Report

Nociceptive Sensitization Reduces Predation Risk

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Summary

Sublethal injury triggers long-lasting sensitization of defensive responses in most species examined, suggesting the involvement of powerful evolutionary selection pressures [1]. In humans, this persistent nociceptive sensitization is often accompanied by heightened sensations of pain and anxiety [2]. While experimental [3] and clinical [4] evidence support the adaptive value of immediate nociception during injury, no direct evidence exists for adaptive benefits of long-lasting sensitization after injury. Recently, we showed that minor injury produces long-term sensitization of behavioral and neuronal responses in squid, *Doryteuthis pealei* [5, 6]. Here we tested the adaptive value of this sensitization during encounters between squid and a natural fish predator. Locomotion and other spontaneous behaviors of squid that received distal injury to a single arm (with or without transient anesthesia) showed no measurable impairment 6 hr after the injury. However, black sea bass given access to freely swimming squid oriented toward and pursued injured squid at greater distances than uninjured squid, regardless of previous anesthetic treatment. Once targeted, injured squid began defensive behavioral sequences [7, 8] earlier than uninjured squid. This effect was blocked by brief anesthetic treatment that prevented development of nociceptive sensitization [6, 9]. Importantly, the early anesthetic treatment also reduced the subsequent escape and survival of injured, but not uninjured, squid. Thus, while minor injury increases the risk of predatory attack, it also triggers a sensitized state that promotes enhanced responsiveness to threats, increasing the survival (Darwinian fitness) of injured animals during subsequent predatory encounters.

Results

Because minor injury in the squid, *Doryteuthis (Loligo) pealeii*, induces hypersensitivity to visual and cutaneous stimuli without affecting general activity levels or foraging behavior [5], we hypothesized that nociceptive sensitization in these animals functions primarily to offset increased risks of predation. To test this hypothesis, we staged a series of controlled interactions with a fish predator, black sea bass (*Centropristis striata*), asking whether prevention of nociceptive sensitization by transient anesthetization during surgery influenced the course and outcome of the predatory interaction. Black sea bass are benthic, visual hunters [10] that prey on *D. pealei* as they migrate into shallow, inshore areas [11]. Our observations confirmed that these predatory interactions follow a distinct series of stages that are diagrammed in Figure 1 [5, 7, 8, 12], beginning with a primary defense of crypsis (avoiding detection) and progressing swiftly to secondary defenses against pursuit by the predator that begin with deimatic behavior of rapid body patterning appearance and/or avoidance swimming, followed by escape jetting and protean defense (ink release and erratic, unpredictable jetting directions). When a fish targets a squid, its chase sequence progresses in stages from orientation to pursuit, attack, and capture [8, 13, 14], with many encounters abandoned prior to completion.

Fish Predators Target Squid with Injuries

Minor injury to a single arm 6 hr earlier produced no effects on spontaneous swimming or other behaviors detectable by human observers during video analysis, regardless of whether the animal had been anesthetized during the injury (see also [5]). Nevertheless, black sea bass selectively targeted squid in both injured groups (injured [I] and injured treated with anesthetic [IA]) compared to the uninjured groups (uninjured [U] and uninjured treated with anesthetic [UA]) (for anesthetic details see legend of Figure 2 and Supplemental Experimental Procedures available online). In mixed-treatment trials (n = 4 trials, containing 2 each injured and uninjured squid), squid in the I group were more likely to be captured than squid in the U group present at the same time (five of eight I versus one of eight U squid, odds ratio = 11.7, z = 1.89, p = 0.05). In trials involving single-treatment groups of squid (e.g., all four were uninjured or all four were injured), fish both oriented to and pursued squid in the I and IA groups from longer distances compared to squid in the U and UA groups (post hoc t tests, p = 0.02 and 0.001, respectively; Figures 2A and 2B), indicating that even minor injuries make squid either more conspicuous or more attractive targets to a natural predator [15].
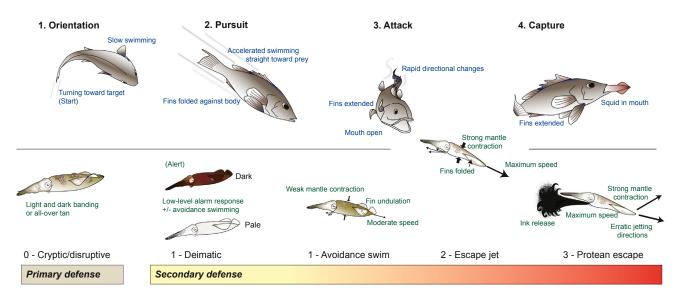
Sensitized Squid Are More Responsive to Predation Threats

Squid in the I group (n = 20) had longer alert distances [16, 17] than squid in the IA, U, or UA groups (n = 16, 20, and 16, respectively), indicating earlier initiation of secondary defense (I versus U, p = 0.03; Figure 2C). Similarly, I group squid showed alert behaviors at earlier stages of encounters than IA, U, or UA squid (p = 0.004; Figure 2D), despite fish orientation and pursuit usually occurring at greater distances from injured than uninjured squid (Figures 2A and 2B). I group squid also had longer flight initiation distances (versus U, p = 0.008; Figure 2E) compared with squid in the other three groups.

Nociceptive Sensitization in Prey Affects the Likelihood that a Predatory Encounter Will Escalate

Squid in the I group were less likely to be pursued after orientation by fish than U group squid (p = 0.046; Figure 2F), compared with squid in the IA group (p = 0.017). In encounters that escalated to pursuit, squid in both the I and IA groups had higher probabilities of being attacked (I versus U, p = 0.042; IA versus U, p = 0.023; Figure 2G). However, only attacks

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Figure 1. Escalation Pattern in Predator-Prey Interactions between Black Sea Bass and Squid

Top: four stages of predator behavior. Orientation is the first change in direction toward a squid from an ongoing swimming trajectory, and the distance from fish to squid is the "start distance" of the predation attempt. Pursuit is an accelerated, direct approach toward a squid, with the fish's dorsal, pectoral, and caudal fins folded. Attack is close-proximity "grappling," with the fish's mouth open and fins extended to facilitate rapid directional changes. Capture is defined as any part of the squid's body caught in the mouth of the fish. Bottom: defensive responses of squid to the fish. Primary defense (avoiding detection via crypsis) escalates to secondary defenses once the squid is alerted. Crypsis, via chromatophore patterns of disruptive banding while sitting on the substrate or all-over beige when swimming, occurs in the absence of encounters and often during early encounter stages; it received an escalation score of 0. Distance between squid and fish at the first secondary defensive behavior is the "alert distance." Secondary defenses were scored based on their typical progression. Deimatic chromatophore displays that distract or startle a predator were scored 1, as were slow avoidance swimming evoked by distant threat. Escape jetting without inking was scored 2. This typically (but not always) followed expression of behaviors scored 1. Ink release, which was almost always combined with erratic escape jetting, was scored 3. The highest escalation score was recorded for each predatory encounter.

involving IA squid had a significantly greater probability of capture (p = 0.048; Figure 2H). Similarly, IA squid made fewer successful escapes (indicated by the total number of encounters that fish aborted prior to capture) than U squid (data not shown, p = 0.028).

Predation Risk Is Highest among Squid that Are Injured but Not Sensitized
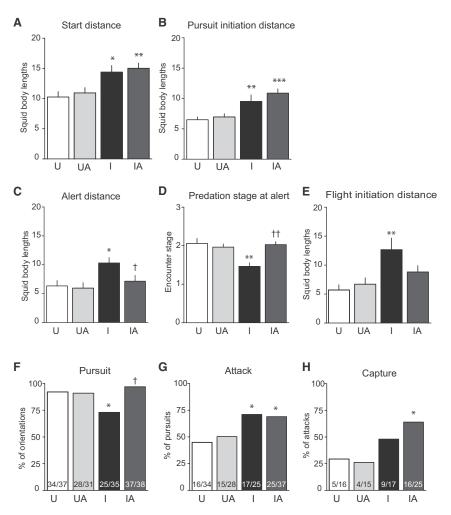
Prior injury reduced survival of attacked squid, with both the I and IA groups having lower odds of surviving the 30 min encounter compared with squid in the U group (I versus U; odds ratio = 4.89 (large effect), z = 2.212, p = 0.026, IA; odds ratio = 17.33 (large effect), z = 3.35, p = 0.008; Figure 3). Based on our earlier findings that injury induces behavioral sensitization [5] and that neuronal sensitization in squid is prevented by injection of anesthetic at an injury site [6], we predicted that survival in the IA group would be less than that in the I group (i.e., that anesthetic treatment would prevent nociceptive sensitization and its later survival benefit). This prediction was supported by the higher survival rate of I group squid compared to the nonsensitized IA group squid (odds ratio 3.54 [medium effect], z = 1.618, p = 0.05). This apparent benefit of nociceptive sensitization (Figure 3) is not accounted for by deficits caused by the anesthetic treatment, because there was only a slight, statistically insignificant difference in survival between the UA and U groups.

Discussion

This study provides the first experimental support for the hypothesis that nociceptive sensitization is an adaptive response to injury. Although neural mechanisms of nociceptive

sensitization have received extensive study in the contexts of pain [18, 19] and aversive learning [20, 21], and its adaptive value for recuperative behavior, contextual learning, and defense against potential attackers has been conjectured [1, 22–24], until now there has been no direct evidence for a contribution of nociceptive sensitization to Darwinian fitness. Indeed, in clinical contexts long-lasting sensitization and pain are often assumed to be maladaptive rather than adaptive [19, 25].

Injury and other types of noxious stimulation produce several forms of long-lasting behavioral and neural sensitization in many animals [5, 9, 26–29]. At the behavioral level, this includes general sensitization (hypervigilance, anxiety-like states) and site-specific sensitization near sites of injury (expressed in mammals as primary hyperalgesia) [1]. Behavioral sensitization is known to occur after predatory attacks (including conspecific and parasitoid assaults) [3, 6, 30–32]. Even in some invertebrate animals, noxious stimulation can produce long-lasting motivational effects with possible functional similarities to mammalian pain and fear states [33–35]. The existence of nociceptive sensitization and related motivational states in diverse species suggests that long-lasting, injury-related defensive states—which in humans are associated with pain and anxiety—are the product of strong and widespread evolutionary selection pressures [1].

Sublethal injury in animals is ubiquitous and costly [13, 36, 37], arising from failed predatory attacks [38], intraspecific conflicts [39], and injury unrelated to predatory attempts [40]. A significant fitness cost of injury is increased risk of subsequent attacks by predators [41, 42], which often target compromised prey [15, 43, 44]. Injury may also lead to less successful avoidance of predation because of biomechanical [45] or energetic sequelae of injury [39, 46].

Figure 2. Encounters Are Affected by Previous Injury to Prey and by Nociceptive Sensitization

Squid received one of four treatments 6 hr prior to exposure to fish. Uninjured (U, n = 20) squid were handled but not injured. Uninjured under anesthetic (UA, n = 16) squid received general and local anesthesia (immersion in 1% ethanol and local injection of isotonic $MgCl_2$ into an arm) but no arm injury. Injured (I, n = 20) squid were handled and the tip of one arm was removed with a surgical scalpel. Squid injured under anesthetic (IA, n = 16) received general and local anesthesia before arm injury.

(A and B) Fish targeted injured prey. Orientation to squid (start distance; A) and initiation of pursuit (B) occurred at greater distances in both the I and IA groups compared with U group. The UA group was not different from the U group. Bars show mean + SEM. Two-way ANOVA with post hoc, two-tailed t tests. *, comparisons to U group; +, comparisons between the I and IA group. *p < 0.05, **p < 0.01, ***p < 0.001.

(C, D, and E) Squid in the I group had longer alert distances (earlier initiation of secondary defenses; C), were alerted at earlier encounter stages (D), and initiated flight (escape jetting with or without ink) at greater distances compared to the other groups (E) (Kruskall-Wallis test with post hoc, Bonferroni-corrected Mann-Whitney U tests, significance indicators as for B).

(F, G, and H) The probability of encounter escalation was increased by prior injury and by prior anesthetic treatment. Encounters were less likely to escalate from orientation to pursuit for the I group than for the other groups (F), were more likely to escalate to attack for the I and IA groups (G), and were more likely to escalate to capture for the IA group (H). Fisher's exact tests, significance markers as for (D). Ratios indicate number of escalations/number of encounters.

The adaptive value of nociceptive sensitization was revealed in our study by using brief anesthetic treatment at the time of injury—which prevents the development of nociceptor sensitization in *D. pealeii* ([6] see also [9])—to prevent ensuing hypervigilance. Adaptive value was indicated by reduced responsiveness of these squid to the predator compared with squid injured without anesthetic (Figures 2C–2E). Most importantly, the previously anesthetized, injured squid had the highest likelihood of capture (Figure 2H) and mortality during the predatory interaction (Figure 3), revealing that the full cost of injury is partially compensated by sensitized behavior. Several considerations indicate that these effects result from anesthesia preventing the development of generalized nociceptive sensitization [5, 6], rather than nonspecific, lingering effects on the condition of the animal. Anesthetic treatment by itself did not compromise behavioral function because the UA group showed no significant differences from the U group in any of the behavioral measures (Figures 2C–2E) or survival (Figure 3), nor did previously anesthetized animals show other signs of motor or sensory impairment during the predatory encounter. In addition, very brief, light general anesthesia by immersion in 1% ethanol [47] was combined with injection of isotonic $MgCl_2$ locally at the site to be transected, where the restriction of relaxed chromatophores to skin near the injection site showed that the $MgCl_2$ did not travel to the base of the treated arm. Moreover, we have shown previously that $MgCl_2$ injected near a wound on a fin remains localized to the injury site while completely blocking the development of generalized sensitization of primary nociceptors [6]. Therefore, the prevention of hypervigilance and the increase in mortality can be attributed to our anesthetic procedures selectively blocking the induction of long-lasting nociceptive sensitization during arm injury. An interesting possibility is that the adaptive, injury-induced hypervigilance may be promoted by persistent spontaneous activity generated in peripheral terminals of primary nociceptors of the squid [6].

Higher mortality rates among injured animals might also be explained by debilitating physiological consequences of injury ([46, 48] but see [49]). However, consistent with our earlier results [5], we found no evidence that injured squid had shorter flight distances or flight durations after encounters were aborted by fish, which would be expected if the experimental injury were debilitating. Loss of maneuverability due to arm injury is also unlikely, as only the injured squid lacking sensitization were less effective at averting predatory escalation from attack to capture, the stage when rapid changes in direction would be most critical.

Although we observed no deficits in swimming ability, squid with injuries, whether or not they were sensitized, had lower survival rates compared with uninjured squid. This confirms that even minor injuries carry significant costs [50], but it is not yet clear which consequences of the arm injury were
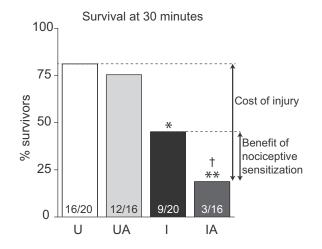
Figure 3. Injured Squid Lacking Nociceptive Sensitization Had the Lowest Odds of Survival

At the conclusion of a 30 min trial with free interaction of squid and fish, squid in the I and IA groups had lower overall survival than in the U group, and IA group squid were most likely to be killed. The difference in survival between the U and the IA group can be considered the cost of being injured, while the difference in survival percentage between the IA and I groups (p = 0.05) reveals the benefit that nociceptive sensitization provides to injured animals. Odds ratios, *p $\leq$ 0.05, **p < 0.01. U, uninjured; UA, uninjured with anesthetic treatment; I, injured; IA, injured with anesthetic treatment.

responsible for this increased risk. The differing survival odds among squid with and without injuries or sensitization resulted from changes in behavior of both the predator and prey. In squid, sensitization produced changes in antipredator responses that resulted in increased alert distances and flight initiation distances and increased reliance on escalated defensive behaviors such as inking and jetting at earlier stages of encounters (Figure 1) [8]. Theoretical and empirical studies have shown that flight initiation distance should be longer when prey perceive risks to be greater [16], which is consistent with our observations. However, these same studies also predict that flight initiation distance should be longer when initial fitness (survival probability at the start of an encounter) is higher. This prediction differs from our finding that injured squid had longer flight initiation distances despite the demonstrated fitness cost of injury. Therefore, in some species, early flight and related effects of nociceptive sensitization may occur instead of or in opposition to other responses of prey animals to substandard conditions such as previous injury.

Fish predators also behaved differently in encounters with injured squid, regardless of prey sensitization. Fish targeting injured prey had longer start distances and initiated pursuits from greater distances compared with controls, suggesting that they may invest more effort in encounters with prey perceived to be more vulnerable. Encounters with injured squid were also more likely to escalate to attack compared with encounters with control squid. Previous studies have shown that predators are adept at detecting and targeting prey animals in substandard condition [15, 44, 51]; an interesting question is whether fish targeted injured squid because they detected signs of injury directly via visual or olfactory cues, or whether injured squid made themselves more conspicuous at early encounter stages by abandoning crypsis sooner [48]. Although video analysis of the injured squid did not reveal deficits in swimming speed or maneuverability, it

is likely that predators that have evolved to select the most vulnerable prey are capable of identifying weaknesses that are not apparent to human observers.

The necessarily artificial conditions of our experimental encounters, particularly the relatively small size of our experimental arena, may have produced dynamics that are uncommon in the wild. Nonetheless, these experimental conditions of high, unremitting predation risk, combined with an intervention that prevented the development of injury-induced hypervigilance, were sufficient to demonstrate opposing effects of injury and nociceptive sensitization and thereby to reveal an adaptive function for the sensitization. Moreover, our findings strongly support the possibility that mitigation of heightened predation risk after sublethal injury has been an important selection pressure shaping the evolution of persistent neural hyperexcitability mechanisms underlying injury-related states of behavioral hypersensitivity.

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Supplemental Experimental Procedures

Animals

Male and female adult squid (*Doryteuthis [Loligo] pealei),* mantle length 14-22

cm), were collected from local waters around Woods Hole, MA and maintained in

groups of 5-10 in holding tanks containing ambient-temperature (19-21°C), flow-

through seawater. Squid were fed once daily on small fish, *Fundulus* spp. Only

animals with no evidence of previous injury were used.

Four black sea bass (*Centropristis striata*, total length 36 - 48cm) were used as

squid predators. Fish were caught by trawl net and acclimated to captivity for at

least 1 month prior to being used in experiments. Fish were rotated out of the

experimental tank and replaced with naïve fish as their experience with the trial

procedure increased. Fish were fed on alternate days on chopped squid,

supplemented with live squid captured during predation trials.

Ethical Note

In the US invertebrate animals are not covered under IACUC regulations, thus no

approval for procedures on squid was required for this study. Manipulations of

live squid followed the guidelines of the Animal Behaviour Society for

predator/prey studies 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 predatory interactions in wild marine animals is extremely difficult. Squid were anesthetized whenever possible during surgical manipulations, except when anesthesia conflicted with the aims of the experiment. Animals were monitored daily and squid with any evidence of compromised health were euthanized. All squid that were not consumed at the conclusion of the 30-minute trial were euthanized immediately by immersion in isotonic $MgCl_2$ followed by decapitation and decerebration. 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 tank enabled effective squid camouflage (reducing escape swimming), and trials were kept short to reduce overall stress. All procedures on vertebrates (capture and housing of black seabass) were approved by the MBL IACUC committee, and fish were released into local waters at the study's conclusion.

Procedure

Treatment groups: Squid received one of four treatments 6 hours prior to interaction with fish.

1. Uninjured (U): Squid were restrained briefly in a net.
2. Injured (I): Squid were restrained as above, and the tip (5-10mm) of one of the third arms (see Fig 1) was removed using a sterile scalpel, approximating common natural injuries to the arm tips that occur during intraspecific aggression and hunting. This injury model produces robust, long-lasting behavioral sensitization [S1].

3. Injured while treated with anesthetic (IA): Squid were immersed in 1% ethanol in seawater, which produces light general anesthesia for a few minutes, [S2], 2 minutes before injury. Approximately 1 minute before injury, local anesthesia was produced by injection of 0.5-1 ml isotonic $MgCl_2$ into muscle and skin in the middle of the treated arm. Arms were ligated prior to cut to prevent excessive bleeding. Focal injection of isotonic $MgCl_2$ blocks the development of local and remote nociceptor sensitization in squid [S3, see also S4]. In contrast to the effect on chromatophores at the arm injection site, the lack of relaxed chromatophores at the base of the injected arm and head demonstrated that the injected solution remained at the injected site, as previously observed with injections into the fin [S3]

4. Uninjured and treated with anesthetic (UA): Squid received the same anesthetic combination but no arm injury.

Predator/Prey interactions: Interactions were staged in a 360 cm diameter tank filled to 90 cm deep with ambient (~21 degrees C), filtered SW. The bottom of the tank was covered in a 5 cm layer of sand and small pebbles to facilitate effective squid camouflage. In the center of the tank an opaque, 90 cm diameter divider ring provided a temporary holding area for squid, while four predatory fish were free to swim around the outside of the divider. One hour prior to the interaction, four squid were placed in the central ring. Beginning 10 minutes before starting the trial, the tank was lit from above with three halogen lamps, and video

cameras were positioned at two windows in the tank walls, providing triangulated

underwater recordings of all interactions. To start the trial, the divider was lifted

by a pulley system operated by an experimenter out of view of the animals, and

squid and fish were allowed to interact. After 30 minutes any remaining squid

were netted from the tank and euthanized by immediate immersion in isotonic

$MgCl_2$.

Trials were run on alternate days. Fish behavior changed over the course

of multiple trials as they learned to anticipate the release of squid. Trials of squid

with different experimental treatments were counterbalanced each week, and fish

were changed three times over the 3-month experiment run to reduce the effect

of predator experience.

Data analysis

We synchronized recordings from each camera using Final Cut Pro (Apple

Computer). Behaviors of fish and squid were analyzed from both angles

simultaneously. Measurements of distances between predator and prey at

various encounter stages were estimated based on measured distances of

landmarks placed on the tank walls and base, and expressed as squid body

lengths. Encounters were divided into five stages as shown in Figure 1. Most

trials contained multiple encounters that were aborted prior to the attack or

capture stages. We tracked individual squid across time whenever possible, but

in some cases it was not clear which squid were the focus of the fish. Previous

studies using a similar experimental set-up have shown that repeated encounters

within minutes do not have an appreciable effect on either predator or prey behavior [8], thus we did not compute within-animal measures and considered encounters independent for analysis purposes.

We recorded behaviors of squid at each of the predation stages and measured the distance between squid and fish for the first response made by squid (the measure of alert distance, [S5, S6]) and for each new behavior observed. Squid defensive behaviors were ranked based on escalation following previous studies [S1, S7–9], and scored as described in Figure 1.

Kruskall Wallis tests were applied to ranked variables, and factorial ANOVA was followed by single factor ANOVA for measurement variables identified as significant in the factorial analysis. Categorical variables were compared with Fisher's exact tests and risks of predation were calculated with odds ratios. Because sample sizes were intentionally small, we protected weak effects by minimizing the number of planned comparisons. Post-hoc tests were two-tailed and the critical alpha was set at 0.05, with one exception. On the basis of our previous demonstration of injury-induced behavioral sensitization [S1] combined with our finding that injection of isotonic $MgCl_2$ blocks the development of neural sensitization [S3] we made the *a priori* hypothesis that survival in the IA group would be lower than survival in the I group, and this hypothesis was tested with one-tailed odds ratios. Statistical analyses were performed using Prism 6.0 and SAS 9.1.

Two observers (RC, KD) analysed video data, with ~40% overlap. Inter-observer reliability was computed for measurement variables and was considered acceptable (76% agreement with 2 body lengths of tolerance).

Definitions of squid behavior (Fig 1) and descriptions of measurements (Fig 2)

Primary defense: Crypsis is a common form of visual camouflage that hinders detection or recognition of prey by predators, thus decreasing prey encounters with predators. General background resemblance (aka background matching) is often used by squids on dark uniform or mottled backgrounds, or in the water column. On some light benthic backgrounds and those with larger objects, squid produce dark transverse bands that are characteristic of disruptive coloration, which presumably retards recognition by breaking up the longitudinal shape of the squid.

Secondary defense: When squid perceive that they have been detected by a predator, they rapidly transition to brief deimatic displays and then a series of protean behaviors. Collectively, these sequences of behavior confuse the predator, make it hesitate in its initial attack sequence, and confound predictability via erratic escape behaviors.

Diematic behavior: These are defined as highly conspicuous visual displays and postures that intimidate or bluff the predator, causing the predator to hesitate or abandon its attack. In squid, this includes rapid darkening of the entire body followed often by equally rapid (i.e. <1 sec) blanching of the entire body.

Protean behavior: These are sequences of behavior that are sufficiently unsystematic in appearance to prevent a predator from predicting in detail the position or actions of the prey. In squid, these entail erratic, unpredictable body pattern changes, inking and jetting.

Start distance: The distance from the fish to the squid (expressed in squid body lengths) when the fish made its first orientating movement toward the squid.

Alert distance: The distance between squid and fish when squid first switched from primary to secondary defense.

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