RESEARCH NOTE

A noninvasive method to remove kleptocnidae for testing their role in defence

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Aeolid nudibranchs are delicate, shell-less marine snails that are often well defended against predators, and many authors attribute this to the nematocysts that aeolids sequester from their cnidarian prey (kleptocnidae). However, we still lack direct experimental evidence for the defensive efficacy of kleptocnidae, even after over 100 years of study (Edmunds, 1966, 2009; Harris, 1973; Thompson, 1976; Todd, 1981). Although many aeolids also possess chemical defensive glands (Edmunds, 1966), their potential contribution to defence is typically ignored (Cimino & Ghiselin, 2001). Experimental designs have failed to test separately the relative defensive roles of secondary chemicals *vs* kleptocnidae, leading to both historical and recent debates (Edmunds, 2009; Greenwood, 2009; Marin, 2009; Penney, 2009). Tests directly comparing the palatability of unmodified aeolids with individuals of the same species lacking kleptocnidae would provide clarity (Edmunds, 2009), but such studies have proved difficult because the cerata that house the kleptocnidae also bear the chemical defensive glands (Edmunds, 1966, 2009; Greenwood, 1988, 2009; Hand, 1994–1996). Therefore, ablative experiments cannot produce nudibranchs lacking kleptocnidae, but with unaltered chemical defence.

To circumvent this problem, we adapted a noninvasive method used on cnidarians to strip nudibranchs of their kleptocnidae, while keeping cerata attached and intact, and confirmed that such treatment did not noticeably alter behaviour or survival of the animal. *Flabellina verrucosa* (M. Sars, 1829) were caught by hand using SCUBA near Appledore Island in the Gulf of Maine, USA (42°59.24″N, 70°37.53″W) and maintained at Shoals Marine Laboratory in tanks with flowing natural seawater in communal plastic containers (15 × 15 × 8 cm) with mesh sides. Prey hydroids (*Tubularia* species) were provided *ad libitum*. Cnidarians have been shown to fire nematocysts upon treatment with 3.5% KCl – roughly the same osmotic potential as seawater (Ruch & Cook, 1984). To test whether such treatment would led to ejection of kleptocnidae and not other material, several cerata were removed from each of three nudibranchs using forceps, mounted on a slide in seawater under a coverslip and examined at 100–400× on an Olympus BX-60 microscope and photographed using an Olympus DP-71 camera. After cerata were confirmed to be whole, 3.5% KCl solution was drawn under the coverslip by capillary action, leading to ejection of kleptocnidae (Fig. 1). Whole nudibranchs dipped for 15 s in 3.5% KCl ejected a cloud of nematocysts ('stripped') while control nudibranchs dipped in seawater did not ($n = 5$ for each group). Less immersion time resulted in incomplete ejection, while longer immersion damaged the animals. Observations of several cerata using the methods above but a stronger (5%) KCl solution confirmed stripped nudibranchs (but not control nudibranchs) were free of nematocysts. All individuals behaved normally within several minutes of return to seawater and survived for at least 1 week after treatment. To monitor for reappearance of nematocysts in nudibranchs that had been stripped, cerata were removed from individuals every 4 h for 16 h, then every day for a week; no functional nematocysts were seen in their cerata again for 4 days, within the normal time for this species, 4–5 days (Day & Harris, 1978). KCl has been used to elicit natural behaviour for neurobiological experiments (Lawrence & Watson, 2002). These observations suggest that nudibranchs were stripped of their kleptocnidae by KCl treatment but were otherwise unaltered, and we are confident that the effects of brief immersion in KCl are fully reversible, as are those of $MgCl_2$ commonly used as an invertebrate anaesthetic.

As a test case, we assayed the relative roles of kleptocnidae and secondary chemicals in defence of *Flabellina* against cunner (*Tautogolabrus adspersus*), a fish known to consume some nudibranchs (Harris, 1987). Cunner of over 100 mm total length were caught using baited hand nets, traps or trawls. Fish were housed in acrylic tanks (37 × 22 × 26 cm) with aeration and a standardized refuge structure, and fed a 1-cm^3 mussel piece on a half mussel shell every other day to produce predators that were hungry, but not starving, for bioassays. For all feeding experiments, cunner were starved for 1 day prior to experiments, randomly assigned to treatments and offered food in no-choice assays. During each assay, cunner were kept in their tank and offered a food type on a half mussel shell; their reactions to food were scored and videotaped for 15 min. We recorded the number of times each item was sampled (taken into mouth, crushed with pharyngeal teeth and expelled) and whether the fish eventually consumed or rejected the food item. After the assay, fish were offered a 1-cm^3 piece of mussel to test for hunger; any fish rejecting this piece were excluded from data analysis as being insufficiently motivated to feed.

In our first set of assays, the cunner sampled both unmodified *Flabellina* and control (mussel) prey, but significantly fewer *Flabellina* were consumed than mussels (Table 1A). To test the effect of kleptocnidae on palatability, we offered cunner either a 'stripped' nudibranch or a control nudibranch. On the morning of assays, nudibranchs were taken off food and treated. Stripped nudibranchs had their nematocysts removed with a 3.5% KCl solution ($n = 5$), control nudibranchs ($n = 3$) were dipped in seawater to simulate handling. All nudibranchs were attacked and sampled, and stripped nudibranchs were typically sampled 1–2 more times than control nudibranchs (Fig. 2; Mann–Whitney U-test, $U_{3,5} = 0.0$, $P = 0.031$). No nudibranchs were actually consumed in either treatment (Table 1B), indicating that nudibranchs lacking kleptocnidae have other defences.

To test for potential chemical defence, we offered cunner artificial food treated with either *Flabellina* extract or a control

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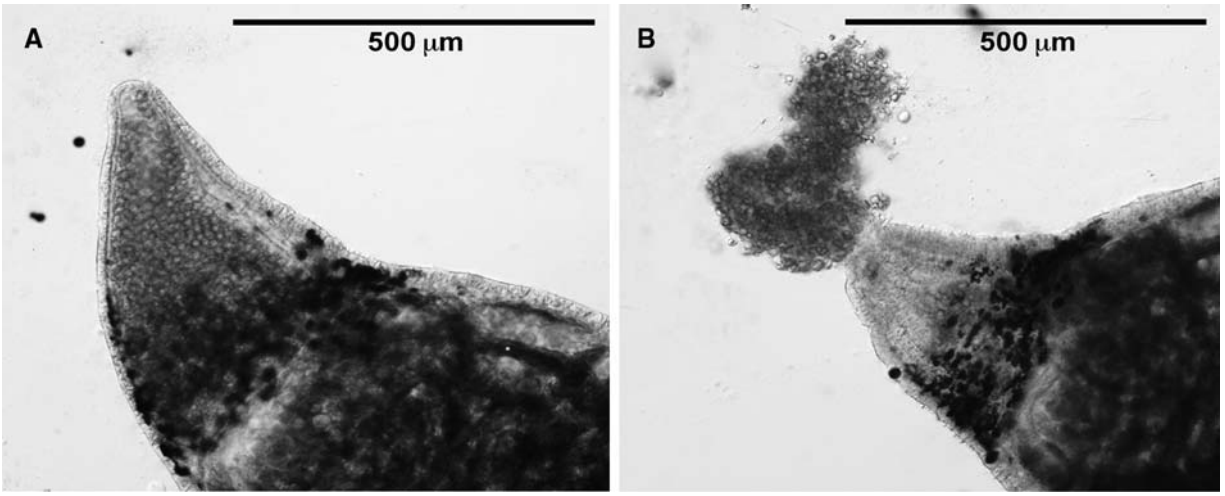

Figure 1. Ceras from *Flabellina verrucosa* before and after exposure to 3.5% KCl solution. **A.** Ceras before treatment. **B.** Photomontage of the same ceras immediately after treatment with KCl.

Table 1. Consumption of prey or food by cunner in bioassays.

	Treatment		Control		*P*
	Description	Consumed (*n*)	Description	Consumed (*n*)	
A	Unaltered *Flabellina*	1 (5)	Mussel mantle	5 (5)	0.024
B	'Stripped' *Flabellina*	0 (5)	Unaltered *Flabellina*	0 (3)	1.000
C	Artificial food with *Flabellina* extract	1 (5)	Artificial food with Acetone	5 (5)	0.024

n, sample size; *P*, exact probability using Fisher's exact test (one-tailed).

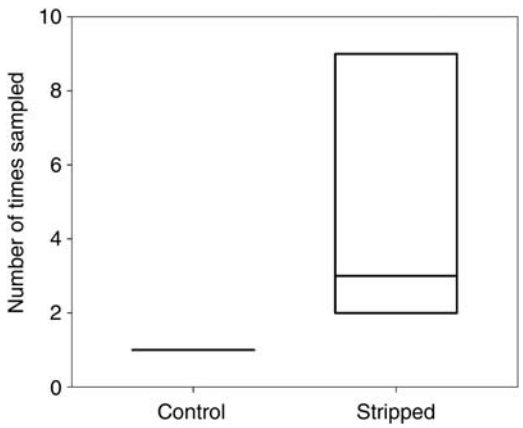

Figure 2. Number of samplings by cunner of *Flabellina verrucosa* with and without nematocysts. Boxes represent 25% and 75% confidence intervals, midlines represent median values for each treatment.

with solvent alone. For extracts, 19 *Flabellina* (4.96 g) were thrice extracted in three times volume acetone for 1 day each and the total extract volume reduced using a rotary evaporator at room temperature to the minimal volume in which all compounds still dissolved (*c.* 7 ml). Nematocyst venoms are proteinaceous (Hessinger, 1988) and should be denatured in acetone, and nematocyst venom seems only to be effective if injected (Bullard & Hay, 2002). Therefore, even if venom was present in the acetone extract, it should not have contributed to any deterrent effect. Artificial foods were made by adding 1 g nudibranch equivalent of extract per 1 g of food (control: equivalent volume of acetone) to a standard artificial food recipe (Hay *et al.*, 1998) of pureed cuttlefish mantle and alginic acid. The solvent was allowed to evaporate, then food was solidified in 0.25 M/l $CaCl_2$ solution and cut into 0.8 × 0.8 cm pieces, roughly the size of an average *Flabellina*. All cunner ate the control food and all sampled the treated food. However, only one cunner consumed the treated food, suggesting that this nudibranch species possesses an effective chemical deterrent (Table 1C).

Combined, these results indicate that *F. verrucosa* uses both nematocysts and secondary chemicals to deter cunner. We were unable to ascertain whether nudibranchs possessing kleptocnidae but lacking chemicals would still be rejected, as this would require more information about what chemicals are present and how they are obtained. It is surprising that kleptocnidae do not seem crucial to defence in this case, as this nudibranch selectively sequesters prey nematocysts and modifies which types it keeps in response to predator presence (Day & Harris, 1978; Frick, 2003). One possible explanation is that other nematocyst types might be more effective against cunner; *F. verrucosa* increases microbasic mastigophores retention in response to cunner and this nematocyst type is not found in *Tubularia* (Frick, 2003). An alternate possibility is that, in some cases, kleptocnidae only supplement chemical defence by speeding the process of rejection.

To ascertain more fully the defensive role of kleptocnidae, these experiments should be repeated using other nematocyst sources and against other predators for a variety of aeolid species. Aeolids vary not only in their prey sources, but also in their tendency to release nematocysts when provoked (Edmunds, 1966; Todd, 1981). The per cent of kleptocnidae exploding on ejection can vary by type even when coming from the same nudibranch individual; some nematocyst types may function better as kleptocnidae than others (Edmunds,

1966). Aeolids are consumed by a number of predators in field and laboratory (Greenwood, 1988; Harris, 1973, 1987; Todd, 1981) and different predators may respond differently to the same defences (Edmunds, 2009; Greenwood, 1988). Some aeolids possess kleptocnidae capable of injuring humans, while others possess no functional nematocysts (Harris, 1973; Todd, 1981). Aeolid defence strategies may be as diverse as those of dorids (Edmunds, 2009) and future work should seek to explore this diversity by testing separately for effects of kleptocnidae, secondary chemicals and other defences.

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