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The use of a non-invasive tool for capture–recapture studies on a seahorse *Hippocampus guttulatus* population

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In this study, the spot pattern in *Hippocampus guttulatus* was analysed using a computer programme algorithm that allowed individual comparison. This methodology was first tested in a controlled environment using 51 adult and 55 juvenile *H. guttulatus*. Positive matches were obtained in 86·3 and 83·6% of the adults and juveniles, respectively. In a second experiment, monthly surveys were carried out in five selected locations in the Ria Formosa Lagoon, south Portugal, over the course of a year and a total of 980 photographs were analysed. Photographed *H. guttulatus* were re-sighted one to nine times during the course of the survey period with an overall re-sight record of over 30%. Photo-identification was therefore shown to be a useful tool for non-invasive mark–recapture studies that can be successfully used to survey the population abundance of *H. guttulatus* aged 6 months or older in consecutive years. This could be of great value when considering the assessment of *H. guttulatus* populations and understanding changes over time.

Key words: long-snouted seahorse; mark–recapture; photo-identification; population abundance.

INTRODUCTION

Estimates of population size, survival, reproduction and movement rates using capture–recapture models require the ability to identify previously marked or sighted individuals (Nichols, 1992). Individual identification is important in conservation studies when considering species distribution, habitat use and population status (Williams *et al.*, 2002) and will allow tracking of individual movements and model population estimates. Tags have been used as a tool for studies in fish ecology with a wide variety of applications (Winner *et al.*, 1999; Baras *et al.*, 2000; Pine *et al.*, 2003; Holm *et al.*, 2007; Hutson *et al.*, 2007; Jiang *et al.*, 2007). The use of artificial tags, either internal or external, is widely recognized as an effective method of marking animals but has been associated with physical and behavioural disruptions (Mellas & Haynes, 1985; Marty & Summerfelt, 1986; Moore *et al.*, 1990; Murray & Fuller, 2000; Welch *et al.*, 2007). Ideally, tags should have minimal effects on mortality, growth and reproduction of the target species so that the results

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from tagging studies may accurately reflect the variables of the study population (Willis & Babcock, 1998). Nevertheless, methods for identifying individual animals that rely on artificial markings such as neck collars, transponders, tissue removal, dyes and chemical markers can be categorized as invasive (Silvy *et al.*, 2005). The most commonly used artificial tags in the Syngnathidae (seahorses, pipefish and seadragons) are external tags (necklaces) and internal tags (visible implant fluorescent elastomer, VIFE) (Monteiro *et al.*, 2005; Curtis, 2006; Sogabe *et al.*, 2007; Palma *et al.*, 2008; Caldwell *et al.*, 2011; Harasti *et al.*, 2012). VIFE tags have been used as a valuable tool in fisheries research and management (FitzGerald *et al.*, 2004) because they are cost-effective, have low rates of tag loss and have negligible effects on survival, growth and behaviour (Willis & Babcock, 1998; Willis *et al.*, 2001; Goldsmith *et al.*, 2003; Woods & Martin-Smith, 2004; Curtis, 2006). Lately, the use of electronic tags has been proven as an effective method of collecting data on individual long-snouted seahorses *Hippocampus guttulatus* Cuvier 1829, although over a shorter time period (Caldwell *et al.*, 2011).

Natural marks or patterns can provide an alternative method of individual identification provided that there is enough polymorphism and information content in the characteristic in question (Anderson *et al.*, 2007). The use of natural marks using photo-identification is a non-invasive technique and has been successfully used to study different marine species (Meekan *et al.*, 2006; Van Tienhoven *et al.*, 2007; Barker & Williamson, 2010; Kitchen-Wheeler, 2010) including syngnathids (Martin-Smith, 2011). In addition to enabling individual identification, the use of natural marks in capture–recapture studies must comply with several requirements. Markings must remain consistent over time and identifiable in order to be usefully used as an estimate tool for studying population variables (Anderson *et al.*, 2007; Martin-Smith, 2011). In fact, several studies have reported that markings may persist well over 10 years after their initial recording in species such as *Carcharias taurus* Rafinesque 1810 (Bansemer & Bennett, 2008), *Stegostoma fasciatum* (Hermann 1783) (Dudgeon *et al.*, 2008) and *Phyllopteryx taeniolatus* (Lacépède 1804) (Martin-Smith, 2011).

Hippocampus guttulatus is a European species that occurs in the Ria Formosa Lagoon, south Portugal. The greatest population size recorded for this species throughout its range was recorded in the early 2000s (Curtis & Vincent, 2005); recent field data, however, showed a significant decrease of 94% in the *H. guttulatus* populations within this lagoon (Caldwell & Vincent, 2012). The use of non-invasive techniques for seahorse monitoring is of paramount importance for studying these dwindling populations. *Hippocampus guttulatus* has a distinct mark pattern composed of white spots scattered throughout the entire body surface. The aim of this study was to evaluate the potential of using photo-identification as a monitoring tool when surveying *H. guttulatus* populations.

MATERIALS AND METHODS

PRELIMINARY EXPERIMENT IN A CONTROLLED ENVIRONMENT

A total of 51 adult *H. guttulatus* (24 females and 27 males; F2 captive-bred generation) and 55 juveniles (30 females and 25 males; F3 generation) were used in this preliminary trial.

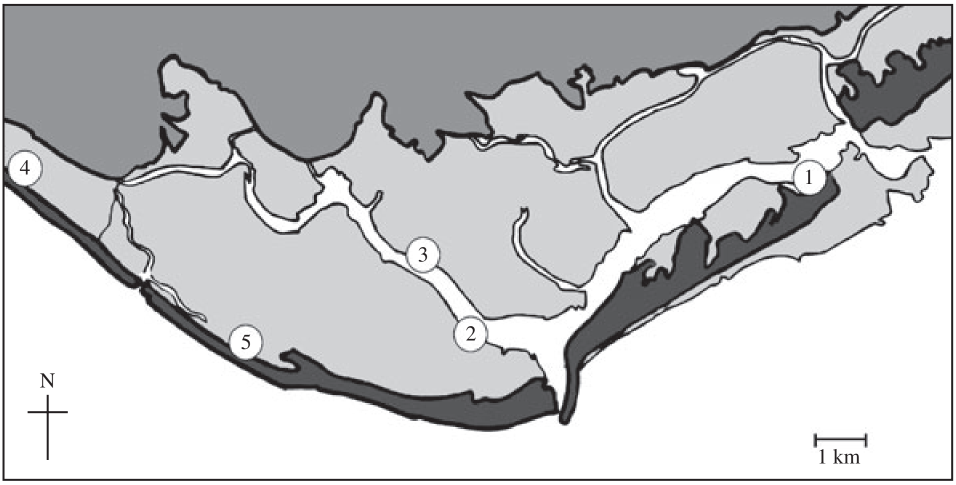

FIG. 1. Location of the *Hippocampus guttulatus* survey sites (sites 1–5) in the Ria Formosa Lagoon, south Portugal.

Hippocampus guttulatus were reared and kept at the Aquaculture Station of the Centro de Ciências do Mar and separated according to their generation in 250 l tanks, in a flow-through system, with moderate aeration and fed on frozen shrimp *Palaemonetes varians*. At the start of the experiment, adults and juveniles were aged 18 and 6 months. The mean ± S.D. of total length (L_T) was 17·5 ± 1·8 cm in adults and 12·3 ± 0·9 cm in juveniles.

On the photographic recording day, *H. guttulatus* were removed from their culture tanks and individually placed in a clear 10 l glass tank. Each *H. guttulatus* was then gently placed close to the wall of the aquarium with their left side facing the glass to allow full visibility and thus optimize the photo quality. After these adjustments were made, a digital camera (Sealife DC1200; www.sealife-cameras.com) was used to photograph each *H. guttulatus*.

A second set of photographs was taken 2 months later, using the exact same *H. guttulatus* and protocol as mentioned earlier, in a random order. Again, photographs were taken of the left side of the *H. guttulatus* for consistency. The same individuals were used in both photo sessions under controlled conditions to determine the viability and accuracy of photo-identification in this species.

IN SITU OBSERVATIONS

This experiment was conducted at five sites (sites 1–5) (Fig. 1) in the Ria Formosa Lagoon, south Portugal (36° 59′ N; 7° 51′ W). The sites were surveyed on a monthly basis over a 12-month period in 2012. Each month, at each sampling site, a modified underwater visual census (UVC) technique (unpubl. data), based on the UVC previously used by Caldwell & Vincent (2012), was used to survey an area of 240 m^2 (30 m × 8 m). On some occasions, sampling was not possible due to poor weather conditions and low visibility, thus creating gaps in the monthly scheduled dives. A GPS unit was used to determine the locations of each study area, and during the site delineation, the same bearing was taken while laying each transect so that the same area could be consistently covered on each sampling occasion. Each site differed in its habitat complexity (Table I).

During each survey, *H. guttulatus* were counted, sexed and photographed on their left side using the same underwater camera (Sealife DC1200) as in the control trials. When possible, photographs were taken with no direct interaction with the *H. guttulatus*. When this was not possible, however, *H. guttulatus* were gently handled for the minimum time possible to reduce stress from the procedure. As the surveys were carried out over a 1-year period, only adults aged 1+ year-old were photographed and all juveniles were discarded. *Hippocampus*

TABLE I. Site description considering substratum type, depth and habitat complexity for *Hippocampus guttulatus*. Sites were located in the Ria Formosa Lagoon, south Portugal

	Substratum	Depth (m)	Habitat complexity
Site 1	Sandy	5–6	High: mostly shells and sessile invertebrates (*Ascidia* sp., *Sabella* sp. and sea urchins)
Site 2	Sandy	6–9	Low: scattered rocks, mostly barren
Site 3	Muddy	3–5	High: *Ascidia* sp. and shells
Site 4	Muddy	2–3	High: seagrass bed, shells and *Sabella* sp. tubes
Site 5	Sandy	2–3	Low: scattered *Codium* sp. and small seagrass patches

guttulatus were measured according to Lourie *et al.* (2004) and those that were at least 15 cm L_T were considered as 1+ year-old adults, as suggested by Curtis & Vincent (2006). Although laboratory results indicated that photo-identification methodology could potentially be used on juveniles older than 6 months, this study only focused on the breeding population. As ageing juvenile *H. guttulatus* can be very subjective, this bias was removed by only working on larger *H. guttulatus* considered to be fully mature.

IMAGE ANALYSIS

Photographs were downloaded and computer labelled to include the individual fish and photograph number, sex, date and site (*in situ*) information. In this study, it was decided to focus on analysing the spot pattern on the head of each *H. guttulatus* for individual identification (Fig. 2). As the spots were clearly distinguishable, no further photo adjustments were necessary. Each spot pattern was recorded using the computer software algorithm, I3S Manta 2.1 (www.reijns.com/i3s) and analysed to determine eventual matches.

The identification procedure assumed that the spot pattern of each individual was a unique distinguishing feature. The user points out the most distinguishing spots of each image and draws an ellipse around each spot after choosing three landmark points on each image, *i.e.* the centre of the eye ball and the upper and lower limit of the pectoral fin. This information

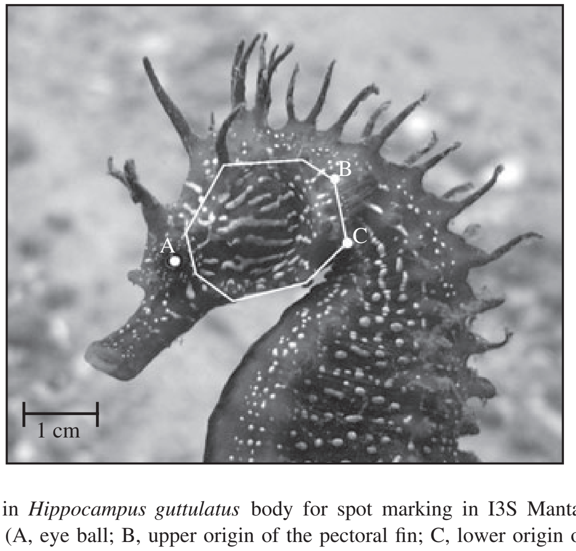

FIG. 2. Selected area in *Hippocampus guttulatus* body for spot marking in I3S Manta software with three reference points (A, eye ball; B, upper origin of the pectoral fin; C, lower origin of the pectoral fin).

provides relative position and size of each spot. This spot pattern is then stored in a fingerprint file. The programme's algorithm (Van Tienhoven *et al.*, 2007) then processes each file providing the user with a ranked list of possible matches. The final decision on a true match is left to the observer through image visual comparison. Each comparison batch was done using all photographs recorded in captivity (preliminary experiment) or the same sampling site (*in situ*). Considering that each site was at least 1 km apart from any other and that low mobility and sedentary nature of *H. guttulatus* were previously recorded at this location (Curtis & Vincent, 2006), the probability of matches between different sampling sites was considered to be very limited. Also, the photographs were divided by sex prior to comparison as this allowed a smaller batch of photographs to be compared and therefore increased the possibility of finding matches. The list of possible matches was then analysed and a visual comparison was made to confirm each match.

WILD POPULATION PARAMETERS

To estimate population abundance at each site, and considering the annual recruitment period of this species reported by Curtis & Vincent (2006), a Cormack–Jolly–Seber (CJS) open model population was used (Cormack, 1964; Jolly, 1965; Seber, 1965). This model assumes that animals retain their tags throughout the experiment and the tags are read properly; catchability is constant for all animals (marked and unmarked) for all sampling periods; sampling periods are instantaneous and recaptured animals are released immediately. Abundance ($\hat{N}$), capture probability (p) and apparent survival (φ) parameters were determined using a CJS open population model in the MARK software (White & Burnham, 1999). Finally, a CAPTURE goodness-of-fit test was run inside the MARK software to test whether the model was an adequate fit to the data. In cases of low recapture data, a cumulative non-linear model [$y = ax(b + x)^{-1}$] was used to fit the data and estimate abundance according to Kohn *et al.* (1999).

RESULTS

PRELIMINARY EXPERIMENT IN A CONTROLLED ENVIRONMENT

Using the I3S Manta software, there was a high matching accuracy when comparing the two sets of photographs. From the 212 photographs analysed, positive matches were classified as first rank in 86·3% for adults (84·0% males and 83·3% females) and 83·6% for juveniles (92·6% males and 79·2% females) in the overall output rank list. Inconclusive matches that were classified as second rank or below were caused by poor photograph quality, incorrect positioning of *H. guttulatus* or algorithm errors. These inconclusive matches were then analysed by direct visual comparison and a corresponding positive match was found.

There were no statistical differences related to sex either in adult or juvenile *H. guttulatus* (Fisher's exact test, d.f. = 1, $P > 0{\cdot}05$). The number of spots varied between each individual from 37 to 71 with a mean ± S.D. of $53{\cdot}7 \pm 9{\cdot}8$ and $48{\cdot}1 \pm 5{\cdot}9$ for males and females, respectively. No statistical difference was found in number of spots between males and females ($\chi^2 = 25{\cdot}2$, d.f. = 22, $P > 0{\cdot}05$).

IN SITU OBSERVATIONS

A total of 980 photographs were recorded at all sites, which was the pooled value for first-sighted and re-sighted *H. guttulatus* (217 in site 1, 363 in site 2, 181 in site 3, 134 in site 4 and 85 in site 5). Recapture percentage, *i.e.* percentage of

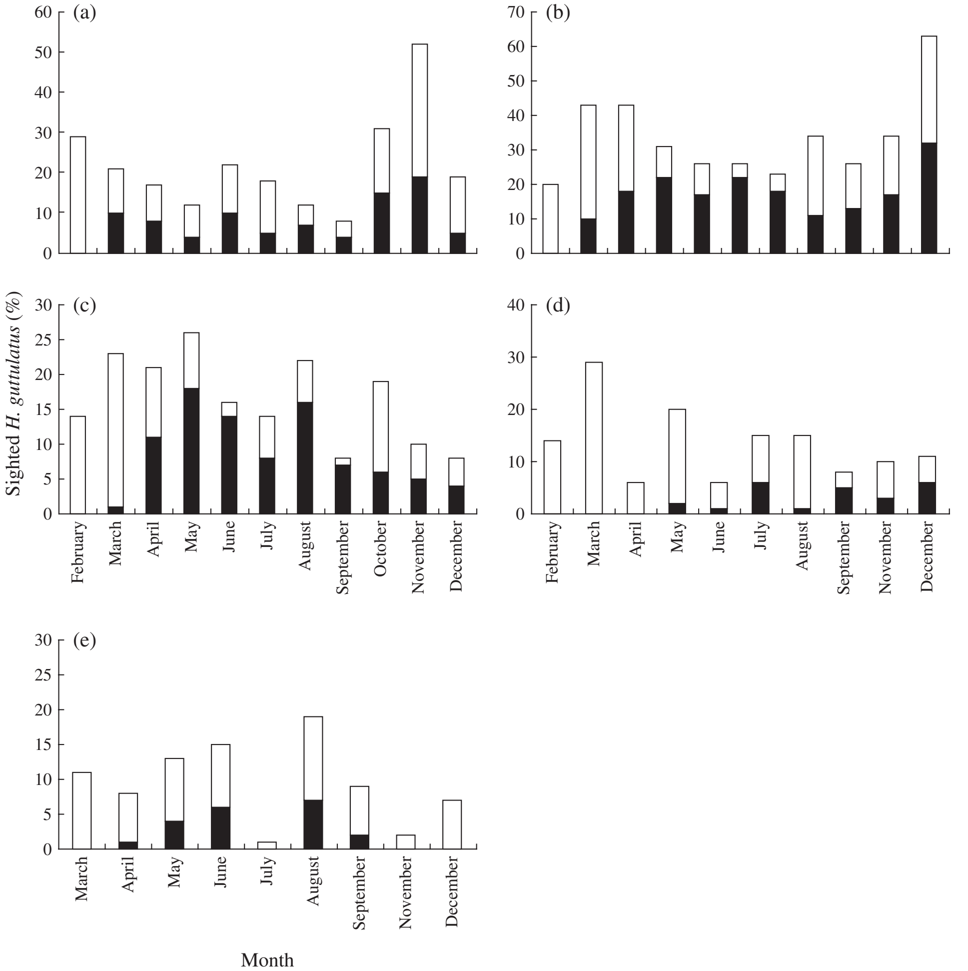

FIG. 3. Re-sight history of *Hippocampus guttulatus* (■, first sighted; □, re-sighted) for sites (a) 1, (b) 2, (c), 3, (d) 4 and (e) 5 surveyed on a monthly basis throughout the experiment.

H. *guttulatus* re-sighted at least once in a sampling period, varied from a minimum of 13·6% in site 4 to a maximum of 44% in site 3, with an overall mean ± S.D. of 31·3 ± 13·1% of the total sighted *H*. *guttulatus* at all the five sites (Fig. 3). The total number of unique individuals found during the sampling period was 131 for site 1, 209 for site 2, 91 for site 3, 110 for site 4 and 65 for site 5.

The overall mean ± S.D. *H. guttulatus* densities (n m^{-2}; considering the full sampling period) was 0·091 ± 0·051, 0·140 ± 0·051, 0·069 ± 0·026, 0·056 ± 0·029 and 0·039 ± 0·024 individuals m^{-2} for sites 1–5, respectively, with maximum densities varying between 0·004 (site 5) and 0·263 individuals m^{-2} (site 2). Most re-sighted *H*. *guttulatus* were only found in one subsequent sampling occasion (mean ± S.D. of 69·1 ± 11·2%), while the remaining were re-sighted in two to nine surveying events. Overall relative number of re-sights per site is presented in Fig. 4.

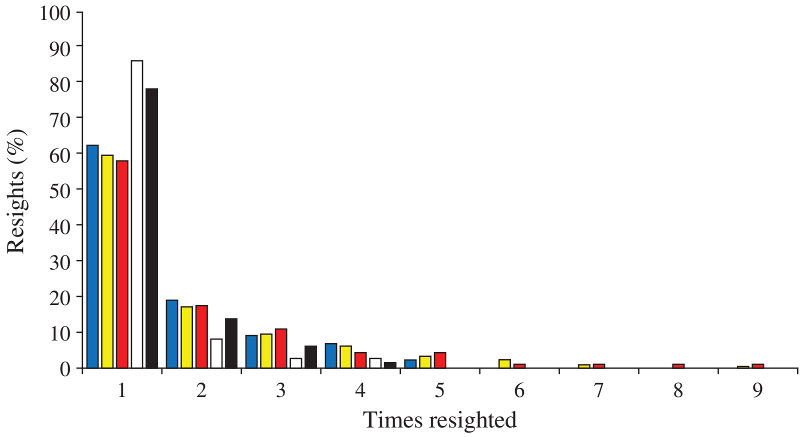

FIG. 4. Overall relative resight history for each site surveyed (■, site 1; ■, site 2; ■, site 3; □, site 4; ■, site 5) considering all photographed *Hippocampus guttulatus*.

WILD POPULATION PARAMETERS

Due to the low recapture data at site 4, abundance was estimated using the cumulative non-linear model. Abundance estimates varied from 108 (site 3) to 257 (site 2) (Table II).

DISCUSSION

The results obtained in the preliminary control experiment fully supported photo-identification as an individual non-invasive marking technique that is applicable to *H. guttulatus*. Photo-identification has been successfully used as a mark–recapture

TABLE II. Abundance estimate $(\widehat{N})$ obtained by population analysis (POPAN) model, apparent survival (φ) and re-sight probability ($\widehat{p}$) for each site were obtained using the Cormack–Jolly–Seber (CJS) open population model and cumulative curve model [$y = ax\ (b + x)^{-1}$]

	CJS open population model						
	$\widehat{N}$	φ	C.I. (95%)		$\widehat{p}$	C.I. (95%)	
Site 1	131	0·92	0·83	0·97	0·19	0·10	0·33
Site 2	209	0·82	0·52	0·91	0·40	0·25	0·57
Site 3	91	0·71	0·51	0·87	0·46	0·37	0·55
Site 5	65	0·89	0·43	0·99	0·19	0·05	0·48

		Cumulative curve		
		C.I. (95%)		r^2
Site 4	217	166	267	0·9866

technique in many studies and has proven to be an effective non-invasive technique for larger marine species such as the bowhead whale *Balaena mysticetus* (Rugh *et al.*, 1992), polar bear *Ursus maritimus* (Anderson *et al.*, 2007), long-finned pilot whale *Globicephala melas* (Auger-Méthé & Whitehead, 2007), *C. taurus* (Bansemer & Bennett, 2008) and whale shark *Rhincodon typus* Smith 1828 (Holmberg *et al.*, 2009). In addition, photo-identification has been used to obtain accurate data in mark–recapture studies in syngnathid species such as *Phycodurus eques* (Günther 1865) (Connolly *et al.*, 2002), *Nerophis lumbriciformis* (Jenyns 1835) (Monteiro *et al.*, 2005), *P. taeniolatus* (Martin-Smith, 2011) and *Hippocampus reidi* Ginsburg 1933 (Freret-Meurer *et al.*, 2013). This technique uses very little or no handling, thus causing reduced stress in wild populations studied when compared to other methods. From previous seahorse tagging techniques, VIFE appears to be the most widely used and is effective for individually identifying wild seahorses (Sanchez-Camara & Booth, 2004; Woods & Martin-Smith, 2004; J. M. Le Cheminant, unpubl. data). This methodology, however, has some implications for animal welfare as it involves lengthy handling and elastomer injection on individuals (Curtis, 2006). In addition, the VIFE must be purchased and takes time to apply, therefore requiring more resources.

The I3SM Manta 2.1 software used in this study has already proven to be effective in mark–recapture studies for other species, including sharks (Meekan *et al.*, 2006; Wilson *et al.*, 2006; Bradshaw *et al.*, 2007), manta rays (Marshall *et al.*, 2008), *U. maritimus* (Anderson *et al.*, 2007) and seadragons (Martin-Smith, 2011) with high accuracy results. The correct contour of the spots is crucial for optimal identification accuracy. Also, in some cases, the shape of the spots is neither perfectly elliptical nor circular, thus creating the potential for some variation due to each user criteria while selecting the spots within the programme I3SM Manta. Therefore, as in other sampling methods, data should be processed by as few researchers as possible to minimize bias. In this study, this process was performed by only one user, the criteria for the selection of each spot remained consistent and was confirmed by the high match percentages obtained when processing the photographs of *H. guttulatus* in controlled conditions, similar to those obtained by Martin-Smith (2011) with *P. taeniolatus*. Another equally important detail to consider for the success of this methodology is the photo quality of the target animal. Underwater photography in the Ria Formosa can be difficult due to low visibility. Visibility in most dives varied from 0·5 to 5 m, but in most cases, it was <2 m which had an effect on the final quality of the resulting photographs, making it hard to identify each spot on some occasions. Also, as observed in the preliminary control experiments, the *H. guttulatus* should be ideally positioned parallel to the camera in order to get a straight profile photograph. Even though it was necessary to handle most of the fish to take the photograph, the handling was carried out using gloves. Each *H. guttulatus* was gently gripped to avoid skin injuries and handling time was kept to only a few seconds per *H. guttulatus*. If the photographs were blurred, the software I3SM Manta was ineffective and each photograph was then needed to be validated by visual comparison. Visual comparison was a good means to check individual identifications, not only when photograph quality was poor, as it allowed the researcher to search for other distinctive marks or spots that could identify possible matches.

This study also proved that photo-identification is valid when used for juveniles aged 6 to 8 months, suggesting that the spot pattern and shape have low or negligible modifications with age. This is in agreement with Martin-Smith (2011) who found that *P. taeniolatus* had a unique spot pattern that did not undergo significant changes over a period of at least 18 months. When considering tagging methods for mark–recapture studies, researchers must verify whether the tags in use comply with the assumptions of their mark–recapture models. For open population models, those assumptions include that tagged fishes are a random sample of the population of interest, numbers of releases are known, tagging is accurate with no tag loss or misread tags, animals are released within a brief time period and the fate of individual fish and the fates of fish in differing cohorts are independent (Jolly, 1965; Seber, 1965). The body area chosen for identification purposes seemed to be adequate as it allowed the correct identification of each individual with no sex variation and no significant changes were observed in spot shape and pattern in all *H. guttulatus* photographed, during the entire survey period. Considering that the spot pattern is present throughout the *H. guttulatus* body, however, the area of focus could be expanded in order to increase the matching accuracy.

Overall, roughly one third (mean ± S.D. = 31·3 ± 13·1%) of all photographed *H. guttulatus* were re-sighted. This result suggests a sedentary behaviour of this species, when compared with other fish species that are migrant (Templeman, 1984; McGovern *et al.*, 2005), and agrees with their well-documented small home range (Curtis & Vincent, 2005, 2006; Caldwell *et al.*, 2011). Although working with smaller sample sizes, Connolly *et al.* (2002) and Martin-Smith (2011) have reported re-sighting rates over 90%. Other species of seahorse such as *Hippocampus comes* Cantor 1849 or *Hippocampus whitei* Bleeker 1855 may also have high re-sighting rates (Vincent, 1995; Perante *et al.*, 2002; Harasti *et al.*, 2012).

Several *H. guttulatus* were re-sighted on multiple occasions throughout the year, up to nine times of the 11 surveys performed for each site. Although Curtis & Vincent (2006) reported that the home range for this species after settlement was *c.* 20 m, *H. guttulatus* have a patchy distribution that may be conditioned by the level of habitat complexity and population density (M. Correia, I. R. Caldwell, J. Palma, H. Koldewey & J. P. Andrade, unpubl. data). Caldwell & Vincent (2013) reported that *H. guttulatus* can move >100 m from its original location during a relatively short period of time and display an individual habitat preference. This behaviour could explain the absence in the survey area of some re-sighted *H. guttulatus* in specific months, as they might adjust their location depending on holdfast and food availability, density, fishing activities and weather conditions. Nevertheless, the area covered in each survey (240 m^2) seems to be adequate as it overlaps the equivalent of several home ranges reported for this species (Curtis & Vincent, 2006; Garrick-Maidment *et al.*, 2011; Caldwell & Vincent, 2013). After completion of the monthly surveys, the individual re-sight occasions were *c.* 20 and 10% for *H. guttulatus* re-sighted two and three times. This could suggest that even though *H. guttulatus* have small home ranges, they can move over larger areas, returning to the original area. This behaviour was observed during the tidal peaks when the current was weak and *H. guttulatus* undertake hunting and feeding behaviour. The highest re-sight number in this study was registered in site 2, which could be due to the habitat characteristics. This area is very hydrodynamic, has low habitat complexity and the available holdfast is only provided by an abandoned metal chain from an old signalling buoy that is laid straight on the bottom, in an otherwise barren area of sandy substratum. This structure is necessary for *H. guttulatus* to cope with the high water currents that occur during the high-tide to low-tide transition period. This low complexity might cause a sedentary behaviour in the local *H. guttulatus* population and increase re-sighting.

The highest *H. guttulatus* density was seen at site 5 (0·263 *H. guttulatus* m^{-2}) and was well above that reported by Caldwell & Vincent (2012), which was of 0·004 individuals m^{-2} on average, considering the 33 sites surveyed, and these authors found a maximum density of 0·035 fish m^{-2}. This high density could be responsible for some migration amongst the adult population to cope with competition for food and holdfasts. Apart from mortality, this could explain why some *H. guttulatus* were sighted only once throughout the entire survey period.

In this study, the preliminary experiment proved that photo-identification is an efficient methodology to successfully identify juvenile *H. guttulatus* aged from 6 months. In younger individuals kept in captivity, spot patterns are already present and start to manifest at 3 months of age (J. Palma, pers. obs.). Nevertheless, the spots are still too small and difficult to distinguish. In order to accurately assess the spot pattern at this age, a longer period of handling would have been necessary, thus increasing handling stress at a very sensitive life period. In addition, the reported migration of juveniles before settlement can bias the assumptions for abundance estimates that require that catchability is constant for all fish (marked and unmarked) and for all sampling periods. Hence, the abundance for each site was estimated considering only the adult population.

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