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Abstract

The ability to identify individual free-living animals in the field is an important method for studying their behavior. Apart from invasive external or internal tags, which may cause injury or abnormal behavior, most cephalopods cannot be tagged, as their skin is too soft and delicate for tag retention. Additionally, cephalopods remove many types of tags. However, body markings have been successfully used as a non invasive method to identify individuals of many different species of animals, including whale sharks, grey whales, seals, and zebras. We developed methods to sex and individually identify Caribbean reef squid, *Sepiotheuthis sepioidea*. Males showed distinct bright dots on their fins on a Basic Brown background and have a light line at the fin edge while the females had a gradual transition from Brown to Pale towards the edge of their fins without showing distinct fin-dots or lines. In the field we used four characters to distinguish individual *S. sepioidea* from each other – sex, relative size to each other, scars, and patterns of light-colored dots on their mantles and fins. These dot patterns are individually unique and constant in location through time. Observations in the field were backed up by an image database using illustrations and photography.

Introduction

Individual animals can be recognized by artificial marks e.g. tags, in mammals and fishes (Fedak, Lovell & Grant 2001; Willis et al. 1995), leg bands in birds (Wayne & Shamis 1977), spraying tiny color particles into fish skin (Jacobsen et al. 2001), injection of colored elastomers in squid (Replinger & Wood 2007), implantation of electronic tags under the skin of fish, dogs (Jefferts, Bergman& Fiscus 1963; Lord et al. 2007) or for species that exhibit sufficient phenotypic variation, by natural markings.

Artificially marking/tagging animals usually involves capturing and handling, which can stress individuals and may alter their behavior. Tagging often creates a wound, which is a potential site of infection. The use of natural features or markings to identify individuals within a population is non-invasive, and therefore does not pose the same risk as invasive artificial marking techniques. Individuals (or a particular region of their bodies) can be either drawn or photographed, and the resulting images compared with the images of previous observations. This technique has been employed most frequently in studies on mammals, both marine (e.g. humpback whales, *Megaptera novaeangliae,* Glockner & Venus 1983; southern right whales, *Eubalaena australis*, Payne et al. 1983; Mediterranean monk seals, *Monachus monachus*, Forcada & Aguilar 2000) and terrestrial (e.g. zebras, *Equus burchelli*, Petersen 1972; lions, *Panthera leo*, Schaller 1972 in Kelly 2001; chimpanzees, *Pan troglodytes*, Goodall 1986; badgers, *Meles meles*, Dixon 2003). It has also been applied to birds (e.g. Bewick's swan, *Cygnus columbianus*, Scott 1978; ospreys, *Pandion haliaetus*, Bretagnolle, Thibault & Dominici 1994; lesser white-fronted geese, *Anser erythropus*, Øien et al. 1996); reptiles (e.g. adders, *Vipera berus*, Sheldon & Bradley 1989; common garter snakes, *Thamnophis sirtalis* sirtalis, Hallmen 1999; five species of Central European lacertid lizards, Steinicke et al. 2000), amphibians (Archer's frogs *Leiopelma archery*, Bradfield, 2004*)* and fish (e.g. pipefish, *Corthoichthys intestinalis*, Gronell 1984; leafy seadragons, *Phycodurus eques*, Connolly, Melville & Keesing 2002).

As with any technique, field identification of individuals must be efficient. Individuals can be identified by eye in the field and this data can then be correlated with other behavioral variables at the time of observation. Additionally, subjects can be photographed or sketched in the field, and identifications made at a later stage in the laboratory. When the catalog of previous captures is relatively small, manual identification (i.e. identification entirely by eye) is rapid, but when the catalog is large, it can take substantially longer. Computer-assisted matching can be used if photo-matching entirely by eye is too time-consuming or difficult (e.g. Whitehead 1990; Kelly 2001). Dividing individuals into subgroups can facilitate rapid identification when there are a large number of previous captures, because the observer has to photo-match to a small subgroup rather than to all previous captures. For example, Gill (1978) was able to identify individual red-spotted newts, *Notophthalmus viridescens*, within 30 seconds, despite a catalog of over 8500 individuals, because individuals could be assigned to subgroups based on the number of spots on each side of the dorsal surface. Another advantage of this approach is that it results in a higher degree of accuracy, as the larger the catalog of photographs, the more likely it is that mis-identification will occur.

Image quality influences error rates, with poor images resulting in a higher number of incorrect identifications than high-quality images (e.g. Agler 1992; Forcada & Aguilar 2000; Gowans & Whitehead 2001; Stevick et al. 2001). Digital photographs have a number of advantages over traditional slide or print film images, even when photo-matching is conducted entirely by eye. Markowitz et al. (2003) compared digital and slide film images of New Zealand dusky dolphins (*Lagenorhynchus obscurus*), and reported a higher proportion of digital images were of suitable quality for use in photographic identification than slide film images taken by the same photographers. That is because digital images are available for inspection immediately after they are taken (i.e. directly in the field), and they can be archived, accessed, and printed easily and rapidly.

It is possible that two or more individuals in a population may have such similar natural markings that they cannot be distinguished from one another (Pennycuick 1978), resulting in false positive errors. The likelihood of this occurring increases with increasing population size, but decreases with increasing pattern complexity. The probability that a pattern will be repeated in a particular population was estimated (Pennycuick 1978). Variability in the degree of distinctiveness of individuals means that 'marked' individuals (i.e. those who have previously been photographed/sketched) do not necessarily all have the same probability of being recognized, and this can potentially have serious effects when estimating abundance (Hammond 1986 in Friday et al. 2000). Only individuals distinctive enough to have equal probabilities of recognition should be considered as "marked." An assumption common to capture-recapture methods is that marks do not change over time. However, natural markings do have this potential, which would also result in population overestimates.

Cephalopods are an interesting group with highly developed sense organs and a complex brain that rivals the complexity found in vertebrates (Hanlon & Messanger 1996). Their ability to change color and texture of their skin in fractions of seconds provides them with the means of visual communication with each other and their environment. The skin display of squid and octopuses is quite complex and subject to constant change (Messenger 2001). There are both expandable colored chromatophores in the skin surface and reflective leucophores and iridophores in deeper layers of the skin that reflect specific wavelengths of ambient colors when the chromatophores are contracted. Nevertheless, there are patterns of iridescent small spots and areas of few chromatophores on the skin and fins that can be used to identify individuals.

The Caribbean reef squid *Sepioteuthis speioidea* (Blainville, 1823) are a model species of cephalopod for generating a catalog of individuals because they live in easily accessible inshore, small, semi-permanent groups. Moynihan & Rodaniche (1982) observed that this species of squid has individual marks and can thereby be individually identified. A method to identify individuals opens the door to a much deeper understanding of their behavior.

The objectives of this study were to determine whether identification by eye in the field and photographic identification of naturally marked animals could be used to identify individual Caribbean reef squid *Sepioteuthis sepioidea* and if the markings used for identification are stable over time.

Methods

Field data for this project was collected during an eight-year observational study of *S. sepioidea* in a small near shore location off the west coast of the Caribbean island of Bonaire. The project was carried out in the months of May and June from 1998 to 2005. Total underwater observation time was over 1000 hours. This island is an ideal location for such a project because the waters around *Bonaire* are a marine park and the squid are habituated to recreational divers. A school of adult squid stays more or less in the same area and easily accessible groups can thereby be followed over periods of weeks. The main times for field observation were the early morning (0700 to 1000) and late afternoon (1500 to 1800) because the squid were most active during these times. Data were collected by snorkelers or divers who recorded notes and sketches on underwater slates and filed them shortly thereafter. For additional documentation squid were photographed with Sea&Sea, Nikonos, Fuji S2 and CoolPix cameras and filmed with a housed Sony Handycam. In the base camp a database for dot patterns of identified squid was drawn onto paper copies of the body outline of a squid and newly identified squid were named.

As verification that squid can be identified according to our method, two sets of 26 pictures each of different squid were shown to seven people. Each picture of set 1 had a match (= same squid) in set 2. Three of the seven volunteers worked on the project in Bonaire, and thus, had experience with squid in the water and four were naïve as they had never seen squid in the field before.

To answer the question if squid dot patterns, which we use for individual identification, have a stable location on the squid's body over time a lab experiment was conducted in 2004 on the island of Bermuda. A school of 10 *S. sepioidea* was caught in Whalebone Bay and brought to the wet lab of the Bermuda Biological Station for Research. There squid were housed in a flow-through system and fed with live silversides and live shrimp. As a control, subjects were tagged with Visible Implant Elastomers (VIE, Northwest Marine Technology) (Zeeh & Wood 2009). During a period of 56 days squid were measured and photographed six times. Six of the original 10 squid provided useful data for this experiment: their picture series were

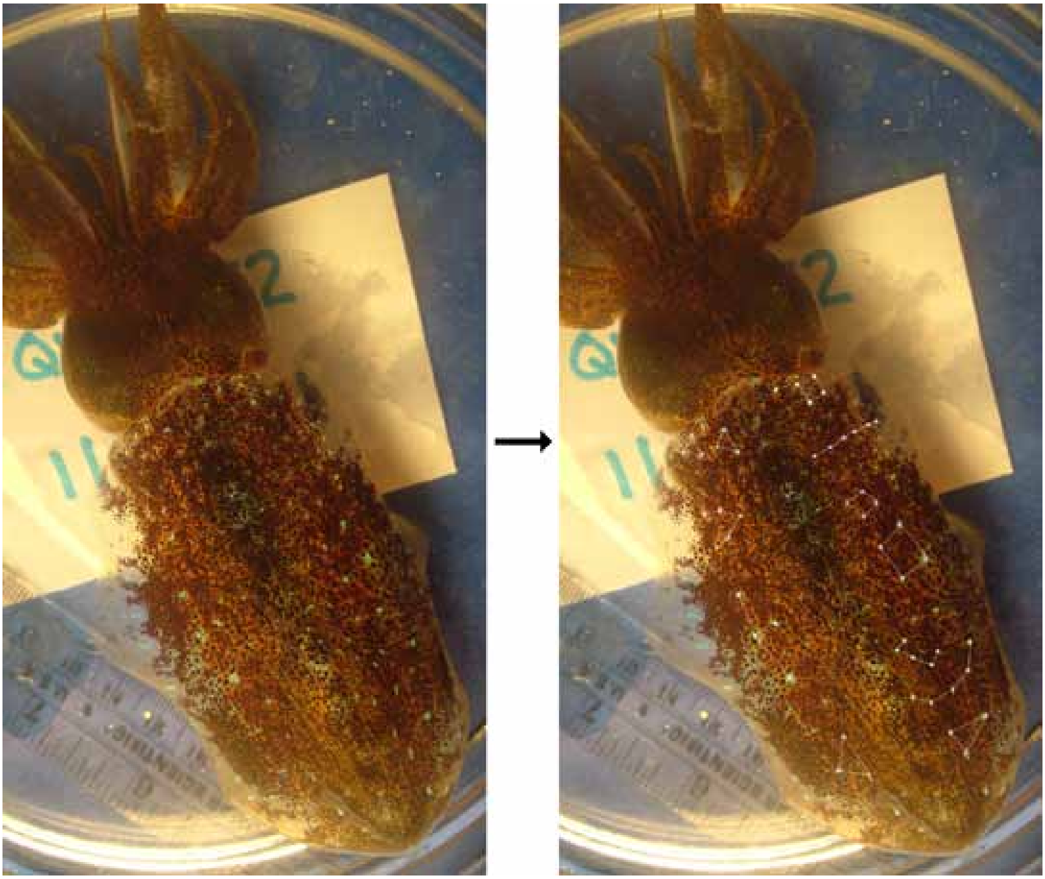

Fig. 1: To test if dot patterns on the dorsal mantle of a squid stay stable over time, we assigned 50 dots into 10 star-sign-like patterns. Photo: James Wood

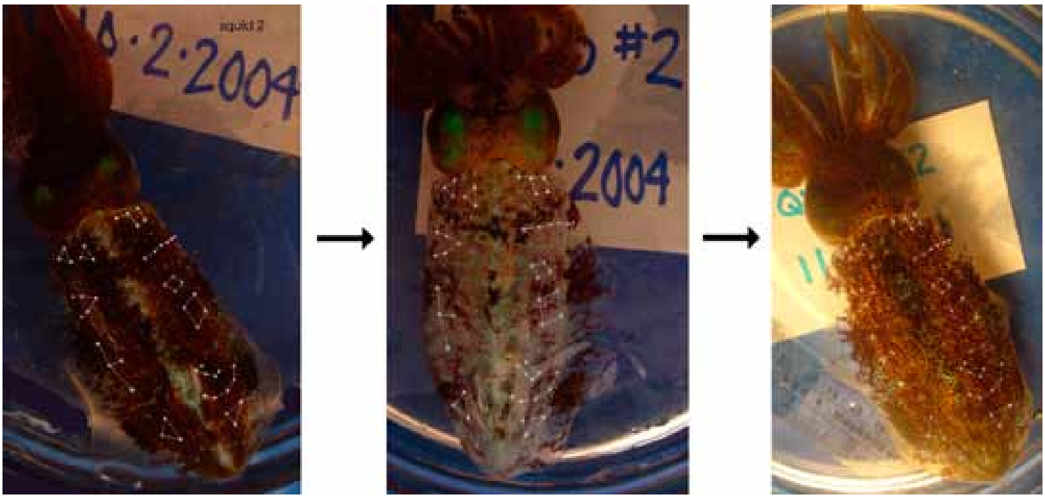

Fig. 2: Over the course of two months subjects were photographed six times. Picture 1, 3, and 6 of one subject's series show that the assigned dot patterns are stable over time. Photo: James Wood

analyzed for dot pattern retention by assigning 50 dots into 10 star-sign-like patterns (Fig. 1). These patterns were then followed on the subsequent five pictures of the six picture series (Fig. 2).

Results

Squid could best be identified when they were showing Basic Brown pattern which most often occurred during times of the day with high light intensity. Both scars and mantle and fin dots show most clearly against the dark brown background coloration. In the field we used four characters on how to distinguish individual *S. sepioidea* from each other, which are discussed in order of detail. They are sex, size, scars and dot patterns:

We were able to distinguish between adult male and female *S. sepioidea* in the field. The first observations of distinct sex dimorphism were made based on the squid's behavior. Instances of mating were observed with attention to which squid are involved and which one transferred spermatophores to the other one. During mating behavior male and female squid show very distinct sex-related displays (Moynihan & Rodaniche 1982; Hanlon & Messenger 1996; Griebel, Byrne & Mather 2002). However, after these activities, when they return to their basic coloration, the sexes can still be distinguished according to the following markings:

Males showed distinct bright dots on their fins on Basic Brown background as well as a light fin edge line (Fig. 3a), while the females had a gradual transition from Brown to Pale towards the edge of their fins without showing distinct fin dots. This does not mean that females do not have fin dots, but that the female fin dots are smaller in size and they cover them by opening the brown chromatophores above them (Fig. 3b).

Although *S. sepioidea* tend to school with squid of similar maturity stage (Boom, Byrne & Mather 2001), they can be distinguished by relative size towards each other. The groups consist of squid of different sizes and they often school in size sorted formations (Moynihan & Rodaniche 1982). The most common formation for squid groups including adults is a one-dimensional line formation (Boom, Byrne & Mather 2001). Here the largest squid tend to swim on either one or both

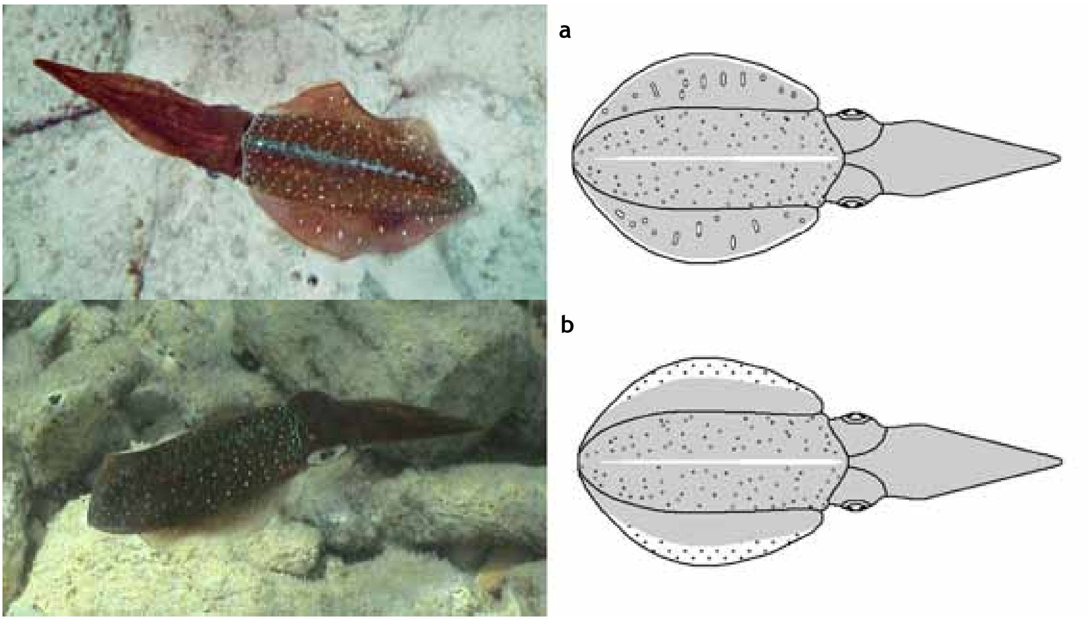

Fig. 3: Male and female sex dimorphism. a) Males can be recognized by looking at the pronounced fin dots as well as the light fin edge line. b) Females do not show clearly visible fin dots and have a transition from brown to translucent towards the fin edge. Photo: Ruth Byrne

ends of a line whereas the smaller ones are size sorted either towards the middle or to the other end of the line. Especially when identifying the larger squid in such a school knowledge of the most likely position of a specific squid within the group can be helpful.

Some squid have scars from predation attempts which can also be used as identification marks. Scars can be found in form of scratches on the mantle where the skin was hurt as there is no more pigmentation from the chromatophores visible. Such areas show up as pale or pink on a basic brown background. Fin scars include fins with missing portions or are visible as pale or lighter areas with no chromatophores (Fig. 3a). Also arms and tentacles can show marks of predation attempts as there are squid with missing or miss-built arms. To identify sub-adult and juvenile squid it is necessary to rely mostly on scars, as they do not show such distinct patterns of bright mantle and fin dots as the adults. Scars are good identification marks as long as they are fresh, but they have the disadvantage of healing and disappearing within a few weeks. This period of time has to be used to find other identification marks such as mantle and fin dots.

Mantle and fin dots are permanent marks that have an individual pattern on each squid's dorsal surface and most likely come about through iridophore reflection (Hanlon & Messenger, 1996). According to our data they do not change their location over longer periods of time in the squid's lifetime. Out of 300 dots (50 dots x 6 squid) we followed over the course of 6 time points for approximately 2 months we were able to track on average 99%. During this time frame the subjects grew on average 25 mm (Std = 4.4 mm) in mantle length. Only 10 dots were not found once or twice, but reoccurred in following images of the series. Reasons for missing dots were either the quality of

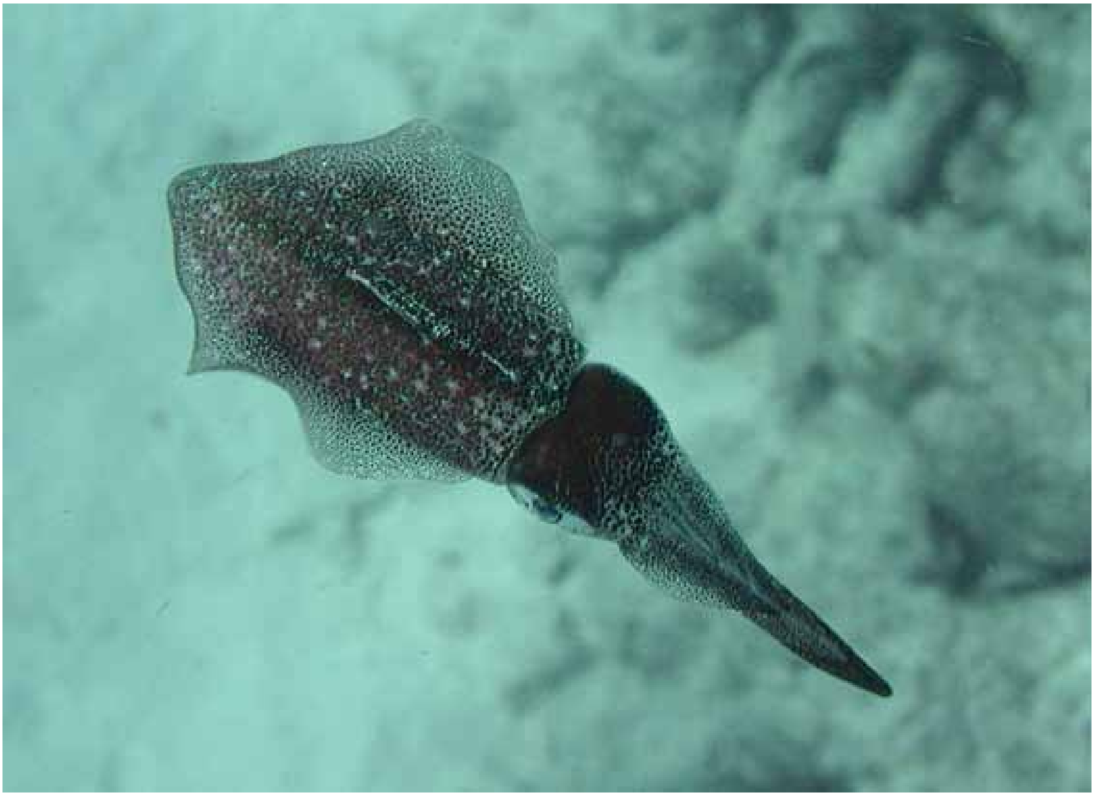

Fig. 4: Juvenile and sub-adult squid do not show clear dot patterns on the dorsal mantle. However, some of them have "freckles" that can be used for identification, as can be seen here on the anterior part of the mantle. Photo: Stephanie Bush

the image (i.e. the angle of picture (n = 4)), or the squids skin display (i.e. covered by a more intense Mid Dorsal Line (n = 1)). Five dots were missing without an obvious reason.

In the field the dots are clearly visible in the basic brown body coloration during the day, but less visible in mornings and evenings when the squid show basic intermediate or basic pale body coloration (Griebel, Byrne & Mather 2002). These dots show up in different intensities, some bright and clearly visible and some smaller, and thereby they form individual star-sign-like combinations on the dorsal surface of the squid. Although the location of fin and mantle dots does not change at least over the course of two months, if not much longer, the brightness of the dots can change, especially in the period of switching from being a sub-adult squid to an adult. A good example for this is that sub-adults do not show distinct fin dots, whereas adult males display bright and large fin dots. These very distinct dots are probably the best way to individually identify adult males. When fin dots are not distinct, as is the case in some still small males and especially in females, it has been useful to look for combinations of dots on the anterior and posterior tip of the mantle (Fig. 3b).

Sub-adult *S. sepioidea* are in general more difficult to identify than adults, as their dorsal surface is smaller and the patterns of bright fin-dots are less clearly visible. In contrast to adults, some sub-adult squid and juveniles show "freckles" (Fig. 4), different types of dots on their head, which can also be used for identification. Freckles are most likely areas of unevenly distributed chromatophores, most likely in location where mantle dots will appear later on, probably because of the skin stretching during this growth period. Unfortunately these freckles disappear in the transition to adulthood.

The three trained squid observers did really well in matching the two series of images of 26 different squid (3 x 100%). The four naïve observers reached on average 80% (100%, 81% and 2 x 69%).

Discussion

The ability to non-invasively identify individual squid is a useful tool for field and lab research on *S. sepioidea*. To track individual squid allows us to follow the development of their behavior patterns as they become mature, assess the mating success of different behaviors, assess mate fidelity, investigate group composition and stability, investigate group schooling behavior (Boom, Byrne & Mather 2002), and track them without altering their behavior over periods of up to several weeks at a time. Zeeh & Wood (2009) report that using VIE tags is one of the least stressful methods to artificially tag squid and that it did not affect the subjects growth rates. However, during their capture and tag process it became clear that this method influenced the subject's subsequent behavior. Squid tagged once were much harder to capture a second time compared to naïve conspecifics (Wood pers. com.). Thus, for the study of behavior a completely non-invasive technique, like the here presented dot pattern identification, seems to be the best choice.

Why should anyone care about identifying individual cephalopods? Cephalopods are an important and growing part of world's fisheries and they are an important prey for commercially and environmentally significant animals such as marine mammals (whales, seals, dolphins), fish (sharks, cod, etc), and birds (albatross, penguins). Yet little is known about their population dynamics and growth rates in the field. A number of invasive methods have been applied to answer these questions, some depending on dead caught animals, e.g. size analysis (Suguyama et al. 1989, tagging (Replinger & Wood 2007), and statolith work (Jereb, Ragonese & Boletzky 1991). These methods work well, but like all methods have their limits; i.e. assumptions about the subjects' size at time one in the case of statolith analysis. In addition, few species are validated with lab work. Individual identification would, at least theoretically and depending on the accessibility of the species, allow continuous observations of subjects living both in the field or in the lab. It also allowed us to make an ethogram of *S. sepioidea* (Griebel, Byrne & Mather 2002), similar to that of Jantzen & Havenhand (2003).

Contrary to Boal & Gonzales (1998) for *S. lessoniana*, not only could we discriminate the sex of adult S. *sepioidea* individuals, we could discriminate individuals over a period of weeks – a significant part of their rather short lifespan. We performed our investigations both in a natural setting as well as in the lab, while they did theirs in the lab only.

Boal & Gonzales (1998) describe the behavior of the squid as abnormal; according to their observations their subjects tried to mate with males and females indiscriminately. Perhaps because of our ability to individually identify the subjects, we never noted such behavior, either in the lab nor in the field. Or maybe in *S. sepioidea* there was an exchange of obvious visual sex-related signals among the squid that may have prevented such behaviors.

The identification of mature male squid by their dot patterns on the fins was relatively easy for observers, and this raises the question of whether such identification was being used by the squid themselves. The fin dot patterns were emphasized during male-male Zebra agonistic contests particularly during the stereotyped Formal Zebra display contest (Mather 2006) and this would be an opportunity to indicate one's identity to a rival (see Norman (2000), page 142-143; despite being mis-identified as a mating sequence, the photographs clearly show the dark background and pattern of dots). *S. sepioidea* and other loliginid squids are highly visual so a visual identification of others could be possible.

This unique identification of cephalopods, despite their underlying variability, gives hope that others of the group could also be individually identified. Research on the skin display system has focused on its variability (Messenger 2001), and its pattern production is certainly dazzling (Moynihan & Rodaniche 1982), but the observation that individuals can be identified nevertheless offers a new insight into this complex system, as well as gives us a tool to help understand the behavior, life history and population dynamics of these animals.

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