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Incorporated nematocysts in *Aeolidiella stephanieae* (Gastropoda, Opisthobranchia, Aeolidoidea) mature by acidification shown by the pH sensitive fluorescing alkaloid Ageladine A

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ABSTRACT

The sequestration of nematocysts (a special group of cnidocysts) from cnidarian prey with subsequent use in defence is described for few metazoan phyla. Members of the taxon Aeolidoidea (Nudibranchia, Gastropoda) are well-known for this. Questions regarding the reasons some nematocysts do not discharge when the gastropod feeds and how these same nematocysts can be transported along the digestive tract into specialized morphological structures called cnidosacs, remain unanswered. Within the cnidosac, nematocysts are incorporated in cells and finally be used for defence against predators.
The most plausible explanation for this phenomenon suggests there are immature and therefore non-functional nematocysts in the food. A recent study by Berking and Herrmann (2005) on cnidarians suggested that the nematocysts mature by acidification via proton transfer into the nematocyst capsule. According to this hypothesis only immature nematocysts are transported into the cnidosac where they are then made functional through an accumulation of protons. In this study we present a fluorescence staining method that tests the hypothesis by Berking and Herrmann (2005) and detects changes in the pH values of incorporated nematocysts, interpreted as changes in maturation stages. This marker, the fluorescent dye Ageladine A, stains nematocyst capsules according to their pH values. With Ageladine A we were able to show that kleptocnides indeed change their pH value after incorporation into the aeolidoidean cnidosac.
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1. Introduction

Acquisition and storage of nematocysts from cnidarian prey is known from several phyla, including Ctenophora, Plathelminthes and a few gastropod groups like Aeolidoidea (see reviews of Greenwood, 1988, 2009; Wägele, 2004; Putz et al., 2010), the nudibranch *Hancockia* and the genus *Embletonia* with unknown affiliation (Martin et al., 2008, 2010). While little is known from the first two groups, literature is abundant concerning the investigation of nematocyst incorporation in the Aeolidoidea. Several hypotheses on function and mechanisms of these so-called kleptocnides have been formulated with few experimental studies underlying these assumptions. One of these questions asked why some nematocysts do not discharge during feeding and how they remain undischarged as they are transported to the cnidosac, a specialised structure, typical in aeolids. Here they are incorporated into cells (phagosomes) lying at the base of the cnidosac and can finally be used for defence against predators (Martin, 2003; Greenwood et al., 2004; Wägele and Klussmann-Kolb, 2005; Martin et al., 2008; see review of Greenwood,

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2009). Naville (1926) and later Greenwood and Mariscal (1984b) suspected that only morphologically immature nematocysts are stored in the cnidosacs and somehow mature in the storage cells of the cnidosac. Some authors (Martin, 2003; Schlesinger et al., 2009) stated that intact and mature nematocysts can be found in the digestive tract and even in the faeces. Others (Mauch and Elliot, 1997; Greenwood et al., 2004) investigated the possibility that mucus inhibits nematocyst discharge during the feeding process, implying that mature nematocysts can also be incorporated.

Nematocyst maturity in nudibranchs was investigated by Greenwood and Mariscal (1984a, 1984b), by analysing the ultrastructure of the nematocysts in the cnidosac of *Spurilla neapolitana* (Delle Chiaje, 1841). They considered capsules with a higher electron dense thread and a more granular appearance to be immature, a feature that is difficult to distinguish using normal light microscopy.

In former times, the maturation of the highly complex organelles formed by the Golgi apparatus (Fautin, 2009) was ascribed to the formation of γ-polyglutamate and a finally high osmotic pressure that allowed discharge of the nematocysts in cnidarians (Holstein, 1995; Anderson and Bouchard, 2009; Özbek et al., 2009). Only recently Berking and Herrmann (2005) described an alternative mechanism for the build-up of pressure. According to these authors, high amounts of protons are imported into the capsule of the nematocyte binding to the carboxyl groups of the poly-γ-glutaminacids and forming hydrogen bonds. Hence a mature nematocyst is characterized by a high proton concentration. This acidification was indirectly shown by Berking and Herrmann (2005) due to lack of adequate vital staining methods at that time. Ageladine A, a secondary metabolite of marine *Agelas* sponges (Fujita et al., 2003), is a highly membrane permeable and pH sensitive fluorescence marker (Bickmeyer et al., 2008). When protonated, the Ageladine molecule can be excited with UV light, and its fluorescent intensity depends on the charge of the molecule (Bickmeyer et al., 2010). The intensity of the fluorescence reaches its maximum at pH 3–4 and its minimum at pH 9 with the greatest variation between pH 6 and 7. Here we show for the first time in vivo that the nematocysts in cnidarians, especially in the acontia and the tentacles, indeed exhibit low pH values and that acidification within the cnidosacs of aeolidoidean gastropods might be connected with maturation of the nematocysts.

2. Material and methods

2.1. Culture and breeding of Aiptasia spec. and Aeolidiella stephanieae

Aiptasia spec. was kept and bred in larger aquaria in aerated artificial seawater at room temperature (22.0 ± 1.0 °C). The water was partly changed every week and anemones were fed every second to third day with *Artemia salina*.

Adult *A. stephanieae* Valdés, 2005 (Fig. 1A) were kept in bowls with 200 ml non-aerated artificial seawater at room temperature (22.0 ± 1.0 °C). The water was changed and gastropods were fed every second day with at least one tentacle of *Aiptasia* spec. Freshly laid egg masses were separated in petri dishes with artificial seawater, which was changed every second day. Four days after oviposition, tentacles of *Aiptasia* spec. were added to the egg masses to induce hatching and metamorphosis. These breeding methods were adopted from the protocol by Carroll and Kempf (1990).

2.2. Experiments

Whole anemones (size of scapus less than 1 cm) as well as tentacles from larger anemones were stained with Ageladine A in seawater (1:1000 from a stock solution of 10 mM in MeOH) for 60–90 min in the dark, to document nematocysts within *Aiptasia* spec. Because of their high mobility, the anemones were anaesthetized in 7% $MgCl_2$ solution for 10 min to ensure proper analysis during the experiments.

To track nematocysts in the digestive system during the feeding process, a stained anemone was offered to an unstained gastropod. This experiment was performed twice.

To state the initial situation in a gastropod kept under natural conditions, cerata of adult *A. stephanieae* were investigated after staining with the fluorescent dye Ageladine A.

To analyse the maturation process in *A. stephanieae*, several individuals were starved for four days to ensure that each cnidosac contained only mature nematocysts. This time period was stated by Day and Harris (1978) as the time required for cnidosacs to refill with functional nematocysts. Starved individuals were then immersed for 5–7 s in 3.5% KCl. This treatment caused the gastropods to eject all kleptocnides from their cnidosac without autotomizing their cerata (Penney et al., 2010). Several minutes after returning to seawater, the animals behaved normally. 60 min after the KCl treatment, the animals were fed with tentacles of *Aiptasia* spec. The exact time each animal started feeding and ingesting new nematocysts was documented, and analyses of the maturation process of incorporated nematocysts were performed 7, 24, 48, 72 and 96 h respectively after feeding. An additional animal was investigated after 5 days starvation.

To document nematocysts maturity states, intact living *A. stephanieae* individuals were stained with Ageladine A and seawater (1:1000 from a stock solution of 10 mM in MeOH) for 60–90 min in the dark. After the staining process, each gastropod was anaesthetized in 7% $MgCl_2$ solution for 10 min. This ensured that no kleptocnides were ejected during the preparation of four to five cerata positioned in the anterior body. Single cerata were mounted in seawater on a microscope slide and gently covered by a coverslip, for further analyses under the microscope. Each animal was only used in one interval.

2.3. Analysis and statistics

The autofluorescence of cnidosacs and adjacent tissue was tested separately in unstained animals under the same excitation wavelengths as in stained samples (see below),

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