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Peripheral injury alters schooling behavior in squid, *Doryteuthis pealeii*

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ABSTRACT

Animals with detectable injuries are at escalated threat of predation. The anti-predation tactic of schooling reduces individual predation risk overall, but it is not known how schooling behavior affects injured animals, or whether risks are reduced equally for injured animals versus other school members. In this laboratory study we examined the effects of minor fin injury on schooling decisions made by squid. Schooling behavior of groups of squid, in which one member was injured, was monitored over 24 h. Injured squid were more likely to be members of a school shortly after injury (0.5–2 h), but there were no differences compared with sham-injured squid at longer time points (6–24 h). Overall, the presence of an injured conspecific increased the probability that a school would form, irrespective of whether the injured squid was a member of the school. When groups containing one injured squid were exposed to a predator cue, injured squid were more likely to join the school, but their position depended on whether the threat was a proximate visual cue or olfactory cue. We found no evidence that injured squid oriented themselves to conceal their injury from salient threats. Overall we conclude that nociceptive sensitization after injury changes grouping behaviors in ways that are likely to be adaptive.

1. Introduction

Many animals aggregate to reduce their individual predation risk (Hoare et al., 2004; Thünken et al., 2014). In groups, risks are decreased due to increased scanning rates (Beauchamp, 2015; Roberts, 1996), predator confusion (Landeau and Terborgh, 1986; Olson et al., 2013) and decreased targeting risk for individuals (Creel et al., 2014). However, there are also costs to aggregation, including increased chances of detection by predators, increased parasite and disease transmission, and increased competition for food and mates (Herczeg et al., 2009). Thus, choosing to be a part of a group represents a dynamic risk/benefit tradeoff that each individual should make based on its own perceived vulnerability to predation versus other costs. Empirical studies of grouping behavior typically assume that individuals within groups have similar initial fitness; thus their cost/benefit tradeoffs are likely to be relatively homogenous. However, in any group of prey animals, initial individual fitness will vary based on previous life history, incorporating a range of conditions that may make individuals more or less vulnerable to predation relative to other group members.

The longfin inshore squid, *Doryteuthis* (*Loligo*) *pealeii*, is a mid-sized (14–30 cm adult mantle length) pelagic species that, like many of the *Loliginid* species, often swims in groups. Groups may be either shoals (opportunistic aggregations) or schools (synchronized, directionally polarized groups), both of which occur across juvenile and adult life stages (Sauer et al., 1992).

Schools and shoals vary greatly in size, from fewer than 10 individuals to thousands, with large breeding aggregations of non-kin adults occurring annually during summer months off Cape Cod, Massachusetts (Shashar and Hanlon, 2011). Observations of schooling dynamics in wild populations show that directional polarity and belt-shaped (i.e., linear) groups are common (Sugimoto and Ikeda, 2012). Composition of schools and individual position is highly fluid, with squid moving within, entering and leaving established schools readily, with seemingly little interaction between school members and 'enterers' or 'leavers' (Adamo and Weichelt, 1999).

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This suggests that school membership and position are primarily determined by the individual, and not by other members of the school or the school as a whole.

In captivity, 3–10 individuals readily congregate into small schools, with individuals typically matching each other's swimming speed and orientation. Captive squid also exhibit group resting in close proximity to each other, with similar cryptic coloration on sandy or rocky substrates. Squid are highly vulnerable to predation at all life history stages, typically from diurnally active fish predators (Staudinger and Juanes, 2010). Unusually for grouping species, squid are also under pressure from conspecific predation (cannibalism) (Adamo and Weichelt, 1999; Sauer and Smale, 1991) especially when a squid appears compromised. Thus the anti-predator advantages of schooling in squid are likely to be at least partially offset by significant costs, represented not only in terms of food and mate competition, increased detection by predators and increased parasitism risk, but also in risks from conspecific predation within a school or shoal.

We have demonstrated previously that in *D. pealeii*, minor injury produces a sensitized behavioral state characterized by visual and tactile hypervigilance (Crook et al., 2013, 2011). In many species (including squid), individuals who have experienced injury display a range of neural and physiological changes that produce long-lasting effects on behavior (Walters, 1994). After bodily injury in molluscs, plasticity in nociceptive sensory neurons produces a complex of sensitized behaviors that may include increased cutaneous sensitivity (Walters, 1987a), increased anxiety-like vigilance (Crook et al., 2011), decreased appetitive behavior (Walters et al., 1981) and enhanced defensive behavior (Crook et al., 2014). This sensitized behavioral state partially offsets the elevated risk of predation that occurs with sub-lethal injury (Crook et al., 2014). Despite extensive study of behavioral sensitization in both vertebrate and invertebrate species, few studies have addressed the functions of these changes in wild animals, and none have examined how nociceptive sensitization influences aggregation decisions in grouping species.

Here we examine how schooling behavior, a defensive tactic in some prey animals, is altered when one member of a small group has recently experienced a minor injury. Using the same species of squid (*D. pealeii*), which we have shown previously to gain a fitness advantage from nociceptive sensitization after minor peripheral injury (Crook et al., 2014), we ask whether an individual's decision to school with a group of uninjured conspecifics is changed by its minor injury. We also investigate whether injury affects schooling decisions when groups are presented with either a visual or olfactory predation threat.

2. Methods

2.1. Animals

Male and female adult squid ((*D.* [*Loligo*] *pealeii*), mantle length 9–22cm), were collected from local waters around Woods Hole, MA and maintained in groups of 10–15 in holding tanks containing filtered ambient-temperature (16–19 °C), flow-through seawater. Fluorescent overhead lighting maintained a 12:12 day-night cycle. Squid were fed twice daily to satiation on small fish (*Fundulus* spp). Only animals with no evidence of previous injury were used. Squid were sexed by direct observation of the pink accessory nidamental gland through the ventral mantle (present in females and absent in males). Typically groups were composed of all females or females with one smaller male. Large males were excluded as they tended to disrupt schools with aggressive courtship behavior.

2.2. Ethical note

In the US invertebrate animals are not covered under IACUC regulations, thus no approval was required for this study. Manipulations of live squid followed the guidelines of the Animal Behavior Society for predator/prey studies (ABS, 2012) and the International Association for the Study of Pain for vertebrates undergoing potentially stressful and painful procedures. We chose to stage interactions and use a controlled injury procedure on selected captive squid because wild adult squid often have pre-existing injuries of unknown age and varying severity, and observation of defensive interactions in wild marine animals is extremely difficult.

Animals were monitored daily and squid with any evidence of compromised health were euthanized immediately. All squid were euthanized at the conclusion of each trial by immersion in isotonic $MgCl_2$ solution followed by decapitation and decerebration, and tissue was used in other physiological studies. To minimize animal numbers the study was designed only to detect large effects. Experimental injuries were minor compared to observed survivable injuries in the wild, the trial arena enabled effective squid camouflage (reducing escape swimming), and trials were kept short to reduce overall stress.

2.3. Procedure

2.3.1. Arena

The trial arena was a rectangular fiberglass tank, measuring 245 × 120 cm, filled with flow-through, filtered natural seawater to a depth of 45 cm. The tank bottom was lined with a 3 cm deep bed of sand and small pebbles. Inflow and outflow pipes located on either side of the trial arena provided constant, predominately laminar flow through the tank with an exchange rate of approximately 40 l/min. Air pumped through an airstone provided constant aeration except during video recording.

2.3.2. Focal animal treatment

One squid in each group was selected as the focus of treatment and observation. It received one of two treatments 10 min prior to being placed into a schooling group of 4 or 5 other squid.

1. Control (C): squid (n = 13) were restrained briefly in a net, placed onto a submerged surgical tray and then released. One fin (L or R) was arbitrarily designated as treated.
2. Injured (I): squid (N = 29) were restrained as above, and either the left or right fin received three closely spaced, 1 s duration crushes with serrated forceps. The injured area extended ~10 mm rostral-caudally and ~15 mm from the edge of the fin toward the body. This procedure approximates common natural injuries to the fins that occur during intraspecific aggression and hunting, and we have shown previously that this injury produces robust, long-lasting neural and behavioral sensitization (Crook et al., 2013, 2014).

All focal squid received a marking procedure, while other squid in the school were unmarked. A small volume (0.1 ml or less) of filtered (0.02 μm pore diameter) seawater colored with Fast Green vital dye (Sigma-Aldrich) was injected subcutaneously into the distal midline tip of the mantle with a narrow (27 gauge) needle. Typically this injection produced no obvious behavioral response from the squid, and the dye remained visible for the 24 h observation period with minimal spreading.

2.3.3. Threat cues

Trials were conducted under one of three conditions.