

Possible function of discharge newly discovered from the hedgehog seahorse *Hippocampus spinosissimus*

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Abstract

Three types of discharge were observed to be released from captive specimens of the hedgehog seahorse *Hippocampus spinosissimus*: feces and two other newly discovered types. Of the latter two, one (“type 2 discharge”) was a translucent mucus that was expelled from the anus and was clearly *not* feces; upon microscopic observation, it remained in the water column longer than feces and was more likely to adhere to artificial holdfasts. Seahorses were significantly more likely to perch on holdfasts containing this discharge ($p < 0.01$), possibly via their detecting of its smell (*i.e.*, olfactory behavior). Preliminary tests revealed that the active components of type 2 discharge are significantly water-soluble ($p < 0.05$). We surmise that this discharge expulsion behavior may be the mechanism by which seahorses “mark their territory” and establish site fidelity.

Key words: defecation, holdfast, *Hippocampus spinosissimus*, ichthyology, olfactory cues, seahorse, site fidelity, Taiwan

Introduction

Many territorial species establish their home ranges with “marks.” Canine species do so with urine (Sillero-Zubiri and Macdonald, 1998), and Western banded geckos (*Coleonyx variegatus*) establish their preferred defecation sites away from their diurnal resting sites, typically in areas that have already been

marked by conspecifics (Carpenter and Duvall, 1995). However, with relatively few exceptions, fish exhibit neither discrete glands for generating such chemical markers, nor do they present readily apparent marking behavior (Liley, 1982).

Seahorses maintain small home ranges over several breeding cycles,

making them among the most sedentary of vertebrates; many species exhibit site fidelity (Vincent et al., 2005). How they establish their territories, though, is unclear, and we aimed to uncover this herein. The lack of 1) paired pelvic fins and 2) a caudal fin means that seahorses cannot swim far or fast. Instead, they use their prehensile tails to attach firmly to holdfasts, such as the soft coral *Dendronephthya australis* (Lourie, 2016). Seahorses can be found in a wide range of habitats, including seagrasses (Bell et al., 2003; Vincent et al., 2004), mangroves (Rosa et al., 2007), coral reefs (Perante et al., 2002), and artificial structures (Harasti et al., 2010), as well as “faunal substrata,” such as sponges, soft corals (Kuitert, 2009), and macroalgae (Moreau and Vincent, 2004). A threatened seahorse does not swim away, but instead instinctively holds more tightly to its holdfast and tucks its head into its chest. Often, they simply choose whichever holdfast is readily available, but some seahorses show more discrimination (Harasti et al., 2014). *Hippocampus whitei* prefers sponges and soft corals, and females and males of this species can stay on the same holdfast for up to 10 and 17 months, respectively (Harasti et al., 2014).

Hippocampus spinosissimus is distributed throughout Australia,

Cambodia, China, Taiwan, Indonesia, Malaysia, Myanmar, the Philippines, Singapore, Sri Lanka, Thailand, and Vietnam (Lourie et al., 2004); although difficult to maintain in captivity due to the potential for air-bubble disease, it is nevertheless a popular species in the aquarium trade. It is typically found at depths > 8 m (with a maximum of 70 m reported by Lourie et al., 2004), near coral reefs on sandy bottoms. As is the case in other seahorses, *H. spinosissimus* lacks teeth and a true stomach; food is sucked whole into the mouth cavity and then passed down through the esophagus into a simple, tubular foregut. Undigested waste materials pass through the gut and out of the anus (Lourie, 2016). We previously observed a variety of non-fecal discharge being expelled from the anuses of *H. spinosissimus* and hypothesized that it may play in role in site fidelity determination, an idea we explored in more detail herein with the additional hope of gaining insight into means of improving the success of long-term seahorse culture.

Materials and Methods

The entire *Hippocampus* genus is listed in Appendix II of CITES (as of May 2004). Since *H. spinosissimus* is listed as vulnerable by the IUCN (Lourie et al., 2004), only local Taiwanese

specimens were used herein. All fish purchasing and handling procedures complied with the 1) Animal Protection Act of the Government of Taiwan and 2) the ethical rules for vertebrate research of the National Taiwan Science Education Center (NTSEC; approval ID: 2018 Taiwan International Science Fair No. 050011). The NTSEC considered the target species, the necessity for research, the number to be potentially sacrificed, and the conservation status, and decided that eight was a sustainable number for use in scientific research; therefore, five females and three males were purchased from a local aquarium pet shop between 2016 and 2018 and re-identified using the taxonomic keys outlined by Lourie et al. (2004) and Lourie (2016).

All seahorses were sub-adult

(determined by dissection), with a total length of 11 to 15 cm (Table 1). They were housed separately in 46 x 20 x 30 cm glass tanks (27.6 L) filled with mixed natural and artificial seawater and exposed to natural sunlight. Submersible motors were used to maintain water flow. Air bubbles were produced and bubbled in a separate tank to serve as an oxygen supply. Seahorses were fed daily with cherry shrimp (*Neocaridina denticulata*), with an occasional supplement of brine shrimp (*Artemia salina*). The concentrations of nitrate, nitrite, and ammonia, as well as the pH value, were monitored, and the water temperature was automatically maintained at 25-27 °C by an iSTA heater. A 1 x 1 x 55 cm plastic sponge was placed in each tank to serve as a holdfast. Improper aquarium aeration can cause under- or

Tab. 1. General information on seahorses used in this study.

	Purchase date	Gender	Length
Seahorse #1	2016-08	Female	4.3 cm
Seahorse #2	2016-08	Male	5.0 cm
Seahorse #3	2016-09	Female	5.0 cm
Seahorse #4	2016-11	Female	4.5 cm
Seahorse #5	2016-11	Female	4.6 cm
Seahorse #6	2016-11	Male	4.8 cm
Seahorse #7	2017-05	Female	4.8 cm
Seahorse #8	2017-05	Male	4.9 cm

over-inflation of seahorse gas bladders, which results in the aforementioned air-bubble disease. As such, special care was taken herein to monitor the presence of gas bubbles under the seahorse scales. No seahorses were sacrificed for experiment use, though some eventually died of presumably natural causes; their corpses were autopsied and stored at the National Experimental High School at Hsinchu Science Park (Taiwan) while those that survived were moved to the National Museum of Marine Science and Technology of Taiwan (Keelung).

Examination of the discharges of *H. spinosissimus*

All tanks were checked daily for monitoring of 1) water quality and 2) the health status of the seahorses. All seahorse discharge was collected by eye droppers and stored in separate test tubes once a day for microscopic examination. To determine whether the feeding frequency affected the frequency of discharge, one specimen (female) was fed once a day with *N. denticulata* (dry weight: $\mu \pm SD = 0.06 \pm 0.02$ g) and another specimen (female) was fed once every three days. The seahorses were housed in

an environment consisting of three tanks connected by “communicating” tubes, and the frequency of discharge release from each specimen was recorded daily for 20 consecutive days.

The drifting pattern of the discharge in the aquarium water column was compared using ImageJ. A 1-L beaker was filled with seawater, and all discharges were carefully dispensed onto the surface of the water via a dropper. The sinking pattern for each sample was recorded using a camera. The video data were then converted into pictures-per-second data using Free Video to JPG v.5.0.99, and the sinking pattern was plotted using ImageJ and Spotfire software. Both the total elapsed sinking time and sinking velocity were calculated. The discharge expulsion events were recorded on video, and an artificial light source was used when needed. Continuous 8-hr (16:00-24:00) observations were recorded each day to gain further insight into the discharge emitted.

Effect of discharge on holdfast selection

To determine whether seahorses have a higher probability of perching on holdfasts coated with discharge, an experiment was undertaken (Fig. 1) in a 35 x 55 x 35-cm tank. Six of the eight *H. spinosissimus* specimens (seahorses #1-6 of Table 1) were tested. Discharges from the seahorses were collected and stored on a small plastic sponge (2 cm in diameter). Two opaque, U-shaped water pipes (60 cm in length) were used as artificial holdfasts. The plastic sponge containing the discharge was placed at the end of one of the water pipes, and a clean plastic sponge was placed at the end of the other. The two water pipes were placed at either side of a tank filled with seawater to a depth of 30 cm. A seahorse was then positioned in the middle of the tank and allowed to choose a holdfast. When the seahorse chose a water pipe on which to perch, it was removed from the pipe, and both

water pipes were cleaned and washed before the next trial. The positions of the holdfasts were switched every two trials. If a seahorse made no choice after five minutes, the experiment was paused for at least four days to 1) allow the seahorse to rest and 2) replenish the seawater in the tank. If the seahorse showed symptoms of air-bubble disease, the experiment was stopped, and the seahorse was allowed to recover. For the control group, neither water pipe was coated with discharge. Using a null hypothesis that discharge does not affect seahorse holdfast selection, a Chi-squared (X^2) test was used to determine whether proportions were significantly higher than the “no preference” percentage of 50%. A Mann-Whitney *U*-test was also used to determine whether there was significant difference between the experimental and control groups. Both means and standard

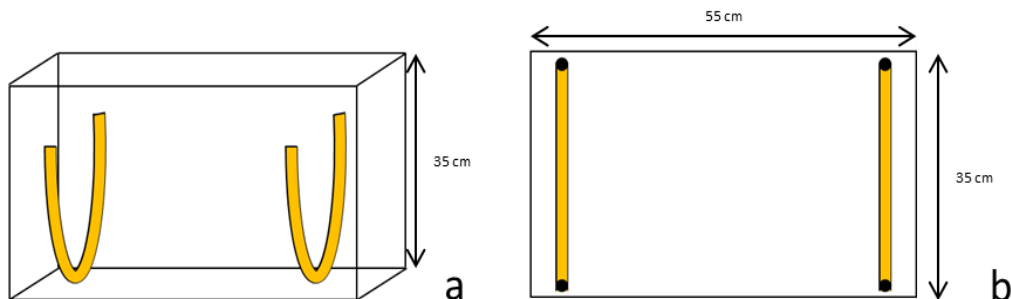


Fig. 1. The tank settings for the holdfast preference test: (a) side view; (b) top view. U-shaped water pipes were used as artificial seahorse holdfasts, and the black dots in (b) indicate where the discharge-soaked plastic sponges were placed. The tanks were 35 x 55 cm x 35 cm.

deviations of the selection percentages were calculated, as well.

To determine whether the constituents of the type 2 discharge were water-soluble, distilled water and ether were used to extract the high- and low-polarity substances, respectively, in an ultrasonic oscillator (Sonic Wave CD-2800, Ultrasonic Jewelry & Eyeglass Cleaner, USA). Water, ether, and non-soluble layers formed, and the former was isolated, dispensed onto plastic sponges, immersed into aquaria (after excess water had evaporated), and tested as above with four of the eight seahorses (#3, #5, #7, and #8 of Table 1). Under the null hypothesis that the partial extract from the discharge would not affect seahorse holdfast selection, a Mann-Whitney *U*-test was used to analyze the data ($\alpha=0.05$ for this and all other statistical tests mentioned above).

Results

Examination of the discharges of *H. spinosissimus*

Three types of discharge

H. spinosissimus emitted three types of discharge from the anus; although their shapes and textures were different, all particles were approximately 2 mm in diameter. “Type 1” discharge was the most common (Fig. 3) and seemed to be influenced by food intake. Feeding with *N. denticulata* and *Artemia salina* led to discharges with orange and white hues, respectively. Because undigested food particles (*i.e.*, *N. denticulata* and *A. salina*) were also seen, we concluded that type 1 discharge was feces (Fig. 2a-b). Type 2 discharge consisted mostly of mucus and appeared translucent (Fig. 2c); its color was not influenced by food intake, nor were *N. denticulata* or *A. salina* found within it. Type 3 discharge appeared white and opaque and lacked mucus (Fig. 2d). Its color was also not related to the aquarium feed, nor was food residue observed under microscopy. The expulsion of type 3 discharge was not recorded on video given how infrequently it presumably occurred.

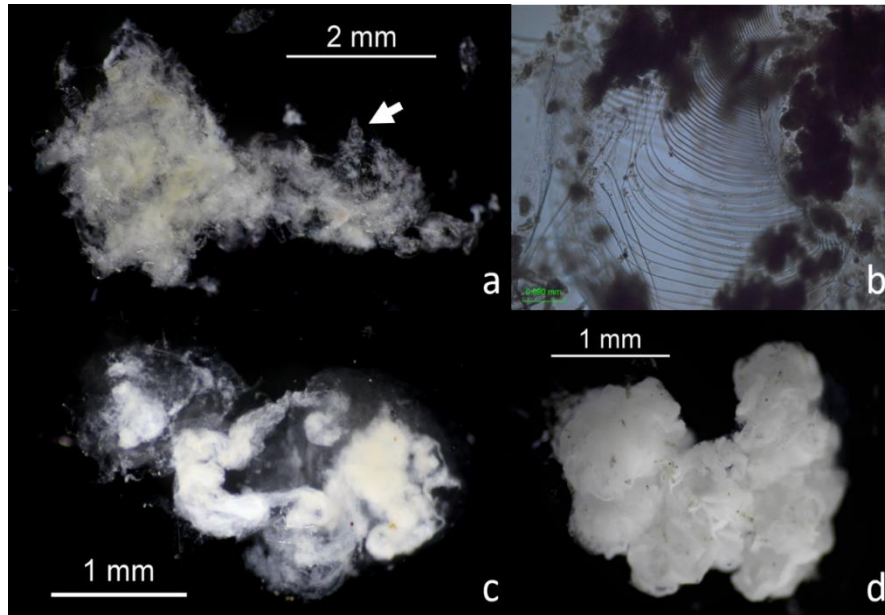


Fig. 2. Three types of discharge expelled by *Hippocampus spinosissimus*: (a) Feces, as identified by undigested shrimp (*Neocaridina denticulata*; denoted by arrow), (b) feces (type 1), as identified by undigested brine shrimp (*Artemia salina*), (c) “type 2” discharge, and (d) “type 3” discharge.

During the 20-day observation period, 29 fecal samples were collected from seahorses that were fed daily, and 21 samples were collected from seahorses that were fed once every three days (Fig. 3). Seahorses fed daily defecated on each of 17 days and those that were fed once every three days defecated on only 11 days. Type 2 discharge was collected from seahorses fed once a day (n=14) and once every three days (n=10), and the former and latter expelled type 2 discharge on 10 and 8 days, respectively (Fig. 3). Feeding frequency, then, had a relatively greater effect on the expulsion of feces

than on type 2 discharge for seahorses fed with *N. denticulata*.

Comparison of discharge drifting patterns

The migration plots for feces, type 2 discharge, and type 3 discharge are shown in Fig. 4, and 6, 52, and 8 s, respectively, were required for each substance to fall from the surface of the water to the bottom of the beaker. The drifting velocity for type 2 discharge was clearly slower than the other two, perhaps suggesting that its composition differs. As further evidence for this, the defecation and expulsion behavior of

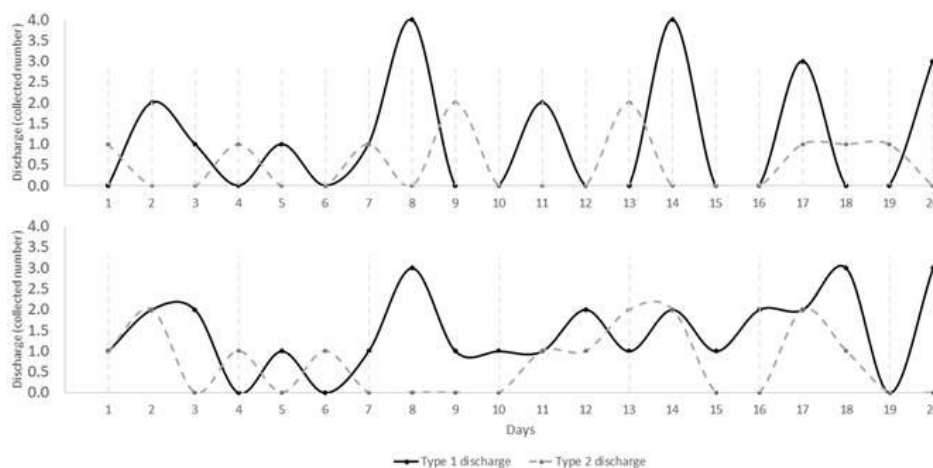


Fig. 3. The frequency of discharge of *Hippocampus spinosissimus*. The vertical dashed lines show the days on which the seahorses were fed. The solid black and dashed grey lines show the discharge frequency for types 1 and 2 discharge, respectively.

Tab. 2. Comparison of the three types of discharge emitted from *Hippocampus spinosissimus*, all of which were expelled from the anus. Type 3 discharge was less effectively characterized herein given how infrequently it was documented/collected. NA=not applicable/observed.

	Feces (type 1)	Type 2 discharge	Type 3 discharge
Location	bottom of tank	holdfast	bottom of tank
Color	depends on feed	white	white
Transparency	opaque	translucent	opaque
Size	depends on feed	2-3 mm	2-3 mm
Mucus	not observed	observed	not observed
Post-expulsion	disperses	maintains shape	NA
Food residue	observed	not observed	not observed
Frequency	depends on feed	once in 2 days	NA
Sinking velocity	fast	slow	intermediate

two seahorse specimens were tracked; after defecation, feces dispersed and sunk directly to the bottom of the tank. Type 2 discharge, on the other hand, did not disperse and instead stuck to the most proximate seahorse holdfast (see online supplemental video.). However, it did not appear from direct observations that the seahorses made deliberate efforts to expel the discharge onto holdfasts; in the seahorses' natural habitats, where water flow is more diverse, type 2 discharge would likely drift in the water before encountering a holdfast. A summary of the differences among the three types of discharge can be found in Table 2.

Discharge effects on holdfast selection

Seahorses prefer holdfasts coated in type 2 discharge

Since type 2 discharge was the only type that stuck to holdfasts after release, additional tests were carried out to determine its function. Upon coating water pipes with type 2 discharge, seahorses did not immediately migrate towards them; instead, they made slow visual inspections of both holdfasts, and they used their prehensile tails to touch the holdfasts before perching on them. After this period, they wrapped their tails around the chosen pipe. This test was repeated over 30 times with each

Table 3. Seahorse preference for holdfasts coated with type 2 discharge. Chi-squared (X^2) tests were used to compare the percentage of times that holdfasts were selected for each seahorse experimental group vs. the expected value (*i.e.*, null hypothesis) of 50% (no significant preference). Across all six organisms, there was a significant preference for holdfasts coated in type 2 discharge (Mann-Whitney U -test, $W=36$, $p=0.009$).

Seahorse	Experimental group		Control group	
	data	X^2 test	data	X^2 test
#1	69/103 (67%)	***	10/20 (50%)	NS
#2	30/44 (68%)	**	10/20 (50%)	NS
#3	38/60 (63%)	*	11/20 (55%)	NS
#4	41/70 (59%)	NS	7/20 (35%)	NS
#5	18/30 (60%)	NS	11/20 (55%)	NS
#6	25/43 (58%)	NS	10/20 (50%)	NS
Mean±SD	63±4%		51±7%	

NS: Not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

individual (#1-6 of Table 1). Seahorses perched on holdfasts that contained their own type 2 discharge $63 \pm 4\%$ of the time (mean \pm SD). In the control group for the same organisms (n=20 trials/organism), the preference of one holdfast over the other was essentially equal: $51 \pm 7\%$ of the time (mean \pm SD) for one vs. 49% for the other (Table 3). A X^2 test revealed that three of the six seahorses showed a significant preference for holdfasts coated with type 2 discharge, and a Mann-Whitney U -test detected a significant difference in holdfast preference between the experimental and control groups ($W=36$, $p<0.01$; Table 3); *H. spinosissimus* tended to select holdfasts coated with their own type 2 discharge. In contrast seahorses demonstrated no significant preference for holdfasts coated with feces (data not shown).

Partial extraction of type 2 discharge

Across 120 trials, the seahorses selected holdfasts coated with an aqueous extract of type 2 discharge 73 times (61%); this is significantly higher than then 50% associated with random selection ($W=16$, $p<0.05$). However, the results for the ether layer, as well as the other partially purified fractions after seven days of incubation, did not influence holdfast preference (data not shown).

Discussion

Three types of discharge were released from the seahorses observed herein (Table 2): feces and two never-before-described ones. The easiest method to distinguish between the three types was to determine whether they contained mucus or food residue: feces lack mucus but contain food particles, type 2 discharge features mucus but lacks food residue, and type 3 discharge contains neither mucus nor food residue. It is worth noting that type 2 discharge was too large to be urine, as particles were larger than the seahorses' renal tubules. A comparison of the drifting patterns also revealed that type 2 discharge behaves distinctly from feces, only the latter of which rapidly disperses upon contact with seawater. Collectively, then, we propose that type 2 discharge is not fecal material.

H. spinosissimus emitted type 2 discharge into the water from their anuses, rather than directly expelling it onto nearby holdfasts. After expulsion, type 2 discharge maintained its shape more so than feces and adhered to holdfasts, potentially signifying that it is an olfactory marker for holdfasts. This phenomenon should be verified in the wild. Seahorses prefer stable, structurally complex habitats in which

they can hide from predators (Harasti et al., 2014); therefore, even though type 2 discharge was not typically emitted directly onto holdfasts, it is likely that, in structure-rich marine environments, the discharge would quickly encounter potential holdfasts.

Given their preference for 3D environments in the wild, *H. spinosissimus* have restricted vision and may face issues with finding prey or holdfasts at night (Martinez-Cardenas and Purser, 2012). We found that seahorses prefer to select holdfasts coated in type 2 discharge, a potential demonstration of olfactory behavior. Given the aforementioned poor swimming ability of seahorses (Lourie, 2016), such marking of holdfasts may not only allow seahorses to find their preferred holdfasts at night, but also in strong currents. More generally, marking of holdfasts with discharge may allow for the creation of a home territory, thereby accounting for the site fidelity observed *in situ*. Although Liley (1982) proposed that fish do not engage in marking behavior, more recent works have found that scents can influence the homing behavior of coral reef fish (Doving et al., 2006). Furthermore, wild cardinalfish (*Cheilodipterus quinquelineatus*) exhibit homing behavior (Gardiner and Jones, 2016), and it is hypothesized that they do

so by using olfactory cues (Doving et al., 2006). Perhaps the seahorses observed herein, then, also rely on olfactory behavior to locate holdfasts they have previously “marked” with type 2 discharge.

References

- Andersson, G.E. & P.M. Sharp. 1996. Codon usage and base composition in *Rickettsia prowazekii*. *Journal of Molecular Evolution*, 42: 525-536.
- Bell, E.M., J.F. Lockyear, J.M. McPherson, A.D. Marsden & A.C. Vincent. 2003. First field studies of an endangered South African seahorse, *Hippocampus capensis*. *Environmental Biology of Fishes*, 67: 35-46.
- Carpenter, G. & D. Duvall, D. (1995). Fecal scent marking in the western banded gecko (*Coleonyx variegatus*). *Herpetologica*, 51(1): 33-38.
- Doving, K.B., O.B. Stabell, S. Ostlund-Nilsson & R. Fisher. 2006. Site fidelity and homing in tropical coral reef cardinalfish: are they using olfactory cues? *Chemical Senses*, 31: 265-272.
- Gardiner, N.M. & G.P. Jones. 2016. Habitat specialisation, site fidelity and sociality predict homing success in coral reef cardinalfish. *Marine Ecology Progress Series*, 558: 81-96.
- Harasti, D., T.M. Glasby & K.M. Martin-Smith. 2010. Striking a balance between retaining populations of protected seahorses and maintaining swimming nets. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 20: 159-166.
- Harasti, D., K. Martin-Smith & W. Gladstone. 2014. Ontogenetic and sex-based differences in habitat preferences and site fidelity of White's

- seahorse *Hippocampus whitei*. *Journal of Fish Biology*, 85: 1413-1428.
- Kuiter, R.H. 2009. Seahorses and their Relatives. Seaford: Aquatic Photographics.
- Liley, N.R. 1982. Chemical communication in fish. *Canadian Journal of Fisheries and Aquatic Sciences*, 39: 22-35.
- Lourie, S.A., S.J. Foster, E.W.T. Cooper & C.J.V. Amanda. 2004. A guide to the identification of seahorses. Project Seahorse and TRAFFIC North America. Washington D.C: University of British Columbia and World Wildlife Fund.
- Lourie, S.A. 2016. Seahorses: a life-size guide to every species. University of Chicago Press.
- Martinez-Cardenas, L. & G.J. Purser. 2012. Effect of stocking density and photoperiod on growth and survival in cultured early juvenile pot-bellied seahorses *Hippocampus abdominalis* Lesson, 1827. *Aquaculture Research*, 43: 1536-1549.
- Moreau, M.A. & A.C. Vincent. 2004. Social structure and space use in a wild population of the Australian short-headed seahorse *Hippocampus breviceps* Peters, 1869. *Marine and Freshwater Research*, 55: 231-239.
- Perante, N.C., M.G. Pajaro, J.J. Meeuwig & A.C.J. Vincent. 2002. Biology of a seahorse species, *Hippocampus comes* in the central Philippines. *Journal of Fish Biology*, 60: 821-837.
- Rosa, I.L., T.P. Oliveira, A.L. Castro, L. Moraes, J.H. Xavier, M.C. Nottingham, T.L. Dias, L.V. Bruto-Costa, M.E. Araujo, A.B. Birolo, A.C. Mai & C. Monteiro-Neto. 2007. Population characteristics, space use and habitat associations of the seahorse *Hippocampus reidi* (Teleostei: Syngnathidae). *Neotropical Ichthyology*, 5: 405-414.
- Sillero-Zubiri, C. & D.W. Macdonald. 1998. Scent-marking and territorial behaviour of Ethiopian wolves *Canis simensis*. *Journal of Zoology*, 245: 351-361.
- Vincent, A.C.J., K.L. Evans & A.D. Marsden. 2005. Home range behaviour of the monogamous Australian seahorse, *Hippocampus whitei*. *Environmental Biology of Fishes*, 72: 1-12.
- Vincent, A.C.J., A.D. Marsden, K.L. Evans & L.M. Sadler. 2004. Temporal and spatial opportunities for polygamy in a monogamous seahorse, *Hippocampus whitei*. *Behaviour*, 141: 141-156.