Lateral line analogue aids vision in successful predator evasion for brief squid

*Lolliguncula brevis*

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SUMMARY

Cephalopods have visual and mechanoreception systems that may be employed to sense and respond to an approaching predator. While vision presumably plays the dominant role, the importance of the lateral line analogue for predator evasion has not been examined in cephalopods. To test the respective roles of vision and the lateral line analogue, brief squid *Lolliguncula brevis* were observed in the presence of summer flounder *Paralichthys dentatus* under light and dark conditions with their lateral line analogue intact and ablated. Hair cell ablation was achieved through a pharmacological technique used for the first time on a cephalopod. Proportion of predator-prey interactions survived was significantly higher in the light non-ablated and light ablated groups compared to the dark ablated group. The mean number of interactions survived varied across treatment groups with the light non-ablated group having significantly more success than the light ablated, dark non-ablated and dark ablated groups. These findings demonstrate that although vision is the primary sense, the lateral line analogue also contributes to predator evasion in squid.

INTRODUCTION

Cephalopods rely on multiple sensory systems for detection of predators (Budelmann, 1996). The eyes are the most prominent sensory feature of cephalopods with the optic lobes being the dominant region of the brain (Young, 1962). The highly evolved visual system of cephalopods likely plays a large role in predator detection and initiation of an escape response. However, to date, the role of vision relative to other sensory modalities in predator evasion has not been examined in any cephalopod.

In addition to their complex visual system, cephalopods have a sensory system that resembles the lateral line system of fishes (Bleckmann et al., 1991; Budelmann, 1996; Budelmann and Bleckmann, 1988). The cephalopod lateral line analogue consists of polarized epidermal hair cells that have several kinocilia and an axon extending from their base (Budelmann and Bleckmann, 1988). Polarization occurs in a precise pattern (e.g., anteriorly, posteriorly, left and right), allowing the animals to respond to water movements as low as 18.8 µm/s, which is equivalent to the sensitivity of fish lateral lines (Bleckmann et al., 1991). Behavioural responses have also been elicited in cuttlefish (*Sepia officinalis*) by stimulating their lateral line analogue using a wide range of frequencies (10-600 Hz) (Komak et al., 2005). While fish can react swiftly to the flow field produced by an attacking predator using their lateral line system (Stewart et al., 2013), it is unknown if the lateral line analogue of cephalopods plays a similar role in predator detection. The goal of this study was to test the hypothesis that the lateral line analogue of squid (*Lolliguncula brevis*) aids vision in successful predator evasion.
RESULTS AND DISCUSSION

To test the respective roles of vision and the lateral line analogue, brief squid *Loligouncula brevis* were observed in the presence of summer flounder *Paralichthys dentatus* under light and dark conditions with their lateral line analogue intact and ablated. Hair cell ablation was achieved through a pharmacological technique which was validated for the first time in a cephalopod. All of the squid that were tested in the light non-ablated group (N=10) successfully evaded the predators with a total number of 60 interactions survived (4-11 interactions survived per individual). In the light ablated group (N=10), however, only 6 squid successfully evaded the predators with a total of 34 interactions survived (0-5 interactions survived per individual). Successful evasion was further reduced in dark condition with only 5 squid surviving in the non-ablated group (N=10; 22 total interactions survived; 0-4 interactions survived per individual) and 2 squid surviving in the ablated group (N=10; 15 total interactions survived; 0-4 interactions survived per individual).

The proportion of interactions survived significantly differed across treatment groups (F3,36=6.16, p=0.002; Fig. 1). Tukey post-hoc comparisons show the light non-ablated group (mean=1.00 ± 0.00 s.d.) had higher proportion of interactions survived than the dark ablated group (mean=0.33 ± 0.44 s.d., p=0.001). Additionally, the light ablated group (mean=0.78 ± 0.34 s.d.) showed higher survivability from the dark ablated group (mean=0.33 ± 0.44 s.d., p=0.046). While not significant at α = 0.05, the light non-ablated group exhibited a trend in higher proportion of interactions survived, relative to the dark non-ablated group (mean=0.57 ± 0.50 s.d., p=0.056). The mean number of interactions survived differed significantly across treatment groups (F3,36=8.69, p<0.001). Tukey post-hoc comparisons of the four groups indicate that the light non-ablated group (mean=6.00 ± 2.20 s.d.) had significantly higher mean number of interactions survived than the light ablated group (mean=2.90 ± 1.52 s.d., p=0.031), the dark non-ablated group (mean=2.20 ± 1.62 s.d., p=0.002) and the dark ablated group (mean=1.50 ± 1.27 s.d., p<0.001).

The results of this study indicate that both vision and the lateral line analogue provide sensory information for successful predator evasion. The light non-ablated group survived a higher number of interactions than the light ablated and dark treatment groups, indicating the importance of both sensory systems. The observed higher proportion of interactions survived for the light non-ablated group relative to the dark ablated group and the trend in higher proportion of interactions survived for the light non-ablated versus the dark non-ablated group demonstrate that vision is the most important modality (of the two tested here) for predator avoidance. The higher mean proportion of interactions survived in the light ablated than dark ablated group provides further support for this conclusion. Increased number of
interactions survived, in the light non-ablated than the light ablated group, however, suggests that the lateral line analogue also plays a role in predator evasion even when vision can be used. Although the dark non-ablated and dark-ablated groups were not significantly different, there was a 30% decline in the total number of animals that survived when the lateral line analogue was ablated, indicating that having the sensory hair cells intact aided survival in dark conditions as well.

The lateral line analogue appears to play a similar role to the fish lateral line, serving as a sensory component to predator evasion (Stewart et al., 2013). Although vision and the lateral line analogue clearly contribute to survival, there are other sensory modalities that could potentially add to successful predator evasion. Olfactory cues were likely present and could have alerted the squid to the predator’s presence. Additionally, mechanoreceptors within the muscle or on the fins could have detected a pressure wave created by an approaching predator (Budelmann, 1996). It is possible that several sensory components not tested here contribute to successful predator avoidance; therefore further research is needed to examine other potential sensory modalities involved in predator detection and evasion in cephalopods. This study, however, demonstrates for the first time that mechanoreceptor ablation techniques can be performed successfully on cephalopods and the lateral line analogue, together with the visual system, is important for predator detection and survival.

MATERIALS AND METHODS

Animal collection and maintenance

*Lolliguncula brevis* ranging from 2.5-6.0 cm dorsal mantle length (DML) were used in this project. Squid were captured by otter trawl in Wachapreague, VA, USA and kept in 1.2 m diameter circular tanks using protocols described in Hanlon (1990). Animals were allowed to acclimate for at least 2 h before experiments. Only animals that appeared healthy and exhibited normal behaviour were considered for the experiments. For the lateral line analogue ablation validation, *Doryteuthis pealeii* paralarvae were purchased from the Marine Biological Laboratory, Woods Hole, MA, and maintained in a recirculating seawater system at a salinity of 30-32‰ and at temperatures of 19-24°C until hatching.

Lateral line analogue ablation validation

Antibiotic solutions have successfully been used for lateral line ablation in fish studies (Harris et al., 2003; Stewart et al., 2013), however the technique had never been performed on cephalopods and therefore validation of this approach was required. To determine the appropriate concentration of the neomycin sulphate solution for lateral line analogue ablation, a series of antibiotic trials were conducted. *Doryteuthis pealeii* paralarvae were used in the antibiotic trials because of the large number available and
small body size, which is conducive for scanning electron microscopy (SEM) prep. Paralarvae were
divided into four treatment groups (N=15 per group): 0 µM, 150 µM, 250 µM and 500 µM neomycin
sulphate. Paralarvae were placed into the antibiotic solution for one hour. Prior to fixation, squid were
over-anesthetized using water at 5°C for approximately 15 minutes.

SEM was employed to survey the success of ablation within each of the experimental groups. For
SEM, the squid were placed overnight in a fixative (concentrations: 3% glutaraldehyde, 6% sucrose, 0.5%
tannic acid, 0.065M Sörensen’s buffer), rinsed and stored in a buffer (concentration: 1% glutaraldehyde,
6% sucrose, 0.065M Sörensen’s buffer) and then dehydrated in a graded ethanol series. Specimens were
dried using the chemical drying agent hexamethyldisilazane and mounted on aluminium stubs with
double-stick tape. Specimens were sputter-coated with 15-30 nm gold and examined with a Hitachi S-
3400N JEOL 6300-F field emission scanning electron microscope at an accelerating voltage of 15kV.
SEM images revealed consistently successful lateral line ablation after being treated with 500 µM
neomycin sulphate solution (Fig. 2). Similar protocols were used with L. brevis juveniles to confirm
lateral line ablation at this treatment concentration. Based on successful ablation in D. pealeii hatchlings
and L. brevis juveniles, a 500 µM concentration was selected for predator-prey experiments. Although
this treatment was effective at decreasing the number and integrity of lateral line hair cells, the lateral line
regeneration capabilities of squid were not tested and are not currently known. Observation of both D.
pealeii and L. brevis after treatment showed that squid maintained normal behaviours. Ablated squid
maintained typical pitch, roll, and yaw behaviours during swimming in the holding tanks, were able to
hover effectively, and were able to successfully approach and attack prey, indicating that ablation does
not impact the function of the statocysts or impact routine swimming and feeding behaviours.

Predator-prey experiments

Predator-prey interaction experiments were used to evaluate the use of vision and
mechanoreception in predator evasion. Trials took place in a 1.2 m diameter x 0.76 m deep round tank
lined with gravel substrate. The arena was lined with curtains to avoid disturbing acclimating animals.
Black plastic sheeting was used to block light during the dark trials. For each experiment, a single squid
was placed in an arena with two flounder (Paralichthys dentatus) (13.2 cm and 15.5 cm total length).
Flounder have shown successful captures for the relative prey size presented in this study (Staudinger and
Juanes, 2010), and were chosen as predators due to their exceptional vision in both bright and dark
conditions (Horodysky et al., 2010). Multiple predators were used to increase the odds of a predation
event. The flounder were fed live squid prior to the trials so that they could become proficient in squid
capture before data collection. Food was withheld 24 h prior to the start of all trials to standardize predator hunger.

One hour prior to trial acclimation (read below), squid were placed in a container, which either held the neomycin sulphate solution for ablation groups or untreated water for the non-ablation groups. Prior to the start of each trial, a cylinder made of 5 mm plastic mesh was lowered into the experimental tank and a single squid was placed inside for a 30 min acclimation period. The trials commenced when the partition was raised above the tank and the flounder and squid were allowed to interact. Each trial ran for 10 min before surviving squid were removed. Four different conditions were tested: 1) light non-ablated, 2) light ablated, 3) dark non-ablated, and 4) dark ablated. Ten separate squid were tested in each treatment condition. Each group contained squid of similar sizes (light non-ablated: mean= 4.2 ±0.3 cm DML; light ablated: mean= 3.9 ±0.3 cm DML; dark non-ablated: mean= 3.9 ±0.4 cm DML; dark ablated: mean= 3.9 ±0.3 cm DML). All interactions were recorded ad libitum by a single observer. In darkened conditions, the observer was easily able to identify interactions by tracking the flounder silhouette against a light-colored tank bottom and observing surface water disturbances, which were frequently present during interactions. After each interaction, a flashlight was turned on briefly to confirm squid capture or escape.

Interactions used for subsequent statistical analysis were defined as: (1) successful predator strikes, (2) unsuccessful predator strikes, and (3) approaches toward the squid but where a strike was not initiated because of an escape response by the squid.

**Statistical analysis**

Statistical analysis was performed in SPSS (v. 18 SPSS Inc., Chicago, IL, USA). The proportion of interactions survived for each squid was calculated to show success relative to the number of capture attempts. Since this measure does not reveal the total number of interactions survived, the sum of interactions survived for each squid in each treatment group was also calculated. All data were tested for normality using Shapiro-Wilk tests. Data from several groups varied from normality (all p ≤ 0.02) and therefore all data were transformed prior to parametric analysis. The proportion of interactions survived was adjusted via arcsine transformation, while the sum of interactions survived was adjusted with a square-root transformation. A regression was performed on the total number of interactions survived and the mantle length of the squid in each condition to determine the relationship between size and survivability. No significance was found (all p ≥ 0.10), and thus all sizes were pooled for further analysis. Analysis of variance (ANOVA) was performed on the transformed values of the total number of interactions survived and the proportion of interactions survived in each treatment group. Although
ANOVA tests were performed on transformed data, the means reported in the results and figures are not transformed to facilitate easier interpretation of the data.

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AUTHOR CONTRIBUTIONS

C.A. York and I.K. Bartol collaborated on the experimental approach, data analysis and preparation of this manuscript. C.A York performed the experiments and collected the data.

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REFERENCES


**FIGURES**

Figure 1: The proportion of interactions survived (a) and number of interactions survived (b) in each treatment group. Non-transformed means ± S.E. are presented, as opposed to transformed data used in statistical analyses, to facilitate easier interpretation of data. None overlapping lines above the bars represent significant difference detected using Tukey posthoc comparison tests.

Figure 2: SEM images of the lateral line analogue shown on a *Doryteuthis pealeii* paralarvae. Lines of hair cells on the head highlighted with arrows (a); close-up views of sensory hair cells of the lateral line analogue (b); sensory hair cells after treatment with a 500 µM solution of neomycin sulphate (c). The majority of hair cells were destroyed completely after treatment with the remaining hair cells being porous and heavily damaged. Scale bar for a = 400µm; b, c =5µm.