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ORIGINAL PAPER

Postembryonic development of *Nymphon australe* Hodgson, 1902 (Pycnogonida, Nymphonidae) from Antarctica

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Abstract

Developmental modes in pycnogonids are variable, even between congeneric species. Most of our previous knowledge of pycnogonid life history stages is based on morphological descriptions of the larval and those postlarval instars still retained on male ovigers, whereas the free-living instars are largely unknown. In this paper, a combination of morphological and molecular tools is used in order to link those postlarval instars retained on male ovigers to free-living specimens representing putatively later stages in the postembryonic development of a sea spider species. Male specimens of *Nymphon australe* carrying postembryonic instars were collected from localities off the South Shetland Islands, the Weddell Sea, the Drake Passage, the Bransfield Strait, and the Ross Sea. Three subsequent free-living instars were collected from Drake Passage. *Nymphon australe* hatch as a postlarval instar with two-articled palps and limb buds of the two first-walking legs. Postlarval instars 1 and 2 (both carried on the ovigers of males), postlarval instars 3 and 4, and a juvenile (all three free living) are all described and illustrated. A molecular phylogenetic based on the *cox1* gene links all free-living stages to *N. australe* populations from the Antarctic Peninsula. The postlarval development of *N. australe* is discussed here and compared with those known from other pycnogonid species with similar development pathways.

Keywords Pycnogonida · Nymphonidae · *Nymphon australe* · Postembryonic development · Postlarval instar · *cox1* · Molecular analyses

Introduction

The pycnogonids, or sea spiders, consist of about 1350 described species (Bamber and El Nagar 2018). They are distributed worldwide, inhabiting all marine benthic environments from intertidal zones to abyssal depths, ranging in size from less than 1 mm to over 70 cm in leg span (Hedgpeth 1947; Arnaud and Bamber 1987). They are particularly diverse and abundant in the Southern Ocean (Child 1995). The family Nymphonidae is the largest and most abundant pycnogonid family in Antarctic and Sub-Antarctic seas (71 species), the most frequently recorded species being *Nymphon australe* Hodgson 1902 (Munilla and Soler-Membrives 2009). This species has a circumpolar distribution, a condition that seems counterintuitive, given the supposedly limited dispersal capabilities of benthic brooding species (Mahon et al. 2008).

Developmental modes in pycnogonids are highly variable, even between congeneric species (Brenneis et al. 2017). Eggs usually hatch as a larval instar known as "protonymphon", characterized by a proboscis and three pairs of larval appendages; in some groups, eggs can also hatch directly as a postlarval instar (PI) (Bamber 2007). The subsequent postembryonic development (PD) is relatively little known, especially in species from Antarctic and Sub-Antarctic seas. The earliest descriptions of PD in pycnogonids were made in

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the late 1800s and early 1900s (Dohrn 1881; Dogiel 1913). But in recent decades, PD studies have increased significantly in number and comprehensiveness (e.g. Bogomolova and Malakhov 2003, 2004, 2006; Bogomolova 2007, 2010; Cano-Sánchez and López-González 2009, 2010, 2013; Mercier et al. 2015; Alexeeva et al. 2017, 2018; Brenneis and Arango 2019). Bain (2003a) summarized five different types of PD in pycnogonids distinguished by different authors: four of them have a protonymphon larva as first instar, but differ in their developmental pathways and morphological features during the subsequent postlarval phases; and the fifth type directly hatches as a PI (see Bogomolova and Malakhov 2003, 2004, 2006; Bogomolova 2007, 2010; Burris 2011; Brenneis et al. 2017).

Most of the previous contributions were based on morphological descriptions of the larvae and PIs still retained on male ovigers, but the rest of their free-living instars were largely unknown. The complete life cycle, including all developmental instars, is known in very few species and all of them were investigated in laboratory conditions (Nakamura 1981; Tomaschko et al. 1997; Vilpoux and Waloszek 2003; Brenneis et al. 2011a; Mercier et al. 2015; Alexeeva et al. 2018). The attribution to a given species of different immature specimens directly collected in natural habitats is difficult, due to our lack of knowledge about morphological changes through successive moults. Molecular analysis could therefore be a useful tool for linking different morphological instars where morphology of developmental stages alone cannot suffice to delimit species.

Here, we present four postlarval and one juvenile instars of *N. australe*, an Antarctic pycnogonid species directly hatching as PI. To date, knowledge of the life cycle of this species has been limited to early postembryonic stages, when instars are still found on the ovigers of the male (Fornshell 2019). We discovered the two first PIs on the ovigers of males, whereas the two subsequent postlarval and the juvenile instar were free living. We applied a barcoding approach to tests species identity, using *cox1* for molecular comparison. Each instar is described and illustrated and compared to the corresponding instars of species with similar patterns of PD.

Materials and methods

Male specimens of *N. australe* Hodgson 1902, carrying eggs and postlarvae of various instars of development, were collected at the South Shetland Islands and Elephant Island during Polarstern ANT-XXIII/8 cruise, at the Ross Sea during the R/V Italica XIX Spedicione, and at the South Shetland Islands, Weddell Sea, Drake Passage, and Bransfield Strait during Polarstern ANT-XXIX/3 cruise. During this last cruise, we also collected free-living postlarval and juvenile specimens at Drake Passage.

The specimens collected during the R/V Italica and Polarstern ANT-XXIII/8 cruises were fixed in 10% buffered formalin in seawater and then transferred to 70% ethanol, whereas the specimens collected during the Polarstern ANT-XXIX/3 cruise were fixed in ethanol 70%. Sampling, voucher information, and deposition data in different museum repositories are given in Table 1 for all males, postlarvae, and juvenile material studied herein. The specimens examined have been deposited in the Museu de Ciències Naturals in Barcelona, Spain (MZB, formerly Museu de Zoologia de Barcelona) and the reference pycnogonid's collection of the research group "Biodiversidad y Ecología Acuática" (BECA-CRP) of the Faculty of Biology at the University of Seville in Spain.

Selected postlarvae and juveniles were stained with Chlorazole black E (Sigma® C-1144), dissected in lactic acid, and examined as temporary mounts in lactophenol. All figures were drawn with the aid of a camera lucida on a Leica DMLB differential interference microscope. Body length measurement was considered the distance between the posterior body end and the bases of the chelifores. In order to detect minute details, some specimens were prepared for SEM study, dehydrated in an alcohol series from 70 to 100%, subsequently critical point dried, mounted on stubs, coated with gold–palladium, and observed and photographed with a Philip XL30 SEM.

The nomenclature used in the description of the PIs mainly follows previous works (e.g., Bain 2003b; Bogomolova 2007; Bamber 2007; Cano-Sánchez and López-González 2010, 2013; Brenneis et al. 2017, among others). In order to match with these published descriptions, we do not consider the terminal claw when the walking leg's article number is indicated. We have adopted the walking leg article nomenclature proposed by Cano-Sánchez and López-González (2010).

Species assignation of free-living postlarvae and juvenile instars was done by a molecular comparison of the *cox1* gene sequences.

Total genomic DNA was extracted from two EtOH-preserved specimens of each instar (PI 3, PI 4, and juvenile), using the Qiagen DNEasy Blood and Tissue kit, following the manufacturer's instructions. The mitochondrial *cox1* region was amplified using the primers LCO and HCO (Folmer et al. 1994). Each PCR used 1 U of Dynazyme II Taq polymerase, 0.2 mM dNTPs, 10 µM of each primer, and approximately 30 ng of genomic DNA and was brought to a final volume of 25 µL with H_2O. PCR was carried out using the following cycle profile: initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 48 °C for 30 s, and extension at 72 °C for 60 s, and a final extension at 72 °C for 10 min. PCR

Table 1 Museum codes and sampling data from *Nymphon australe* specimens

Museum code	Area	Cruise	Station	Gear	Position (lat.–long.)	Depth (m)	Date
MZB 2019-1151 (2 ovigerous males) BECA-CRP-100 (1 ovigerous male)	South Shetland Islands	*Polarstern* ANT-XXIII/8	678–1	Bottom trawl	62°19.36′S 60°27.10′W	108.9–128.8	2 January 2007
MZB 2019-1152 (2 ovigerous males)	South Shetland Islands	*Polarstern* ANT-XXIII/8	679–1	Bottom trawl	62°23.84′S 60°48.79′W	87.1–91.3	2 January 2007
MZB 2019-1153 (1 ovigerous male)	South Shetland Islands	*Polarstern* ANT-XXIII/8	676–1	Bottom trawl	62°11.06′S 60°47.49′W	418–472.4	2 January 2007
MZB 2019-1154 (1 ovigerous male)	South Shetland Islands	*Polarstern* ANT-XXIII/8	680–4	Agassiz trawl	62°24.35′S 61°23.77′W	346	2 January 2007
BECA-CRP-101 (1 ovigerous male)	South Shetland Islands	*Polarstern* ANT-XXIII/8	680–5	Agassiz trawl	62°23.37′S 61°25.58′W	324.3–348.8	2 January 2007
MZB 2019-1155 (1 ovigerous male)	Elephant Island	*Polarstern* ANT-XXIII/8	608–1	Bottom trawl	61°11.34′S 54°43.17′W	284.2–293	20 December 2006
MZB 2019-1156 (1 ovigerous male)	Elephant Island	*Polarstern* ANT-XXIII/8	634–1	Bottom trawl	61°0.98′S 55°56.23′W	274.6–278.3	25 December 2006
BECA-CRP-102 (1 ovigerous male)	Victoria Land (Ross Sea)	Italica XIX	H-in 2	Rauschert dredge	72°16′.9 S 170°12.2′E	388–391	10 February 2004
BECA-CRP-103 (1 ovigerous male)	Victoria Land (Ross Sea)	Italica XIX	C2	Agassiz trawl	73°22.7′S 170°06.9′E	408–410	18 February 2004
MZB 2019-1157 (1 ovigerous male)	South Shetland Islands	*Polarstern* ANT-XXIII/8	680–1	Bottom trawl	62°24.13′S 61°24.06′W	336.3–340.6	2 January 2007
MZB 2019-1158 (1 ovigerous male)	Drake Passage	*Polarstern* ANT-XXIX/3	246–3	Agassiz trawl	62° 0.23′S 60° 3.81′W	260–266	11 March 2013
MZB 2019-1159 (11 ovigerous male)	Weddell Sea	*Polarstern* ANT-XXIX/3	162–7	Agassiz trawl	63° 58.78′S 56° 46.24 ‘W	213.9–215.6	10 February 2013
MZB 2019-1160 (1 ovigerous male)	Bransfield Strait	*Polarstern* ANT-XXIX/3	227–2	Agassiz trawl	62° 55.83′S 58° 41.09′W	562–564	5 March 2013
MZB 2019-1161 (1 ovigerous male)	Bransfield Strait	*Polarstern* ANT-XXIX/3	118–4	Agassiz trawl	62° 25.95′S 56° 17.26′W	434.4–437	27 January 2013
MZB 2019-1162 Postlarval instars 3–4 and juveniles	Drake Passage	*Polarstern* ANT-XXIX/3	240–2	Rauschert dredge	62° 6.92′S 60° 33.86′W	277–278	9 March 2013

MZB Museu de Ciències Naturals in Barcelona (formely Museu de Zoologia de Barcelona) (Spain), *BECA-CRP* Biodiversidad y Ecología Acuática (Seville, Spain)

products were purified using the Qiagen PCR Purification Kit, following the manufacturer's instructions. Purified products were electrophoresed on an ABI PRISM® 3730xl Genetic Analyzer, and sequence traces were edited using Geneious R9. *Cox1* sequences obtained for this work that have been deposited in GenBank with the accession numbers MN954930–MN954938.

Available sequences of *Pentanymphon antarcticum* (EU14400, EU140376, and EU140351) and 14 species of *Nymphon* were used as outgroup taxa, following the result of Mahon et al. (2008). The alignments of the different sets of sequences were carried out using MUSCLE v. 3.8.31 (Edgar 2004). The *cox1* dataset consisted of 266 terminals: 263 *Nymphon* spp. (of which 226 were *N. australe*) and 3 *P. antarcticum*. After trimming of overhanging ends, the final alignment had 554 positions, with 37 parsimony informative sites. Maximum likelihood (ML) analysis was performed in IQ-TREE ver. 1.6.12 (Nguyen et al. 2015), with automated detection of the best-fitting nucleotide substitution model under the Bayesian Information Criterion (BIC). The best-fitting model selected was the TIM2 + F model with corrections for rate heterogeneity (Γ_4) and invariant sites (I), and tree searches were performed using the default heuristic intensity and 1000 ultrafast bootstrap replicates for estimation of nodal support (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017; Hoang et al. 2018). For visualization of the

haplotype network, the multiple sequence alignment was culled to retain only *N. australe* terminals ($n = 226$). The haplotype network of *N. australe* was inferred using PopART (Population Analysis with Reticulate Trees) under a TCS (statistical parsimony) framework (Clement et al. 2002; Leigh and Bryant 2015). The multiple sequence alignment, ML tree topology with nodal support values, and TCS network are provided as Online Resource 1–3, respectively.

Results

Ten males of *N. australe* discovered as carrying postlarvae were collected during the Polarstern ANT-XXIII/8 cruise; fifteen such males were collected during the Polarstern ANT-XXIX/3 cruise; and two such males were collected during Italica XIX cruise. Additionally, free-living specimens (17 PIs and 17 juveniles) were collected during ANT-XXIX/3 cruise. This latter material was attributed to *N. australe* after the molecular analysis above was described.

We distinguished eggs and two different postlarval developmental instars (1 and 2) attached to the ovigers of *N. australe* males. During these successive instars, the structure and number of walking legs increases. Legs of PI 1 appear as primordial limb buds, but in the next instar they are substituted by articled appendages and an additional limb bud appears. In this second instar, the first pair of walking legs has seven articles, the second pair has six articles, and third pair has three articles. Morphological variations were not observed between specimens collected from different Antarctic areas.

We distinguished two free-living PIs (PI 3 and PI 4) of *N. australe*, and we infer them to be instars at the next stage of development from those found on the ovigers of males. During the development of these successive instars, the number of walking leg and oviger articles gradually increases.

Description of postembryonic instars of *Nymphon australe*

Postlarval instar 1 (Figs. 1, 2, 3a, b, 4a)

The average length of this instar is 0.89 mm (range 0.8–0.99 mm), based on 10 specimens. It is characterized by an egg-shaped body, a proboscis, two pairs of larval appendages, a pair of distinctly elongated processes of walking legs 1 and 2, and a small elevation of walking legs 3. Eyes and anus are absent.

The body surface is practically smooth, with scattered multi-furcated sensilla. The body is externally unsegmented

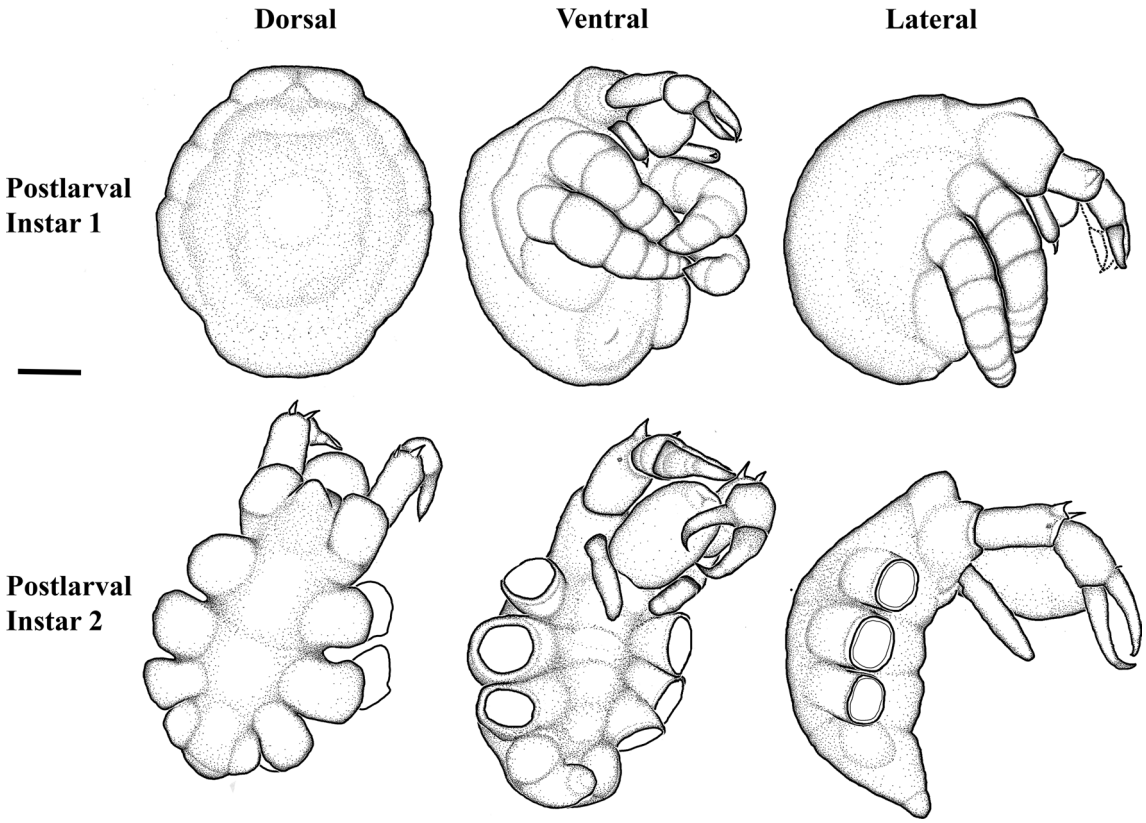


Fig. 1 Postlarval instar (PI) 1 and 2 of *Nymphon australe*. Dorsal, ventral, and lateral view of PI 1 and PI 2. Scale bars: 0. 2 mm

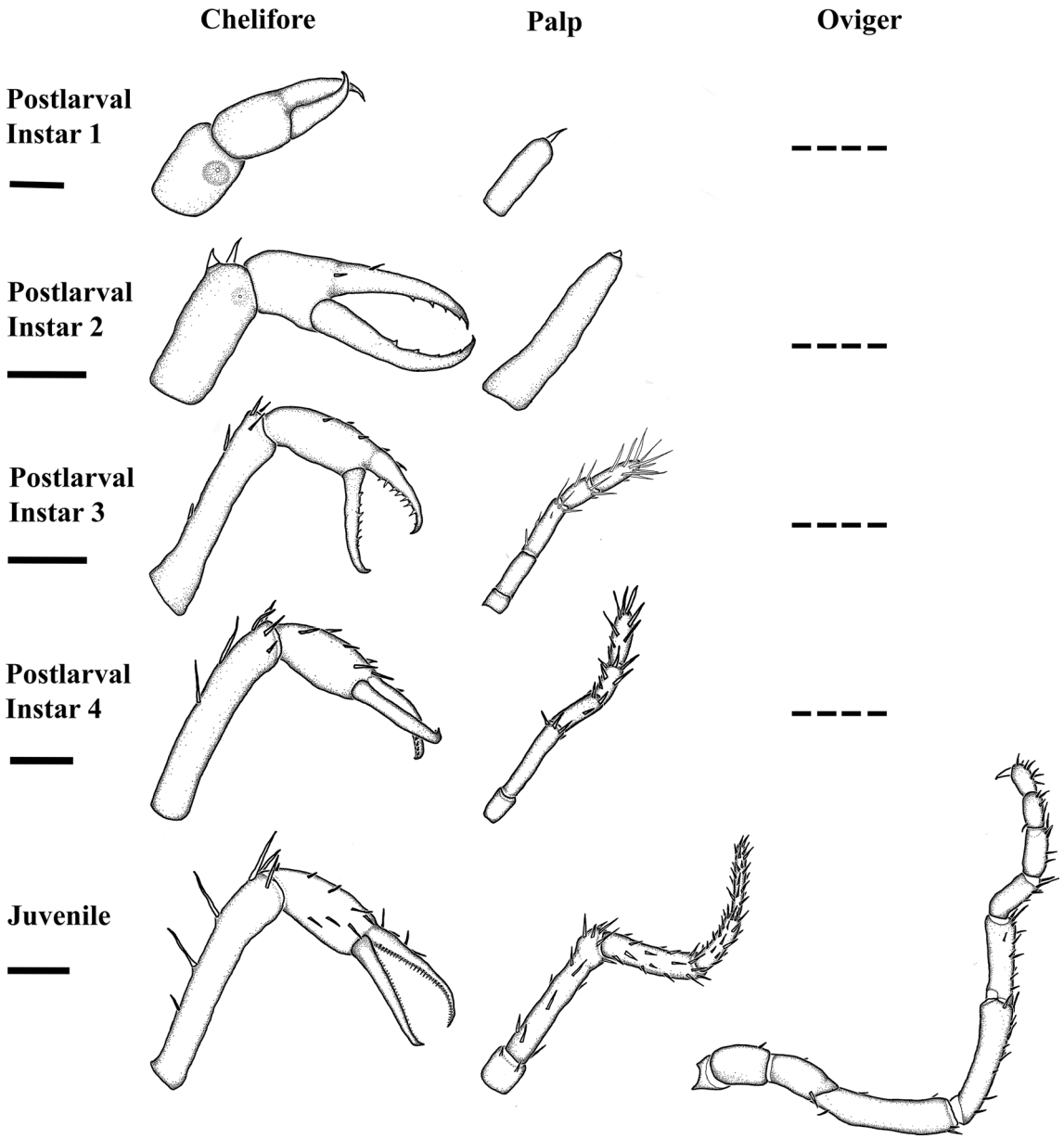


Fig. 2 Chelifores and palps of the described postembryonic instars of *Nymphon australe*. Scale bars: 0.1 mm for Postlarval instar (PI) 1 and 2.5 mm for PI 2, PI 3, PI 4, and PI 5

(Fig. 3b). The proboscis is short, cylindrical, and shorter than the chelifores.

The basal article of the chelifores (appendage pairs I) is shorter than the chelae and bears (on the outer laterodistal margin) a short process with a distal pore of the spinning gland. The fingers are curved at the tip and they bear a few short denticles on the inner surfaces (Figs. 2, 4a).

The palpal limbs (appendage pairs II) have two articles. The first article is cylindrical, similar to proboscis length. The second article is short and claw like.

Appendage pair III (ovigers) is absent.

Walking legs 1 and 2 are unarticulated.

The walking legs 3 are a swollen area posterior to the second walking legs. The posterior region of the body has a swollen area corresponding to the future abdomen.

3.1.2. Postlarval instar 2 (Figs. 1, 2, 5)

The average length of this instar is 0.98 mm (range 0.91–1.02 mm), based on 10 specimens. It is characterized by an enlarged body; the walking legs are seven articled (first), six articled (second), and three articled (third). The fourth pair of walking legs is represented by the presence of a pair of limb buds.

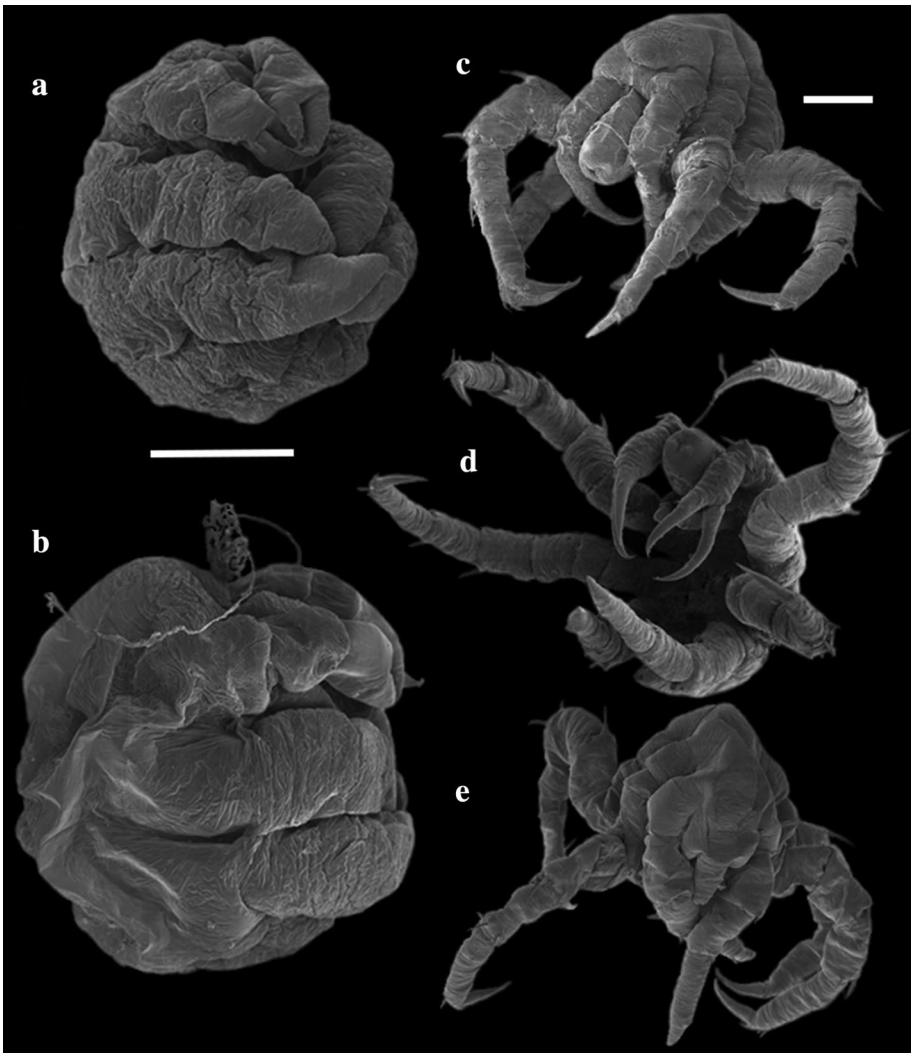


Fig. 3 Postlarval instar (PI) 1 and 2 of *Nymphon australe*. SEM photographs: **a** PI 1, just emerged, latero-ventral view. **b** PI 1, lateral view. **c** PI 2, latero-frontal view. **d** PI 2, ventral view. **e** PI 2, dorso-posterior view. Scale bars 200 µm

The body surface is practically smooth, with scattered simple or bifurcated sensilla. The intersegmental suture between the first and second walking leg segments is slightly defined. Dorsally, the incipient ocular tubercle is distinct (Fig. 3c).

The proboscis is shorter than the chelifores. The basal article of the chelifores is shorter than the chelae, has two spines on the dorso-distal part, and bears a slight elevation on the latero distal part bearing the pore of the spinning gland. The fingers are curved at the tip and they bear several denticles on the inner surfaces; two short spines are present on the outer surface of the immovable finger.

The palpal limbs are larger and elongated, somewhat similar to those described for the previous instar with the second article distinctly reduced.

The appendage III pair (ovigers) is absent.

The first pair of walking legs has seven articles. Coxae I to III are short. Coxae II and III have a ventral spine. The femur and tibia I have three dorso-distal spines. The tibia II and tarsus-propodus have three dorso-distal spines and a short ventro-distal spine. The claw is shorter than the tarsus-propodus article. Short auxiliary claws are present (Fig. 4d).

The second pair of walking legs has six articles. It is similar to first pair of walking legs, except for the presence of a combined femur-tibia I article with three dorso-distal spines and a short dorso-medial spine.

The third pair of walking legs has three articles. The first and second articles have a short ventro-distal spine and a short dorso-distal spine. The third article is the longest and is pointed, with a dorso-distal spine.

The 4th trunk segment bears two lateral swollen areas corresponding to the primordia of the fourth pairs of walking legs, and an incipient abdomen is distinguishable (Fig. 4b). The anus is closed (Fig. 4c).

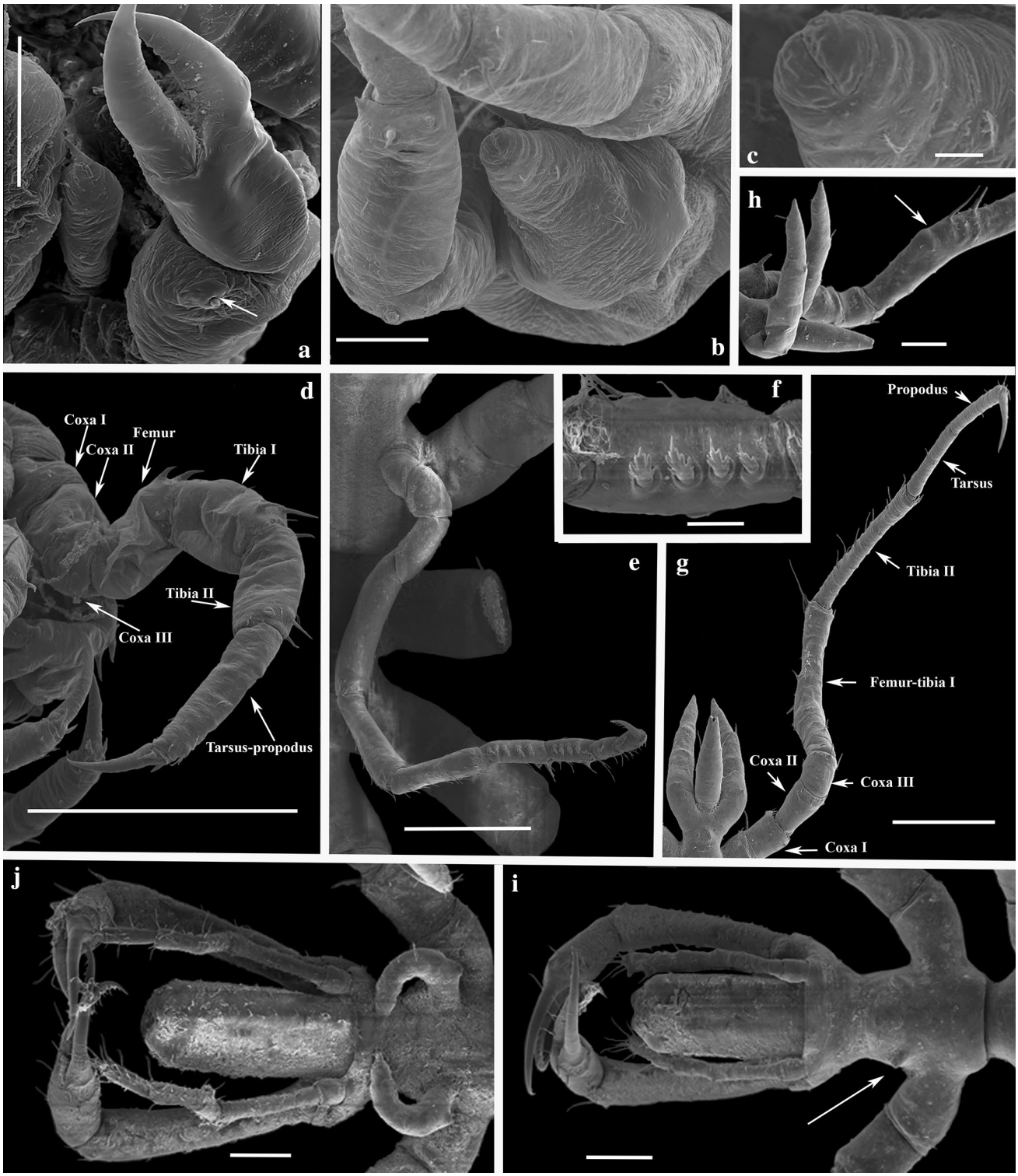


Fig. 4 Postlarvae instars (PI) and juvenile of *Nymphon australe*. SEM photographs: **a** Short process with the distal pore (arrow) of the spinning gland on laterodistal surface of basal segment of chelifore and palpal limb, PI 1. **b** Incipient abdomen and the primordial of the fourth pair of walking legs, PI 2. **c** Detail from **b**, terminal portion of the abdomen. **d** First pair of walking legs (articles are indicated), PI 2. **e** Ovigeral appendage, juvenile. **f** Denticulate spines on the inner surface of the strigilis article. **g** Third pair of walking legs (articles are indicated), ventral view, PI 3. **h** Fourth pair of walking legs (unarticled) and firsts articles of third pair of walking legs (arrow indicates the limit of the future suture between femur and tibia I), PI 3. **i** Anterior body part, ventral view, showing the oviger as tiny cuticular elevation (arrow), PI 3. **j** Anterior body part, ventral view, showing the unarticulated limb buds of the ovigers, PI 4. Scale bars: **a**, **b** 100 µm; **c** 20 µm; **d**, **e**, **g** 500 µm; **h**–**j** 200 µm; **f** 50 µm

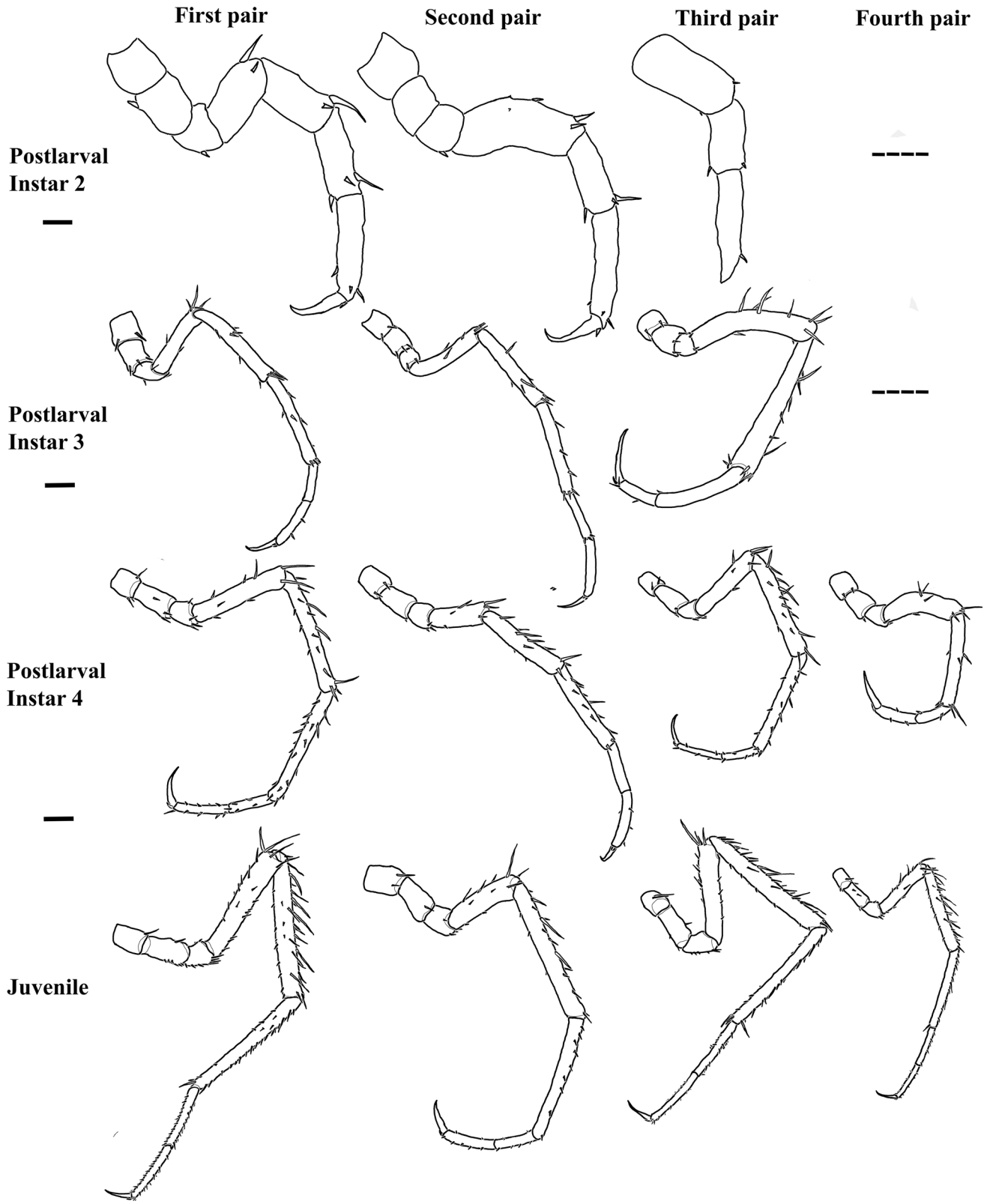


Fig. 5 Walking legs of the postlarvae instars (PI) and juvenile of *Nymphon australe*. Scale bars: 0.1 mm for PI 2, 0.2 mm for PI 3, and 0.4 mm for PI 4 and juvenile 5

Postlarval instar 3 (Figs. 2, 4g–i, 5, 6)

The average length of this instar is 1.24 mm (range 1.18–1.35 mm), based on eight specimens. It is characterized by an enlarged body; the first and second walking legs are eight articled; the third walking legs are seven articled; the fourth walking legs are unarticulated; the ocular tubercle is tall, with distinct eyes; and the abdomen bears an open anus.

The lateral processes and legs have long spines. The intersegmental suture lines between walking leg segments are distinctly delimited. The lateral processes are widely separated, with one to two long dorso-distal spines. The

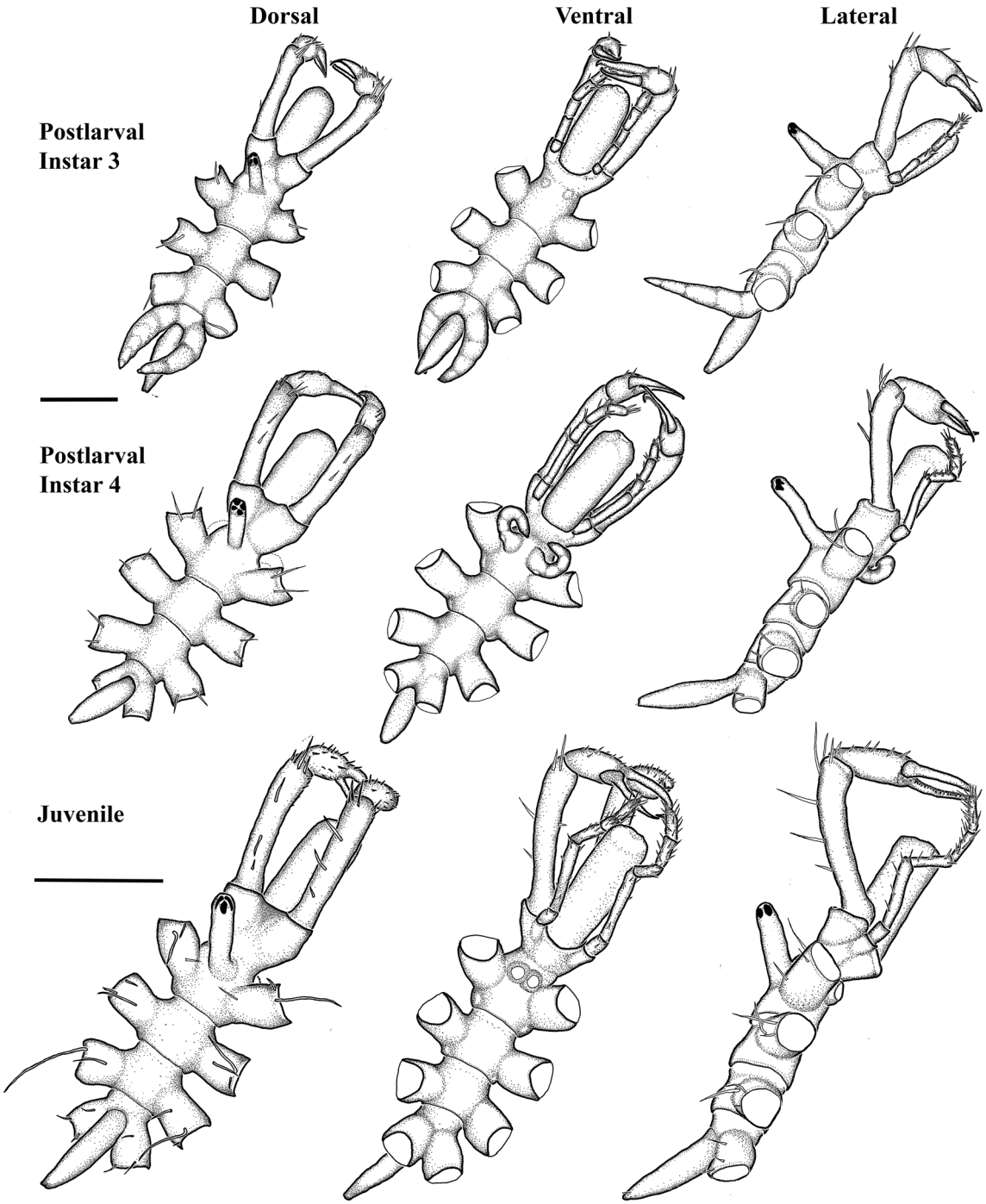


Fig. 6 Postlarval instar (PI) 3, 4, and juvenile of *Nymphon australe*. Dorsal, ventral, and lateral view of the postlarval instar 3, 4, and 5. Scale bars: PI 3 and PI 4 0.5 mm; juvenile 1.2 mm

ocular tubercle is a tall cylinder and the four eyes are at the top of the tubercle.

The proboscis is shorter than the chelifores and nearly straight. The basal article of the chelifores is similar in length to the chelae, it has three short dorso-distal spines and two short dorso-medial spines. The fingers are curved at the tip and they bear numerous short denticles on the inner surfaces, several spines are present on the outer surface of the immovable finger and on the palm.

Appendage pair II (palps) has five articles, the number of spines increasing from the third to the fifth, and it is longer than the proboscis.

Appendage pair III (ovigers) consists of tiny cuticular elevations (Fig. 4i).

The first pair of walking legs has eight articles, with scarce long spines, which are mainly on the three longest articles. Coxae I to III are short. The first and second tibia are similar in length. The tarsus is shorter than the propodus. The claw is about half of the propodus length. The auxiliary claws are present and short.

The second pair of walking legs has eight articles, and it is ,in general, of similar structure to the first pair of walking legs.

The third pair of walking legs has seven articles, and it is ,in general, of similar structure to the two previous walking leg pairs. There are long spines on the two longest articles, mainly on the dorsal surface. Femur-tibia I is curved and longer than tibia II.

The fourth pair of walking legs is unarticulated and upward directed, with three short spines along the ventral surface and a dorso-distal spine (Fig. 4g, h).

The abdomen is developed, straight, with two short lateral spines in the distal third. The anus is open.

Postlarval instar 4 (Figs. 2, 4j, 5, 6)

The average length of this instar is 1.52 mm (range 1.48–1.58 mm), based on eight specimens. It is characterized by an enlarged body, the first, second and third walking legs are eight articled, and fourth walking legs are seven articled; the ovigers are distinct and unarticulated.

The lateral processes and legs have long spines. The intersegmental sutures between walking leg segments are distinctly delimited. The ocular tubercle is a tall cylinder and the four eyes are at the top of the tubercle.

The proboscis is shorter than the chelifores and is nearly straight. The basal article of the chelifores has several long spines on the dorsal surface, more numerous distally. The fingers are curved at the tip and they bear numerous short denticles on the inner surfaces, several spines are present on the outer surface of the immovable finger, and on the palm.

Palps are similar to those described for the previous instar.

The limb buds of the ovigers are unarticulated, hook shaped, and have a prominence on the external surface basally (Fig. 4j).

The first pair of walking legs has eight articles, with long spines, especially on the dorsal surface of the three longer articles. Tibia I and II are subequal in length, they are the longest articles of the walking leg. The tarsus is shorter than the propodus. The claw is longer than half propodus length. Short auxiliary claws are present.

The second and third pair of walking legs also have eight articles and are similar to the first pair of walking legs.

The fourth pair of walking legs has seven articles, with fewer spines than the previous walking legs. Femur-tibia I has long spines on the distal half.

The abdomen is directed upward.

Juvenile (instar 1?) (Figs. 2, 4e, f, 5, 6)

The average length of this instar is 2.43 mm (range 2.1–2.56 mm), based on 8 specimens. It is characterized by an enlarged body; the four pair of walking legs are eight articled; and the ovigers are ten articled.

The body, legs, proboscis, chelifores, and palps are similar in structure to those for the previous instar.

The ovigers are ten articled, plus the terminal claw. The distal most four articles forms a strigilis, each of them bears a row of denticulate spines on the inner surface (Fig. 4e, f). The terminal claw bears a row of tiny denticles on its inner surface.

Species assignation and phylogenetic analyses

All our obtained sequences (two PI-3, three PI-4, and four juvenile specimens) were explored using BLAST and showed unambiguous hits to sequences of *N. australe*. Moreover, the ten closest sequences (100% sequence coverage and sequence identity 100–99.49%) were specimens from Antarctic Peninsula and South Shetland Islands collected in 2004 and 2006 aboard the *ASRV Laurence M. Gould* (stations 57, 117, 123, and 137) (see Mahon et al. 2008: Supplementary Table 1). In a recent and detailed work on population relationships of *N. australe* around Antarctica, Soler-Membrives et al. (2017) delimited 85 *cox1* haplotypes. Our BLAST searches suggested that the juvenile specimens described in this work were related to several of these haplotypes. Specifically, our sequences were inferred to be highly similar to nine haplotypes exclusive to Antarctic Peninsula (see Soler-Membrives et al. 2017, haplotypes 3, 15–17, 20, 22, and 26–28), one haplotype present in Antarctic Peninsula and Eastern Antarctica (haplotype 23), and three haplotypes exclusive from Eastern Antarctica (haplotypes 40, 74, and 79). Consistent with this outcome, the broad biogeographic areas inhabited by these haplotypes are coincident with the

collecting data of our sequenced specimens. These outcomes not only support the assignation of our free-living specimens to the species *N. australe*, but also suggest (tentatively, due the low resolution of basal clades), a South Shetland Island and Antarctic Peninsula origin.

Phylogenetic analysis of 266 Nymphonidae *cox1* sequences recovered the juveniles as nested among the 226 N. *australe* sequences previously sequenced in comprehensive phylogeographic surveys (Mahon et al. 2008; Soler-Membrives et al. 2017) (Fig. 7a). The haplotype network including the new specimens recovered the same population genetic architecture previously mentioned by different authors within *Nymphon* (Mahon et al. 2008; Arango et al. 2011) (Fig. 7b). Specifically, upon coding localities using the biogeographic areas defined by Soler-Membrives et al. (2017), we reproduced similar distributions and connectivity of haplotypes in the network (compare to Fig. 6 of Soler-Membrives et al. 2017), with the juvenile specimens occupying the same part of the network as other Antarctic Pensinsula specimens. Notably, three of the juveniles we sequenced were inferred to constitute new haplotypes (Fig. 7b). We additionally note that one of the two sequences corresponding to the species *Nymphon paucituberculatum* Gordon, 1944 (GenBank accession numbers EU140352-3), was recovered as nested within *N. australe*; we are presently unable to evaluate whether these terminals represent misidentified semaphoronts of *N. australe* or the lack of resolution provided by *cox1* in species delimitation.

Discussion

Postembryonic development

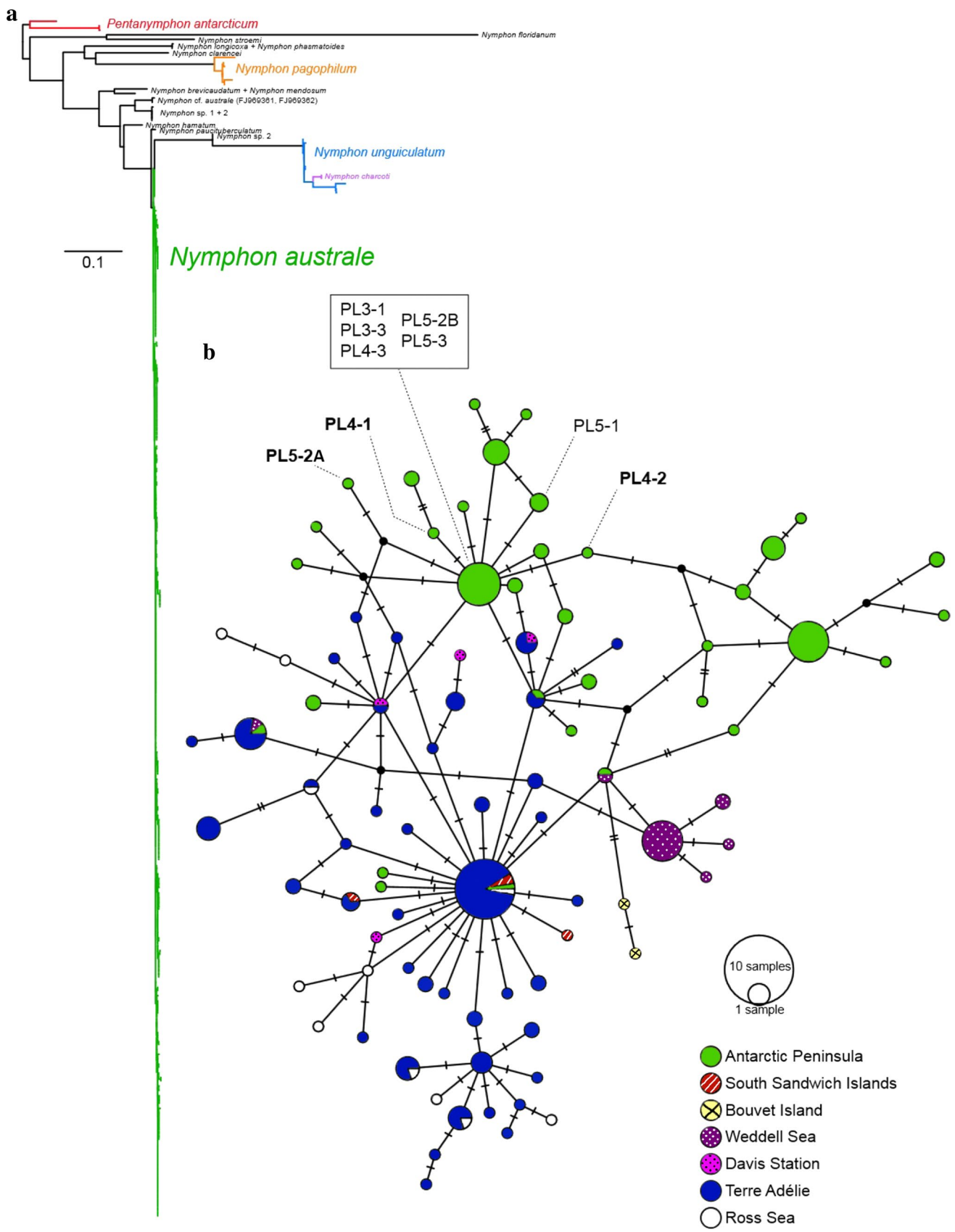
Much of our knowledge of PD in Pycnogonida comes from the study of those species and genera in which larval and postlarval instars are retained on ovigers of brooding males. Species assignation of free-living postembryonic instars is difficult because species diagnoses are based on adult features. These free-living instars are known in only a few species: *Pseudopallene* sp., where males carrying postembryonic instars and free-living postembryonic instars all inhabit bryozoan colonies (Brenneis et al. 2011a); and *Pycnogonum litorale* (Ström, 1762) and *Nymphon hirtipes* Bell, 1955, where all instars were cultured in a laboratory (see Tomaschko et al. 1997; Wilhelm et al. 1997; Vilpoux and Waloszek 2003; Mercier et al. 2015). In this work, we applied a molecular phylogenetic (barcoding) toolkit for assigning free-living PI or juvenile specimens to species.

In some studies, larval instars are all those immature stages without full development of the walking legs (Okuda 1940; Behrens 1984; Vilpoux and Waloszek 2003; Bain 2003a, b; Bogomolova and Malakhov 2003, 2006; Gillespie and Bain 2006; Bogomolova 2007; Cano-Sánchez and López-González 2009; Burris 2011). However, other works treat a single instar as larval (the protonymphon) and successive stages as PIs, wherein walking legs are progressively developed (Bamber 2007; Cano-Sánchez and López-González 2010, 2013; Brenneis et al. 2017; Brenneis and Arango 2019). The latter criterion is adopted in this study.

Different proposals have been presented to characterize PD in Pycnogonida. The criteria used to account for PD emphasize different facets of development. Some proposals considered only the larval life history (Dogiel 1913; Burris 2011) or a combination of the first postembryonic instar, the larval life history, and the sequence in which the walking legs appear (Bain 2003a; Bogomolova and Malakhov 2003, 2004, 2006; Bogomolova 2007; Fornshell 2015). These criteria are still under discussion and can be difficult to apply, given knowledge gaps in the natural history of various pycnogonid species. Recently, Brenneis et al. (2017) have redefined the developmental pathways based on (1) the type and morphology of the hatching instar, (2) the morphological characteristics during subsequent PD, and (3) the selected life history features. These authors have proposed five pathways of PD. Following this last criterion, *N. australe* corresponds to type 5, characterized by hatching as an advanced postlarva.

However, even within Nymphonidae, there are different PD pathways described, most of which hatch as protonymphon larvae. According to Brenneis et al. (2017), PD type 1 includes the following: *Nymphon gracile* Leach, 1814 (see Sánchez 1959), *Nymphon brevirostre* Hodge, 1862 (see Bogomolova 2007; Alexeeva et al. 2017, 2018), *Nymphon micronyx* Sars, 1888 (see Bogomolova 2007), and *Nymphon charcoti* Bouvier, 1911 (see Fornshell 2017); PD type 2: *Nymphon grossipes* (Fabricius, 1780) (see Bogomolova and Malakhov 2003, 2006; Bogomolova 2007), *Nymphon macronyx* Sars, 1877 (see Bogomolova 2010), *N. unguiculatum* Hodgson, 1915 (see Cano-Sánchez and López-González 2010), and *N. hirtipes* (see Mercier et al. 2015). PD type 5 has been reported as personal observations by Brenneis et al. (2017) and shown as general SEM images of the stages attached to the male oviger by Fornshell (2019), or indirectly pointed out by Hoek (1881), who described and illustrated the embryonic development of *Nymphon brevicaudatum* Miers, 1875. Thus, our study represents one of the most complete, illustrated descriptions of PD type 5 in the family Nymphonidae.

Fornshell (2019) has briefly described the PI attached to the male oviger. His PI 2 has the first two walking legs seven articled and a third walking leg with four articles. The discrepancies observed with our study (Table 2) are probably due to the methodology used. Fornshell considered as article divisions those cuticle constrictions observed in SEM images of walking legs. However, in our study not all



◂**Fig. 7** **a** Phylogenetic tree of Nymphonidae inferred by maximum likelihood under a TIM2 + F + Γ_4 + I model. All postembryonic stages analysed in this study were recovered within *Nymphon australe* (green). **b** Haplotype (TCS) network of *N. australe* including newly sequenced postembryonic stages. Collecting localities are indicated by colours (following legend in bottom right). Boldface text indicates unique haplotypes represented only by postembryonic stages. (PL3 and PL4: Postlarval Instars 3 and 4; PL5: Juvenile Instar)

these possible constrictions on SEM images are considered as true article divisions, supporting these observations with temporary mounts for OM observations. In non-dehydrated specimens, it is possible to observe by transparency the segmentation of walking legs of the next instar, and that can be a source of misinterpretation.

Delimiting postembryonic instars

One of the criteria considered to mark the transition from the last PI to the first juvenile instar is based on the establishment of the full number of functional walking legs (even when some of them have not completed their full complement of articles) (Tomaschko et al. 1997; Wilhelm et al. 1997; Brenneis et al. 2017). A second criterion for defining this transition holds that it occurs when all appendages reach the complete external structure of adult forms (in this case also including palps and ovigers, if present) (Brenneis et al. 2011a). A third criterion considers only the establishment of the full number and full article endowments of functional walking legs as the threshold for the transition (Vilpoux and Waloszek 2003). This last criterion is followed herein for *N. australe*, and consequently this species presents four PIs.

Brenneis et al. (2011a) pointed out for *Pseudopallene* sp. that when a walking leg reaches a seven-articled configuration during instar development, the tarsus and propodus are already separated, and a femur-tibia I article is present. On the other hand, according to the current knowledge, *Pallenopsis hodgsoni* Gordon, 1938 and *Pallenopsis villosa* Hodgson, 1907 have PI 1 with seven-articled walking leg 1, the femur and tibia are already separated, and the tarsus-propodus article is present (Brenneis and Arango 2019). The patterns observed in *N. australe* in this work are variable. Although the first-walking leg of PI 2 has seven articles, femur and tibia I are separated, but tarsus-propodus are not yet so (Figs. 4d, 5). However, in successive PIs, walking legs with seven articles exhibit a femur-tibia I and a separated tarsus and propodus (Figs. 4g, h, 5).

Brenneis et al. (2017), in their pathways of PD type 5, considered that the last instar before leaving the male's oviger is (at least) a postlarva with the two first pairs of walking legs being functional. Following this criterion, we have no reason to consider an intermediate instar between our observations on the instars here named PI 2 and PI 3 (Table 2). In *N. australe,* during the transition from PI 1 to PI 2, limb bud 1 moults to a seven-articled appendage, limb bud 2 moults to a six articled leg, and the swollen area of PI 1 results in a three-articled appendage in PI 2. This observed transition is slightly different to that described for other species of PD type 5 (see Table 2). The pattern of acquisition of successive walking leg articles in subsequent free-living PIs is similar to that in other described species of this type of PD (see Table 2).

The observed instar attributed herein to a juvenile stage of *N. australe* (following the abovementioned criteria) has the complete article endowment for the four pairs of walking legs, the palps, and the ovigers. However, we cannot, with certainty, assign this instar to the first or a subsequent juvenile instar because a direct transition (pertaining also to the number of oviger articles) was described for *Propallene longiceps* (Böhm, 1879) (see Nakamura 1981, and Table 2), and a transitional intermediate instar (six articled instead of 10-articled ovigers) was described for *Pseudopallene* sp. (see Bain 2003b; Brenneis et al. 2011a, and Table 2).

Phylogenetic considerations and the evolution of development

PD hatching as advanced postlarvae is described in all studied Callipallenidae (e.g. Hooper 1980; Nakamura 1981; Bogomolova and Malakhov 2003; Bain 2003b; Brenneis et al. 2011a, 2017), as they lack the larval instar (protonymphon) in their development, and their postembryonic moults are epimorphic (Brenneis et al. 2017). This PD has been also observed in some Pallenopsidae (Brenneis and Arango 2019) and in some Nymphonidae (Hoek 1881; Bogomolova, Brenneis, and Arango as a personal observation in Brenneis et al. 2017; Fornshell 2019).

According to different molecular studies (Bain 1992; Arango 2002; Arango and Wheeler 2007; Nakamura et al. 2007; Arabi et al. 2010; Brenneis et al. 2011b, 2017), callipallenids are recovered as a paraphyletic group due to the nested position of Nymphonidae. In this complex scenario, Brenneis et al. (2011b, 2017) suggested a plesiomorphic nature of the larval instar (protonymphon) compared with the apomorphic nature of a postlarval hatching instar. The presence of the apomorphic feature in a species of *Pallenopsis* (Pallenopsidae) (see Brenneis et al. 2017) reveals at least one convergent event in the evolution of pycnogonid hatching instars. Brenneis and Arango (2019) highlighted the fact that the PI 1 of some *Pallenopsis* species has functional palp and oviger larval appendages, in contrast to their lack of differentiation in callipallenid–nymphonids of PD type 5.

Several authors have considered that when eggs hatch as postlarval instars, the instar larva should have developed inside before hatching (Nakamura 1981; Bain 2003a, b; Bamber 2007). However, the observations carried out by Brenneis et al. (2011b) suggest that, at least in a species

Table 2 Summary of appendage structure in successive stages of postembryonic development of pycnogonids with type 5 postembryonic development (according to Brenneis et al. 2017)

	Postlarval instars				Juveniles	
Austropallene cornigera Möbius, 1902 (Bain 2003b)						
Chelifore (S.g.p.)	Present	Present	–	–		
Palp	–	–	–	–		
Oviger	–	–	Tiny elevation	Limb buds		
Walking legs						
1	Limb buds	8 articled	8 articled	8 articled		
2	Limb buds	6 articled	8 articled	8 articled		
3	Swollen area	Limb buds	6 articled	8 articled		
4	–		Limb buds	6 articled		
Pseudopallene sp. (Brenneis et al. 2011b)						
Chelifore (S.g.p.)	Present	Present	–	–	–	–
Palp	–	–	–	–	–	–
Oviger	–	–	Tiny elevation	Limb buds	6 articled	10 articled
Walking legs						
1	Limb buds	8 articled	8 articled	8 articled	8 articled	8 articled
2	Limb buds	6 articled	8 articled	8 articled	8 articled	8 articled
3	Swollen area	Limb buds	6 articled	8 articled	8 articled	8 articled
4			Limb buds	6 articled	8 articled	8 articled
Propallene longiceps (Nakamura 1981)						
Chelifore (S.g.p.)	n.d.	–	–	–		–
Palp	–	–	–	–		–
Oviger	–	–	Tiny elevation	Limb buds		10 articled
Walking legs						
1	Limb buds	8 articled	8 articled	8 articled		8 articled
2	Limb buds	7 articled	8 articled	8 articled		8 articled
3		Limb buds	7 articled	8 articled		8 articled
4			Limb buds	7 articled		8 articled
Nymphon austral (This paper)						
Chelifore (S.g.p.)	Present	Present	–	–		–
Palp	2 articled	2 articled	5 articled	5 articled		5 articled
Oviger	–	–	Tiny elevation	Limb buds		10 articled
Walking legs						
1	Limb buds	7 articled	8 articled	8 articled		8 articled
2	Limb buds	6 articled	8 articled	8 articled		8 articled
3	Swollen area	3 articled	7 articled	8 articled		8 articled
4		Swollen area	Limb buds	7 articled		8 articled
Pallenopsis hodgsoni (Brenneis and Arango 2019)						
Chelifore (S.g.p.)	Present	Present				
Palp	3 articled	3 articled				
Oviger	3 articled	2 articled				
Walking legs						
1	7 articled	8 articled				
2	Limb buds	8 articled				
3	Swollen area	Limb buds				
4		Swollen area				
Pallenopsis villosa (Brenneis and Arango 2019)						
Chelifore (S.g.p.)	Present					
Palp	3 articled					
Oviger	3 articled					

Table 2 (continued)

	Postlarval instars	Juveniles
Walking legs		
1	7 articled	
2	Limb buds	
3	Swollen area	
4		
Pallenopsis vanhoeffeni Hodgson, 1915 (Brenneis and Arango 2019)		
Chelifore (S.g.p.)		Present
Palp		3 articled
Oviger		3 articled
Walking legs		
1		8 articled
2		8 articled
3		Limb buds
4		Swollen area

Species, sources of information, and appendages (no. articles) are indicated

n.d. no data available, *S.g.p.* Spinning gland process

of Callipallenidae, there is no embryonized "egg-protonymphon" because of the absence of limbs of palpal and ovigeral and the advanced level of of the "post-protonymphon region". This last consideration raises the question of whether there is an embryonized egg-protonymphon in Nymphonidae or Pallenopsidae. Nymphon australe hatches as an advanced PI similar to Callipallenidae, but with the second larval appendage developed (two articled) and the third larval appendage absent, whereas Pallenopsis sp. possess all larval appendages fully developed. It is necessary to increase our knowledge of the embryonic development of pycnogonid species with PD type 5, in order to clarify these controversial questions.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals have been followed.

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