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Fluorescence distribution pattern allows to distinguish two species of *Eugymnanthea* (Leptomedusae: Eirenidae)

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The auto-fluorescence patterns of the medusae observed under a fluorescent microscope with blue light excitation allows to distinguish two species of Eugymnanthea*, this even when they are still attached to the hydroid as small medusa buds despite the occurrence of a sex-dependant pattern in* E. japonica*. A total of four distribution patterns of green fluorescence, including non-fluorescence, could be found. Three of them are found in* E. japonica*, called 'subumbrellar fluorescence type' except for non-fluorescence, while another type is found in* E. inquilina*, called 'umbrellar margin fluorescence type'. During the short life of the medusa the latter type remained invariable for up to six days in* E. inquilina*, while the pattern observed for up to seven days in* E. japonica *changed sometimes, but it always remained distinguishable from the pattern found in* E. inquilina*. Therefore, the fluorescence pattern is a reliable taxonomic character. Fluorescence was not found in unfertilized eggs, planulae 2–8 days old, parthenogenetically produced larvae, or in the hydroids of the two species. The auto-fluorescent and possible bioluminescent tissues of these* Eugymnanthea *medusae could have some unknown biological significance.*

Keywords: fluorescence distribution patterns, *Eugymnanthea* species, various developmental stages

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INTRODUCTION

Eugymnanthea is a commensal hydroid associated mainly with *Mytilus galloprovincialis* and other bivalves inhabiting shallow waters. It occurs in the Mediterranean Sea (*E. inquilina*) and around the Japanese Sea (*E. japonica*) (Kubota, 1989, 1992, 2004; Piraino *et al.*, 1994; Rayyan *et al.*, 2002; Baba *et al.*, 2007). The life cycle of both species comprises a benthic hydroid and a planktonic medusa stage. Species of *Eugymnanthea* have a simplified medusa that is released with already formed gonads. They only spend a very short time in the plankton and they die after spawning. The two species of *Eugymnanthea*, which evolved as the most derived bivalve-inhabiting hydrozoans from an ancestral *Eirene* and/or *Eutima*-like progenitor, resemble each other both in the hydroid and medusa stages. The similarity is likely due to a parallel, progenetic evolution, which also explains some of the subtle morphological differences (Kubota, 2000). Individuals from the Mediterranean and Japan are very similar at any developmental stage, but by examining a large number of specimens of several populations, Kubota (1991, 2004) found some morphological differences of the medusae that permit to discriminate the two species. These are: presence of a manubrium and a smaller number of statoliths per statocyst (usually 1) in the Japanese form, versus the absence of a manubrium and several statoliths per statocyst (usually 2 or 3) in the Mediterranean form. Govindarajyan *et al.* (2005) demonstrated that they are two distinct species by using a cross-fertilization test, a mesogloeal adhesion and spreading test, and by 16S rDNA sequence comparisons. In the present study, we describe a new morphological character that allows both species to be distinguished, i.e. different distribution pattern of green auto-fluorescence.

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MATERIALS AND METHODS

A total of 77 medusae of both sexes of *Eugymnanthea inquilina* were examined, originating from 53 specimens of *Mytilus galloprovincialis* Lamarck from Taranto (39 specimens) and Lago Fusaro, the type locality (14 specimens), one specimen of *Mytilaster minimus* (Poli) from Taranto, and eight specimens of *Chylamys glabra* (Linnaeus) from Taranto. They were collected from October 1999 to January 2000 (Kubota, 2004). *Mytilus galloprovincialis* associated with hydroids were kept in the biological laboratory of Lecce (University of Salento) from mid-October 1999 to mid-March 2000 at 23°C and a 15hL:9hD photoperiod. They were kept in several thousand ml glass or plastic containers with natural seawater taken from rocky coasts near Porto Cesareo or from inside the port of Porto Cesareo and fed with newly hatched *Artemia* nauplii. The seawater (38–40 ppt) was changed nearly every day and almost all of the released mature medusae were examined before they spawned in early morning. A Zeiss Standard Axioplan microscope equipped with a halogen lamp (Hg 100) light and blue light excitation with a BP 485/20 excitation filter, an FT 510 chromatic beam splitter, and an LP 520 barrier filter were used to observe the slides prepared from

each sample. Hydroids with or without a medusa bud were removed from the bivalves, and reared and examined under the microscope as described above.

A total of 474 medusae of both sexes of *Eugymnanthea japonica* Kubota, 1979 were examined, originating from 47 specimens of *Mytilus galloprovincialis* from Shirahama (27 specimens) and Atami, near to the type locality Shimoda (20 specimens), Japan, which were reared and examined as described above (Kubota, 2004).

Medusae of both species (12 *E. japonica* and 4 *E. inquilina*) were reared until they died and the change of the fluorescence pattern was examined every day. Unfertilized eggs that were not older than a few hours and planulae obtained after conspecific crossings (see Govindarajan *et al.*, 2005) of both *Eugymnanthea* species were examined similarly together with parthenogenetically produced larvae if present.

RESULTS

The presence and type of green fluorescence is described in relation to the developmental stage (Tables 1 & 2). The changes of the fluorescence pattern observed during the life span of the medusa, maximally for seven days, are summarized in Table 3.

Medusa

The auto-fluorescence pattern observed in the medusae of *E. inquilina* (Figure 1A, B) and *E. japonica* (Figure 1C, D) was constantly different, showing 'umbrellar margin fluorescence type' in the former and a 'subumbrellar fluorescent type' in the latter. In addition, in *E. inquilina* only the peripheral part of gonads showed an auto-fluorescence (Figure 1B).

Two subtypes of fluorescence distribution were found in *E. japonica*, namely the subumbrellar fluorescence type confined to individual auto-fluorescent photocytes (Figure 1D) and another type with a uniform overall auto-fluorescence (Table 2). No pattern-variation was found for *E. inquilina* (Tables 1 & 2; Figure 1B). Only a small fraction of *E. japonica* medusae (2.7% in 474) showed no fluorescence at all (Table 2). Summarizing, we observed four different patterns in the medusae of *Eugymnanthea*.

The type of subpattern found in *E. japonica* seems to depend somewhat on the sex as females had no auto-fluorescent particles, thus the subumbrella has a brilliant, uniform fluorescence (92.6% in 394 examined females), while the auto-fluorescence in males tends to be concentrated into auto-fluorescent particles (63.6% in 33 males examined).

During the short life span of the medusa of *E. japonica*, maximally a week, a change of fluorescence pattern was detected in three out of four examined males (from the subumbrellar type to absence type or vice versa), and the changes were all irreversible (Table 3). The changes did not correlate with the age of the medusa or with senescence. Females of *E. japonica* did not change their pattern (examined in eight individuals; Table 3). In *E. inquilina* the fluorescence pattern was constant in all of the examined individuals, i.e. in two males and two females.

Table 1. Presence of fluorescence in various developmental stages of two species of *Eugymnanthea*.

Developmental stage	Appearance of fluorescent type in	
	E. inquilina	***E. japonica***
Medusa (see Table 2)	1 type (77, 3*, 2**)	2 types + absent (474, 1, 2)
Unfertilized eggs within several hours after spawning	Absent (82 from 9 females, 1, 1)	Absent (203 from 26 females, 1, 2)
Planula	Absent (8 3–8 days old, 1 pair, 1, 1)	Absent (2 2–3 days old, 1 pair, 1, 1)
Parthenogenetic larva	Not appeared	Absent (2 1-day-old, 1 pair, 1, 1)
Hydroid	Absent (137, 3*, 2**)	Absent (116, 1, 2)
Medusa bud	Same as medusa (97, 3*, 1)	Same as medusa (103, 1, 2)

*, *Mytilus galloprovincialis* (53 specimens), *Mytilaster minimus* (1 specimen), and *Chlamys glabra* (8 specimens); **, Taranto and Lago Fusaro. N, number of host sp. examined, number of localities surveyed.

Table 2. Fluorescent subtype of medusa and its frequency in the number of individuals examined in the two species of *Eugymnanthea*.

Distribution of fluorescence	***E. inquilina*** **collected from 3 host spp. from 2 localities**	***E. japonica*** **collected from 1 host sp. from 2 localities**
Subumbrella with photocysts	0	21 males + 26 females + 12*
Subumbrella without photocysts	0	4 males + 365 females + 33*
Umbrellar margin (marginal bulbs with photocysts) and peripheral part of gonads	34 males + 16 females + 10* + 17 hermaphrodites	0
Non-fluorescent	0	8 males + 3 females + 2*

*, sex undetermined.

Table 3. Change of fluorescent type in the life span of the medusa of the two species of *Eugymnanthea*.

Fluorescence	Number of medusae examined	Age of change in days and/or state continuation
E. japonica from *Mytilus galloprovincialis* collected from two localities		
Changes		
from subumbrella without photocysts to non-fluorescent	1 male	Change on the 4th day, then continued until the 5th day
from non-fluorescent to subumbrella without photocysts	2 males	Change on the 2nd day, then continued until the 3rd day
No changes		
subumbrella with photocysts	1 male	For up to 3 days
subumbrella without photocysts	8 females	For up to 4–7 days
E. inquilina from *Mytilus galloprovincialis* and *Chlamys glabra* collected from Taranto		
No changes		
umbrellar margin	2 females	For up to 3 days
umbrellar margin	2 males	For up to 6 days

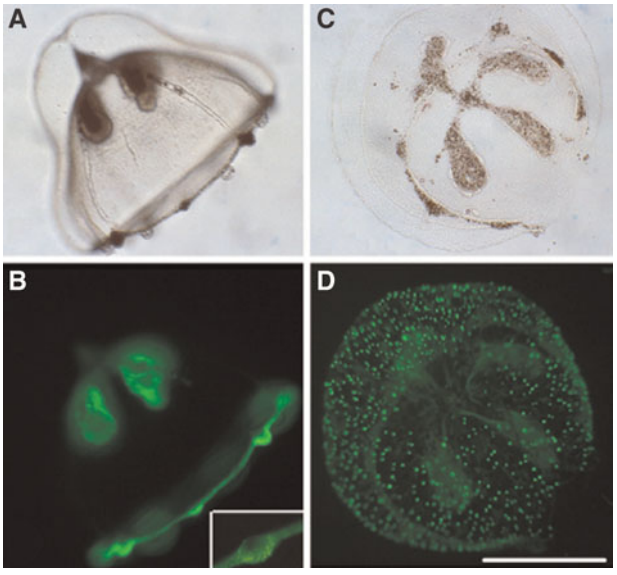

Fig. 1. Photomicrographs of medusa of (A, B) *Eugymnanthea inquilina* and (C, D) *E. japonica*. Auto-fluorescence image under blue light excitation (B, D) and transmitted light image (A, C) of same individuals. Scale bars: 0.5 mm except for inset in (B) that shows a marginal bulb.

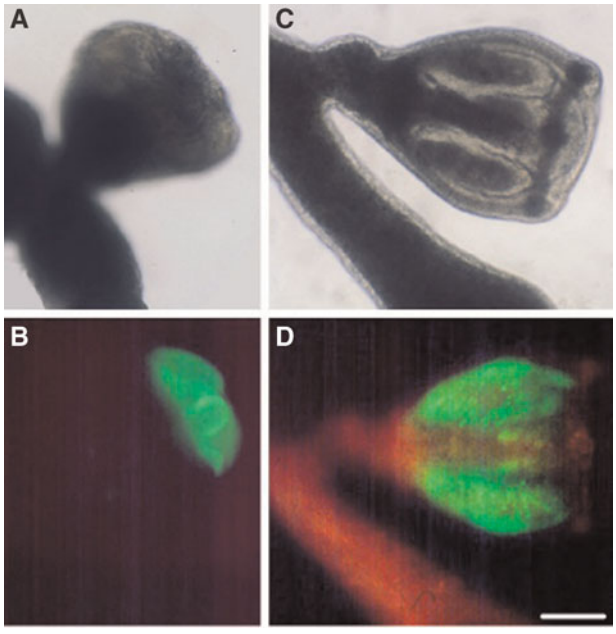

Fig. 2. Photomicrographs of medusa buds of (A, B) *Eugymnanthea inquilina* and (C, D) *E. japonica*. Auto-fluorescence image under blue light excitation (B, D) and transmitted light image (A, C) of same individuals of respective species. Scale bars: 0.1 mm.

Medusa bud

In *E. inquilina* medusa buds we could detect auto-fluorescence in the distal part of the bud in every one of the 97 examined individuals (Figure 2A, B), while in *E. japonica* the whole subumbrella showed a uniform fluorescence in all 103 examined medusa buds (Figure 2C, D; Table 1). The auto-fluorescence of the gonads in *E. inquilina* appeared only later, when the gonads were well developed.

Eggs, planulae and hydroids

The auto-fluorescence was completely absent in 285 (203 in *E. japonica* + 82 in *E. inquilina*) unfertilized eggs, 10 (2 + 8) 2–8 days old planulae, even in two parthenogenetically produced larva of *E. japonica*, and 253 (103 + 97) hydroids in the two *Eugymnanthea* species (Table 1).

DISCUSSION

We found that the auto-fluorescent distribution pattern of the medusa bud and the free medusa is a reliable taxonomic character, which permits to distinguish the two *Eugymnanthea* species, despite the occurrence of a sex-dependant pattern in *E. japonica*. Such an auto-fluorescence pattern might also be useful for distinguishing other similar hydrozoan species, particularly for species evolved convergently or sibling species. As was pointed out by Morin & Reynolds (1969, 1974), fluorescence microscopy is easily applied to living material without complicated, time-consuming techniques. It is noteworthy that the fluorescent pattern is different in the two *Anemonia* species common to the Mediterranean Sea (Leutenegger *et al.*, 2007).

Several studies have been carried out on natural bioluminescence produced by Cnidaria and Ctenophora due to the presence of natural bioluminescent compounds (Haddock & Case, 1999; Haddock *et al.*, 2001). Some photoproteins have been isolated from Hydrozoa: aequorin (Harvey, 1935; Davenport & Nicol, 1955; Nicol, 1962; Shimomura, 2005) and obelin (Campbell, 1974; Vysotskii *et al.*, 1990, 1993, 1995). Morin & Reynolds (1974) found that in *Obelia*

hydroids the distribution of the fluorescent regions correlates with the ability to produce bioluminescence and that it may have some functional significance. One eminent ecological example of fluorescent structures serving as lures is reported in an *Erenna* siphonophore that preys upon fish in deep waters (Haddock *et al.*, 2005). The auto-fluorescent and possible bioluminescent tissues of present *Eugymnanthea* medusae could likewise have some unknown biological significance as pointed out by Morin (1983). It is noteworthy that in contrast to complete absence of auto-fluorescence in the eggs and planula larvae of the two species of *Eugymnanthea*, the eggs and embryos of many kinds of sea urchin display auto-fluorescence and the pluteus larvae emit green fluorescence (Nakamura *et al.*, 2005) though their function is unknown.

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