposite sex, encouraging the performance of courtship behavior.
“Alteration of the original color" (M/F)	An individual, when in the presence of another that aroused reproductive interest, changes its original color from dark brown/dark green to pale yellow/cream.
“Tail Interlacing” (M/F)	One of the individuals entwines its tail with the tail of the partner it is interacting with.
"Resume original coloring" (M/F)	One of the interacting individuals (courtship behavior) returns to its original coloration.

“Walks away",” (M/F)	One of the interacting individuals (courtship behavior) withdraws from the partner, abandoning the behavior.
“Pairing” (M/F)	One of the individuals stands side by side with the partner, initiating a synchronized behavior where both walk around the tank, close to the bottom.
“Pointing” (F/M)	One of the individuals raises its head, pointing to the surface of the water column.
“Pumping” (M)	The male bends his body forward, bending over himself, in order to open his hatching bag, hydrate it and empty it again.

The following behaviors were also considered in situations where more than one male was available to a single female (1♀:3♂ sex ratio).

Table (2.3): Description of standard behaviors of males (M) and females (F) of *H. hippocampus* in the 1♀: 3♂ sex ratio.

Standard Behaviors	Description
"Interaction between more than one male in courtship behavior"	A male starts a behavior with the female after the intervention of another or other males present in the tank.
"Direct competition between males"	The males involved in the behavior compete intensely for access to the female, exhibiting aggressive behaviors such as pushing with the snout and “fighting” with the tail.
"Only male Interaction"	Males involved in courtship behavior compete for access to the female, but without showing aggressive behavior.

2.5. Delimitation of the Stages of the Reproductive Cycle

Once the observation of the courtship period and description of the standard behaviors were concluded and a potential pregnancy was detected, the animals were removed from the observation tanks and placed in smaller 40 L tanks, assembled in the same rearing system, which allowed to keep the groups of animals together for subsequent observations. For the delimitation of the phases of the reproductive cycle, the groups of *H. hippocampus* were observed daily in order to monitor all the phases of their reproductive cycle, such as gestation, parturition and recovery from parturition by the male. To define and characterize the complete reproductive cycle of the species under study, the occurrence dates of each of the mentioned stages were recorded for each of the observed groups.

2.6. Analysis of the Reproductive Monogamy/Bigamy

The existence of an eventual reproductive monogamy in *H. hippocampus* was tested using the sets of individuals in the proportions (1♀:3♂) and (3♀:1♂). The individuals were kept together for more than one complete reproductive cycle, in order to verify if the female in the ratio (1♀:3♂) and the male in the ratio (3♀:1♂) selected the same partner in different consecutive reproductive cycles, or mated with different partners in each reproductive cycle, as there was no limitation of sexually active individuals.

2.7. Determination of Reproductive Potential for *H. hippocampus*

Since the reproductive potential is related to the time interval that the individuals are available for mating during the reproductive cycle, it was necessary to determine the latency intervals, before and after the mating. This quantification was adapted from the study carried out by Masonjones & Lewis, (2000), according to the behavior exhibited by *H. hippocampus*. The total mean duration of the reproductive cycle (T) used was that corresponding to the first reproductive cycle, where it is possible to quantify the time interval that individuals take to prepare for mating, in which competition and mate selection takes place.

2.7.1. Latency Interval ("time out") Before Mating

This time interval was quantified considering the individuals' display of certain courtship behaviors that showed a predisposition to mate. Latency time was determined by quantifying the number of days from which individuals were placed together until the male exhibited "Pumping" behaviors or when the female exhibited "Pointing" behaviors.

2.7.2. Latency Interval ("time out") Between Consecutive Mattings

This time interval was quantified from the moment males mated for the first time, to the moment they mated again. This quantification was performed separately for males and females since the females had the possibility of mating with another sexually available males in the 1♀:3♂ sex ratio.

2.8. Behavior Quantification and Statistical Analysis

To verify which of the sexes usually takes the initiative in courtship behavior and which one ends it, Chi-square tests (χ^2) (Zar, 1999) were applied. Comparisons between groups were made in terms of courtship times and intervals between behaviors using a one-way ANOVA (Zar, 1999). Afterwards, in cases with significant differences between the means, a post-hoc test, the Tukey Test, was performed to verify in which groups there were these differences. In 1♀:3♂ ratio, the interaction differences (in percentage) between directly competing males and the ones that do not exhibit this behavior were analyzed using a chi-square test ($\chi 2$).

The overall duration of the complete reproductive cycle, as well as the different stages that make up the reproductive cycle, were calculated and expressed as mean values (mean ± st. dev.), always considering the imminent temperature variation.

To calculate the reproductive potential of each sex, the average intervals of latency duration were determined, before mating and between consecutive mattings.

Statistical analyses were conducted using the software package RStudio.

At last, the analysis of the reproductive strategy presented by this species was carried out through the analysis of the behavior of individuals in the proportions (1♀:3♂) and (3♀:1♂), to which it was not necessary to perform any type of statistical test.

2.9. Recording and Analyzing Behaviors along with Sounds

In order to obtain a comprehensive overview of the *H. hippocampus* courtship behavior, and to identify if one or both genders display other behavior traits alongside the visual performance exhibited during courtship, the acoustic behavior was also analyzed. After observations of the different behavior traits have been logged in the ethogram, sound analysis allowed to make a positive or negative association between sound and behavior, that is, to match a sound to a behavior whenever these happen simultaneously. During the observation periods, alongside the video camera, one hydrophone (digitalHyd SR-1 hydrophone; MarSensing Lda, Faro, Portugal) was placed inside the observation tanks. The hydrophone was placed in view of the video camera, so any potential sound production could be linked to the visual behavior traits. The hydrophone technical features (sampling frequency, 50,781 samples per second; cutting frequency, 25 kHz; programmable gain of 1, 2, 4, 8, 16, 32, or 64; converting analog/digital 16-bit; data memory, MMC card 2 GB; autonomy of memory of about 5 h 40 min (in continuous acquisition)) ensure the recording of any eventual sounds produced by the fish under observation.

Sound and video recording took an average time of 3 hours a day. The recorded audio files were analyzed in the free audio editor *Audacity*®, and for a comprehensive analysis, sound intensity was measured in decibels (dBs).

3. Results

3.1. Courtship Behavior

A total of 195 hours of observations (180 hours in the morning, and 15 hours in the afternoon period) were obtained during this experiment. As fish remained inactive for most of the observation periods, a total of 5 hours and 32 minutes of effective courtship behavior in the 1♀:1♂ sex ratio, 7 hours and 6 minutes in the 1♀: 3♂ sex ratio, and 6 hours and 9 minutes in the 3♀:1♂ sex ratio, were obtained for each of the observed sex ratios. During these time lapses, a total of 157, 246, and 223 records of courtship behavior were respectively observed for the 1♀:1♂, 1♀:3♂, and 3♀:1♂ sex ratios. The comparison between the total time of courtship behavior and the number of records obtained during the observations are shown in Table 1.

Table (3.1) - Comparison of data obtained during observations made in the morning and those made in the afternoon

Ratio	**Total courtship time**		**Nr^{o} of records**	
	Morning	**Afternoon**	**Morning**	**Afternoon**
1♀:1♂	5 hours 22 minutes	10 minutes	150	7
1♀:3♂	7 hours 3 minutes	3 minutes	245	1
3♀:1♂	5 hours 37 minutes	32 minutes	199	24

As the observed individuals were more active soon after the start of the light period, courtship time and number of records observed during the afternoon was much smaller than in the morning period (Table 3.1).

The table 3.2 shows the comparative contribution of each sex in the courtship behavior for the three observed sex ratios, referencing the behaviors of initiative and suspension of courtship activity. This was verified, considering the behaviors of “Approach”, “Change of color” and "Entwine the tail" that initiate courtship. Such behaviors were mostly performed by females in 1♀:1♂ (60.7%) and 3♀:1♂ (60%) sex ratios, contrasting with what was observed in the 1♀:3♂ sex ratio where the number of times these behaviors were initiated by males was significantly higher (59.1%) ($p<0.05$). Subsequently, the behaviors "Resume initial coloring" and "Withdrawal" that end the courtship behavior were performed mainly by males only in one sex ratio (1♀:1♂) and by females in the other two sex ratios, (1♀:3♂ and 3♀:1♂).

Table (3.2) - Roles played by each of the sexes during the characteristic interactions of the courtship behavior, in the 1♀:1♂, 1♀:3♂ and 3♀:1♂ sex ratios (α = 0,05).

	Interactions	**% of interactions initiated by the female**	**% of interactions initiated by the male**	**P-value (Significance)**
(1♀:1♂)	"Approach"	62	38	0.0031
	"Change of color"	59	41	0.0206
	"Entwine the tail"	61	39	0.0052
	"Resume initial coloring"	36	64	0.0006
	"Withdrawal"	43	57	0.0664
(1♀:3♂)	"Approach"	43.5	56.5	0.0413
	"Change of color"	40	60	0.0022
	"Entwine the tail"	39	61	0.0006
	"Resume initial coloring"	62	38	0.0002
	"Withdrawal"	65	35	< 0.0001
(3♀:1♂)	"Approach"	53	47	0.384
	"Change of color"	49	51	0.7378
	"Entwine the tail"	51	49	0.7378
	"Resume initial coloring"	51	49	0.8408
	"Withdrawal"	58	42	0.0191

Comparing the participation of each sex in courtship behavior for the three sex ratios, (1♀:1♂, 1♀:3♂ and 3♀:1♂) (Table 3.3), no significant differences were observed, except for the "Withdrawal" behavior ($P < 0.05$).

Table (3.3) - Comparison of the roles played by each sex in courtship behavior between the three different sex ratios (1♀:1♂, 1♀:3♂, and 3♀:1♂); (α = 0.05); (**NS -** Non significant; **S -** Significant)

Interactions	**Comparison (1:1) - (1:3) - (3:1) P-value**	**Significance**
"Approach"	0.4719	(NS)
"Change of color"	0.3374	(NS)
"Entwine the tail"	0.5758	(NS)
"Resume initial coloring"	0.4719	(NS)
"Withdrawal"	0.0006	(S)

During the observation of the courtship period, other behaviors were also observed, which, in an identical way to those described, are part of the standard behavior of courtship, being these the "Pairing", "Pointing", displayed by both sexes and "Pumping", performed only by the male.

The frequency at which each sex takes the "Pairing" initiative is shown in figure 2. Except in the proportion (1♀:1♂), it is the male who, most of the time, takes the initiative in this behavior, with the most significant difference being observed in the proportion in which the males are in greater number (1♀:3♂) (χ^2: P (1:1) = 0.1749, Q (1:1) = 1.84; P (1:3) < 0,0001, Q (1:3) = 26.02; P (3:1) = 0.05214, Q (3:1) = 3.77). When comparing these values for the three propositions, it was observed that there is a significant difference between them (χ^2: P=0.00009576, Q=18.507).

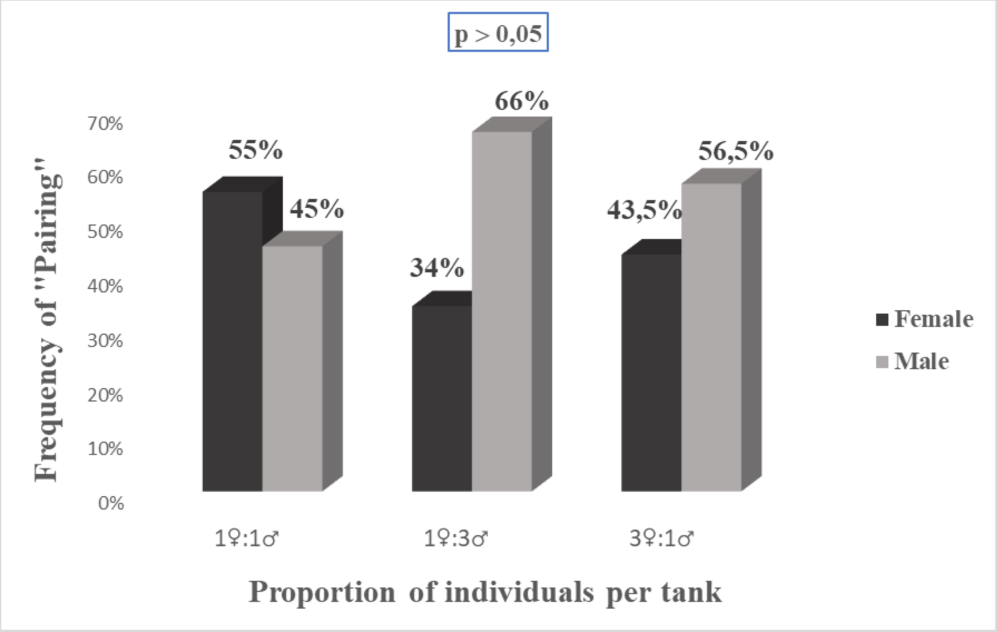

Figure (3.1) - Frequency of occurrence of the “Pairing” behavior for each of the sexes and comparison between the proportions 1♀:1♂, 1♀:3♂ and 3♀:1♂.

Figure 3.2 shows the frequency of occurrence of the remaining behaviors, in the total number of observations made. The “Pointing” behavior was observed infrequently 3%, 4% and 6% in the 1♀:1♂, 1♀:3♂, and 3♀:1♂ sex ratios respectively and found to be female exclusive. Finally, the “Pumping” behavior, exclusively performed by males, was observed in only 10% for the ratio (1♀:1♂), 17% for the ratio (1♀:3♂) and 13% for the ratio (3♀:1♂).

When analyzing the possible existence of differences between the three sex ratios in terms of frequency of occurrence of the referred behaviors, it was observed that only the “Pumping” behavior varied significantly (χ^2: Q = 4.23; P = 0.1207), with this behavior being more frequent in situations where the proportion of males in the tank was bigger.

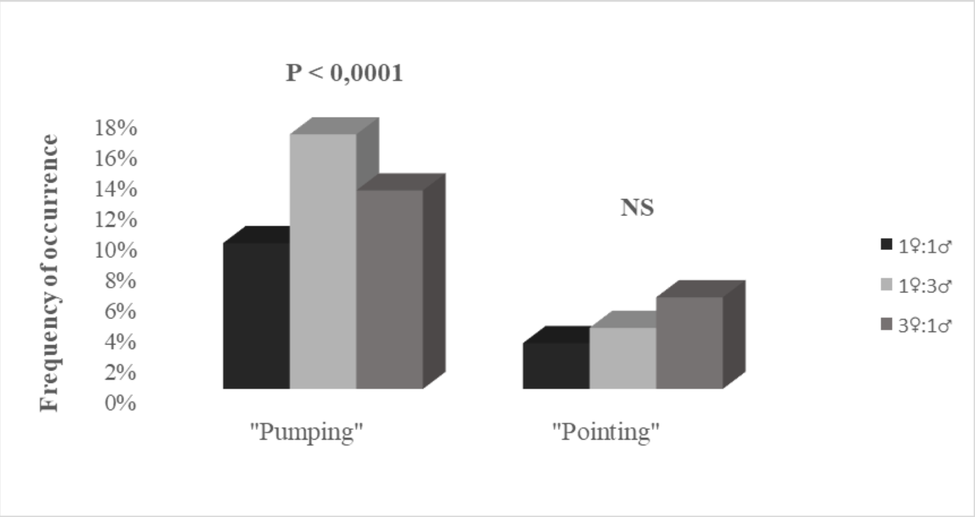

Figure (3.2) - Frequency of occurrence and comparison between behaviors of “Pumping” and “Pointing” for all ratios, (1♀:1♂), (1♀:3♂) and (3♀:1♂).

Regarding the activity and latency periods, it was observed in the 1♀:1♂ sex ratio that each courtship behavior had an average duration of 2 minutes and 7 seconds, (127.4 ± 68.9 seconds), with an average latency period of 14 minutes and 31 seconds (871.4 ± 880.9 seconds) between behaviors. In the 1♀:3♂ sex ratio, it was observed that each behavior had an average duration of 1 minute and 44 seconds (104.3 ± 69.1seconds), with an average latency period of 4 minutes and 35 seconds (275,4 ± 233,1 seconds). Lastly, for (3♀:1♂ sex ratio), the average duration of each observed behavior was 1 minute and 39 seconds (99,4 ± 68,9), with an average interval of 9 minutes and 41 seconds (581,9 ± 633,3). Comparing the results in terms of average duration of courtship behavior (Figure 3.3) it was observed that there was a significant variation (P<0.05) between the different proportions, with the 1♀:1♂ sex ratio being the one with the longest activity time.

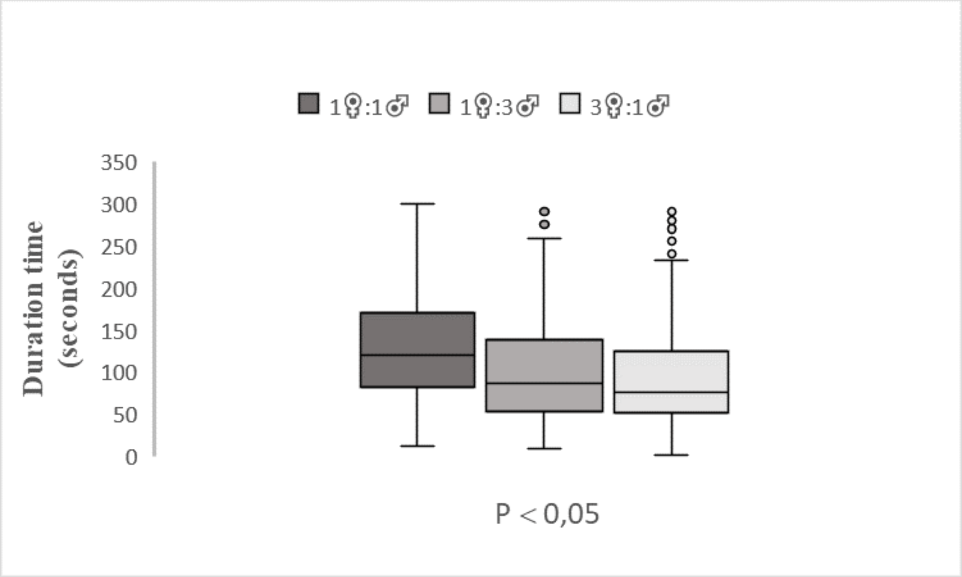

Figure (3.3) - Comparison of the average duration of courtship behavior for the different proportions of individuals per tank (1♀:1♂) and (1♀:3♂).

As in the previous results, the average interval or latency period between courtship behaviors also shows a statistically significant variation (ANOVA: P = 2.51 e^{-16}; α = 0,05) (Figure 5). After the post-hoc test (Tukey's test), it was possible to confirm a significant difference (P < 0.05) in the duration of the latency period among all observed sex ratios

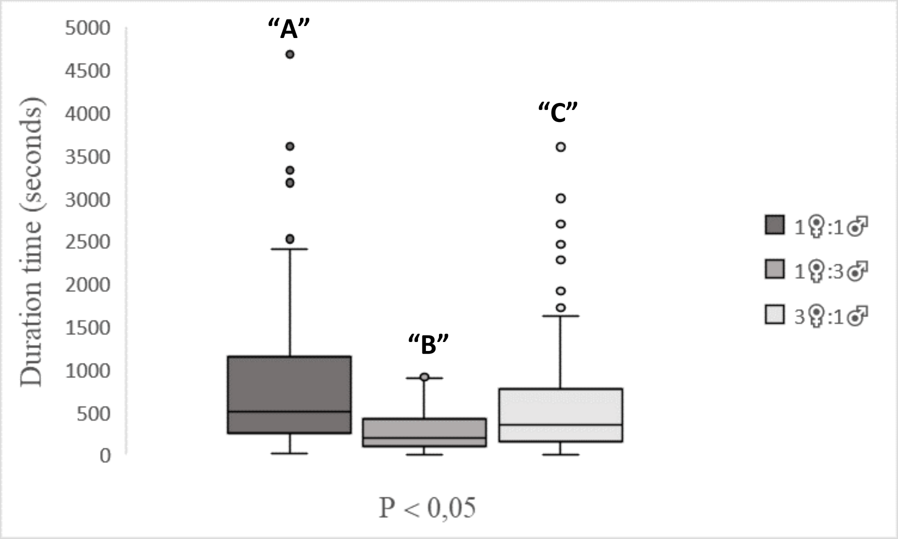

Figure (3.4) - Comparison of the average time of interval between the courtship behaviors for the different proportions of individuals per tank.

As for male competition, it was observed that when there was more than one male available to a single female, it was generally found that more than one male, on average 2 (1.7 ± 0,64) interacted with the female (Figure 3.5), and therefore, there is a probability of competition between males for access to the female. Accordingly, in 49% of the observed behaviors, two of the males have intervene in courtship behavior (figure 6) and in only 9% of the cases the 3 available males interacted in the courtship behavior. This can be translated into a high probability of direct competition between males (two or more) (58% of observed behaviors) and a smaller percentage where only one of the males interacted with the female.

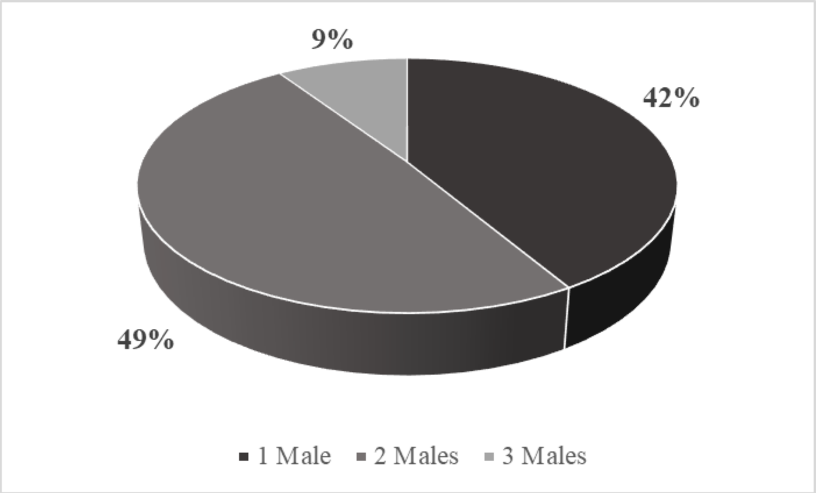

Figure (3.5) - Frequencies of behaviors, in which 1, 2 and 3 males are involved.

When more than one male interacted with the female, the percentage of males that engaged in direct competition with each other were compared to the percentage of males that relied only on one interaction (Figure 3.6). The latter was identified through an extension of the courtship behavior, after abandonment by the female. As a result, it was observed that the percentage of cases where direct competition occurred was significantly higher ($\chi2$: Q = 38,03; P < 0,0001), compared to the percentage of cases where there was only interaction between males.

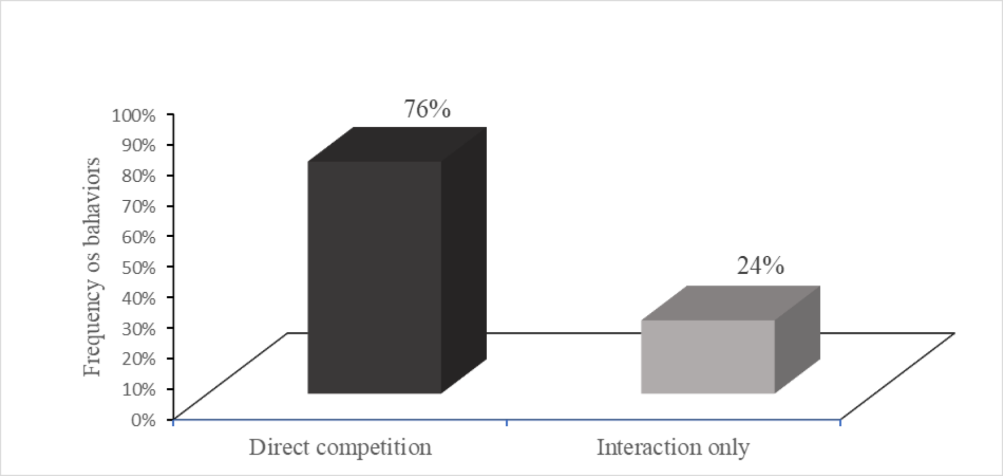

Figure (3.6) - Comparison of the frequency of occurrence of direct competition between males and the interaction between them.

3.2. Description and Delimitation of the Various Stages of the Reproductive Cycle

After completing the description of the courtship behavior and its differentiation between the three sex ratios, an extension of the time of observation was made with the objective of recording all the information that allowed a descriptive delimitation of the various courtship phases that constitute the reproductive cycle of *H. hippocampus.*

The description of the stages of the reproductive cycle will be presented together for all proportions due to the lack of relevant differences between the observed durations of each sex ratio.

During the entire experimental period, 10 pregnancies were observed, 4 in the 1♀:1♂ sex ratio, 4 pregnancies in 1♀:3♂ sex ratio, and 2 pregnancies in the 3♀:1♂ sex ratio. The average length of the reproductive cycle, which includes the gestation period, was 30.4 ± 11.2 days.

3.2.1. Copula

Of the 10 copulas that took place during the experimental period, egg transfers were only observed in two occasions, one in the 1♀:1♂ sex ratio and one other in the 1♀:3♂ sex ratio. Both took place in the afternoon, around 7 to 8 hours after the start of the light cycle. Once ready to pass her oocytes to the male, the female began to exhibit the “Pointing” behavior, ascending in the water column to the surface, followed by the sexually receptive male, ready to receive the oocytes. Ascents in the water column became consecutive and synchronized, with both partners in a facing position. After reaching the limit of the water column, the pair moved away from each other and returned to the bottom of the tank. Although the ascent was repeated several times, the transfer of oocytes to the male's brood pouch was not recorded during the observation period. In the standard situation (1 ♀ 1♂), this copulating behavior led to a pregnancy. The same did not happen in proportion (3 ♀ 1♂) where unfertilized oocytes were observed at the bottom of the tank the day after the mating attempt.

When copulation was not observed, it was very difficult to accurately determine the onset of pregnancy. This was due to the small postures that led to a small increase in the volume of the male's pouch and only a slight decrease in the abdominal volume of the female, causing the pregnancy to go unnoticed. To this extent, the onset of pregnancy is counted from the day that courtship behaviors ceased after continued observation.

3.2.2. Gestation

The mean duration of the gestation period was estimated simultaneously for the three sex ratios, since this is a physiological characteristic of individuals, not depending on the proportion of individuals distributed per tank. It was observed that the mean gestation period was 15.9 ± 2.5 days. Since the temperature varied only very slightly over the course of the experiment, no correlation between gestation time and temperature was observed.

During the gestation period, colorless eggs were found at the bottom of the tank on five occasions. In the 3♀:1♂ sex ratio, colorless eggs were found lost at the bottom of the tank for 3 times, once just moments before parturition and twice after several attempts for copulation. In the two other sex ratios, colorless eggs were found only once in each.

During the gestation periods, in the 1♀:1♂ sex ratio, it was observed that the individuals were located relatively close to each other with sporadic interactions between them, displaying a behavior labeled as "Daily greetings". In the two other sex ratios, interactions between the pregnant male and the female were also observed, many of these with the intervention of one or more males present in the tank.

3.2.3 Parturition

Parturition proved to be a discontinuous process, with a duration that varied between 1 and 3 days (1.3 ± 0.6), for all sex proportions considered. All the registered parturitions started during the dark period or a few moments after the start of the light period, when several juveniles were observed dispersed throughout the tank when the tank lights came on. Due to this no births were observed during the course of the observation period.

The number of juveniles per pregnancy varied between 123 and 350, with an average of 173 ± 71.1, again with no perceptible correlation with the temperature, which remained stable.

3.2.4. Postpartum Recovery

The delimitation of this period was made through the quantification of the number of hours from the end of the juveniles' release until the moment when the male reverts to courtship behavior towards the female. Of the total of 10 pregnancies, 5 consecutive pregnancies were observed (50%), that is, the individuals started another reproductive cycle after the conclusion of the previous one, which is marked by the release of the juveniles. In the remaining cases of pregnancy after the end of the reproductive cycle, it did not start again, despite the fact that in many cases there was an attempt, without response, by the male or the female. So, the recovery period of the male after calving, that is, the period of time required for the male to be able to mate again, it took an average of 43 ± 26.0 hours. It is important, however, to point out that in one case there was no manifestation of courtship behavior between successive pregnancies, with mating occurring in the same day, just a few hours after the previous one ended. In the remaining recurrent pregnancies, there was a less intense exhibition of courtship behavior which occurred between 2 and 3 days after the release of the juveniles.

3.3. Analysis of Reproductive Monogamy/Bigamy

The results presented in this section refer only to the sets of animals observed in the 1♀:3♂ and 3♀:1♂ sex ratios.

Of the 6 reproductive cycles, 4 occurred in the 3♀:1♂ sex ratio. Since the passing of the eggs was not observed, it was difficult to determine which of the females had passed the eggs. The method used was the observation of which of the females remained closer to the male and if this coincided with the female with the smallest abdominal perimeter, which is an indicator of the absence of eggs. It was then observed that the male possibly performed each of the 4 reproductive cycles with different females, being that the couple's bond ended with the end of the cycle with the release of the juveniles.

3.4. Same Sex Interaction

During the observation period in the proportions with 4 fish, competitive interactions between individuals of the same sex were observed several times. The aggression observed in competitive behaviors involves some type of physical confrontation, such as hitting, and use of the tail to limit the competitor's movements.

In the 1♀:3♂ sex ratio, competitive behavior between males was repeatedly observed. This male-to-male interaction accounted for 53% of the total observed interactions. On the other hand, in the 3♀:1♂ sex ratio, females also display typical competitive behaviors. This female-to-female interaction was observed in 45% of the total

3.5. Reproductive Potential

3.5.1. Latency Interval Before Mating

For the males, the established latency interval was 8.5±0.9 days and 13±11.2 days for the females. This difference in latency period between both sexes indicates that the female needs a longer time interval to prepare for the mating, that is, to give up her eggs than the male to receive them.

3.5.2. Latency Interval Between Consecutive Mattings

This interval is classified as the time interval between the first mating and the following mating. Even though in situation where there was no limitation of sexually receptive males (1♀:3♂) and the female had the possibility to mate with another male present before completion of the gestation period of the initial male it was observed that females only had consecutive copulations in the proportion (1♀:1♂).

Of the 10 observed pregnancies, 5 were consecutive pregnancies. The 2 consecutive mattings of the proportion (1♀:1♂), were the only ones in which the couple remained together for more than one reproductive cycle. In this proportion with 2 individuals, one of the pregnancies was separated by 1 day and the other by just 9 hours. The remaining consecutive pregnancies were observed in the proportion (3♀:1♂), always with a different female, one being 2 days apart, the other 3 days apart and the last one not being discernable. The proportion (1♀:3♂), was the only one without consecutive mattings.

3.5.3. Reproductive Potential and Relative Reproductive Time-out

The only females that showed predisposition and performed consecutive mattings were those observed in the 1♀:1♂ sex ratio. It was observed that those females, having to wait for the male's pregnancy to end, ended up having the same “time out" as the male. Thus, for females in the proportion with two individuals, the total mean of the reproductive “time out” during a reproductive cycle was 17 days, being male-dependent. It was then observed that for the males in general the total average reproductive “time out” was about 16 days.

3.6. Recording and Analyzing Behaviors along with Sounds

Sound data failed to identify any produced sounds related to courtship behavior. The only association observed was with feeding behavior, with the recording of the noise made during prey sucking.

4. Discussion

4.1. Courtship Behavior

This study provided a quantitative description of the standard courtship behavior of the short-snouted seahorse, *H. hippocampus*, and how it changes in the presence of different sex ratios. The fact that the experiment was carried out under artificial conditions, with the development of behavioral patterns according to the patterns of the species, made it possible to carry out this study, which, in a natural environment, would be practically impossible.

To the present, seahorse reproductive behavior has not been fully described for most of the species, and it is most often assumed to be identical for all *Hippocampus* species. As it may be expected that the seahorse reproductive behavior follows a similar pattern, is also assumable that different species may endorse variations in their reproductive behavior, proportional to the differences among species. In the present study, it was observed that *H. hippocampus* presents an extensive courtship period, characterized by ritualistic behaviors, and identical to that observed for *H. whitei* (Vincent & Sadler, 1995), *H. zosterae* (Masonjones & Lewis, 1996) and *H. guttulatus* (Fialho, 2009).

According to Vincent (1994b), *H. fuscus* only needs two full mornings of courtship behavior before mating occurs. In comparison, *H. hippocampus* showed an extended courtship period, alternating between days of full activity with days when the reproductive activity was not evident. Thus, *H. hippocampus* needs a long courtship period, consisting of daily interactions between predisposed individuals, until they are ready to mate.

As observed in wild populations of *H. hippocampus* by Curtis *et al*. (2017), courtship behaviors occurred more frequently in the morning. These behaviors were also observed in the afternoon, but much less frequently. As described for *H. whitei* (Vincent & Sadler, 1995), courtship interactions continued to be observed throughout the male's gestation period, although at a much lower rate. In species that establish pairs that last a reproductive season (*e.g., H. fuscus*, Vincent, 1995; *H. zosterae,* Masonjones & Lewis, 1996), the extension of greetings beyond the courtship period aims to reinforce the affective bonds between the pair and facilitate the reproductive synchronization between the sexes. Concordantly, could be expected that the *H. hippocampus* couples under observation would remain together for more than one reproductive cycle, but that was not observed.

The sexual selection theory described by Wilson & Martin-Smith (2007) predicts that relative parental investment is positively correlated with mate choice. Nevertheless, the present study agreed with the sexual selection theory presented by Darwin (1871) which was later proved by Amundsen & Forsgren (2001) and Foster & Vincent (2004), among others, for the *Hippocampus* genus, describing it as having conventional sex roles. This theory argues that males play the most active role during courtship, being the ones that compete for access to the female, who, in turn, is the one who chooses the best male as a reproductive partner. Despite this, previous studies have shown that competition and selection are not mutually exclusive behaviors and can occur simultaneously in both sexes (Owens & Thompson 1994; Bergstrom & Real, 2000; Amundsen & Forsgren 2001; Kokko & Johnstone, 2002; Berglund *et al.,* 2005). In contrast, recent studies on wild populations of *H. abdominalis* (Wilson & Martin-Smith 2007) reveal a sex role reversal, with females exhibiting high levels of competition, leaving the male responsible for mate selection. To better understand the changing roles of each sex during courtship, Naud *et al.,* (2009) demonstrated that the evolution of mating systems can be influenced by the difference between the proportions of sexually active males and females in a population. Forsgren *et al*. (2004) also stated that the availability of partners or existing ecological resources have the ability to rapidly change sex roles during a breeding season. Thus, according to the available information to the *Hippocampus* species, it can be observed that the sex roles are not the same for all species, and that these may change according to circumstances.

In the present study, it was commonly observed in the 1♀:1♂ sex ratio, an inversion of the traditional courtship roles as predicted by the sexual selection theory of Wilson & Martin-Smith (2007). It was often observed that it was the female who took the initiative to display courtship behaviors (in 60.7% of the observations), thus trying to engage the male's reproductive interest, contradicting the theory of sexual selection presented by Darwin (1871). Contrary to the results obtained in the study carried out by Fialho (2009) with *H. guttulatus* under similar circumstances, the predominant sex competed more intensely for the opposite sex due to the existence of more potential competitors and the limitation of potential reproductive partners. Thus, the proportion of individuals that interact with each other seems to influence the sex that initiates courtship behavior (Naud *et al.*, 2009). In the present study, in the groups where the proportion of females was higher (3♀:1♂), it was also the female that most frequently approached the male to initiate the courtship behavior and the opposite in the proportion where males were higher (1♀:3♂).

As detailed for other seahorse species, the performance of courtship behaviors implies sudden color changes, a behavior used to entice the opposite sex to courtship. In the wild this behavior can lead to a possible loss of camouflage and an increased risk of predation. According to Faleiro *et al.*, (2008) despite the greater susceptibility to being preyed upon, the risk seems to be reduced in relation to the benefits obtained with reproduction. As described for *H. whitei* (Vincent & Sadler, 1995) when approaching individuals of the opposite sex, the individual's original color changes rapidly from dark green/dark brown, to soft yellow/white, maintaining a dark band on the back. The same author described that the receptive male also changes the color of his pouch, as he begins to fill it with water to prepare to receive the eggs. During the color change, pectoral fin display was not observed for individuals of *H. hippocampus*, a behavior described by Mattle & Wilson (2009), for wild populations of *H. abdominalis*. Most often it is also the male who entwines his tail with his partner's tail, although there is not much difference between the number of times each sex initiates this behavior. This tail entanglement initiated by the male induces mating with the female with the onset of synchronized behavior (dancing), which may take a few minutes. In turn, it is the female that assumes the main role in the finalization of these behaviors, returning to its original color and moving away from the male. Although it is the female that most often approaches the male, it is he that plays the most active role during courtship, similar to what was described by Mattle & Wilson (2009) where males show a high level of activity in courtship behavior compared to female, who spend most of their time without activity. As described by Masonjones & Lewis (1996; 2000) for *H. zosterae*, in the present study females presented a significantly higher level ($P < 0.05$) of activity in the days closer to mating, showing a greater predisposition to mate.

After the beginning of the courtship period, individuals of both sexes start to present characteristic behaviors that show their predisposition to mate, and with that, a possible approach to the mating moment (Vincent, 1994a; Woods, 2000). Males begin to exhibit the "Pumping" behavior a few days before mating or when the male is receptive to receive eggs from the female. Such behavior aims to prepare the brood pouch to receive the eggs. “Pointing” is a behavior that has been observed by both sexes in some seahorse species such as *H. zosterae* (Masonjones & Lewis, 1996). Regarding *H. hippocampus*, despite the observation of this behavior in males, it was mainly performed by females when closer to copulation, not being a very common behavior.

Vincent (1994b) demonstrated that are the females that impose the long periods of courtship, possibly to confirm the predisposition of males before the final hydration of the eggs, as these, once prepared and not transferred to the male's brood pouch, will be discarded (Vincent, 1990). Accordingly, it was observed that the male is, in general, the first to show a predisposition to mate, exhibiting the “Pumping” behavior before the “Pointing” by the female.

When comparing courtship behaviors between the three sex ratios, the only behavior that showed significant variation in frequency of occurrence was the “Pumping” behavior. This characteristic behavior of males (Masonjones & Lewis, 1996) was more frequently observed in the 1♀:3♂ sex ratio, where was commonly observed more than one sexually active male competing for the one female. With an increased competition between males to gain access to the single female, males used the “Pumping” behavior as a way to display their brood pouch and their readiness to mate. As only similar sized males were selected to eliminate any eventual female preference based on other traits either than the actual male courtship performance, the hierarchy between the males was not well defined and, therefore, the competition between them to gain access to the female was quite intense until the moment of copulation.

The time spent on courtship was noticeably different, with a negative correlation between the number of individuals per tank and the time spent on courtship behavior. It was observed that the individuals in the 1♀:1♂ sex ratio spent more time performing courtship behaviors than those in the ratios with 4 individuals. In the case of the 1♀:3♂ sex ratio, having only one available female for a greater number of males with sexual predisposition, that made the individuals to strive to display their potential. This led to a higher frequency (shorter intervals) of intense competition among them for access to the female, but of shorter durations. In contrast, the 1♀:1♂ sex ratio had the most extensive intervals given the absence of competition.

Vincent (1994a; 1995) revealed the existence of competition with aggressive behavior between males to gain access to female. However, in this study, agonistic behaviors were less frequent despite a greater number of courtship behaviors involving the participation of more than one male. Contrary to aggressiveness, males revealed a prolongation of courtship interactions between them after the female had withdrawn. Females normally entices males so that they compete for her, putting each one's reproductive potential to the test (Vincent, 1994a).

The average initial courtship period was very similar between the 1♀:1♂ and 1♀:3♂ sex ratios, differing considerably from the 3♀:1♂ sex ratio. These differences lead to the assumption that the duration of this period varies according to several aspects intrinsic to each individual. Among these aspects, the preparation of the male's incubation pouch to receive the oocytes and the stage of maturation of the female's oocytes can be highlighted, which, were certainly not the same, for all sets of individuals used at the beginning of each treatment. Sex ratio was also a factor believed to have led to a variation in the total duration of the initial courtship period due to the existence of competition in situations where the ratio of males per tank was higher. These observations suggest that the reproductive process can be accelerated by competition for mating, decreasing the courtship period established by individuals.

4.2. Description and Delimitation of the Stages of the Reproductive Cycle

Observations carried out in wild populations of *H. abdominalis* (Poortenaar *et al.*, 2004), refer to the existence of reproductively mature females during almost year round, which reveals that the reproductive season can be quite extensive for some species. According to previous observations of full pouches, the proportion of males of wild *H. hippocampus* able to reproduce varies throughout the breeding season, which may extend between April to October (Wheeler, 1985; Whitehead, 1986; Reina-Hervás, 1989). However, consistent with the observations of Curtis *et al.* (2007), during the present study, the breeding season of this species peaked in July and August and within a temperature range between 20.7 and 23ºC. According to Otero-Ferrer *et al.*, (2012) successful reproduction in *H. hippocampus* close to the Canary islands occurs when temperatures vary between 22-23ºC. Considering the results obtained during the experiment, it is possible to observe that this species can have reproductive success at temperatures below 22ºC, denoting a geographic adaptation.

As described for *H. guttulatus* (Planas *et al.*, 2010) and *H. zosterae* (Strawn, 1958; Masonjones & Lewis, 1996, 2000), *H. hippocampus* females transfer the whole set of eggs to a single male in each reproductive cycle. After mating, even if the available space in the male's brood pouch exceeds the number of eggs provided by the female, the male cannot simultaneously incubate more than one batch of eggs. During egg transfer, ovulation represents an irreversible commitment from the female, so, if the pair is separated during this period, the female will eventually release her batch of unfertilized eggs in the water column (Vincent, 1990; Masonjones & Lewis, 1996). During the experimental period, in some pairs/sets, eggs were observed at the bottom of the tank with colorations varying from the normal orange color to colorless. Although the pairs were not separated during the reproductive cycle, the possible non-predisposition of the male after the preparation/hydration of the eggs, imposed a mating asynchrony, which may have led the female to get rid of the eggs. In the case of the observed colorless eggs, it can be due to intentional discharges of nonviable eggs by the male(s), moments before parturition.

The hatching occurs at night, which can be considered an advantageous strategy since it determines, under natural conditions, the reduction of the risk of visual predation (Vincent, 1990; Vincent & Sadler, 1995; Woods, 2000). However, in captivity, predation is non-existent which may pre-dispose differences in this behavior. In this study, births were observed to start at night, but sometimes continue during the day, or even for several consecutive days, an observation that aggress with Faleiro *et al.* (2008), who reported that *H. guttulatus* parturition proved to be a discontinuous process. As described for other seahorse species (Foster & Vincent, 2004), the juveniles of *H. hippocampus* swim directly to the water surface, soon after leaving the paternal incubation bag. According to Woods (2000), this behavior occurs through the inflation of the gas bladder, giving juveniles immediate access to zooplanktonic prey, and acting as a dispersal mechanism.

The range of brood sizes (173 ± 71.1) was smaller than reported in most of the previous studies: with means of 509 in Boisseau (1967), 50 in Golani & Fine (2002) and

468 in Otero-Ferrer *et al*. (2010); and ranges of 200–300 in Cabo (1979) and 250–300, as reviewed by Foster & Vincent (2004). A factor that can greatly influence brood size is the quantity/quality of food that will condition parental fitness, either by affecting sperm motility, ovary development or fertilization (Forteath, 1997; Wong and Benzie, 2003; Foster & Vincent, 2004; Lin *et al*., 2007; Fernandez-Palacios *et al*., 2011).

In this study, the individuals under observation were fed a variable mix of two wild captured mysid shrimp species, prior to the start of the experiment, and throughout it. Although this feeding protocol is fitted to this species and allows to maintain the adults with good fitness condition, it may lack specific nutritional elements (*e.g*. vitamins and carotenoids) deemed to produce high-quality eggs and sperm and could play an important role in explaining the smaller brood size.

4.3. Analysis of Reproductive Monogamy/Bigamy

The monogamous mating strategy is well documented in several *Hippocampus* species (*H. fuscus*, Vincent 1994a, b, 1995; *H. whitei*, Vincent *et al*., 1995, 2005; *H. zosterae*, Masonjones & Lewis, 1996; *H. angustus*, Jones & Avise, 1998; *H. subelongatus*, Kvarnemo & Avise, 2000). According to Vincent & Sadler (1995), during a reproductive season, *H. whitei* couples mate repeatedly and exclusively, and interactions with individuals other than the usual sexual partner are extremely rare. However, *H. abdominalis* (Wilson & Martin-Smith, 2007) and *H. guttulatus* (Naud *et al.*, 2009), evidence behaviors of social promiscuity, despite being considered to have a monogamous reproductive strategy. Furthermore, according to Wilson & Martin-Smith (2007) the true frequency of multiple mattings is often underestimated in investigations of social mating systems, while genetic studies reveal that in socially promiscuous species, it is rare for the mating strategy to be strictly monogamous. The observations for *H. hippocampus* are in agreement with Colwell and Oring (1989) who suggest that promiscuous mating behaviors after copulation may provide an opportunity to assess mate quality for subsequent mattings.

Monogamy can be advantageous as it reduces the physiological costs associated with finding a new reproductive partner and the time span required for it to reach a mating predisposition, thus increasing reproductive potential (Sogabe *et al*., 2007). The reproductive potential of both sexes tends to increase in monogamous species, because after consecutive mattings, the couple ends up acquiring a high level of reproductive synchrony. According to the results obtained for the 1♀:3♂ sex ratio (the only where more than one male was available), consecutive mattings with the same male were not observed. Likewise, in situations where more than one female was sexually available to a single male, the individuals under study did not show monogamy during the reproductive cycle. Consequently, the formation of pairs and their maintenance during the reproductive season does not seem to be the only mating strategy adopted by the species, since the male, when predisposed to mate, always received eggs from another female present in the tank. A possible conclusion for choosing different females in each reproductive cycle could be that females may have a lower reproductive potential than males, causing the couple's bonds to disappear after the reproductive cycle.

The fact that the experience was developed in captivity may have contributed to the lack of strictly monogamous reproductive strategies in any of the proportions. In a captive environment, relationships with individuals other than the usual reproductive partner occur naturally without the usual waste of time and energy in finding a new sexual partner, which may be more common in wild populations. The non-establishment of monogamous relationships between individuals was supported by differences in the rate of reproductive potential of each sex. As observed in males, the sex with the highest reproductive potential is expected to mate polygamously unless there are temporal or spatial restrictions on mate availability (Evans *et al.,* 2004). In these captive situations, polygamy could be advantageous to increase the rates of reproductive potential on the part of the males, since they appeared to have greater potential to mate again, after the end of the gestation period, than the initial female. According to the results obtained in this study, it was found that the interval between mating is different for males and females. This was observed in situations in which, even though there was no limitation of sexually receptive females, the male, after the end of pregnancy, always ended up choosing another female that was predisposed and prepared to transfer the eggs. As so, the fact that the interval between pregnancies is shorter in males than in females reinforces the probability of bigamy.

The lack of synchrony between the first pair formed may be another possible explanation for the non-occurrence of strict monogamy. This could possibly be caused by the presence of more than one sexually available male (in the 1♀:3♂ sex ratio) or female (in the 3♀:1♂ sex ratio) in a same restricted environment. Males only take a few hours to get fully ready to mate, and they can stay that way for as long as the female takes to prepare, which usually takes at least 2 days until they become sexually receptive after the preparation of a new clutch of eggs (Masonjones & Lewis, 2000). Since in the 3♀:1♂ sex ratio there were more females that could be receptive before the initial one, the male could have chosen a new ready to mate female. On the other hand, after hydration of the eggs, the females have little time to transfer them to the male. So, if in the 1♀:3♂ sex ratio this process does not coincide with the availability of the males, it can be assumed that the female could choose to avoid the risk of losing her eggs and the physiological investment associated by choosing another partner. Like so, one can consider that the sexes, in these situations, chose another partner to avoid losses or increase their reproductive potential in terms of the number of offspring. The *H. hippocampus* presented a similar mating system to that of *H. abdominalis* (Woods, 2000), that was observed mating with more than three different individuals during the same breeding season. Later on, Wilson & Martin-Smith (2007) contradicted the theory of Woods (2000), stating that, genetic studies revealed their monogamy despite the social promiscuity presented by this species.

Given the ambivalence between the results obtained in the current study and those carried out to date, it is reasonable to question the strict monogamy that has been assumed for the *Hippocampus* species. In view of the results, it can be concluded that monogamy can occur during a reproductive cycle but, in the presence of more than one member of the opposite sex, monogamy does not occur.

4.4. Same Sex Interactions

The operational sex ratio (OSR) concept is defined as the sex ratio of individuals ready to mate in a population (Emlen, 1976; Emlen & Oring, 1977). The intensity and variation of mating competition can be explained by the OSR theory, as it predicts that the level of intrasexual competition increases with the scarcity of potential mates (De Jong, 2011). In a biased OSR, the mate-limited sex is expected to compete more intensely for access to mattings. It is therefore assumed that males and/or females can both chose and compete for a mate, depending on the OSR of a given population at a given time (Lawrence, 1986).

In the observation trials with 4 individuals, a switch in sex roles coincided with the change of OSR from male to female-biased. In situations of male-biased OSRs (1♀:3♂ sex ratio), interactions between males were the most prevalent behavior, as males competed more intensely for mates due to the existence of potential competitors. Concordantly, as speculated in previous studies (Emlen & Oring, 1977; Rosvall, 2011; Myhre *et al*., 2012), in the situation of female-biased OSRs (3♀:1♂ sex ratio), due to male limitation, female-female competition was very frequent, as observed in sex role reversal systems.

These results support the hypothesis that the number of available partner’s influences both the sexual response and mating strategy of females and males (Thornhill, 1984; Lawrence, 1986; Souroukis & Murray, 1995).

4.5. Differences in the Reproductive Potential of Males and Females

The determination of the reproductive potential for each sex, and the exact definition of the “time out” of each one of them, has been a difficult subject in previous studies. According to Clutton-Brock & Parker (1992) the “time in” is the period in which an individual is able to reproduce in the presence of sexually available reproductive partners. On the other hand, “time out” is the time involved in reproductive tasks such as gamete preparation, courtship, copulation, and parental care (Clutton-Brock & Parker 1992; Parker & Simmons 1996).

In the present study, the exact time each sex spent in “time in” and in “time out” during the courtship period before mating was difficult to calculate. This difficulty was verified several times, mainly regarding the females as for the males it was easier to determine these time frames through the observation of more evident behaviors that showed a predisposition to mate, as previously described.

Differences in latency periods (“time out”) can lead to differences in their reproductive potential, as they cause an inequality in the average number of offspring produced by each sex during a breeding season. In this study, the latency period of females before mating proved to be longer than that of males, meaning that females need more time to prepare their eggs than males need to prepare to receive them (Masonjones & Lewis, 2000). On the other hand, the latency interval between consecutive mattings was not discernible between the sexes. In the 1♀:3♂ sex ratio despite multiple sexually receptive males were available, females were not observed to remate during the gestation of their partner, but males were observed mating with a different female only 2-3 days postpartum in the 1♀:3♂ sex ratio. A possible conclusion to the fact that the male always mated with a different female is that females may have a lower reproductive potential than males, causing the couple's bonds to disappear after the reproductive cycle. Despite being males that provide osmoregulation, oxygenation, removal of residues, protection and, possibly, nutrients for the development of the embryos in their brood pouch during pregnancy periods (Foster & Vincent, 2004) the reproduction costs appear to be higher for females, as egg production is physiologically more cost full than sperm production. The same was observed by Vincent (1994b) in unpaired *H. fuscus* seahorses, where males were seen preparing to mate faster and remaining sexually receptive for a longer period of time than females, causing the reproductive potential male to exceed that of females and therefore being the most competitive sex.

5. Final Considerations

The objective of conceiving this work was to approach some aspects of the reproductive behavior of the short-snouted seahorse, *H. hippocampus.* The lack of previous studies on this subject, led to the assumption that the reproductive behavior of this species would be the same or similar to that of other seahorse species studied to date. To test this assumption, an experimental protocol was defined in order to vary the proportion of one sex in relation to the opposite sex, with the objective of defining the reproductive behavior in each of these sex ratios.

According to the courtship behavior results obtained in this study, *H. hippocampus* presents both conventional and reversed sex roles, the latter meaning that females are responsible for competition and males are for the selection. The courtship roles of each sex were influenced by the sex ratio, with the most numerous sex also being the most competitive. On the other hand, in the proportion of 2 individuals that aimed to define the reproductive behavior of the species, an inversion of the conventional sexual roles was observed. In situations with 4 individuals, the time spent in courtship tends to decrease, as the behaviors are more frequent but shorter and with smaller intervals between them. The decrease in the interval between courtship behaviors observed in situations with 4 individuals is induced by the existence of competition for partners. This can be observed in the situation with 2 individuals, where, due to the absence of competition, the intervals between courtship behaviors were longer.

Copulation was difficult to observe during the experimental period as it is a late-day/early-night process. However, the moment of copulation is characterized by the display of a synchronized behavior between the male and the female, with consecutive ascents in the water column, until the moment when the female manages to transfer all her eggs into the male's brood pouch. Although to a lesser extent, behavioral interactions are maintained throughout the male's gestation period, with the aim of reinforcing the couple's bond, in addition to contributing to reproductive synchronization between the sexes.

Parturition is a discontinuous process, having been observed with durations between 1 to 3 days, and a variation between 123 and 350 juveniles. All births started during the dark period or a few moments after the start of the light period. Male postpartum recovery generally lasts an average of 43 hours, and, in many cases, no manifestation of courtship behavior was observed between consecutive mattings.

Regarding the reproductive strategy adopted by this species, a strictly monogamous reproductive strategy was not observed. Taking as an example, the record of consecutive mattings of one of the males always with different females. Although the observed cases of bigamy are not enough to assume this as the main reproductive strategy of this species, the results obtained in this study refute the long-standing theory of strict monogamy for the genus *Hippocampus*.

In future studies, we suggest the continuation of the present work, giving special attention to bigamy since the number of cases in which it was found to exist was not sufficient to assume this reproductive strategy as the main. It will also be important to repeat some parts of the experimental protocol used, in order to confirm the results obtained. It is also suggested an experiment where several factors are tested (e.g., temperature) in order to understand the causes of oocyte losses by females, and abortions by males

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