

# Psychedelics sea slugs: Observations on colour ontogeny in two nudibranch species from the genus *Nembrotha* (Doridina: Polyceridae)

Marta Pola<sup>1,2</sup>, Yara Tibirićá<sup>3,4</sup>, Juan Lucas Cervera<sup>4,5</sup>

<sup>1</sup>Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain.

<sup>2</sup>Centro de Investigación en Biodiversidad y Cambio Global (CIBC-UAM), Madrid, Spain.

(MP) (Corresponding author) E-mail: [marta.pola@uam.es](mailto:marta.pola@uam.es). ORCID iD: <https://orcid.org/0000-0003-0518-346X>

<sup>3</sup>Stazione Zoologica Anton Dohrn, Italy. Res. Marina Biology Station of Inhaca, Maputo. Province, Mozambique.

(YT) E-mail: [yara.tibirica@szn.it](mailto:yara.tibirica@szn.it). ORCID iD: <https://orcid.org/0000-0003-3955-8186>

<sup>4</sup>Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Campus de Excelencia Internacional del Mar (CEI-MAR), Universidad de Cádiz, Puerto Real, Spain.

<sup>5</sup>Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI•MAR), Universidad de Cádiz, Puerto Real, Spain.

(JLC): E-mail: [lucas.cervera@uca.es](mailto:lucas.cervera@uca.es). ORCID iD: <https://orcid.org/0000-0002-8337-2867>

**Summary:** In recent decades, thanks to the use of integrated taxonomy, the traditional recognition of a nudibranch species based on observation and colour pattern variation has become increasingly questioned, mainly due to the presence of cryptic and pseudocryptic species complexes. Individuals with the same colour pattern can be genetically identical, but individuals with different colour patterns may also be genetically identical and this variation may instead represent different life stages. But things can get even more complicated. What happens when the same species changes its colour pattern radically as it ages? Here we present two extraordinary examples in species of the genus *Nembrotha* based on laboratory observation. Specimens of *Nembrotha livingstonei* Allan, 1933 and *Nembrotha yonowae* Goethel and Debelius, 1992 were collected in Mozambique and kept in captivity as long as feeding was possible. The results showed that colour patterns in both species changed over time and that this change was linked to diet. Furthermore, species delimitation analysis and comparison of the uncorrected COI pairwise distances of examined specimens from Mozambique and others downloaded from GenBank confirmed that *N. yonowae* Goether and Debelius, 1992 is a junior synonym of *N. cristata* Bergh, 1877. Similar studies with laboratory observations are needed on other species of the genus, as they were described on the basis of different colouration, but integrated taxonomy may show different results.

**Keywords:** colour changes; morphology; Nembrothinae; nudibranch; ontogeny; taxonomy.

**Babosas de mar psicodélicas: observaciones sobre la ontogenia del color en dos especies de nudibranquios del género *Nembrotha* (Doridina: Polyceridae)**

**Resumen:** En las últimas décadas, gracias al uso de la taxonomía integrada, el reconocimiento tradicional de una especie basado en la observación y la variación del patrón de color en los nudibranquios se ha vuelto cada vez más cuestionado, principalmente debido a la presencia de complejos de especies crípticas y pseudocrípticas. Por lo tanto, los individuos con el mismo patrón de color pueden ser genéticamente idénticos, pero los individuos con diferentes patrones de color también pueden ser genéticamente idénticos y, en cambio, esta variación puede representar diferentes etapas de la vida. Pero las cosas pueden complicarse aún más. ¿Qué sucede cuando la misma especie cambia radicalmente su patrón de color a medida que envejece? Aquí presentamos dos ejemplos extraordinarios en especies del género *Nembrotha* basados en observaciones de laboratorio. Se recolectaron ejemplares de *Nembrotha livingstonei* Allan, 1933 y *Nembrotha yonowae* Goethel and Debelius, 1992 en Mozambique y se mantuvieron en cautiverio mientras fue posible alimentarlos. Los resultados mostraron que los patrones de color en ambas especies cambiaron con el tiempo y que este cambio estaba relacionado con la dieta. Además, el análisis de delimitación de especies y la comparación de las distancias por pares no corregidas de COI de ejemplares examinados de Mozambique y otros descargados de GenBank confirmaron que *N. yonowae* Goether y Debelius, 1992 es un sinónimo menor de *N. cristata* Bergh, 1877. Estudios similares con observaciones de laboratorio son necesarios en otras especies del género, ya que se describieron en función de una coloración diferente, pero la taxonomía integrada puede mostrar resultados diferentes.

**Palabras clave:** cambios de color; morfología; Nembrothinae; nudibranquio; ontogenia; taxonomía.

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## INTRODUCTION

Nudibranchs are among the most beautiful and colourful animals in the sea. Their incredible colours and shapes, as well as their variety, make them the delight of many divers, and they adorn many dive guides and internet pages with hundreds of extraordinary photographs. Thanks to the numerous studies of integrative taxonomy carried out in recent decades (Sørensen et al. 2020, Toms et al. 2021, Paz-Sedano et al. 2022, among many others), we know that the diversity of nudibranchs, as well as of many other organisms, is much greater than is currently described due to the existence of complexes of cryptic or pseudocryptic species, that is, species that are morphologically identical but only differ molecularly (cryptic) and those that can only be morphologically distinguished after a detailed molecular study (pseudocryptic) (Bickford et al. 2007, Mann and Evans 2008). Many specimens are also identified as different species on the basis of slight differences in colouration (Goodheart and Valdés 2013, Padula et al. 2014, Layton et al. 2018), but this conclusion is based on the assumption that most species have limited colour variation, so individual members of the same species share similar colour patterns (Valdés et al. 2013). However, several molecular studies have shown that, at least in some species, colour patterns are considerably variable within members of the same species (Pola et al. 2008, Padula et al. 2016, Tibiriçá et al. 2018). Finally, it can also occur that the same individual changes colour as it grows and matures (Ortea et al. 1996). Ontogenetic colour changes are non-reversible colour changes associated with normal progressive development of an individual of a species (Booth 1990, de Bruyn and Gosselin 2014, Hultgren and Mittelstaedt 2015). Therefore, in these cases, we may overestimate biodiversity when a single species is identified as a different species on the basis of the colour pattern it exhibits in a certain phase of its life.

The genus *Nembrotha* was introduced by Bergh in 1877. It is distributed throughout the tropical Indo-Pacific, from South Africa to Eastern Australia, but is absent from other tropical areas in the Atlantic and the Eastern Pacific (Pola et al. 2008). Today, the genus includes 12 nominal species (MolluscaBase 2022), most of them originally described on the basis of differences in their colour pattern (Pola et al. 2008). In their reviews of the genus, Pola et al. (2007, 2008) separated the species into two groups on the basis of their external pattern: a group characterized by having large pustules or spots scattered on the body (*N. kubaryana* Bergh, 1877, *N. cristata* Bergh, 1877, *N. yonowae* Goethel and Debelius, 1992, *N. livingstonei* Allan, 1933, *N. milleri* Gosliner and Behrens, 1997, *N. mullineri* Gosliner and Behrens, 1997 and *N. rosannulata* Pola, Cervera and Gosliner, 2008) and a group characterized by having longitudinal lines or bands on the notum (*N. lineolata* Bergh, 1905, *N. purpureolineata* O'Donoghue, 1924, *N. chamberlaini* Gosliner and Behrens, 1997, *N. megalocera* Yonow, 1990 and *N. aurea* Pola, Cervera and Gosliner, 2008). Internally, the “lined” group have a penis with only one type of penial spines and without

penial spines at the base of the penis, as well as a highly convoluted ampulla and vagina, while the “spotted” group have three different types of penial spines and a convoluted ampulla with a straight vagina.

Rudman (1998–2005) showed that the colour pattern of *Nembrotha* species is extremely variable and suggested that most of the currently recognized species may be varieties of the same species, but that there was not enough anatomical evidence to demonstrate that proposal. Later, Pola et al. (2007, 2008) provided anatomical and molecular evidence to synonymize several species, but these authors claimed that since they were not able to examine all different variable forms and intermediate states of each species, or even include all the variable forms in the molecular analyses, they preferred to maintain a conservative point of view and retain most of the nominal species of the genus. Furthermore, since most nudibranchs are diet-specific and rarely survive long enough in captivity (Calado and Dinis 2005, Dionisio et al. 2013, Alqudah et al. 2016), opportunities to follow colour ontogeny are very rare.

In this study, we report ontogenetic colour changes in two species of *Nembrotha* on the basis of laboratory observations. We also provide molecular evidence to synonymize two species of *Nembrotha* with different colour patterns.

## MATERIALS AND METHODS

### Study location and samples information

Specimens of the genus *Nembrotha* were collected in Zavora Bay, Mozambique (Fig. 1A) from 2010 to 2015 by scuba diving (0–20 m deep) as part of a nudibranch diversity assessment by Tibiriçá et al. (2017). In 2015, we were able to set up a laboratory for aquarium observations. At that time, on two occasions, specimens of *Nembrotha* spp. were found on the blue lollipop ascidian *Eudistoma caeruleum* (Sluiter, 1909) (Fig. 1B), brought together with the ascidian to the lab and kept in captivity in a 10 L air-pumped tank for further observation (Fig. 1C). First, two adult specimens previously attributed to *N. yonowae* were kept in captivity for 24 days from 2 April 2015. However, after mating and laying eggs, they were euthanized due to the logistical difficulty associated with bad weather in bringing them fresh food. Second, on 26 May 2015, one specimen of *N. livingstonei* and two specimens of *N. yonowae* were found at Witch's Hat at 17 m depth on a single individual of *E. caeruleum*. These specimens were collected and maintained in the tank for 100 days. To keep the specimens alive and healthy, new *E. caeruleum* ascidians were collected on a regular basis (every 3 to 8 days depending on the logistics and the weather) by scuba diving at Zavora Bay, and half of the tank water was changed every three days. In mid-August, the abundance of *E. caeruleum* on the reef was reduced, and diving conditions were unfavourable for many days, making the collection of fresh ascidians problematic. As a result, fresh food was being given more occasionally, so for logistical reasons the specimens were euthanized by freezing on 4 September 2015 for further

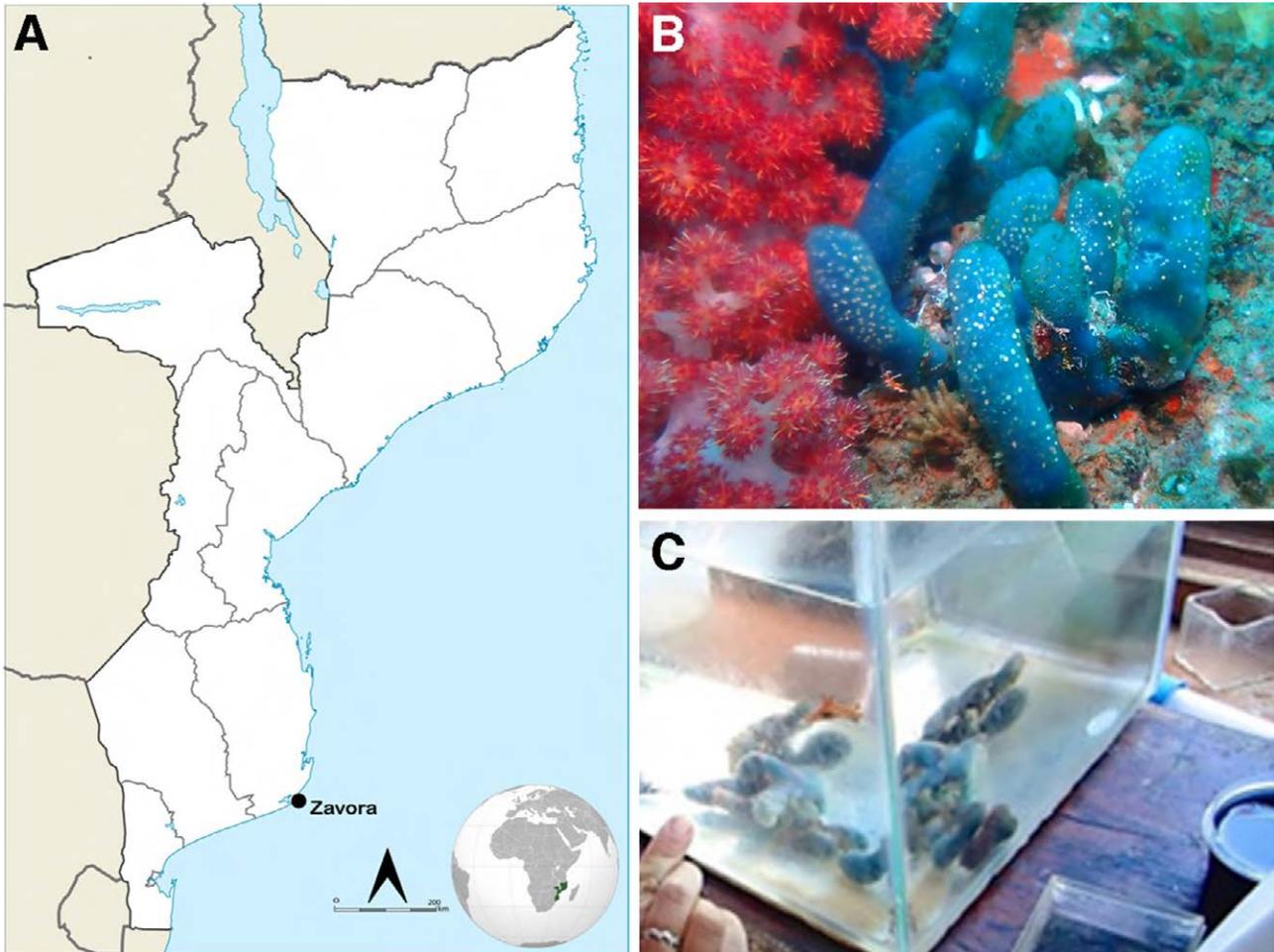


Fig. 1. – A, map of Mozambique showing Zavora Bay. B, blue lollipop ascidian *Eudistoma caeruleum* (Sluiter, 1909). Photograph taken in situ, Zavora, 17 m depth. C, 10 L air-pumped tank where the specimens were kept alive.

study. Photographs were taken with Olympus PEN Lite E-PL5 with a macro lens and Olympus TG-5 cameras. Figures were created in Adobe® Photoshop® CS4. We were unable to make laboratory observations for specimens collected before 2015, and at that time, we were only able to make them on those when conditions were favourable. We tried to sequence all specimens kept in alcohol, but not all amplifications were successful (see Table 1).

### Molecular studies

Tissue samples of a few specimens from Mozambique and one specimen from Bali (Table 1) were taken from the foot, and DNA was extracted using the Dneasy Blood and Tissue Kit (Qiagen, Valencia, CA). Partial sequences of the uncorrected mitochondrial cytochrome c oxidase subunit I (COI) were amplified by polymerase chain reaction (PCR) using LCO1490 and HCO2198 universal primers (Folmer et al. 1994). Polymerase chain reactions were conducted in a 25  $\mu\text{L}$  reaction volume containing 1  $\mu\text{L}$  of both forward and reverse primers (10  $\mu\text{mol L}^{-1}$ ), 2.5  $\mu\text{L}$  of dNTP (2  $\text{mmol L}^{-1}$ ), 3.5  $\mu\text{L}$  of magnesium chloride (25  $\text{mmol L}^{-1}$ ), 0.25

$\mu\text{L}$  of Qiagen DNA polymerase (5 units/ $\mu\text{L}$ ), 5  $\mu\text{L}$  of ‘Q-solution’ (5 $\times$ ), 2.5  $\mu\text{L}$  of Qiagen buffer (10 $\times$ ) (Qiagen Taq PCR Core Kit) and 1–2  $\mu\text{L}$  of genomic DNA. Amplification of COI was performed with an initial denaturation for 5 min at 94 $^{\circ}\text{C}$ , followed by 35–36 cycles of 1 min at 94 $^{\circ}\text{C}$ , 30 s at 45 $^{\circ}\text{C}$  (annealing temperature) and 1 min at 72 $^{\circ}\text{C}$ , with a final extension of 10 min at 72 $^{\circ}\text{C}$ . Successful PCR products were sent to Macrogen, Inc. for purification and sequencing on a 3730XL DNA sequencer (Applied Biosystems). Sequences were edited in GENEIOUS v.10.2.3 (Kearse et al. 2012). All sequences were tested for similarity with the BLAST algorithm and aligned using MEGA7 (Kumar et al. 2016). The software jModelTest2 on XEDE, CIPRES Science Gateway (Miller et al. 2010), was used to select the evolutionary models for each codon position of COI under Akaike information criteria (Schwartz 1978). Evolutionary models for COI were TIM2+G, TPM1uf+I and TIM1+G for the first, second and third codon position, respectively. Bayesian inference analysis was performed using the software MrBayes on XSEDE, CIPRES Science Gateway (Miller et al. 2010), for ten million generations, four independent runs and a sampling frequency of 1000. New generated sequences

Table 1. Specimens of *Nembrotha* included in this study. New generated sequences in bold.

| Species                          | Voucher          | Observations                | GenBank accession number (COI) | Morphology | Photo |
|----------------------------------|------------------|-----------------------------|--------------------------------|------------|-------|
| <i>Nembrotha livingstonei</i>    | MB28-004737      |                             | -                              | External   | YES   |
| <i>Nembrotha livingstonei</i>    | MB28-005019      | Kept in tank 100 days       | <b>OP874951</b>                | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-005020      | Kept in tank 100 days       | -                              | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-005021      | Kept in tank 100 days       | -                              | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-005003      | Kept in tank 24 days        | <b>OP874950</b>                | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-005004      | Kept in tank 24 days        | <b>OP874949</b>                | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-004421      | Formol                      | -                              | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-004422      | Formol                      | -                              | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-004424      | Formol                      | -                              | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-004547      | -                           | <b>OP874948</b>                | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MNCN15.05/91069  | -                           | <b>OP874946</b>                | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MNCN15.05/91070  | -                           | <b>OP874945</b>                | Dissected  | YES   |
| <i>Nembrotha yonowae</i>         | MB28-004707      | -                           | -                              | External   | YES   |
| <i>Nembrotha yonowae</i>         | MHN-YT 1506      | -                           | -                              | External   | YES   |
| <i>Nembrotha yonowae</i>         | MB28-004980      | -                           | -                              | External   | YES   |
| <i>Nembrotha yonowae</i>         | MNCN15.05/91071  | -                           | <b>OP874947</b>                | External   | YES   |
| <i>Nembrotha yonowae</i>         | WAMS11556        | Pola et al. (2007)          | EF142894.1                     | -          | YES   |
| <i>Nembrotha cristata</i>        | CASIZ 191428     | Hallas et al. (2017)        | MF958431.1                     | -          | YES   |
| <i>Nembrotha cristata</i>        | CASIZ 157483     | Pola et al. (2006, 2008)    | DQ231003                       | -          | YES   |
| <i>Nembrotha chamberlaini</i>    | CASIZ 158812     | Pola et al. (2006)          | DQ231006                       | -          | YES   |
| <i>Nembrotha chamberlaini</i>    | WAMS11566        | Pola et al. (2007)          | EF142881                       | -          | YES   |
| <i>Nembrotha chamberlaini</i>    | MNCN15.05/46727  | Pola et al. (2007)          | EF142880                       | -          | YES   |
| <i>Nembrotha lineolata</i>       | CASIZ 158257     | Pola et al. (2006)          | DQ231005                       | -          | YES   |
| <i>Nembrotha lineolata</i>       | WAMS11562        | Pola et al. (2007)          | EF142886                       | -          | YES   |
| <i>Nembrotha lineolata</i>       | MNCN15.05/46724  | Pola et al. (2007)          | EF142885                       | -          | YES   |
| <i>Nembrotha megalocera</i>      | ZSM-Moll20006510 | Pola et al. (2007)          | EF142888                       | -          | YES   |
| <i>Nembrotha megalocera</i>      | MNCN15.05/46729  | Pola et al. (2007)          | EF142887                       | -          | YES   |
| <i>Nembrotha purpureolineata</i> | CASIZ177006      | Knutson and Gosliner (2022) | MZ382785                       | -          | YES   |
| <i>Nembrotha purpureolineata</i> | WAMS11563        | Pola et al. (2007)          | EF142884                       | -          | YES   |
| <i>Nembrotha purpureolineata</i> | C205326          | Pola et al. (2007)          | EF142883                       | -          | YES   |
| <i>Nembrotha purpureolineata</i> | MNCN15.05/46726  | Pola et al. (2007)          | EF142882                       | -          | YES   |
| <i>Tambja verconis</i>           | MNCN15.05/46653  | Pola et al. (2007)          | EF142869                       | -          | YES   |
| <i>Roboastra gracilis</i>        | CASIZ 188582     | Knutson and Gosliner (2022) | MZ382793                       | -          | YES   |
| <i>Polycera quadrilineata</i>    | ZMMU Op-760      | Korshunova et al. (2021)    | MZ425370                       | -          | YES   |

are available in GenBank. For molecular comparison and species delimitation analysis, we used sequences downloaded from GenBank of several specimens of Nembrothinae, highlighting specimens identified as *N. guttata* (synonym of *N. yonowae*) from the Philippines (voucher code WAMS11556, accession number EF142894.1), and two specimens of *N. cristata* from Papua New Guinea (CASIZ191428, MF958431.1) and the Philippines (CASIZ 157483, DQ231003). Specimen details are summarized in Table 1.

Gene pairwise uncorrected *p*-distances for COI were obtained using MEGA7 (Kumar et al. 2016). Analyses of species delimitation were conducted on COI ingroup sequences. Bayesian Poisson Tree Process (bPTP) was carried out with the bPTP webtool (<https://species.h-its.org>), using the following parameters: MCMC generations = 200000, Thinning = 100 and Burn-in = 0.1. Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2021) was also conducted using the webtool (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>), under the Kimura model (K80) ts/tv.

## RESULTS

### *Nembrotha livingstonei* Allan, 1933 (Fig. 2)

*Material examined:* MB28-005019, collected on 26 May 2015 at Witch's Hat, Zavora, depth 17 m, found on *E. caeruleum*, length when collected 2 mm, kept in tank until 4 Sep. 2015, preserved size 18 mm. Sequenced. MB28-004737, 19 Sep. 2013, Rock Pool, Zavora, Mozambique, depth 1 m, length 50 mm, on rock. Studied for external morphology (unsuccessful sequencing).

*Diagnosis.* As described by Pola et al. (2008).

#### *Colour ontogeny*

*Specimen* MB28-005019 (Fig. 2A–G)

When collected, the specimen measured approximately 2 mm (Fig. 2A), and the background colour was blue. A light blue patch was present on the dorsal midline behind the rhinophores. Three thin light blue lines ran from this patch toward a light blue band in front of the gill. A few bright orange pustules were present on the notal border and surrounding the edge of the foot. The rhinophores and the non-retractile multipinnate gill branches were bright orange, especially bright at the tips (Fig. 2A). After one month (Fig. 2B, C), the specimen measured 6 mm, the orange pustules developed in almost complete lines (at the top of the light blue line) surrounded by a thin brown line. The light blue patches became almost bright white, except at the base of the gills, the base of the rhinophores and the end of the tail, where they remained bright light blue. The gill branches and rhinophores became less bright, becoming more reddish-brown in colour, except for the branchial rachises and rhinophoral tips. After two months, at about 10 mm (Fig. 2D), the orange lines/pustules lost their brightness, and the brown lines formed a clearer ridge texture. The bright light

blue was now darker blue, clearly limited to the base of the gill branches, the tip of the rhinophoral sheaths, the oral tentacles, and the border of the entire foot. White-cream patches were limited to the circle around the gill below the blue and the area between the rhinophores, going up the rhinophore sheaths, clearly giving the typical appearance of a cross-shaped mark. In the third month the specimen grew to 12 mm (Fig. 2E, F) and the orange dorsal pustules disappeared almost completely, some becoming cream-coloured. The initial blue colour of the background had completely disappeared. Just before euthanizing, the specimen measured 12 mm long and had orangish pustules only on the anterior and posterior edge of the mantle (Fig. 2G). A much larger specimen (50 mm) was collected on the same reef in September (Fig. 2H). This specimen only had a few scattered orange pustules on its head, but it had many more creamy ones. The brown lines were darker and in greater numbers.

*Ecological notes.* This species is relatively rare in Zavora. It has only been seen on two occasions over seven years of intensive field research in shallow reefs (up to 17 m).

*Remarks and discussion.* Pola et al. (2008) reports different morphotypes for this species, including a dark specimen with green spots. However, the early juvenile is described here for the first time. We confirmed that the pale, cross-shaped mark is present among the rhinophores from juvenile to adult and, at present, it can be considered a diagnosis character regardless of life stage. Similarly, the oral tentacles and foot were lined with blue and did not lose colour with growth. All adults specimens reported for Mozambique show the same blue colouration, but the Philippines specimens observed by Pola et al. (2008) had bright orange oral tentacles and foot edge. Notably, in the specimen observed in the tank, the gill branches and rhinophores changed from bright orange to red and brownish. The edge of the rhinophore sheath was orange-pink pigmented in the large specimens and in the photographs provided by Pola et al. (2008, Fig. 14), but not in the juvenile kept in captivity. Additionally, the background changed from blue to creamy. The species delimitation analysis (Fig. 3) and uncorrected COI *p*-distances clearly indicate that this species is different from *N. yonowae*, with a minimum distance of 9.5% (Table 2).

### *Nembrotha yonowae* Goether and Debelius, 1992

*Material examined:* Thirteen specimens in total. MB28-005020 and MB28-005021, 26 May 2015, Witch's Hat, Zavora, Mozambique, depth 17 m, found on *Eudistoma caeruleum*, length 2 and 30 mm when collected, kept in tank until 4 Sep. 2015, length 28 and 42 mm when preserved, respectively. Both sequenced and dissected. MB28-005003 and MB28-005004, 27 May 2015, Vascos, Zavora, Mozambique, depth 15 m, found on *E. caeruleum*, length 22 mm and 28 mm, respectively, collected by Mona Andskog, kept in tank for 24 days. MB28-004421, 31 May 2010, Witch's Hat, Zavora, Mozambique, depth 16 m, length 42 mm, on *E. caeruleum*. Dissected. MB28-004422, 07 Jun. 2010, Witch's Hat, Zavora, Mozambique, depth 18 m, length 25 mm, substrate not recorded. MB28-004424,

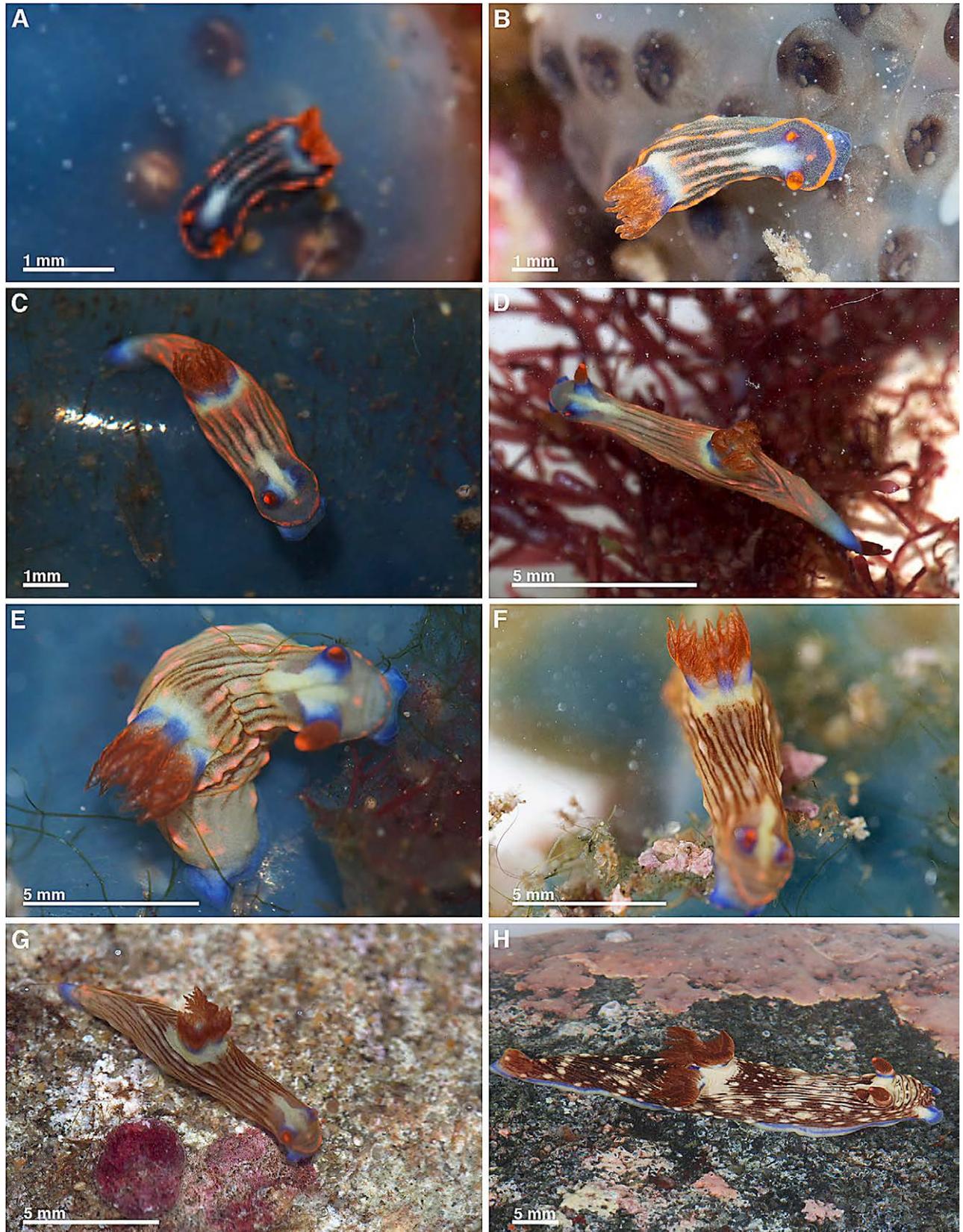


Fig. 2. – *Nembrotha livingstonei* Allan, 1933. A-G, MB28-005019; A, when collected, 2 mm. B-C, after a month, 6 mm; D, after two months, 10 mm. E-F, in the third month grew to 12 mm; G, Just before euthanizing, the specimen measured 12 mm long and had orangish pustules only on the anterior and posterior edge of the mantle. H, MB28-004737, 50 mm.

27 May 2010, Witch's Hat, Zavora, Mozambique, depth 18 m, length 18 mm, crawling on the reef. MB28-004547, 20 May 2012, Launch area reef, Zavora, Mozambique, depth 2 m, length 38 mm, substrate not recorded. Dissected. MNCN15.05/91069, 9 Dec. 2012, Area 51, Zavora, Mozambique, depth 12 m, length 32 mm, on *E. caeruleum*. Sequenced and dissected. MNCN15.05/91070, 12 Dec. 2012, Area 51, Zavora, Mozambique, depth 12 m, length 6 mm, on *E. caeruleum*. Sequenced and dissected. MB28-004707, 06 Aug. 2013, Launch area reef, Zavora, Mozambique, depth 0.5 m, length 16 mm, between blue tunicate (*Clavelina* sp.). MHN-YT 1506, 02 Apr. 2015, Vascos, Zavora, Mozambique, depth 15 m, length 21 mm, on *E. caeruleum*. MB28-004980, 3 Apr. 2015, Vascos, Zavora, Mozambique, depth 17 m, length 16 mm, on *E. caeruleum*.

### Colour ontogeny

#### Specimen MB28-005020 (Fig. 4A-D)

When collected, the specimen measured 2 mm, the background colour was dark blue with bright scattered orange pustules (Fig. 4A). The base of the rhinophores and the four non-retractile multipinnate gill branches were lighter blue. The sheaths, the rhinophore clubs and the oral tentacles were same blue as the body. The foot sole was dark blue with a dark line on the "tail" and light blue edge. At one month, the specimen reached 5 mm (Fig. 4B). The background colour was still blue, but not so dark, and the overall number of spots increased, these being more and more intensely orange in colour; a line of spots was well formed

around the edge of the mantle and on the upper part of the foot. Traces of orange pustules appeared on the rhinophores' sheaths. The outer rachises of the gill branches maintained a light blue tonality, but the pinnae turned dark blue. The rhinophores maintained their blue but a light blue tip appeared. The foot sole was translucent with blue edge and darker tip (Fig. 4B). In the second month the specimen grew to 10 mm (Fig. 4C). The background colour became lighter, with a light purple colouration, and the orange pustules became very bright. The rhinophores' lamellae and the gill pinnae changed to the same colour as the body ground. The rhinophores' tip and stalk, as well as the outer gill rachises turned a lighter blue, almost white. The edge of the elevated anus turned bright orange, as did most of the edge of the rhinophore sheath (Fig. 4C). In the third month, at about 25 mm size, the background colour was light purple, almost brownish, and the pustules stayed orange but less vivid. The pustules were bordered by a darker purple/brownish line. The rhinophores' lamellae and the gill plumes turned the same shade of purple/brownish. The rhinophores were tipped in white and the rhinophores' sheaths lost their pale colour, turning brown but bordered in orange. The tail, oral veil and foot line lost the vivid blue turning into a grey-light blue (Fig. 4D).

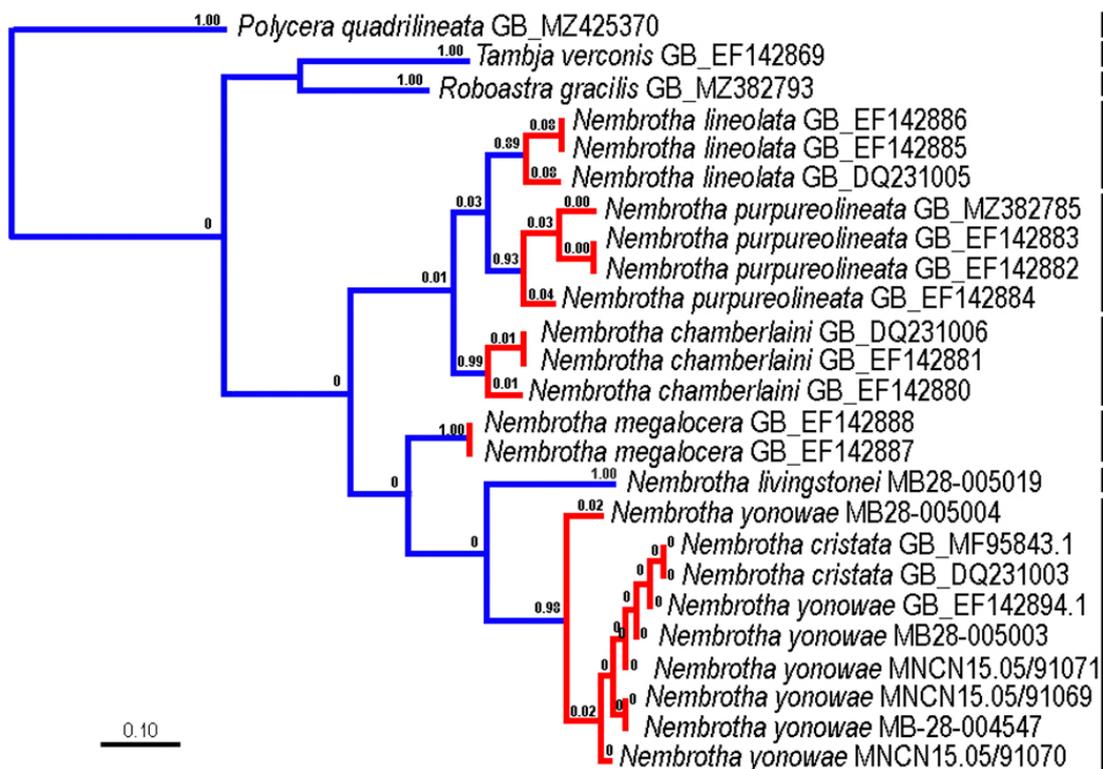


Fig. 3. – COI Bayesian tree showing bPTP species delimitation results. Each red clade is a well-delimited bPTP subset. Black bars represent the same potential different taxa inferred by ASAP.

Table 2. – Uncorrected COI gene pairwise *p*-distances (%) among different specimens of *Nembrotha yonowae* and *N. livingstonei*.

|                                       | 1   | 2   | 3          | 4   | 5   | 6          | 7   | 8   | 9   |
|---------------------------------------|-----|-----|------------|-----|-----|------------|-----|-----|-----|
| 1. <i>N. yonowae</i> MNCN15.05/91070  | -   |     |            |     |     |            |     |     |     |
| 2. <i>N. yonowae</i> MNCN15.05/91069  | 0.5 | -   |            |     |     |            |     |     |     |
| 3. <i>N. yonowae</i> MNCN15.05/91071  | 0.7 | 0.6 | -          |     |     |            |     |     |     |
| 4. <i>N. yonowae</i> MB28-004547      | 0.5 | 0.2 | 0.6        | -   |     |            |     |     |     |
| 5. <i>N. livingstonei</i> MB28-005019 | 9.5 | 9.8 | 9.7        | 9.8 | -   |            |     |     |     |
| 6. <i>N. yonowae</i> MB28-005004      | 0.7 | 0.8 | <b>1.0</b> | 0.8 | 9.9 | -          |     |     |     |
| 7. <i>N. yonowae</i> MB28-005003      | 0.5 | 0.6 | 0.8        | 0.6 | 9.7 | <b>1.0</b> | -   |     |     |
| 8. <i>N. yonowae</i> WAMS11556        | 0.4 | 0.5 | 0.7        | 0.5 | 9.8 | 0.9        | 0.5 | -   |     |
| 9. <i>N. cristata</i> CASIZ 191428    | 0.3 | 0.4 | 0.4        | 0.4 | 9.5 | 0.8        | 0.4 | 0.3 | -   |
| 10. <i>N. cristata</i> CASIZ 157483   | 0.8 | 0.7 | 0.9        | 0.7 | 9.9 | <b>1.3</b> | 0.9 | 0.8 | 0.5 |

#### Specimen MB28-005021 (Fig. 4E-H)

When collected, the specimen measured about 30 mm (Fig. 4E) and was like the one described above after two months (Fig. 4C). Over the days, the specimen gradually lost brightness (Fig. 4F). By the third month (approx. 50 mm), most pustules were pale and the oral tentacles and foot line pale bluish grey. The white tip of the rhinophores and the orange edge of the rhinophore sheath disappeared, and the gills and rhinophores turned dark brown (Fig. 4G-H). The specimen lost their vivid colour when the food supply was reduced, suggesting that colour is probably related to diet.

#### Specimens MB28-005003 and MB28-005004 (Fig. 5A-C)

These specimens, 22 mm and 28 mm in length respectively, were kept in the tank for 24 days. They were very similar to the ones described above with roughly the same size. However, both bore a few creamy marks under the orangish pustules, particularly on the side of the mantle. In one specimen the creamy pigmentation was also present between the rhinophores (Fig. 5A). Moreover, in these specimens, the colouration of the rhinophores and gill was much lighter. They mated in the tank (Fig. 5A) and laid eggs two days later (Fig. 5B). The eggs were photographed under an optical microscope 15 days later (Fig. 5C).

Several other specimens were collected and preserved for further studies (Table 1). These specimens were very similar to those observed in captivity (Fig. 5D-H). We noted that the orange edge on the rhinophore sheath, anus and kidney pore were variable, as was the presence and abundance of creamy spots (Fig. 5E-G). Some of these specimens were sequenced and dissected (Table 1) and, as predicted, they all belonged to the same species, with maximum uncorrected COI *p*-distance of 1% (Table 2). This maximum genetic distance is between the two specimens kept together in the aquarium (MB28-005003 and MB28-005004),

which had mated and laid eggs (Fig. 5A-C), as well as between one of the previous specimens (MB28-005004) and the one from Bali (MNCN15.05/91071). The anatomy of some of them (see Table 1) was also studied, and no internal differences were found. Delimitation analysis of both ASAP and bPTP species also confirmed that these specimens belonged to the same species (Fig. 3).

*Ecological notes.* This species was relatively abundant in the reef in Zavora and most often associated with *Eudistoma caeruleum*. Interestingly, at 15 m (without flash) this species appeared dark brown with glowing red/orange pustules and a glowing rhinophore sheath (Fig. 5E). On the surface or under flash/strobe, the glowing parts were not as vivid. Red was the first depth-filtered colour, suggesting that this specimen fluoresced. On another occasion, a specimen of *N. kubaryana* was collected, observed and filmed in the laboratory in the dark with blue light, revealing red fluorescence on the rhinophores, gill sheath, oral tentacles and end of the gill branches and around the edge of the foot (Supplementary material). The specimen released a purple ink when it was disturbed.

*Remarks and discussion.* The naming history of *N. yonowae* is complicated and is clearly summarized by Pola et al. (2008), who considered *N. guttata* Yonow, 1994 as a junior synonym of *N. yonowae*. This species, with type locality in the Maldives Islands, was described by Goether and Debelius (1992), Yonow (1994, 2012) and Pola et al. (2008) for specimens of 40, 31, 35 and almost 60 mm long, respectively. In this size range, *N. yonowae* were described with black ground colour covered with strong orange-red pustules all over, 30% of them with a green border, black rhinophores with orange-red lamellae and rhinophoral sheaths bordered in orange. Gills black with green rachises. This exact

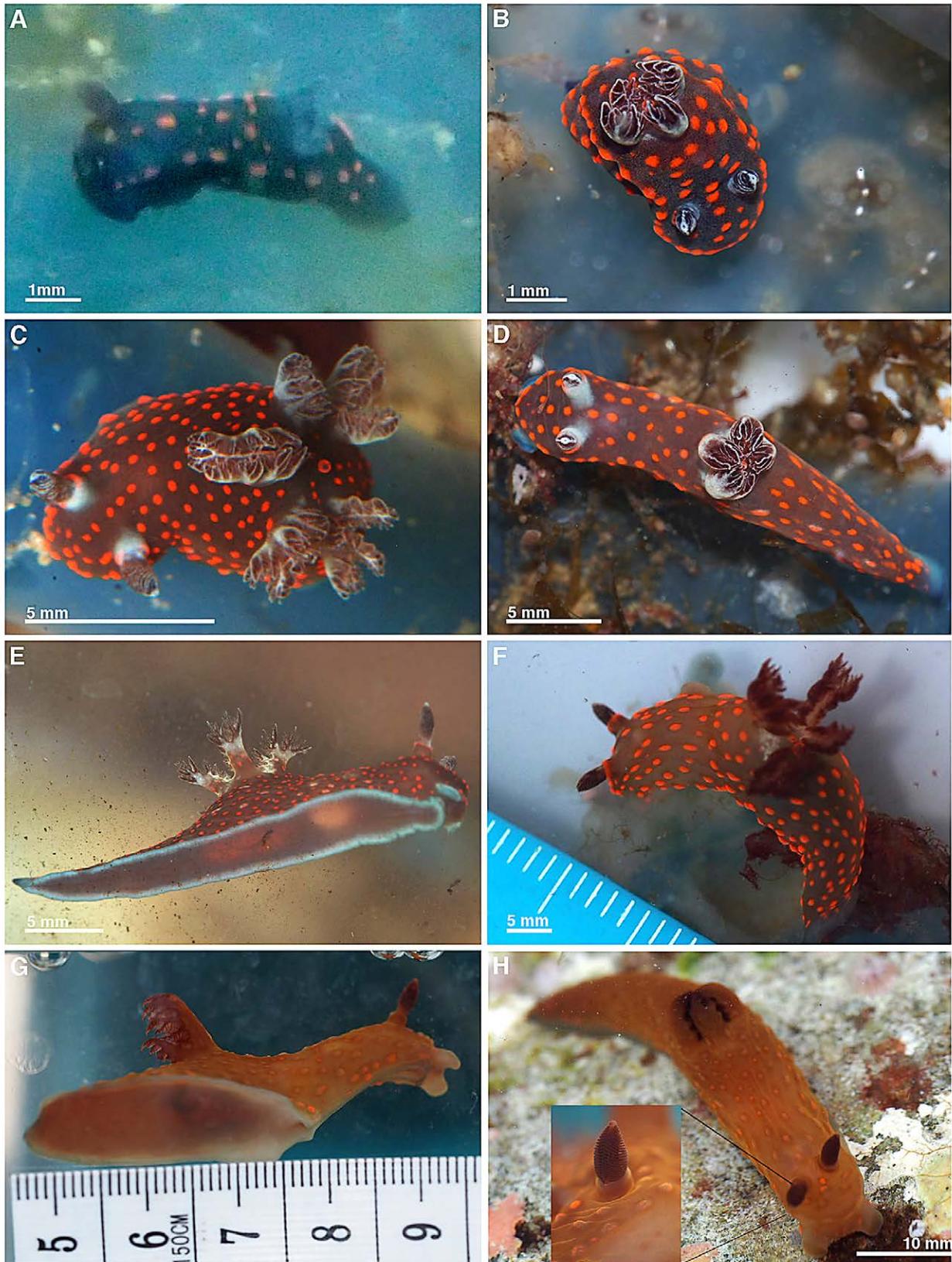


Fig. 4. – *Nembrotha yonowae* Goether and Debelius, 1992. A-D, MB28-005020; A, when collected, 2 mm; B, after one month, 5 mm; C, after two months, 10 mm; D, in the third month grew to 25 mm. E-H, MB28-005021; E, 30 mm, similar to the one in Fig. 4C after two and a half months; F, over the months, the specimen gradually lost brightness; G-H, by the third month (approx. 50 mm) most pustules were pale and the oral tentacles and foot line pale bluish grey. The white tip of the rhinophores and the orange edge of the rhinophore sheath disappeared, and the gills and rhinophores turned dark brown.

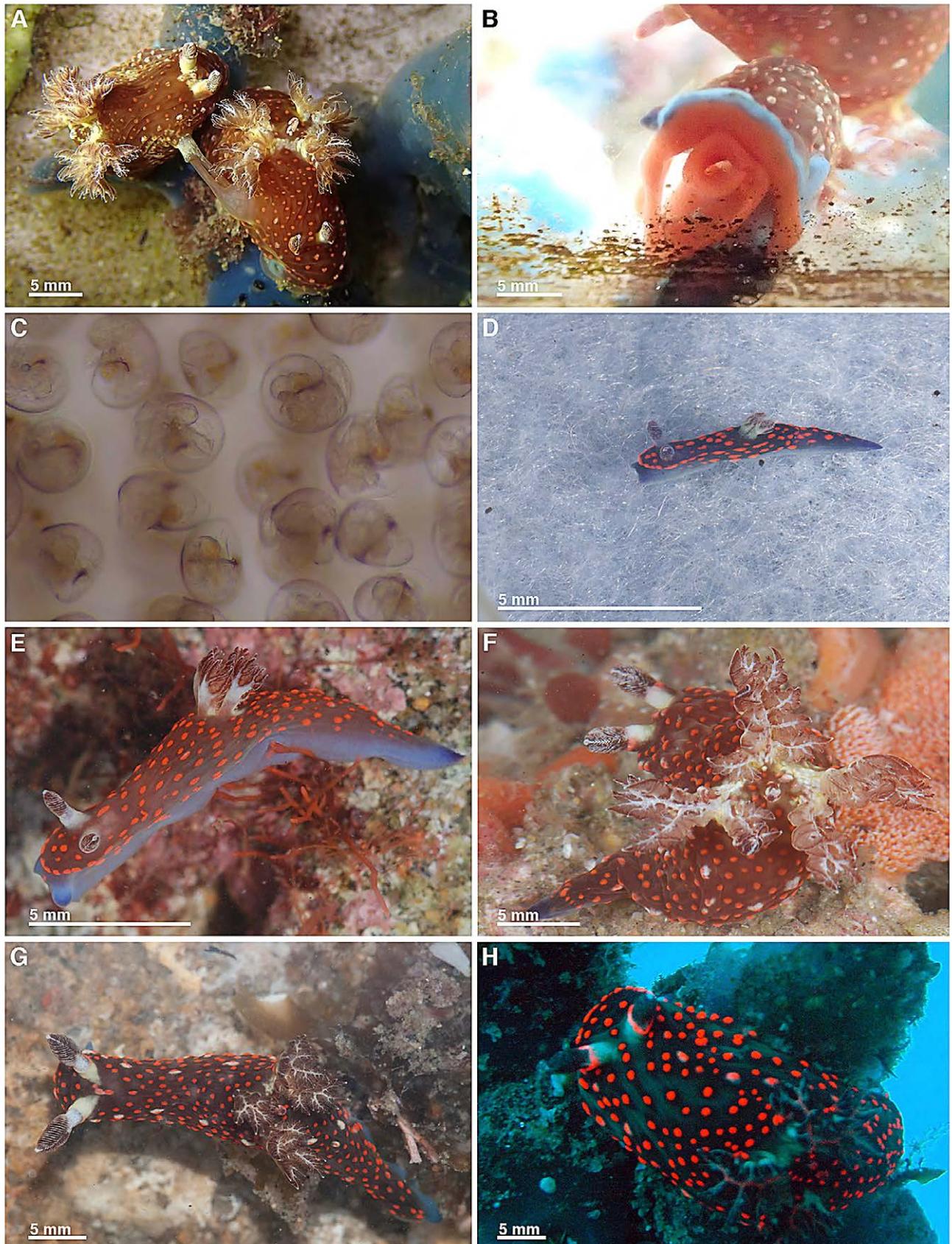


Fig. 5. – *Nembrotha yonowae* Goether and Debelius, 1992. A-C, MB28-005003 and MB28-005004; A, specimens mating on the tank; B, laying eggs two days after; C, eggs were photographed through an optical microscope 15 days later. D, MNCN15.05/91070. E, MB28-004707. F, MNCN15.05/91069. G, MB28-004547. H, MB28-004421.

pattern was always seen in adult specimens reaching at least 30 mm (Fig. 6A). However, other specimens resembling that colouration but not exactly, much more similar to some of the specimens described in this study, have been identified as *Roboastra rubropapillosa* in Ono (1999), *Nembrotha nigerrima* Bergh, 1877 in Debelius and Kuitert (2007), *N. mullineri* Gosliner and Behrens, 1997 in Gosliner et al. (2018), or most commonly as undescribed species (*Nembrotha* sp. 1, *Nembrotha* sp. 2, *Nembrotha* sp. 3 in Gosliner et al. (2008); *Nembrotha* sp. 2, *Nembrotha* sp. 3, *Nembrotha* sp. 4 in Human and DeLoach (2010); *Nembrotha* sp. 2, *Nembrotha* sp. 4 in Nakano (2018); *Nembrotha* sp. 1, *Nembrotha* sp. 3, *Nembrotha* sp. 4, *Nembrotha* sp. 5, *Nembrotha* sp. 6 in Debelius and Kuitert (2007); *Nembrotha* sp. A in Tonzuka (2003) from Bali; *Nembrotha* sp. cf. *guttata* in Nakano (2004) from Japan or even *Gymnodoris* sp. 4 in Wells and Clayton (1993) from western Australia. Coleman (2008, p. 345), identified specimens similar to ours as *Nembrotha guttata* (synonymy of *N. yonowae*).

In this paper we confirm that *Nembrotha yonowae* changes colouration as it grows, and that colouration depends on the diet. As the individuals grow, the number of pustules and their brightness increase, until the food disappears and then their colouration fades away. The studied specimens were found and fed in Mozambique with *E. caeruleum*, a composed blue ascidian with a recorded distribution on the KwaZulu-Natal and Mozambique coasts, the west coast of Madagascar, and Mayotte in the Comoros islands ([http://www.biodiversityexplorer.info/mm/tunicates/eudistoma\\_caeruleum.htm](http://www.biodiversityexplorer.info/mm/tunicates/eudistoma_caeruleum.htm)). Most likely, due to its proximity (the two farthest regions are between 25 and 38 degrees apart in latitude), it is also present in the Maldives, although no records or images are available to date.

*Nembrotha cristata* Bergh, 1877 is very similar to *N. yonowae*, but all its pustules and pigmentation around rhinophores, oral tentacles, gill and foot are consistently green. This species has been reported mostly from the Western Pacific, with fewer observations in the Indian Ocean (Tanzania, Mauritius, Maldives Islands) (Yonow 1994, 2012, Pola et al. 2008, Anderson 2022). In most of its recorded distribution, *N. cristata* has been observed feeding on *Sigillina signifera* (Sluiter, 1909) (<http://www.seaslugforum.net/showall/nemberis>), a dark green colonial tunicate (Fig. 6B). However, it has also been observed feeding on other ascidians (Rudman 2007), suggesting that it may feed on a variety of ascidians. Yonow (2012) includes a photograph of *N. yonowae* together with *N. cristata* in the Maldives (Fig. 6C), and Debelius and Kuitert (2007, p. 58) show a picture of two specimens identified as *Nembrotha* sp. 3 that resemble *N. yonowae* (identified as *Nembrotha guttata*) mating with *N. cristata*, but the latter had a few orange pustules in addition to the green ones (Fig. 6D). Finally, we show a small individual with intermediate colour pattern (Fig. 6E), with some green and some bright yellow-orange pustules. This specimen was studied and sequenced here. Sequences of *N. cristata* specimens previously studied by Pola et al. (2006, 2008) and Hallas et al. (2017) were downloaded

from GenBank and compared with our specimens of *N. yonowae* from Mozambique and Bali, as well as one *N. yonowae* sequence (EF142894.1) from the Philippines. The maximum uncorrected COI *p*-distances between *N. yonowae* and *N. cristata* was 1.3% (Table 2), and species delimitation analysis (Fig. 2) included all *N. yonowae* specimens as belonging to the same species. Based on all the observations and analysis presented in this study as well as the results obtained by Pola et al. (2007, 2008), we finally synonymized *N. yonowae* as a junior synonym of *N. cristata* Bergh, 1877.

It is important to comment on the effect that the fact that these specimens are in an aquarium could have. Obviously, for one thing, this is the only way we can see how these changes are taking place. It could be thought that it is an effect of their maintenance and the conditions in which the aquarium is located, but because these intermediate colorations are frequently observed in nature, we are quite sure that the aquarium effect is minimal, and these changes simply reflect actual ontogenetic changes that depend on growth and diet.

## DISCUSSION

Ontogenetic colour change is typically associated with changes in size, vulnerability or habitat (Booth 1990, Wilson et al. 2007, Kraus and Allison 2009). In nudibranchs some species show chromatic variability, which is often correlated with different ontogenetic stages or different food sources (Padula et al. 2016), but this is not the case in all of them, as shown for *Peltodoris atromaculata* (Ávila 1996). There are very few published studies on ontogenetic colour change in nudibranchs, but, for example, the colour pattern in *Spurilla* depends on the colour of the sea anemones upon which they feed (Gosliner 1980), as was later confirmed by molecular studies (Carmona et al. 2014). Recently, the study by Córdoba et al. (2022) in the heterobranch *Aplysia* showed that specimens from the Catalan coast of Spain, with morphological differences in body colour and shell, radular and jaw morphology, could in fact be different species of this genus, belonging to *A. punctata*. These authors observed that *A. punctata* feed on a variety of red algae, incorporating the pigments of the algae into their body and thus showing the phenotype of the adults of *A. parvula*. They hypothesized that as the individuals increase in size, they must implement alternative defensive strategies because they no longer go unnoticed by predators on the red algae. For example, they can feed on the green algae *Ulva lactuca* Linnaeus, 1753, changing their body to an olive-green hue with many white spots and becoming typical *A. punctata*. They may also move to a different habitat that provides better protection, such as under stones. These *A. punctata* specimens acquire other green or brown tones, in addition to the typical white spots that characterize the species in its adult state, which may help them to go unnoticed by predators, contributing to the ecological success of the species. *Nembrotha kubaryana* and *N. cristata* have been the focus of several natural product studies (Paul et al. 1990, Karuso and Scheuer 2002,



Fig. 6. – A, photograph taken by Winfreid Werzmirzowsky, Maldives, 2020; Available at <http://slugsite.us/bow2007/nudwk1169.htm>. B, photograph taken by David Rolla, Raja Ampat, Papua Barat, Indonesia, January 2019; Photo 38651989 (CC BY-NC), <https://spain.inaturalist.org/photos/38651989>. C, photograph taken by H. Voigtmann in Maaya Tila, Ari Atoll, 6-8 m depth, March 1994 (Yonow, 2012), [https://es.wikipedia.org/wiki/Nembrotha#/media/Archivo:Nembrotha\\_guttata,\\_N\\_cristata.jpg](https://es.wikipedia.org/wiki/Nembrotha#/media/Archivo:Nembrotha_guttata,_N_cristata.jpg), photograph taken by Mike Krampf, Raja Ampat, Dunia Kecil dive site near Wajil island, December 2006. E, photograph taken by Scott Johnson, Seraya, Tulamben, Bali, 10 mm, November 2014.

Bandaranayake 2006). Paul et al. (1990) observed that the secondary metabolites of the ascidians *Atapozoa* spp. were also found in the *Nembrotha* spp. studied, and that these compounds also serve as chemical defences for the nudibranchs. Karuso and Scheuer (2002) confirmed the dietary link between these nudibranchs and their prey species, and Bandaranayake (2006) stated that these pigments are fluorescent in UV light. Therefore, it seems logical to think that the brightness displayed by *Nembrotha kubaryana*, as well as the intense brightness present in *Nembrotha cristata* (= *N. yonowae*), is an aposematic mechanism to warn predators of their toxicity (Bandaranayake 2006, Fisch et al. 2017, Dean and Prinsep 2017). The adaptive significance of colouration may be used to

advertise the distastefulness or hazardousness of a species to putative predators (aposematism) or to mimic aposematic species, but their functional, evolutionary significance is poorly understood (Wilson et al. 2007, Bornancin et al. 2017, Winters et al. 2017, 2022). In this study we showed that both *Nembrotha livingstonei* and *N. cristata* (= *N. yonowae*) change colours as they grow and, in addition, that their colour pattern can differ depending on the diet. This is important to clarify the real number of species within the genus *Nembrotha*, as well as their geographical distribution. It would be most desirable to continue making these aquarium observations, although it is really difficult to find these specimens as well as their food, and to keep them alive.

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## SUPPLEMENTARY MATERIAL

Video of *Nembrotha kubaryana* glowing at dark under blue light. Collected at Sacred Sands, Nuarro, Memba, Nampula (14°11'48"S, 40°59'18"E), Mozambique, 8 m, 38 mm, 11 august 2017. <https://youtu.be/bjWD2uV15d4>