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Sexual dimorphism in Tripedaliidae (Conant 1897) (Cnidaria, Cubozoa, Carybdeida)

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Abstract

The family Tripedaliidae was re-defined and expanded based on a molecular phylogenetic hypothesis by Bentlage *et al.* (2010, *Proceedings of the Royal Society Biological Science,* 277: 497). Additionally, Bentlage *et al.* (2010) proposed that all members of the family Tripedaliidae present dimorphism in gonads and have structures that function as seminal vesicles (at least in males). Until now, no information on *Tripedalia binata* concerning gonad morphology, sexual dimorphism, spermatophore formation or structures that serve as seminal vesicles or spermathecae were published. We studied mature medusae of both sexes of *Tripedalia cystophora, Tripedalia binata* and *Copula sivickisi* in order to compare these structures in their stomach regions. We found sexual dimorphism and spermatophore formation in seminal vesicle-like structures in all three species. In particular, we show that along with the females of *Copula sivickisi,* the females of *Tripedalia cystophora* and *Tripedalia binata* also possess structures that store spermatophores and serve as spermathecae.

The results are in agreement with the morphological synapomorphies for Tripedaliidae outlined in Bentlage *et al.* (2010), but suggest an adjustment of the diagnosis of Tripedaliidae (underlined): All carybdeids that display sexual dimorphism of the gonads, produce spermatophores and in which males <u>and females</u> possess subgastral sacs, <u>pockets or purses</u> which <u>function as seminal vesicles or spermathecae</u>.

Key words: *Copula sivickisi*, cubomedusae, gonads, seminal vesicle, spermathecae, spermatophores, *Tripedalia binata*, *Tripedalia cystophora*

Introduction

Until the beginning of the 1970s, box jellyfish were classified as the order Cubomedusae in the class Scyphozoa (Haeckel 1880; Mayer 1910; Stiasny 1919; Thiel 1936; Kramp 1961). During the 1970's cubozoans were studied with a focus on the development, life cycle and morphology (Werner *et al.* 1971; Werner 1973a), culminating with the proposal of raising the order Cubomedusae to the class level among the cnidarians (Werner 1973b; 1975). The new class Cubozoa was established based mostly on the already well-established morphological distinctions—the cuboid shape of the umbrella, presence of pedalia and velarium, and the development of lens eyes in the rhopalia (e.g. Agassiz 1862; Haeckel 1880; Mayer 1910)—from the other groups (Hydrozoa and Scyphozoa) but additionally including life cycle observations from the species *Tripedalia cystophora* Conant, 1897 (Werner *et al.* 1971). Besides the presence of a polyp stage which undergoes complete metamorphosis instead of strobilation that is characteristic of Scyphozoa, the medusae of *Tripedalia cystophora* also display a mating behaviour, which at that time was considered unique among cnidarians.

Cubozoans are the earliest lineage among metazoans to present any kind of copulatory behaviour including internal fertilization. Among cubozoans only members of the family Tripedaliidae Conant, 1897 present sexual dimorphism in the areas where gametes are formed, produce spermatophores and (at least in males) possess subgastral sacs that serve as seminal vesicles (see Hartwick 1991; Bentlage *et al*. 2010).

Based on previously published studies, Bentlage *et al*. (2010) proposed that the synapomorphies uniting all members of the family Tripedaliidae are dimorphism in gonads and possession of seminal vesicles. These structures exist in both sexes of *Copula sivickisi* (Stiasny, 1926) (Bentlage *et al.* 2010; Straehler-Pohl 2011) and have thus far only been reported for males of *Tripedalia cystophora* (Werner 1976). We compared such structures from both males and females of all members of the family Tripedaliidae (i.e., *Copula sivickisi, Tripedalia binata* Moore, 1988 and *Tripedalia cystophora*) in order to provide further information about sexual dimorphism in cubozoans and especially in the least known species *Tripedalia binata.*

Material and methods

Specimens observed, culturing and documentation

We compared the anatomy of adult medusae of the three known tripedaliid species (*Copula sivickisi, Tripedalia cystophora* and *Tripedalia binata*) focusing on structures that are involved in sexual reproduction. Medusae of *Tripedalia cystophora* (n=10: 5 males, 5 females) were bred and reared in aerated aquaria (28°C, 30 psu) in the laboratory at the Marine Biological Section, University of Copenhagen, Denmark. The polyp stock originated partly from the stock culture established by Werner in 1971 and from larvae obtained from medusae collected by the second author from 8 gravid females in the mangrove near La Parguera, Puerto Rico in 2006.

We sampled 96 medusae (29 immature males, 23 mature males, 11 immature females, 5 mature females, 28 medusae of unidentifiable sex) of *Copula sivickisi* by using light traps and scoop buckets between 7 pm and 9 pm during August 2010 and 2012 in the harbour of Aka on Akajima Island (Japan) as described in Garm *et al.* (2012). The macro-anatomy (Figure 1) of all 96 medusae was examined in vivo but only 5 mature males and 5 mature females were preserved and dissected while the others were set free again after examination.

Type material of the species *Tripedalia binata* (holotype NTM C005858: female medusa; 11 medusae: paratypes NTM C2944 a–i: 5 mature males, 6 mature females) was examined at the collection of the Museum & Art Gallery of the Northern Territory (NTM) in Darwin (see details of sampling in Moore 1988).

Images used in the study were taken either with a digital camera (ColorView, Soft Imaging System, GmbH) connected to a stereo microscope or with a Canon G12 or a Canon EOS 550D through the oculars of the stereo microscope when camera facilities were not available. The pictures taken with the ColorView camera were edited and evaluated by using the program analySIS® (Soft Imaging System GmbH) for enhancing the pictures and adding scales.

"Gonads" in Cubozoa

Campbell (1974: 142) stated that in most cnidarians gonads are not separate organs as found in other animals as the germ cells generally are found in interstitial portions of the body tissue which, prior to formation of germ cells, exhibit no reproductive specialization. However, we will follow Campbell (1974), Marques & Collins (2004), Bentlage *et al.* (2010) and Morandini & Marques (2010) in using the term gonad to refer to areas where gametes are formed.

Disambiguation of subgastral sacs, stomach pockets and stomach purses

Subgastral sacs (male and female medusae of *Copula sivickisi*):

Four, interradial pairs of sac-like structures of the stomach tissue flanking each gastric phacellum. The openings are partly covered by gastric filaments.

Stomach pockets (female medusae of *Tripedalia binata* and *T. cystophora*):

Four, interradial pocket-like structures of the stomach tissue located beneath each gastric phacellum. The openings are covered by gastric filaments.

Stomach purses (male and female medusae of *Tripedalia binata* and *T. cystophora*)

Four deep folds of the stomach tissue which are located above the perradial mesenteries (suspensoria) which connect the upper manubrium and stomach with the subumbrella. The deep stomach folds form a visual connection between the openings of the gastric pouches and the mouth opening.

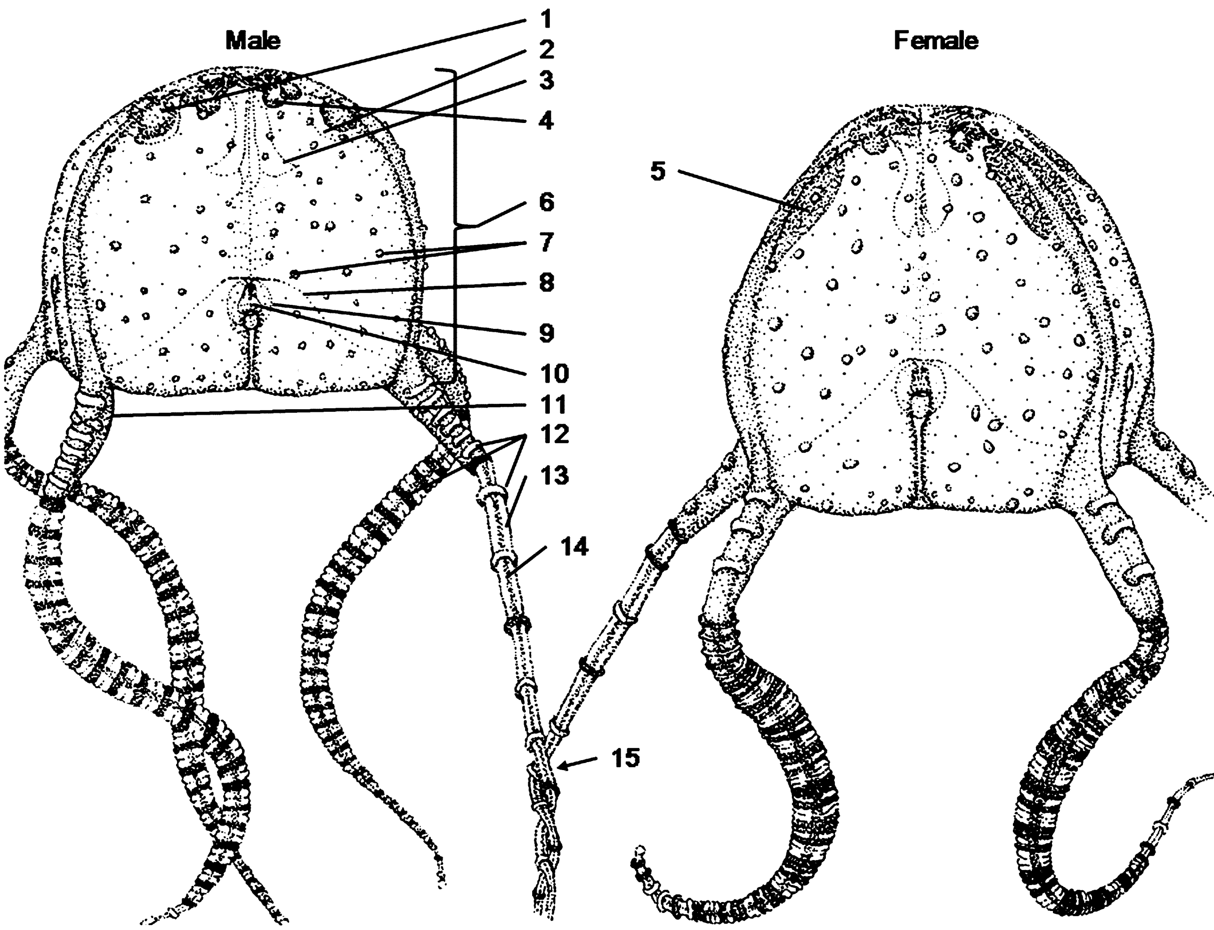

FIGURE 1. Anatomical scheme of tripedaliid medusae: a male (left) and a female (right) medusa of *Copula sivickisi* (after Straehler-Pohl 2011, with courtesy from Midoriishi). Labels within the figure are as follows: 1 = male gonad (testis), 2 = gonad tissue, 3 = mouth tube (manubrium), 4 = gastric saccules, 5 = female gonad, 6 = medusa body (bell), 7 = nematocyst warts, 8 = ring nerve, 9 = rhopalial niche, 10 = rhopalium (sense center including 6 eyes and statolith), 11 = pedalium (uncontractable tentacle base), 12 = nematocyst batteries, 13 = tentacle, 14 = gastric canal, 15 = joined tentacles of male and female in "Mating dance"

Dissection of subgastral sacs, stomach pockets, and purses (seminal vesicle)

For anatomical comparison preserved medusae of *Copula sivickisi* (n=10: 5 mature males, 5 mature females) and *Tripedalia cystophora* (n=10: 5 mature males, 5 mature females) were dissected and the complete stomach regions (apex, stomach and manubrium) were removed and inspected separately using a stereo microscope. To observe the inner stomach structures, the stomach regions were cut in half along the oral-aboral axis, along one perradial median and the apices including the endodermal tissue covering the upper part of the gastrovascular cavity (stomach roof) were removed. The manubrium of *Tripedalia cystophora* was also removed because of its length. The gastric filaments of *Tripedalia cystophora* could be lifted for a better view into the stomach pocket cavity as they were aligned in one horizontal row while the gastric filaments of *Copula sivickisi* were arranged in stacks of horizontal rows and covered the pocket cavity nearly completely. As the pockets are small (ca. 300–500 µm) and the structure is delicate, the gastric filaments were not removed in *Copula sivickisi* to prevent damage to the pocket structure.

Since the only preserved mature medusae of *Tripedalia binata* available were either a rare single specimen from the collection of Avril Underwood or the type material from the Museum in Darwin (NTM C2944 a–i), they were not dissected. Therefore, in this species only pictures of non-dissected animals were taken for comparison with the two other species.

Results

Taxonomy

Phylum Cnidaria Verrill 1865

Subphylum Medusozoa Petersen 1979

Class Cubozoa Werner 1973

Order Carybdeida Gegenbaur 1856

Family Tripedaliidae Conant 1897

Definition: Carybdeids with sexual dimorphism of gonads, spermatophores and subgastral sacs/seminal vesicles present at least in males (following Bentlage *et al.* 2010).

Genus *Copula* Bentlage Cartwright, Yanagihara, Lewis, Richards & Collins 2010

Definition: Tripedaliidae with adhesive pads on exumbrellar apex used to attach to substrates when resting.

Type species: *Copula sivickisi* (Stiasny 1926), by original designation.

***Copula sivickisi* (Stiasny 1926)**

Description of adult medusae. Bell blunt, pyramidal (slightly wider at bell opening and slightly narrower at top compared to bell height), highly transparent, slightly brownish, with white nematocyst warts (Figures 2A, H). Nematocyst warts scattered on bell from apex to velarium. Bell apex flattened with no horizontal constriction near top, with 4 adhesive pads used to attach themselves to substrates when resting. Adhesive pads transparent, almost invisible in live specimens, but slightly opaque and visible in preserved medusae (Figure 2D). Bell height up to 12 mm, bell width ca. 14 mm wide (interpedalial distance).

Pedalium, single, flattened, slender knife blade-shaped, ca. ½ the bell height in length (Figure 2F), situated in each interradial corner, with 3–7 broad, rectangular nematocyst bands on outer keel of pedalium, carrying single tentacle. Tentacles show typical striped pattern (also maintained in preserved specimens) of broad and thin bands of brightly purple (males) or brown-orange colour (males and females) and white nematocyst batteries (Figure 2G). Pedalial canal with rounded knee bend without any angle, hook or thorn appended to the outer knee bend (Figure 2F).

Rhopalium located inside rhopalial niche on each side of bell. Orifice vertical, longish keyhole-shaped with rounded closure at the top and being open at the bottom without any prominent covering scales (Figure 2 E), ca. 1/6 of bell height up from margin. Rhopalial horns thin and short, located above top end of rhopalial niche (Figure 2E, arrows).

Velarium, containing 4 simple, broad velarial canals per quadrant (Figures 2C, J). Four-lobed, cruciform manubrium, ca. 1/3 of bell height in length and connected to flat stomach; stomach communicates perradially with 4 gastric pouches leading into velar canals. Four horizontal, slightly concave gastric phacellae (Figure 2D), each consist of ca. 40, simple, unbranched, vertically stacked gastric filaments. Gonads paired (hemigonads), separated by interradial septum, extending from stomach rim to upper third of the bell about manubrium level (Figure 2H).

Sexual dimorphism:

Males: characteristic gonad pattern, 4 transparent, wasp-head-shaped gonad tissues with two, round to oval shaped, brightly yellow to deep orange male gonads (Figure 2B).

Females: 4 palm leaf-shaped, paired bands of gonads, slightly opaque, whitish to brownish (Figure 2I) Stark white ‘velarial spots’ (pigmented terminal regions of velar canal structures) protruding from velarium (Figures 2J, K) when mature.

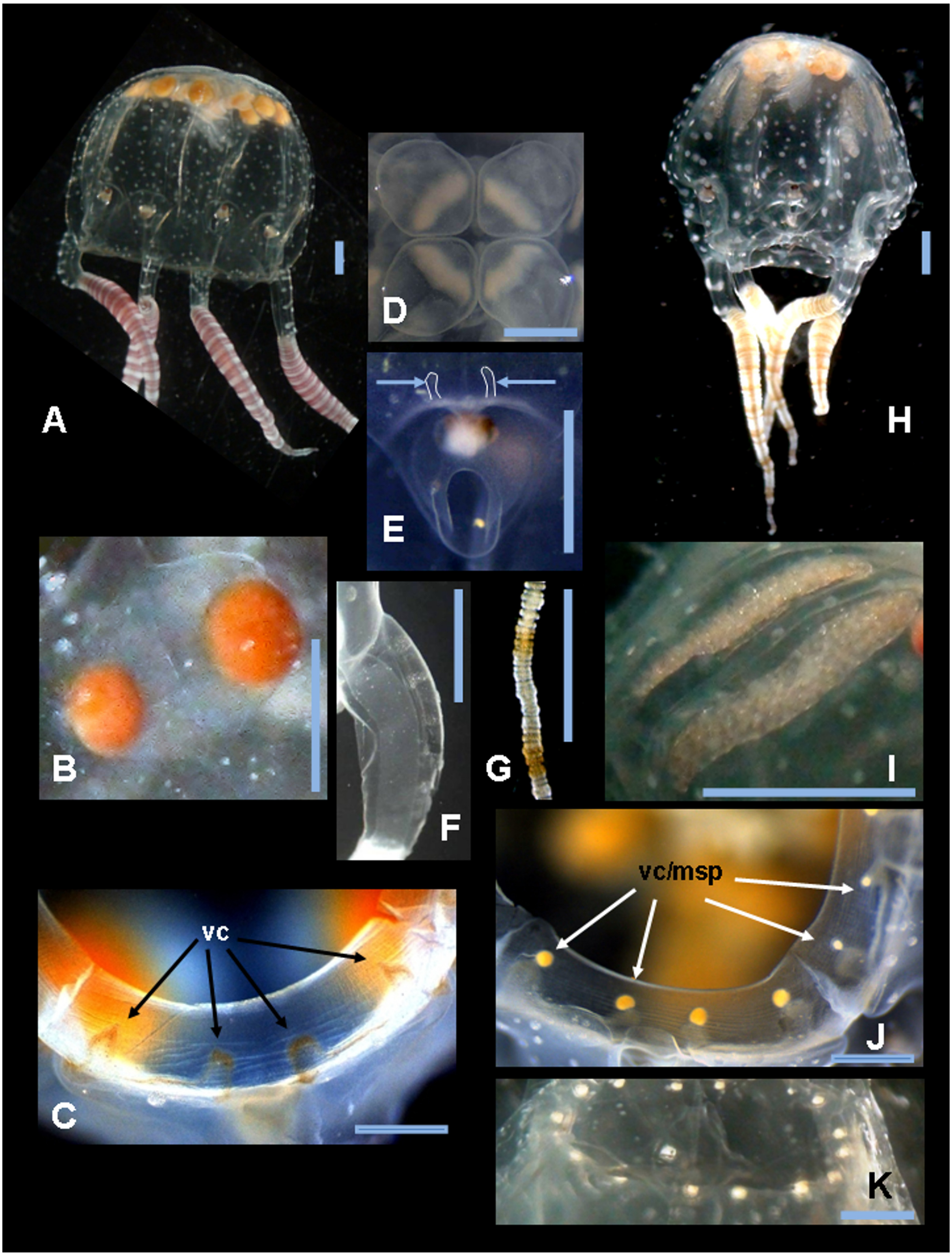

FIGURE 2. *Copula sivickisi* (scales: 1 mm). A: mature male medusa, note purple tentacle stripes (live specimen). B: mature male gonads (live specimen). C: velarium of mature male medusa (vc= velarial canals). D: adhesive pads on apex (preserved), note ivory coloured gastric phacellae shining through. E: rhopalial niche with keyhole-shaped opening and rhopalial horns (arrows, horn contours outlined by hand). F: pedalium. G: tentacle structure (in life). H: mature female medusa (in life). I: mature female gonads (in life). J: velarium of mature female medusa (preserved) with yellow “velarial spots”, colour due to preservation (vc/msp = velarial canals marked as maturity spots). K: velarium of mature female medusa (live specimen) with white “velarial spots” (photo provided by Ronald Petie).

Both sexes possess four, light orange to dark red coloured pairs of small, roughly hemispherical, pocket-like protuberances from the subumbrellar surface of the stomach, located beside the interradial septae above the gonads (Figures 2A,H; see also section 'Spermatophore formation in males of *Copula sivickisi* and *Tripedalia cystophora*' with Figure 5A and section ‘Stomach pocket structures’ with Figures 7B–E below).

Genus *Tripedalia* Conant 1897

Definition (redefined after Moore 1988 and Bentlage & Lewis 2012): Tripedaliidae with four interradial groups of 2 or 3 unbranched pedalia, each bearing a single tentacle. Rhopalia niche forming a roof without covering scale. Larviparous.

Type species: *Tripedalia cystophora* Conant 1897, by original designation.

Tripedalia binata Moore 1988 and *Tripedalia cystophora* Conant 1897

Description and comparison of adult medusae. Both species with cuboid bell, whitish transparent with a yellowish to brownish tinge in colour, with rounded edge (Figures 3A, G; Figures 4A, H). In *Tripedalia binata*, bell sparsely covered with nematocyst warts, in *Tripedalia cystophora* nematocyst warts frame mainly bell outline from apex edge to velarium; apex of *Tripedalia binata* flattened with slight horizontal constriction near the top (Figure 3G, arrows), apex of *Tripedalia cystophora* slightly arched, no horizontal constriction; in *Tripedalia binata* bell heights up to 11 mm, bell width up to 14 mm (interpedalia distance), in *Tripedalia cystophora* bell height up to 12 mm, bell width up to 15 mm (interpedalia distance).

Groups of two (*Tripedalia binata*) or three (*Tripedalia cystophora*) flattened, slender knife-blade-shaped pedalia with one tentacle each located at interradial corner of bell rim (Figure 3D; Figure 4F); pedalia length ca. 1/2–2/3 bell height. In *Tripedalia cystophora* outer wing keel length lined by nematocyst band. In both species, pedalial canals show slight bend, round knee ; tentacles resemble string of beads with white (*Tripedalia binata* and *Tripedalia cystophora*) nematocyst batteries in life specimens (Figure 4G). Rhopalium located inside rhopalial niche cavity on each side of bell, 1/3 of bell height up from margin, (*Tripedalia binata*, Figure 3F; *Tripedalia cystophora*, Figure 4E), niche forms a roof without upper nor lower covering scale. Short, very faint, “viking helmet-horn”-like rhopalial horns extend from top of rhopalial niche in *Tripedalia binata* (Figure 3F, arrows), no rhopalial horns in *Tripedalia cystophora*.

Velarium, measuring ca. 1/5–1/6 width of bell base, containing 6, sharp-pointed-triangular velarial canals per quadrant in both species (Figures 3C, I, J; Figures 2C, J), in *Tripedalia binata* main canals flanking pedalia and/or frenulae can grow branches or side canals in further development (Figure 3J). Mature females in both species show stark white coloured velarial canal tips (Figure 3I; Figure 4J).

In both species, long, four-lobed manubrium, 1/2 to 3/4 bell height in length; small, flat stomach communicating with 4 gastric pouches leading into velarial canals. Gastric phacellae in both species epaulette-shaped (Figure 3E; Figure 4D).

Gonads located in gastric pouches, generally butterfly-shaped and separated by interradial septum, centres of gonad “wings” attached to septum, canal system connects gonad “wings” through septum.

Sexual dimorphism:

Tripedalia binata

Males: gonad "wings" are very slender to stick-shaped, reaching from pedalial level to the upper third of the bell, whitish to bright yellow in preserved specimens (Figure 3B).

Females: gonad "wings" resembling butterfly wings, broad in the upper half, narrowing towards the lower end, reaching from velarial level nearly to stomach level, yellowish in preserved specimens (Figure 3H).

Tripedalia cystophora

Males: gonad "wings" stout, triangular, occupying middle third in height of gastric pouches, yellowish-brown to bright orange (Figure 4B).

Females: gonad "wings" longish oval bands, stretching from stomach to velarium, whitish transparent when alive to slightly yellowish in preserved state (Figure 4I).

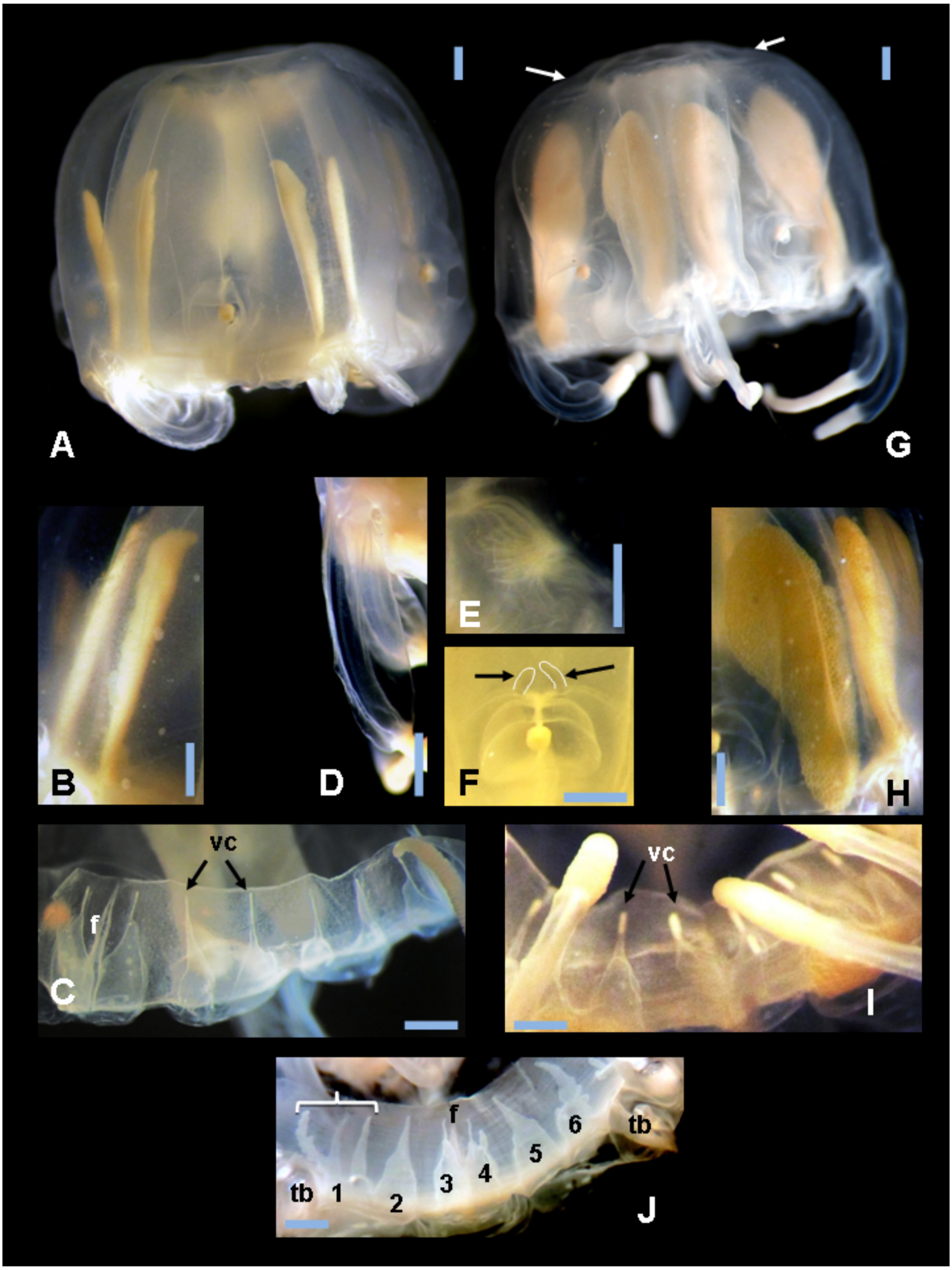

FIGURE 3. *Tripedalia binata* (two medusae from paratype material NTM C2944 a–i; scales: 1 mm). A: mature male medusa. B: mature, stick-shaped, male gonads. C: velarium of mature male medusa (vc = velarial canals). D: pedalium. E: epaulette-shaped gastric phacellum. F: rhopalial niche and rhopalial horns (arrows, horn contours outlined by hand). G: mature female medusa, note horizontal constriction near apex (arrows). H: mature, butterfly wing-shaped, female gonads. I: velarium of mature female medusa, note conspicuously white tips of velarial canals (vc/msp = velarial canals). J: velarium of a specimen with further developed canal system giving the impression of more than 6 main canals per quadrant, note main canal 1 shows three, partly lobed branches (bracket encloses three branches), main canals 2 and 3 simple, main canal 4 shows one side branch, main canal 5 simple, main canal 6 shows lobation and the beginning of a second branch (f = frenulum; tb = tentacle base) (photo provided by A. Underwood).

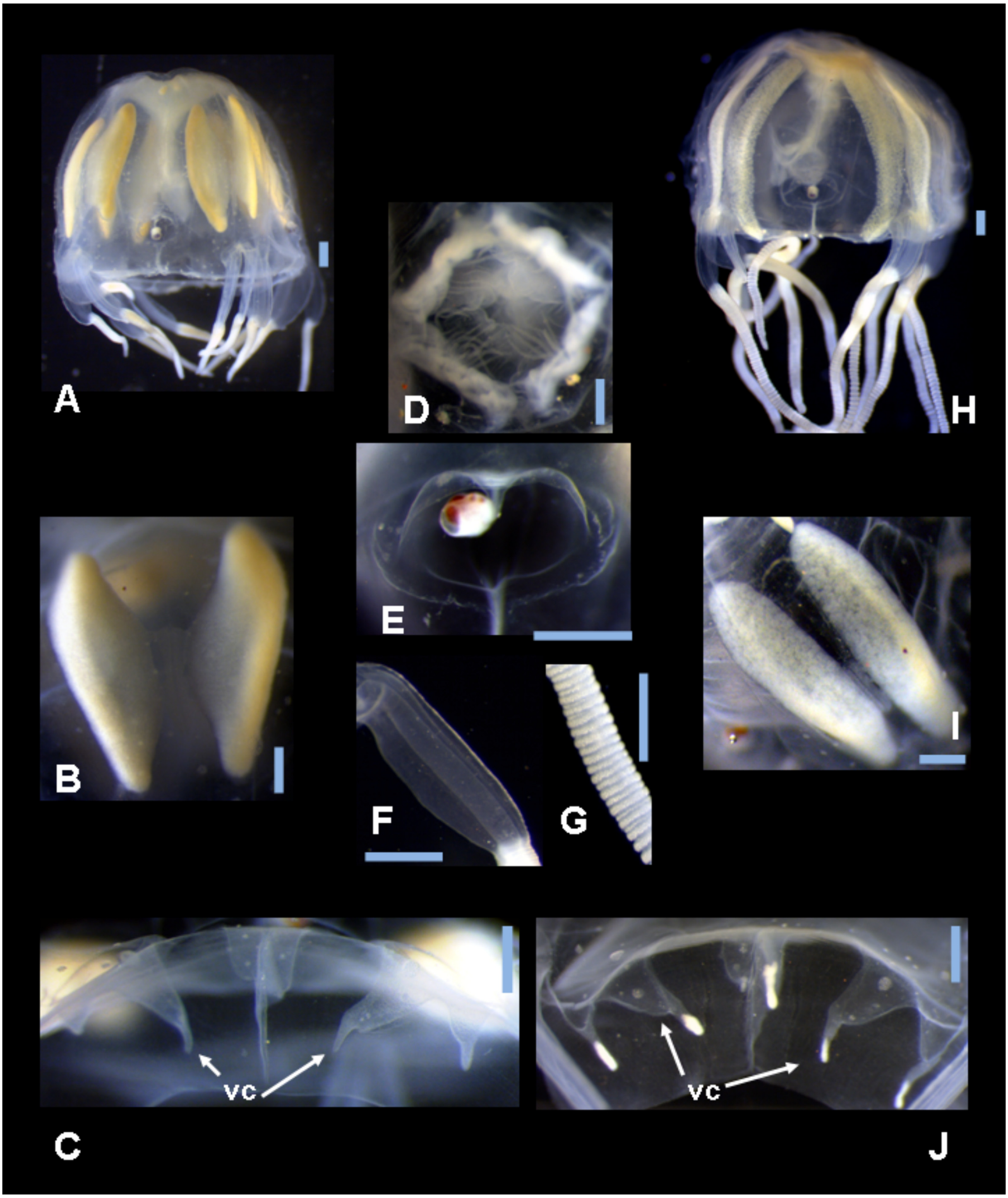

FIGURE 4. *Tripedalia cystophora* (preserved material, scales: 1 mm). A: mature male medusa. B: mature, butterfly wing-shaped, male gonads. C: velarium of mature male medusa (vc = velarial canals). D: stomach with epaulette-shaped gastric phacellae. E: rhopalial niche. F: pedalium. G: tentacle structure. H: mature female medusa. I: mature, longish-oval shaped, female gonads. J: velarium of mature female medusa, note conspicuously white tips of velarial canals (vc = velarial canals).

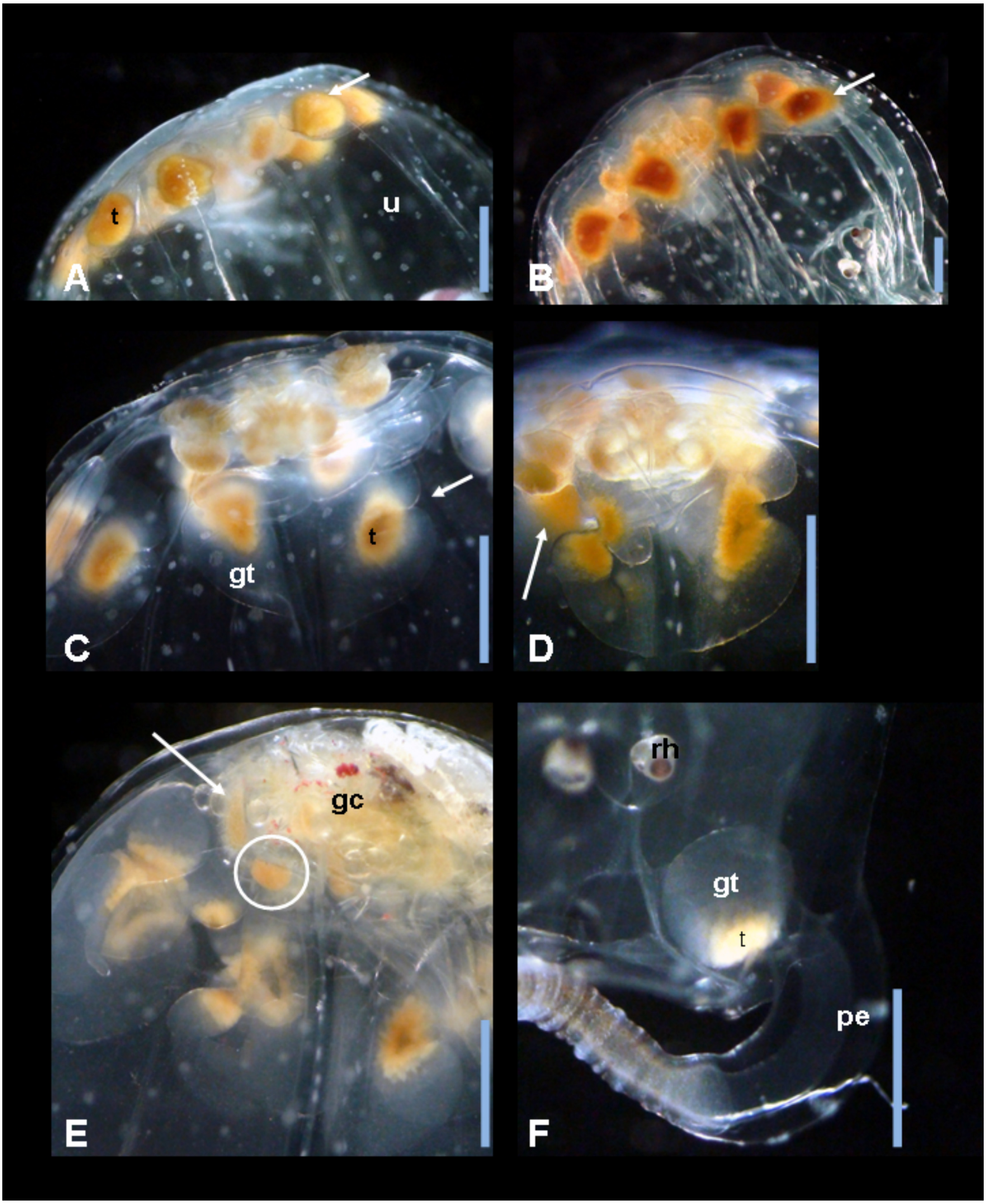

FIGURE 5. Spermatophore formation in *Copula sivickisi*. A: gonad tissue with well defined testis with sharp edges (arrow) in a mature male medusa. B: gonadal tissue with testis with blurred edges (arrow), gonadal tissue cloudy from released sperm. C: gonadal tissue opens up (arrow). D: sperm is transferred into the gastric pouches (arrow). E: gonadal tissue opens more and more, gastric pouches turn cloudy, male gonads pale in colour, sperm is transported into gastric cavity of stomach (white arrow) and collected in the subgastral sacs (white circle). F: sometimes parts of the gonad tissue detach and float freely through the gastric pouches until all sperm is released and the tissue is dissolved. Labels within the figure are as follows: gc, gastric content (food in form of plankton); gt, gonadal tissue; pe, pedalium; t, male gonad tissue; u, umbrella.

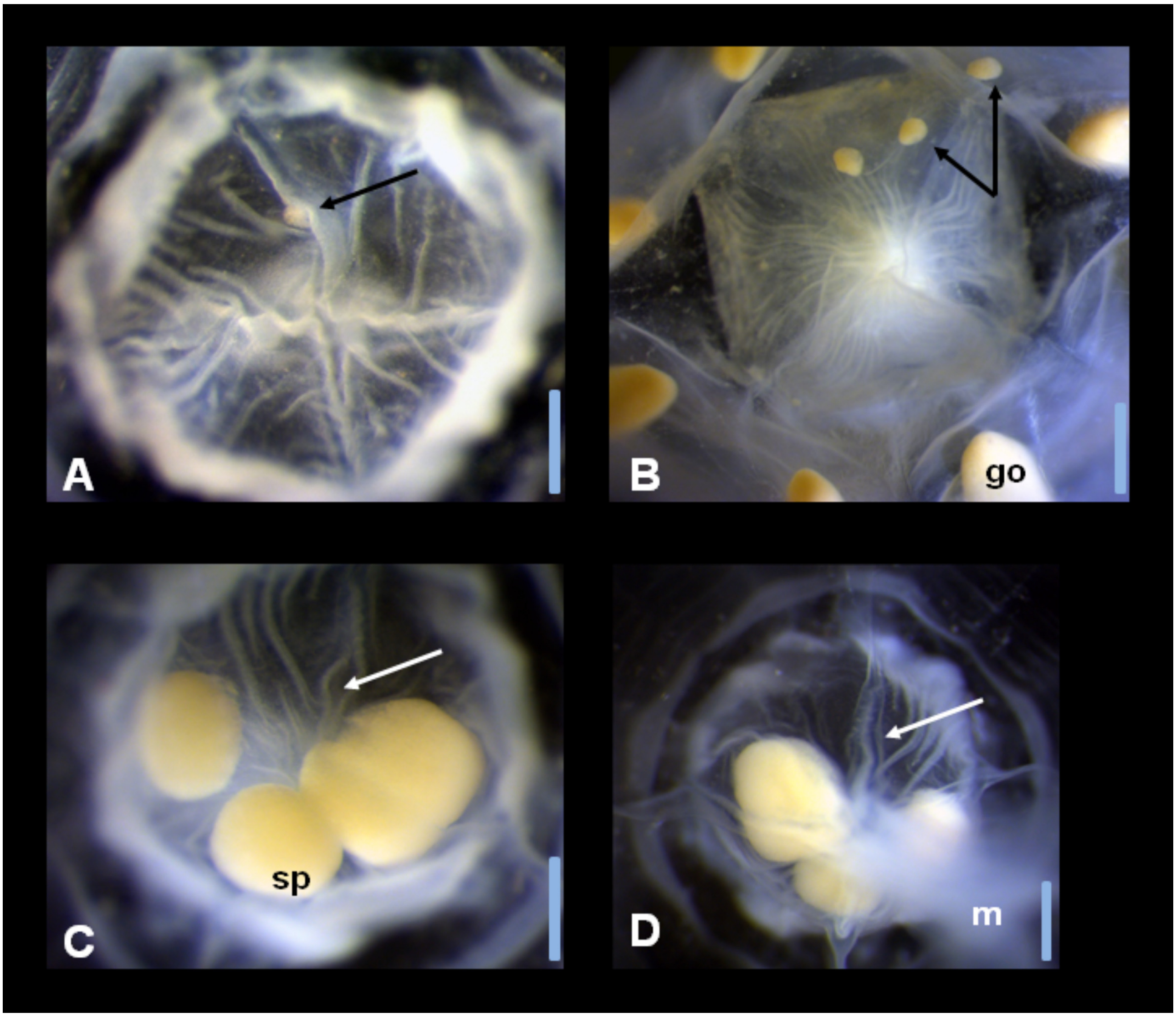

FIGURE 6. Spermatophore formation in *Tripedalia cystophora* (scales: 1 mm). A, B: dorsal view on stomach, note the sperm clusters which are churned from the gastric pouches into the stomach (B, arrows) and nestle into the stomach purses (A, arrow). C: dorsal view on stomach with spermatophores, three purses are full while the upper, fourth one is empty with the opening visible (arrow). D: subumbrellar view on stomach, note the spermatophores occupying the stomach purses, arrow marks the empty purse. Labels within the figure are as follows: go, gonads; m, manubrium; sp, spermatophore.

Both sexes in both species bear 4 perradial purses and/or pocket pairs inside the folds of their stomach (see also section "Subgastral sacs/purse structure" below; Figures 6 and 8).

Spermatophore formation in males of *Copula sivickisi* and *Tripedalia cystophora*

Copula sivickisi. In mature male medusae the membrane enclosing the dark-yellow-coloured hemigonads (Figure 5A) ruptures and the well-defined edges (Figure 5A, arrow) blur (Figure 5B, arrow) due to the release of sperm cells which also turn the former transparent gonad tissue cloudy (Figure 5C). The gonad tissue forms openings (Figure 5C, arrow) to transfer the sperm into the gastric cavity of the gastric pouches (Figure 5D, arrow). The sperm is transported from the gastric pouches into the stomach cavity (Figure 5E, white arrow) and collected into the subgastral sacs (Figure 5E, black arrow) to form spermatophores. The male medusae still feed during spermatophore formation (see stomach content in Figure 5E). The openings sometimes deepen so far that parts of the gonad tissues including parts of the hemigonads break free and float through the gastric pouches (Figure 5F).

Tripedalia cystophora. Spermatophore formation starts like in *Copula sivickisi* with the opening of the tissue of the mature male gonads (Werner 1973a, 1976) and the transfer of sperm into the gastric pouches that communicate freely with the velarial canals and the stomach. The sperm clusters are churned into the stomach

(Figures 6A, B) where it is collected in the four purses which are recessed into the cross-shaped stomach folds. The stomach folds are an extension of the mouth arm chutes (Figures 6A, B). The collected sperm clusters are enclosed by a membrane and form spermatophores, up to one in each purse (Figures 6C, D; Figures 8A). The male medusae do not feed during spermatophore formation (Werner 1973a, 1976).

Subgastral sacs/stomach purse structure

In *Copula sivickisi* both sexes are known to show the same kind of subgastral sacs (Figures 7A–E) for sperm storage (Lewis & Long 2005, Lewis *et al.* 2008). For *Tripedalia cystophora* pit-like structures in the folds of the stomach for sperm storage and spermatophore formation, which we term stomach purses, were only found in males (Werner 1976) but not in females (Hartwick 1991). Nothing was published up to now about the structures involved in sexual reproduction in *Tripedalia binata*.

As shown in Figure 3, the dimorphism also occurs in *Tripedalia binata*. The males of *Tripedalia binata* had spermatophores stored in equal stomach purses similar to the males of *Tripedalia cystophora* (Figures 8A, B). In the females of both *Tripedalia* species openings to potential stomach purses could be made out from the outside (Figures 8C, D).

Sperm filled subgastral sacs could be seen flanking the bundles of gastric filament in *Copula sivickisi* (Figure 9E). In *Tripedalia cystophora* the males showed only stomach purses while the females showed additional pocket-like structures flanking the stomach purses at the edge of the stomach hidden beneath the gastric filaments (Figure 9G–J). Those pockets resembled the subgastral sacs in *Copula sivickisi* but could not be clearly observed in the stomachs of the females of *Tripedalia binata*.

Discussion

Gershwin (2005: 155–156) proposed an amendment to "resurrect" the family Tripedaliidae as the sister group of Carybdeidae (Gershwin 2005: 155–156) because she presumed the relationship of *Copula* (*Carybdea*) *sivickisi* with *Tripedalia cystophora* based on results of Collins' (2002) complete 18S rDNA gene sequence analysis which showed a grouping of both species (Gershwin 2005: 85–86). This hypothesis was corroborated by subsequent molecular analysis, and "Tripedaliidae Conant, 1897 [was amended] to contain all carybdeids that display sexual dimorphism of the gonads, produce spermatophores and in which at least the males possess subgastral sacs/seminal vesicles (see Hartwick 1991)" (Bentlage *et al.* 2010: 497).

The medusae of *Copula sivickisi* from Akajima Island described herein conform to the morphology of the medusae of *Copula sivickisi* reported previously (Stiasny 1926; Uchida 1929, 1970; Hoverd, 1985; Hartwick, 1991; Lewis & Long, 2005; Lewis *et al.*, 2008; Gershwin, 2005; Straehler-Pohl, 2011), with two exceptions. One, the form of the four velarial canals, similar to the New Zealand population (Hoverd 1985), did not have the small velarial side canals that were noticed in specimens from the Philippines, from Okinawa and Australia. Two, the colour of the velarial spots which differed in being stark white from the ones described by the above mentioned authors as being dark (Stiasny 1926), orange (Lewis & Long 2005, Lewis *et al.* 2008) or brown (Hartwick 1991). As *Copula sivickisi* is a shallow water species that needs hard substrates, both as polyp and as medusa (Garm *et al.* 2012), and as many of the *Copula sivickisi* populations (Japan: mainland, Okinawa Island, Akajima Island; Australia: Magnetic Island; New Zealand: Taputeranga Island) are separated geographically by open sea and great distances, they could have developed differences which should be investigated in the future by genetic analysis to check for possible cryptic species.

The macro-morphological inspection of *Tripedalia cystophora* agrees well with the morphological descriptions of Conant (1897, 1898), Kramp (1961), Uchida (1970), Werner *et al.* (1971), Werner (1973a, 1976, 1993), Mehnert (1984), Laska-Mehnert (1985), Stewart (1996), Garm *et al.* (2006, 2007, 2008) and Bentlage & Lewis (2012). The little information that exists on *Tripedalia binata* (Moore 1988; Underwood *et al.* 2013) was is in agreement with our observations with the exception that the velarium does not contain 7–8 velarial canals but exactly 6 velarial canals per quadrant, similar to *Tripedalia cystophora*. The growth of branched side canals in older specimens (Figure 3J) might have caused the misinterpretation by Moore (1988) and Underwood et al. (2013). Further, small and faint rhopalial horns could be observed above the top of the rhopalial niches in *Tripedalia binata* as described by Underwood *et al.* (2013). These are similar to the rhopalial horns observed in *Copula sivickisi* (Gershwin 2005); rhopalial horns are absent in *Tripedalia cystophora.*

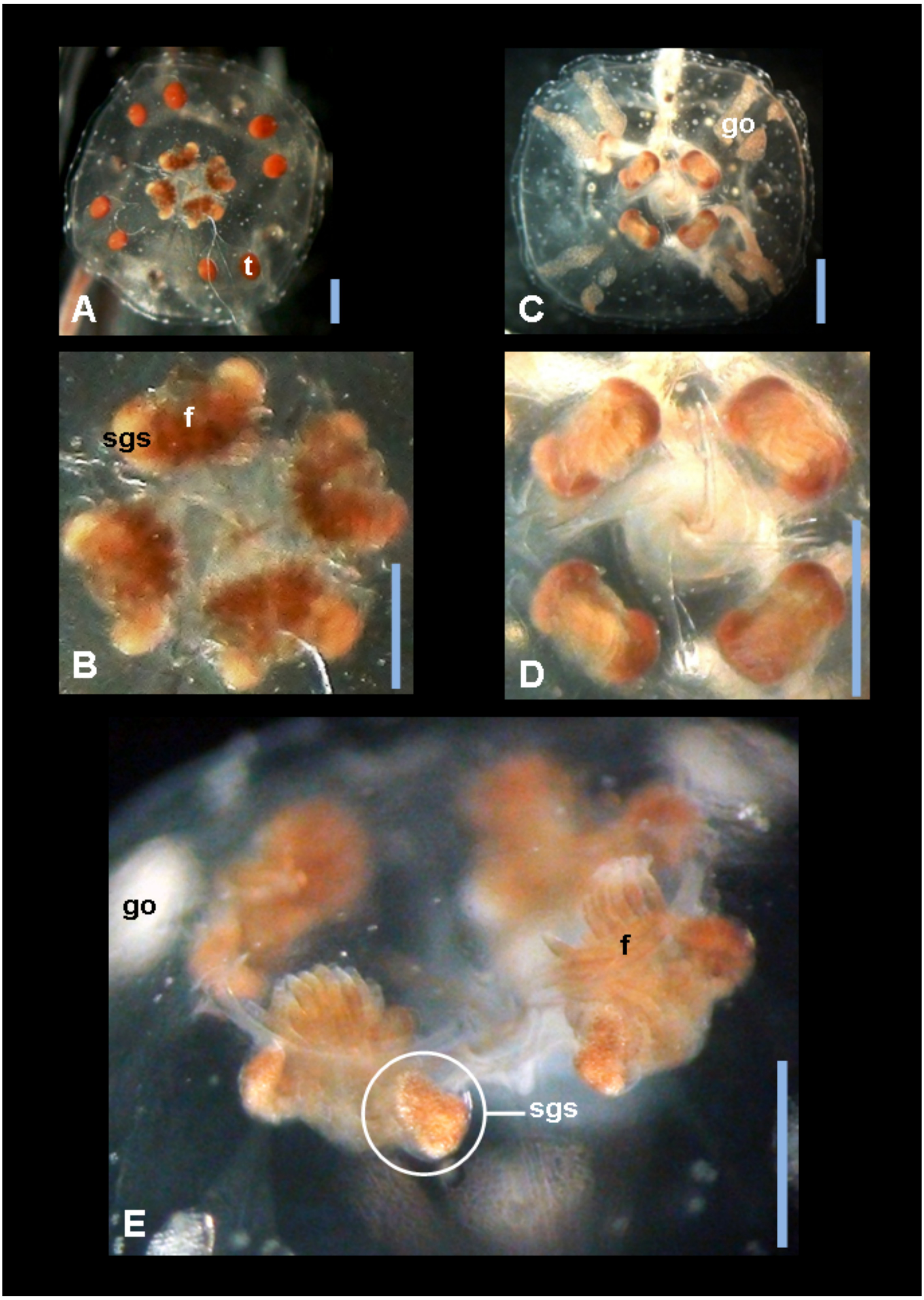

FIGURE 7. Subgastral sacs of *Copula sivickisi* (scales: 1 mm). A: view on apex of mature male medusa, note red-orange male gonad pairs, light flesh coloured subgastral sacs and dark brown coloured gastric phacellae. B: close up dorsal view on subgastral sacs and gastric phacellae of male medusa. C: view on apex of mature female medusa, note beige colour of gastric filaments. D: close up dorsal view on subgastral sacs and beige coloured gastric phacellae of female medusa. E: close up lateral view on subgastral sacs (white circle around one sac) and gastric phacellae of female. Labels within the figure are as follows: f, gastric filaments; go, gonads; sgs, subgastral sacs/pockets with collected sperm; t, male gonad.

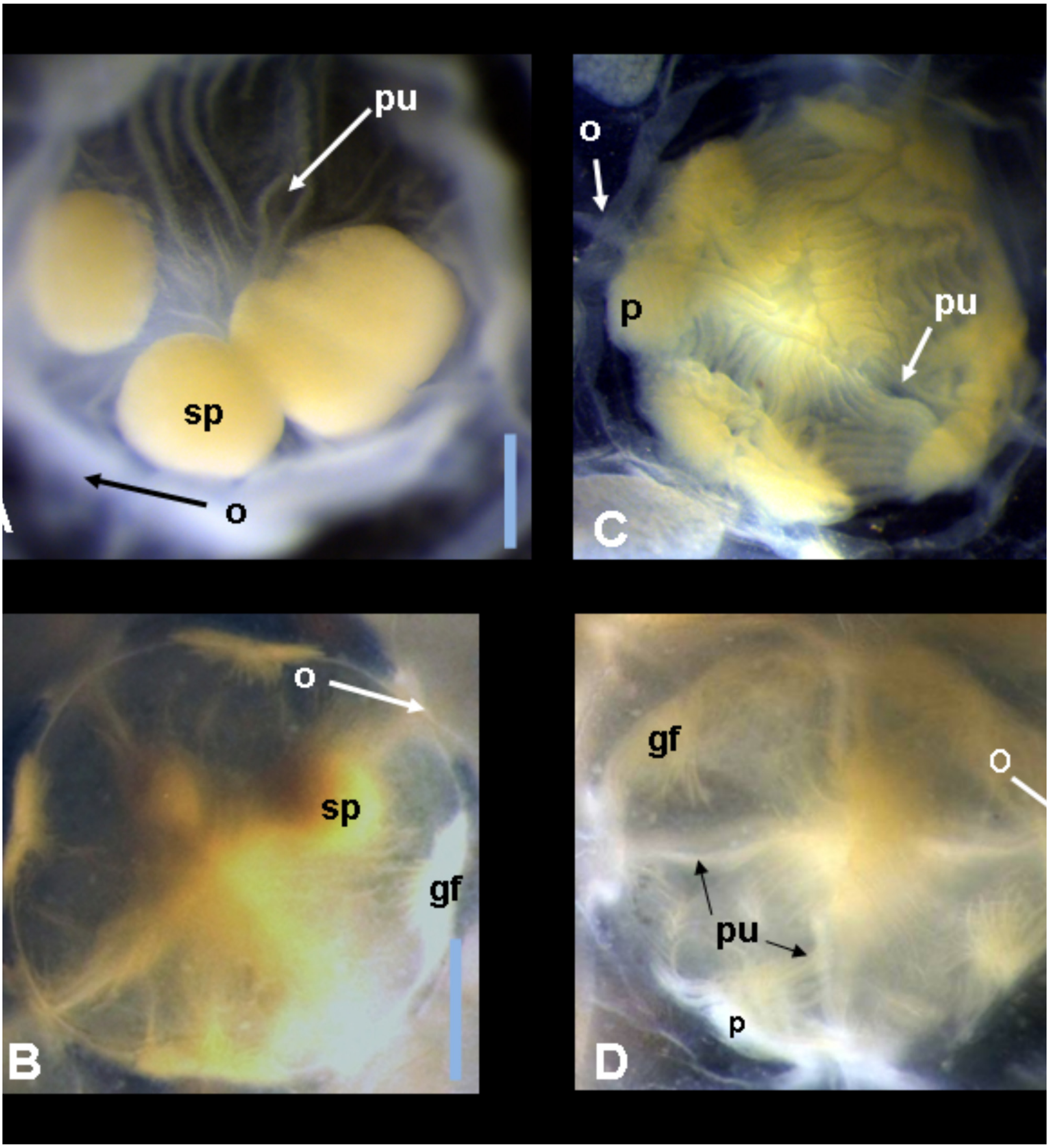

FIGURE 8. Stomach purses and pockets in *Tripedalia cystophora* and *Tripedalia binata* (scales: 1 mm): A: close up of the stomach of a mature male medusa of *Tripedalia cystophora*, note spermatophores. B: close up on stomach of a mature male medusa of *T. binata*, note spermatophores. C: close up of the stomach of a mature female medusa of *T. cystophora*, note openings of empty stomach purses in stomach. D: close up on stomach of a mature female medusa of *T. binata*, note openings of empty purses in stomach. Labels within the figure are as follows: gf, gastric filaments; o, opening of stomach to gastric pouch; p, stomach pocket; pu, stomach purse; sp, spermatophore.

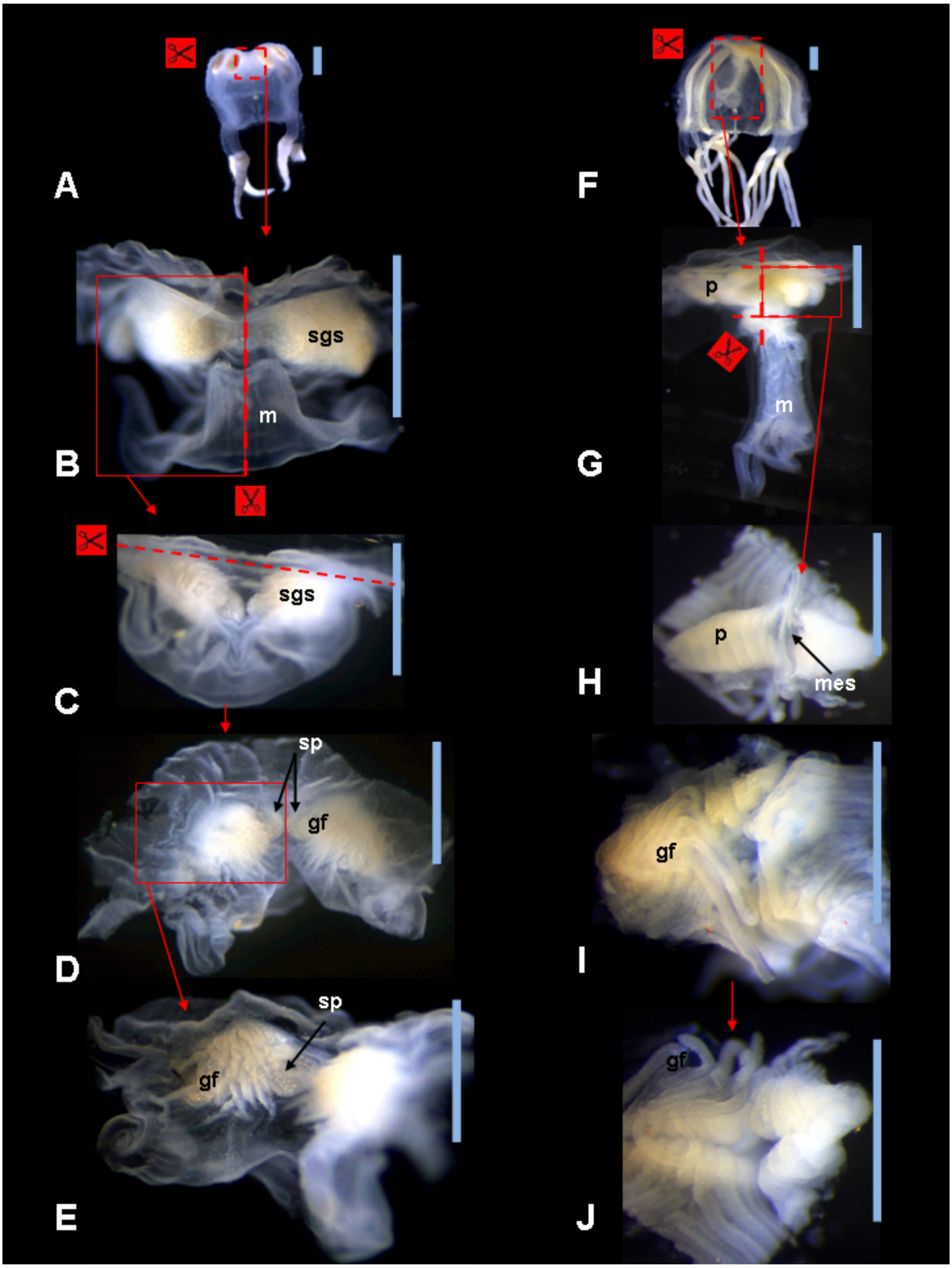

FIGURE 9. Presentation of the stomach pocket dissection in *Copula sivickisi* (male, A–D) and *Tripedalia cystophora* (female, F–J) (broken lines mark cuts or incisions, scales: 1 mm). A, F: mature medusae, scale in A: 2 mm. B, G: removed stomach regions with subgastral sacs and manubrium. C: one stomach region half, pockets laid open. D: dorsal view into the stomach, stomach roof was removed, gastric filaments hiding partly spermatophores inside subgastral sacs which flank the filament bundles. E: stacked rows of gastric filaments which partly hide the spermatophores stored in the pockets on the left and right side of the phacella. H: backside of subgastral sacs divided by a mesentery. I: stomach pocket opening covered by gastric filaments. J: gastric filaments were lifted to allow a view on the opening of the stomach purse which is divided at the stomach edge into a left and a right pocket. gf, gastric filament; m, manubrium; mes, mesentery; p, stomach pocket; sgs, subgastral sacs; sp, spermatophore.

Mature females of all three species have the tips of the velarial canals filled with a stark white substance, these prominent spots in *Copula sivickisi* were called "velar spots" and suggested to mark females as mature or immature (Hartwick, 1991; Lewis & Long, 2005). The sperm release and spermatophore production are quite similar in *Copula sivickisi* and *Tripedalia cystophora* even though *Copula sivickisi* produces up to eight spermatophores due to eight subgastric sacs flanking the bases of the gastric phacellae (Hartwick 1991) while *Tripedalia cystophora* produces only four spermatophores in its four stomach purses located near the stomach entrance (Werner 1976). Whether subgastric sacs and stomach purses are homologous structures cannot be stated here with certainty – further studies on morphological development supported by histological sections are planned for the future. Male medusae of *Tripedalia cystophora* stop feeding during spermatophore production (Werner 1976) which might avoid the sperm or the spermatophores being flushed out as the stomach purses are located near the stomach entrance. In contrast, the male medusae of *Copula sivickisi* still feed during spermatophore production which might be possible because the sperm is collected by the gastric filaments into the subgastric sacs located far away from the mouth opening.

We corroborated the possession of subgastric sacs in *Copula sivickisi* and function analogous to the seminal vesicle in males and spermathecae in females, i.e. to form and/or accommodate spermatophores (Hartwick 1991). Werner (1976) described pit-like structures in the males of *Tripedalia cystophora*, which also form and accommodate spermatophores, but he did not mention the same structure for the females. Hartwick (1991, p. 176) denied those structures in females of *T. cystophora* by stating "*Copula sivickisi* is distinct from *Tripedalia* in that ... similar structures, with similar contents (effectively, spermathecae) ... are found in the female as well". However, the females of *Tripedalia cystophora* have four pit-like stomach purses similar to the ones of the males, which might accommodate spermatophores, also shown in *Tripedalia binata*. Further, we show that the females possess additional pockets flanking mesenteries, which we hypothesize serve as spermathecae when the spermatophore membranes are dissolved as observed in females of *Copula sivickisi*.

Morphological examination of the medusae of *Tripedalia binata* shows that both male and female medusae possess stomach purses. In male medusae the purses were occupied by spermatophores, consistent with this species also producing spermatophores and having a mating behaviour similar to the other two species described by Werner (1973a, 1976), Hartwick (1991), Lewis & Long (2005) and Lewis *et al.* (2008).

Our results support the proposal by Bentlage *et al.* (2010), but suggest an adjustment of their amended diagnosis of Tripedaliidae (underlined): All carybdeids that display sexual dimorphism of the gonads, produce spermatophores and in which males <u>and females</u> possess subgastral sacs, <u>pockets or purses</u> which <u>function as seminal vesicles or spermathecae</u>.

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