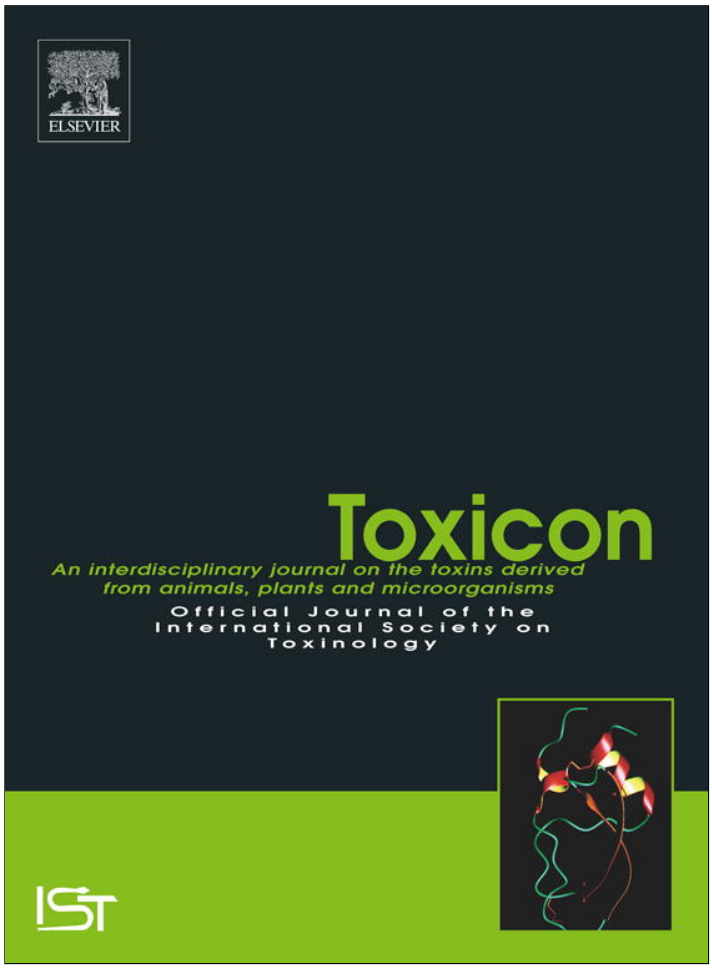

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Incorporated nematocysts in *Aeolidiella stephanieae* (Gastropoda, Opisthobranchia, Aeolidoidea) mature by acidification shown by the pH sensitive fluorescing alkaloid Ageladine A

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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.

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),

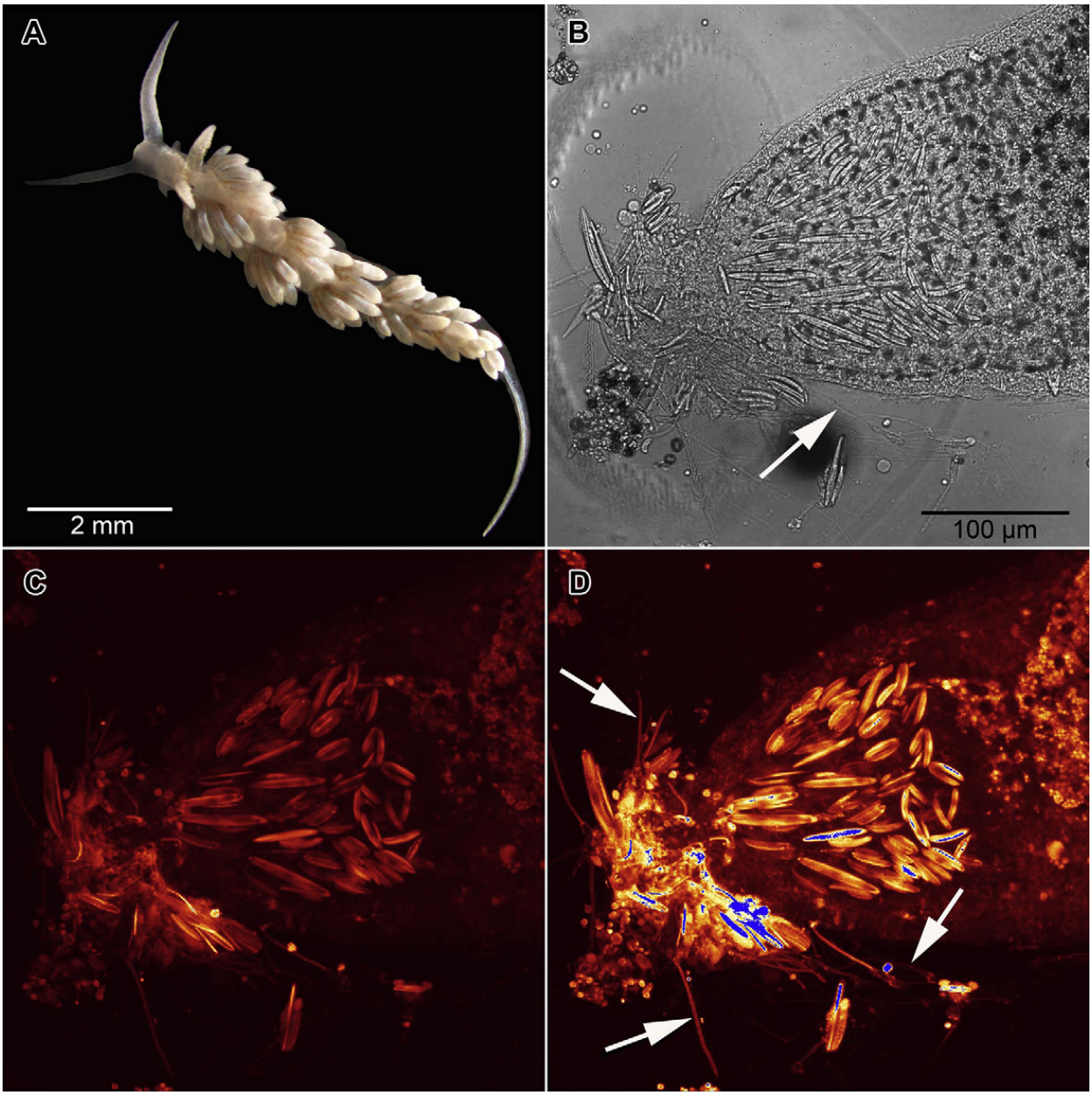

Fig. 1. *Aeolidiella stephanieae* and staining of kleptocnides in cnidosacs with Ageladine A: A, adult specimen of *Aeolidiella stephanieae*. B, transmission image of a cnidosac of *Aeolidiella stephanieae* filled with kleptocnides. Note the extruding and discharged kleptocnides on the left side (arrow). C, same cnidosac as in B stained with Ageladine A and excited optically by UV laser. Fluorescence of the wavelength between 420 and 500 nm was measured with a photomultiplier setting (PMT1) of 450 V. D, same cnidosac as in B and C with PMT1 = 500 V. Bluish colour indicates values outside of range (blue colour for fluorescence intensity higher than 255 i.u.). Note the higher intensities in the same nematocysts and threads (arrows) as depicted in C. The photomultiplier settings were increased to 500 V to make the discharged kleptocnides visible. B–D of same magnification.

without detectable fluorescence. The fluorescence of Ageladine A in the nematocysts of the food organism *Aiptasia* spec. and the kleptocnides of *A. stephanieae* were monitored by a confocal laser scanning microscope Leica TCS SP2 equipped with a UV laser (coherent). Ageladine A was optically excited using UV light of the wavelength 365 nm. The wavelength between 420 and 500 nm of the emitted light was filtered out and made visible on the screen using the "glow over/under" function of the software.

For every mounted cnidosac, as well as for whole mounts of anemones or gastropods, a z-stack of ten optical sections was taken with identical settings (photomultiplier settings PMT1 = 450 V or PMT1 = 500 V, Pinhole 2, LiA = 2, solution 1024 × 1024 pixel). Sections were analysed individually or as maximum projection pictures.

Analyses of *Aiptasia* were mainly performed with photomultiplier settings of 450 V, whereas those of the gastropods were taken with PMT1 = 500 V. These latter accommodations were chosen after preliminary analyses, since lower voltage resulted in low visibility of the freshly-incorporated nematocysts fluorescence.

The fluorescence of the *A. stephanieae* kleptocnides at different times after incorporation was measured with the "region-of-interest" function of the CLSM software (LCS

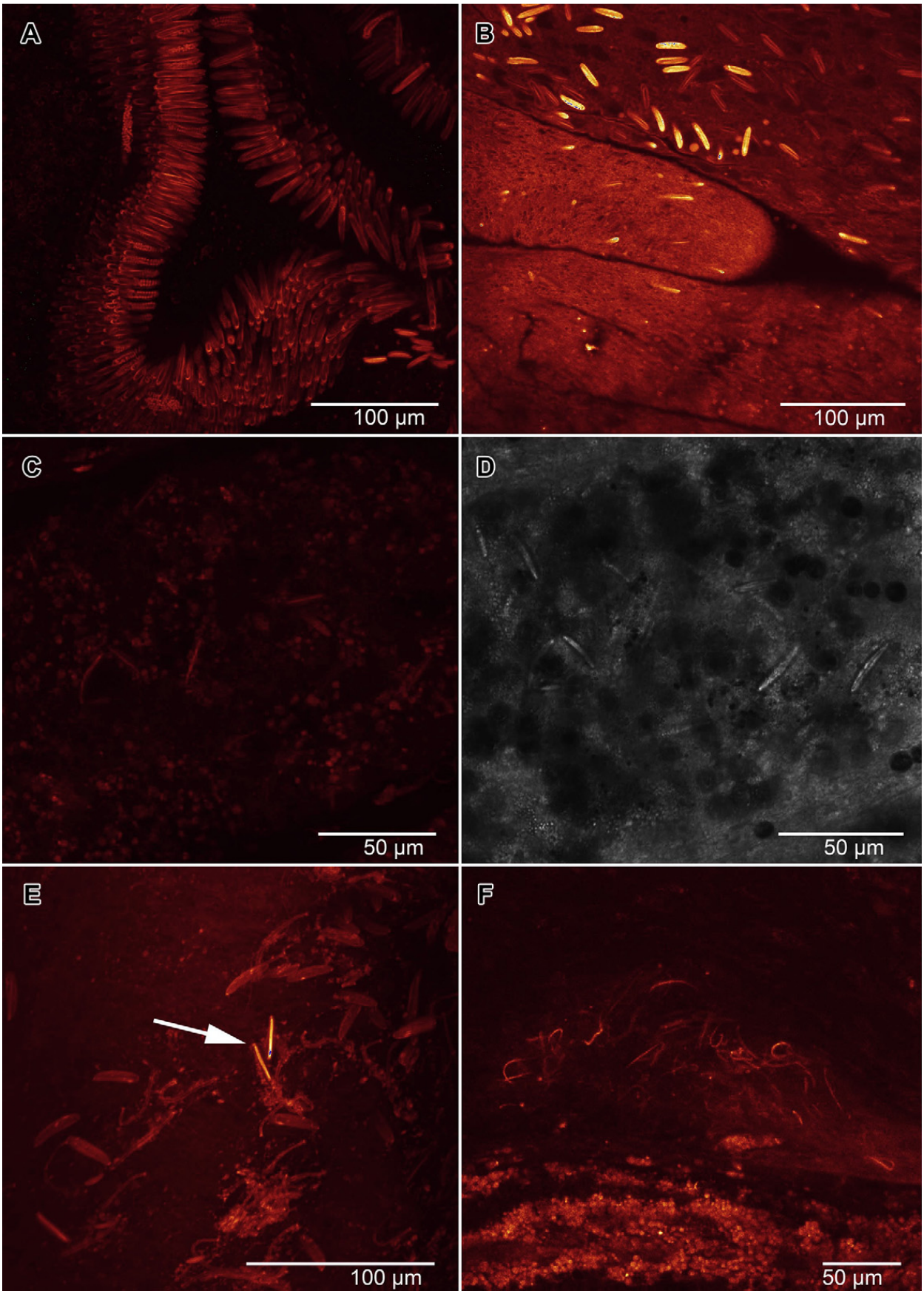

Fig. 2. Nematocysts in different regions of *Aiptasia* spec. and *Aeolidiella stephanieae* stained with Ageladine A: A, acontia of *Aiptasia* spec. Note that fluorescence intensity is similar in all nematocysts; PMT1 = 450 V. B, nematocysts in tentacles of *Aiptasia* spec. Note the differences in fluorescence intensities of the various nematocysts; PMT1 = 500 V. C, digestive tract of *Aeolidiella stephanieae* which fed on stained *Aiptasia* spec. The sequestered nematocysts show a low fluorescence,

Lite). The fluorescence intensity was given in imaginary units (i.u.) with values from 0 to 255. To detect immature kleptocnides having low fluorescence intensities high photomultiplier values (PMT1 = 500 V) were chosen. This caused mature kleptocnides to appear out of the intensity range (blue, the colour for overload of measuring range) with an intensity value of 255 i.u. or higher.

The highest fluorescence intensity of every single nematocyst within the ten sections of a cnidosac was determined (maximum absolute fluorescence of single nematocysts, fNc). In addition the background fluorescence (fBg) in every section was measured to finally calculate a relative value (fNc/fBg). This value was determined by dividing the maximum absolute fluorescence of a nematocyst by the fluorescence of the surrounding tissue of the same section (Fig. 3A). Additionally, the percentage of kleptocnides with a higher fluorescence than 255 i.u. was calculated for every time slot (Table 1).

Statistical analysis was performed with Microsoft Excel and SPSS 15 (IBM). Statistical tests on significance of the changes in fluorescence intensity values over time were performed with the non-parametric Mann Whitney U test (SPSS15).

3. Results

The autofluorescence of unstained nematocysts in *Aiptasia* spec. and in *Aeolidiella stephanieae* was very low and negligible. The nematocysts became slightly visible when excitation was amplified with settings of PMT1 = 900 V, almost twice as much as used in the Ageladine A staining experiments.

Preliminary staining experiments revealed that Ageladine A easily permeates the nematocysts of gastropods and their food (Fig. 1B–D, Fig. 2A, B). Undischarged nematocysts with high fluorescence intensity were present in high amounts within the *Aiptasia* epidermis at the tip of the tentacles and especially in the acontia (Fig. 2A, B). Lower amounts of fluorescing nematocysts were found along the tentacles and throughout the scapus. Nematocysts exhibiting hardly any fluorescence were found in lower numbers along the tentacles, and in higher numbers in the scapus.

Gastropod feeding was used to trigger the discharge of the anemone's nematocysts. Discharged nematocyst capsules, which could be found around the two animals (anemone and gastropod) in high numbers, lost their high fluorescence with time, although their threads continued glowing, especially when PMT1 = 500 V is chosen (Fig. 2E). This was also observed in discharged kleptocnides extruded from the cnidosac (Fig. 1D). In the digestive gland and stomach area of the freshly fed gastropod, non-fluorescing nematocysts outnumbered fluorescing ones by far. This became obvious when transmission pictures were compared to fluorescence pictures (Fig. 2C, D). Since the body with the overlying viscera in the gastropod was very thick and optic measurements were difficult, no statistic values could be obtained.

To investigate properties of incorporated nematocysts in the gastropod at various times, 47 cnidosacs with 1770 nematocysts in total were measured at five intervals (7, 24, 48, 72 and 96 h) after food uptake. For details and explicit numbers see Table 1. All intervals are also documented in Fig. 3(B–F) and Fig. 4. Maximum absolute fluorescence intensity values of nematocysts increased with high significance ($p = 0.000$) over time from about 129 i.u (mean value) after 7 h (Fig. 3B) to 230 i.u. after 48 and 72 h (Fig. 3D, E). After 96 h, the nematocysts fluorescense values decreased significantly (Table 1, Fig. 3F). The percentage of nematocysts with a fluorescence intensity of 255 i.u. or higher was greatest after 48 and 72 h (55% and 51%, respectively) and also decreased after 96 h (27%) (Fig. 4A). 7 h after feeding, no nematocysts with fluorescence intensities higher or equal to 255 i.u. were observed.

Similarly, the ratiometric values (Table 1, Fig. 4B) indicate a significant increase after 24 h after feeding, with an additional highly significant increase after 48 h. After 96 h, they decrease (with low significance $p \leq 0.05$) compared to the values after 72 h. The animal investigated after 5 days starvation as control also revealed kleptocnides, which did not show a high fluorescence (no ratiometric values taken here).

4. Discussion

Ageladine A is a fluorescent marker that allows in vivo staining of complete and living animals or tissues. Its advantage compared to other dyes indicating pH values, such as BCECF or Lysosensor, is its large pH range and its ability to penetrate cellular membranes quickly and easily, allowing the fast staining of entire animals. Bickmeyer (2012) demonstrated methods for the calculation of tissue pH values and showed its ability to highlight regions with low intracellular pH in cnidarians and plathelminthes. The present study presents a comparative analysis of pH changes by comparing fluorescence intensities without calibration procedures. This is the first study to apply this dye on living gastropods, taking advantage of its high penetration abilities. It clarifies a long-standing question regarding how aeolids process incorporated nematocysts rendering them capable for discharge and therefore usable for defence.

The tests on unstained *Aiptasia* spec. and *A. stephanieae* clearly indicated that no relevant autofluorescence occurred in nematocysts and cnidosacs. Analyses of both species prior to the specific experiments gave evidence that nematocysts in situ exhibit various fluorescence intensities after staining. The presence of exclusively high fluorescing nematocysts in acontia, which are the defensive structures

indicating a high pH value; PMT1 = 450 V. D, transmission image of C shows that digestive tract of *Aeolidiella stephanieae* contains several nematocysts which are not or only hardly visible in C. E, *Aiptasia* tentacle and part of scapus on which *Aeolidiella stephanieae* specifically has fed on a minute ago. Only two of the undischarged nematocysts (arrow) show a high fluorescence, whereas all other undischarged and discharged nematocysts show a low fluorescence; PMT1 = 450 V. F, gastric/oesophageal region in *Aeolidiella stephanieae* after feeding on stained *Aiptasia* spec. Note the high amount of discharged nematocysts and the still fluorescing threads which were only visible by applying a PMT1 of 550 V.

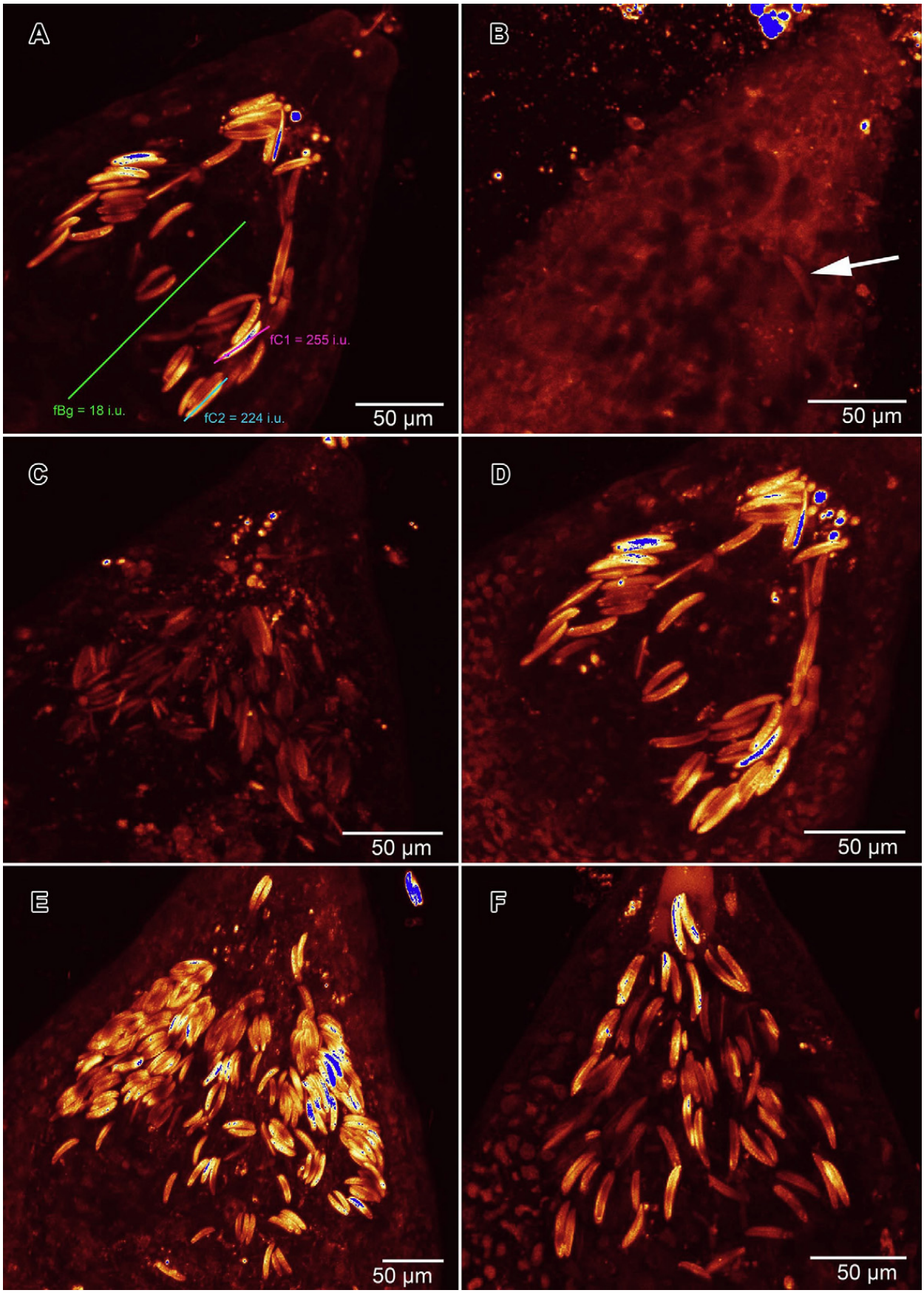

Fig. 3. Ageladine A stained nematocysts (kleptocnides) in *Aeolidiella stephanieae* in different intervals: A, one of ten sections to demonstrate measurement of ratiometric values: background fluorescence (fBg) was measured in every section to calculate a relative value (fNc/fBg, green bar). For every nematocyst the highest fluorescence intensity within the ten sections was determined. In this section here, the two nematocyst fC1 (violet bar) and fC2 (bluish bar) showed

Table 1
Fluorescence intensity values of nematocysts stained with Ageladine A at different time intervals after feeding. #spec = number of specimens measured, #Cs = number of cnidosacs measured, #Nc = number of nematocysts measured, fNc = absolute fluorescence intensity of nematocysts. Mean value ± Standard deviation, i.u. = imaginary units, fBg fluorescence of background.

Time after feeding [h]	#spec	#Cs	#Nc	fNc [i.u.]	fBg [i.u.]	Ratiometric (fNc/fBg)	fNc>255 i.u. [%]
7	3	8	21	128.62 ± 46.69	35.76 ± 12.84	3.76 ± 1.11	0.00
24	4	13	402	125.91 ± 66.50	17.90 ± 4.02	7.17 ± 3.80	2.99
48	3	8	530	229.97 ± 42.03	30.89 ± 7.09	7.81 ± 2.20	55.47
72	2	8	547	229.29 ± 40.07	29.77 ± 5.86	7.88 ± 1.63	51.01
96	3	10	270	188.00 ± 65.63	23.47 ± 7.12	8.37 ± 3.18	27.04

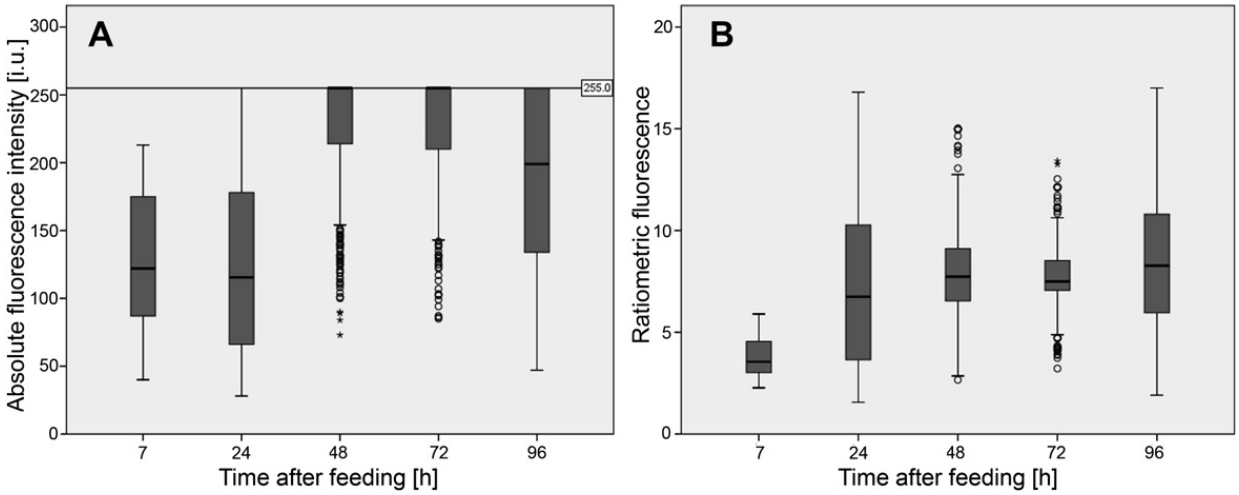

Fig. 4. Boxplots of measured fluorescence values: A, Boxplots of absolute fluorescences of single nematocysts (fNc); B, Boxplots of ratiometric fluorescence values (fNc/fBg).

of *Aiptasia*, indicate that nematocysts capable of discharge show a high fluorescence and therefore a low pH. According to Berking and Herrmann (2005), the maturation of nematocysts is induced by proton transport and accompanied decrease in pH. They observed a lower pH value in the surrounding fluid after explosion of mature nematocysts in eight different species of all four major cnidarian groups and indicated this as the indirect proof of an acidification in maturing nematocysts.

We consider the differing intensities in the observed nematocysts of the anemones and the gastropods as different stages of maturity. A high fluorescence intensity (high acidity), as seen in the acontian nematocysts, is interpreted as indication of being mature and capable of discharge. The lack of fluorescence in the discharged nematocysts (Fig. 1D, arrow to the right) indicates the loss of protons during the explosion process, hence the pH value of the empty nematocyst lumen is assumed to be similar to the surrounding tissue. Nevertheless, the threads still seem to show lower acidity for a while.

The number of discharged nematocysts around the area where the gastropod has fed (Fig. 2E), and within the gastropod's oesophageal area (Fig. 2F) clearly show that many mature nematocysts discharge during the feeding process. Undischarged nematocysts were found in high numbers in the digestive glandular areas, especially in the cerata. These results are supported by unpublished data of E. Tilic and H. Wägele on the aeolid *Flabellina ischitana*. They showed that discharged nematocysts can only be found in the anterior digestive tract, whereas the main bulk of intact nematocysts lay in the stomach and the digestive gland. This does not necessarily contradict former results of Martin (2003) and Schlesinger et al. (2009), who found intact nematocysts in the faeces of aeolids. They may have been unable to discharge yet or were prevented from discharge by other factors not yet known. Nevertheless, this study presents strong evidence showing that undischarged and hardly fluorescing nematocysts in the digestive tract (exhibiting a higher pH value) are immature and not yet ready for use in defence.

Interval analyses showed a continuous acidification in the kleptocnides incorporated in the cnidosacs. Although the number of cnidosacs investigated in the first time period (7 h after feeding) was similar to all others (8 versus 13, 8, 8 and 10 respectively), the number of kleptocnides that could be measured was low (only 21, versus 402, 530, 547 and 270

maximum absolute fluorescence, whereas in all other nine sections fluorescence in these two was lower. Measured fluorescence values are shown in Table 1. B, cnidosac 7 h after feeding. Fluorescence of the nematocyst (arrow) barely higher than fluorescence of the surrounding tissue. C, cnidosac 24 h after feeding. D, cnidosac 48 h after feeding. Few nematocysts appear out of range (blue colour for fluorescence intensity higher than 255 i.u.). E, cnidosac 72 h after feeding. F, cnidosac 96 h after feeding. A–F: PMT1 = 500 V; B–F: maximum projections.

respectively). This was certainly due to the low number of nematocysts that have been transported into the cnidosac. It was apparent that only nematocysts with low or nearly no fluorescence were incorporated and visible after few hours. Increase of fluorescence within the next 48–72 h clearly indicates an acidification process. Nevertheless, the fluorescence intensity variance of kleptocnides observed within a single cnidosac, as well as in the various cnidosacs from the same time period, indicates that either nematocysts had various maturation states when incorporated, or that the acidification process can vary to a certain extent. This variation is also reflected in the observed high standard deviation of measured nematocysts.

Notably the fluorescence in undischarged kleptocnides decreased between 72 and 96 h. Three explanations are outlined here but future investigations will highlight the more probable reasons. First, the proton concentration in the kleptocnides can not be held up for many days. This would imply that kleptocnides are rendered rather useless after a few days and new nematocysts have to be incorporated and matured. Published data on long term retention and maintenance of functional kleptocnides (Greenwood and Mariscal, 1984a; Greenwood et al., 1989; Greenwood, 2009) contradict this hypothesis. Second, Ageladine A is a dye with its highest intensity at around pH 3–4. A further decrease of the pH value hence could imply a subsequent decrease of the intensity. This has not been studied in detail yet. Members of some gastropod taxa are able to produce acids of pH values lower than 2 (Edmunds, 1968; Thompson, 1960, 1988). It seems likely that aeolids are also able to produce high amounts of protons. Therefore the dye's properties in tissues known to exhibit extreme low pH values needs to be tested. Third, according to Berking and Herrmann (2005), the free protons are bound onto the poly-γ-glutaminacids in the capsule matrix after transport into the capsule. This implies a lower number of free protons after 72 h that could bind onto the guanidine moiety of the Ageladine. In consequence a lower fluorescence intensity of the Ageladine A due to a reduced number of free protons is observed after 3 days. It has to be emphasized here that nematocysts in the acontia of *Aiptasia* showed a high fluorescence, and we assume that these are mature and capable of discharge. Nevertheless, some of the nematocysts in the same sample (Fig. 2A and further results) showed a higher intensity. This reflects the same situation we find in the cnidosacs with a high fluorescence after 2–3 days but a decrease after 4 days.

Due to the chosen photomultiplier value of 500 V in the experiments with *Aeolidiella*, many fluorescence values of the measured kleptocnides were out of the maximal range and exhibited fluorescence intensities higher than 255 i.u. at the later time intervals. To show a better resolution of the acidification in later maturation stages, a lower photomultiplier setting is necessary, as can be seen in the first experiments with *Aiptasia*. However, lower photomultiplier setting result in little or no visibility of kleptocnides in the earlier time intervals because of their low fluorescence due to a still rather high pH value. Irrespective of this drawback of chosen accommodations, we were able to show the rising fluorescence and therefore decrease of pH values of kleptocnides after incorporation into the cnidosac. The comparison with control gastropods investigated with higher photomultiplier settings also show, that kleptocnides in the cnidosac exhibit various intensities of fluorescence connected with various stages of maturity. This would explain why only some of the kleptocnides discharge during handling the gastropod and others do not (Fig. 1D).

5. Conclusions

The results of this study support the hypothesis suggested by Berking and Herrmann (2005) that, irrespective of further requirements, the discharge of nematocysts needs a high concentration of protons in the capsule. Furthermore, the results offer an explanation why many of the nematocysts do not discharge during sequestration by *A. stephanieae* and can therefore subsequently be incorporated in the cnidosacs. The sequestered nematocysts probably are not fully functional at the moment of gastropod feeding and therefore are not able to discharge even when they show the same morphology. Acidification in the cnidosac is at least one process to render them functional, so that they can be used by the gastropod for defensive purposes. This does not necessarily preclude that other factors help to avoid discharge during the feeding process of the gastropod, and it does not preclude that even mature nematocysts might pass through the digestive tract or even be incorporated in the cnidosac. Our results mainly show that acidification is a necessary process of nematocysts' and kleptocnides' maturation. The mechanism, how the capsules are triggered for discharge and whether there are further processes in maturation still have to be investigated.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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