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TESE DE DOUTORADO

Estudo sobre os decápodes anomúros *Aegla castro* Schmitt, 1942 (Crustacea: Aeglidae) nas bacias do Paranapanema e Tibagi: trata-se de um táxon amplamente distribuído ou um complexo de espécies crípticas?

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Co-orientador: Prof. Dr. Sérgio Luiz de Siqueira Bueno

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“There are no freshwater Crustacea
at all like *Aegla* anywhere else in the world.”

Waldo Schmitt (1942)

Dedico essa tese a minha família.

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SUMÁRIO

Considerações iniciais……...1

Diversificação biológica.....2

A escolha do modelo: Aegla.....3

Implicações ambientais……...4

Referências bibliográficas……..……..6

Capítulo I: Morphological comparison between *Aegla castro* Schmitt, 1942 (Decapoda, Anomura, Aeglidae) populations as an important source of information for conservation status

Abstract……11

Introduction……12

Material & Methods……14

Results……..15

Taxonomic review of the *Aegla castro* Schmitt, 1942 holotype.....15

Comparison among the "*A. castro"* species.....19

4. Discussion……32

References……34

Capítulo II: Ultrastructure of spermatozoa of *Aegla sp*. (Crustacea, Decapoda, Aeglidae): description of morphological patterns, comparison of the potential cryptic taxon *Aegla castro* Schmitt, 1942 and information for conservation

Abstract……40

1 Introduction……..41

2 Material & Methods…….....44

2.1 Sampling.....45

2.2 Transmission Electron Microscopy (TEM).....45

3 Results……...……46

3.1 Spermatozoa of *Aegla castro* Ponta Grossa (state of Paraná)……46

3.2 Spermatozoa of *Aegla castro* Mauá da Serra (state of Paraná).....51
3.3 Spermatozoa of *Aegla castro* Itatinga (state of São Paulo).....53
3.4 Spermatozoa of *Aegla parana* Schmitt, 1942 União da Vitória (state of Paraná).....55
3.5 Spermatozoa of *Aegla sp.* Tibagi (state of Paraná).....64
3.6 Spermatozoa of *Aegla sp.* Tibagi– Fazenda Pinheirinho (state of Paraná).....63
3.7 Spermatozoa of *Aegla sp.* Tibagi – Puxa Nervos (state of Paraná)..... 65
Discussion……..……...66
References……72
Conclusion……79

CONSIDERAÇÕES INICIAIS

A família Aeglidae Dana, 1852 possui o único gênero na fauna atual *Aegla* Leach, 1820 com mais de 80 espécies descritas (Bueno et al., 2016), e dois gêneros fósseis *Haumuriaegla* (Feldmann, 1984) e *Protaegla* (Feldmann et al., 1998).

Os eglídeos têm sido identificados em maior parte por caracteres morfológicos e no geral possuem semelhança morfológica muito próxima (morfotipo geral conservador), sendo que alguns autores ressaltam a possibilidade da existência de espécies crípticas ou irmãs (Jara et al., 2003). Espécies crípticas fazem parte de um mesmo gênero e são morfologicamente similares ou indistinguíveis, porém geneticamente distintas (Mayr, 1963). Atualmente, as investigações sobre tal questão vêm utilizando a análise genética como ferramenta (Russello et al., 2010), assim como estudos tradicionais que usam a morfologia para fins comparativos. Alguns trabalhos obtiveram informações relevantes sobre diferenças morfológicas pela análise de morfometria geométrica em comparações entre populações de eglídeos residentes ao meio epígeo e hipógeo (Fernandes & Bichuette, 2013) e entre populações isoladas de uma mesma espécie (Trevisan & Masunari, 2010). Para a morfologia, não apenas as informações dos adultos podem ser utilizadas, como também das formas jovens, com o potencial de originar novas perspectivas e potenciais linhas de investigação para os estudos comparativos e a relação evolutiva desse grupo (Moraes & Bueno, 2013). De fato, a existência de um complexo de espécies crípticas já foi evidenciada para *Aegla longirostris* Bond-Buckup & Buckup, 1994 com a genética (Bartholomei-Santos et al., 2011) e morfometria geométrica (Marchiori et al., 2014) genética e taxonomia (Moraes et al., 2016).

Bartholomei-Santos et al. (2011) consideram que espécies com ampla distribuição geográfica, incluindo ambientes heterogêneos ou isolados geograficamente, podem abrigar espécies crípticas. Ademais, essa problemática também vem sendo observada na literatura em

estudos com camarões tanto de águas continentais (Carvalho et al., 2013) quanto marinhos (Terossi & Mantelatto, 2012). Apesar da existência do estudo filogenético realizado por Pérez-Losada et al. (2004) com as espécies do gênero *Aegla*, incluindo *Aegla castro* Schmitt, 1942, não é possível inferir sobre as relações intraespecíficas existentes em uma população. Vale ressaltar que, há problemas taxonômicos relacionados à *A. castro* e indícios de que na verdade constitua um complexo de espécies crípticas (Sérgio Bueno, comunicação pessoal). Portanto, é importante investigar com detalhe espécies que apresentam dúvidas em relação ao seu status taxonômico.

Diversificação biológica

As características morfológicas são utilizadas tradicionalmente como as principais informações empregadas na delimitação e reconhecimento das espécies, por serem consideradas de fácil obtenção, uma vez que não há necessidade do uso de ferramentas que demandam alto custo (Carvalho et al., 2013). No entanto, devemos considerar que os organismos pertencentes a uma mesma espécie podem apresentar variações intraespecíficas, como resultado da variação no comportamento ou nas características morfológicas (fenótipo), que são determinados pelo genótipo em diferentes condições ambientais, ou seja, a plasticidade fenotípica (West-Eberhard, 1989).

De acordo com West-Eberhard (2005), se o fenótipo é o objeto da seleção exposto a uma variação ambiental significativa, essa seleção pode prosseguir para as próximas gerações sem alteração genética e sem efeito evolutivo. Com o passar do tempo, a variação genética que afeta essas características surge como resultado da mutação ou recombinação genética, e a partir desse ponto há imediatamente um efeito evolutivo. Detectar processos como a especiação (evolução do isolamento reprodutivo) (Ridley, 2005) e verificar se há separação das espécies é algo complexo e exige investigações aprofundadas.

Portanto, há duas situações a serem consideradas: de indivíduos pertencentes à mesma espécie, porém, devido à plasticidade fenotípica apresentam algumas diferenças (morfológicas ou comportamentais); ou dos indivíduos já terem passado por esses processos, já estarem em processo de especiação, mas ainda possuem semelhança morfológica considerável a ponto de não ser possível sua separação com base na observação macroscópica.

A escolha do modelo: Aegla

Várias características tornam os eglídeos um grupo peculiar, principalmente em relação a sua história evolutiva, por ser a única família dentro de Anomura restrita à região Neotropical do Sul da América e aos ambientes de águas continentais. Várias das espécies conhecidas estão ameaçadas de extinção (Pérez-Losada et al., 2004) e atualmente muitas espécies de *Aegla* estão sendo descritas, portanto ainda há carência de estudos à fim de elucidar a riqueza de espécies do grupo (Santos et al., 2013).

Recentemente foi proposta a definição de uma superfamília específica para Aeglidae, chamada de Aegloidea (McLaughlin et al. (2007) por comparações morfológicas, sendo que a mesma foi anteriormente inserida à superfamília Galatheoidea. O primeiro trabalho a questionar a inclusão de Aeglidae dentro de Galatheoidea foi de Martin & Abele (1986), que consideraram a existências de características morfológicas distintas entre os grupos, além das demais famílias de Galatheoidea estarem restritas ao ambiente marinho. Tudge & Scheltinga (2002) verificaram que a morfologia dos espermatozoides de *A. longirostri* é única em comparação a outros grupos de Anomura sugerindo, portanto, uma linhagem independente e basal para Aeglidae. Estudos moleculares e morfológicos reforçaram a ideia de retirar Aeglidae de Galatheoidea (Ahyong & O'Meally, 2004; Pérez-Losada et al., 2002b).

Portanto, faz-se necessário solucionar ou esclarecer questões taxonômicas pendentes aos eglídeos como o presente projeto com *A. castro*, que corroborará se a mesma é um caso de complexo de espécies crípticas.

Implicações ambientais

A existência de um complexo de espécies crípticas pode revelar uma biodiversidade oculta e ter implicações na conservação da diversidade biológica (Bickford et al., 2007). Aspectos importantes ao separar corretamente essas espécies estão relacionadas ao conhecimento da efetiva distribuição geográfica das mesmas, grau de endemicidade e, assim, seu real estado de conservação (Bartholomei-Santos et al., 2011).

Existem três trabalhos realizados de âmbito populacional com *A. castro* (Swiech-Ayoub & Masunari, 2001ab; Fransozo et al., 2003), sendo a espécie distribuída no sul do estado de São Paulo e no norte e nordeste do estado do Paraná (Bond-Bockup & Buckup, 1994) com ocorrência registrada em duas bacias hidrográficas pertencentes ao Alto Paraná (bacias do Médio Paranapanema e do Tibagi), compostas por rios de várias ordens e portadoras de um valioso abrigo florestal (Mata Atlântica), formando ricos ecossistemas aquáticos. Porém, a maioria desses ecossistemas esta ameaçado pela destruição das matas ciliares (urbanização e agricultura) e consequente assoreamento das fontes de água, pela introdução de espécies exóticas, poluição da água e pela construção de represas sem um planejamento que vise à conservação desses ambientes (Pérez-Losada et al., 2002a; Bond-Buckup et al., 2010).

A bacia do Tibagi vem sofrendo com a ação antrópica desde 1875 com a ocupação por povos não indígenas (SEMA, 2010). Desde então, as florestas vêm dando lugar principalmente a agricultura, pastagem, reflorestamento e a ocupação humana. Ademais, a área possui grandes polos industriais, com o município de Télemaco Borba contendo uma das maiores indústrias de celulose do país. Com essas atividades, restam apenas 3,8% de sua vegetação original, da qual

uma pequena parte está protegida em reservas como o Parque Estadual Mata dos Godoy e o Parque Estadual de Vila Velha (Soares & Madri, 2002; SEMA, 2010).

A bacia do Médio Paranapanema encontra-se também alterada por uma ampla gama de atividades humanas: usinas hidrelétricas construídas desde meados do século XX (Jorcin & Nogueira, 2008), áreas de pastagens que contabilizam 54,9% do uso do solo, áreas de culturas temporárias (14,8%), áreas de culturas semi-perenes (13,6%), cobertura vegetal natural de apenas 6,2%, área de reflorestamento (4,8%), áreas de culturas perenes (2,2%), áreas urbanas (1,0%) e outros usos (2,5%) (SERHS, 2005).

Portanto, atualmente observa-se que algumas espécies de Aeglidae não são mais encontradas em localidades registradas anteriormente como resultado da rápida degradação da qualidade da água (Bond-Buckup et al., 2010). Por ser indicadora da qualidade ambiental, essas espécies são encontradas apenas em locais com água limpa e bem oxigenada, sendo qualquer alteração no seu habitat desfavorável ao seu estabelecimento (Bond-Buckup, 2003). Alguns estudos já foram realizados no Chile com enfoque conservacionista, priorizando alertar sobre a preocupante situação dos eglídeos, uma vez que várias das espécies chilenas estão ameaçadas ou já extintas (Pérez-Losada et al., 2002a; Pérez-Losada et al., 2009). Para as espécies brasileiras, trabalhos sobre a estimativa do tamanho populacional (Cohen et al., 2013; Bueno et al., 2014) e com espécies pouco estudadas até o momento (Grabowski et al., 2013) vêm sendo realizados a fim de fornecer informações relevantes sobre essas populações. Fica clara, portanto, a necessidade de uma investigação sobre *A. castro* na sua faixa de distribuição diante da carência de trabalhos com a espécie em questão, de sua ampla distribuição geográfica sendo possível a existência de um complexo de espécies, e da degradação ambiental dos rios pela ação humana. Essas informações serão de grande validade para a conservação da espécie ou do complexo de espécies crípticas em questão.

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CAPÍTULO 1

Morphological comparison between *Aegla castro* Schmitt, 1942 (Decapoda, Anomura, Aeglidae) populations as an important source of information for conservation status

Abstract

The *Aegla* species are very similar in relation to the morphological characters and species identification always have been problematic. Indeed, it have been observed the complex of cryptic species that reveals a hidden diversity for this taxon. This have serious influence on the true distribution geographic of the species and consequently the validation as vulnerable, endangered or extinct species, once Aeglidae family represents currently the most threatened freshwater taxon in South America. For this reason, the goal of this research was to investigate the *Aegla castro* species and verify if they are in fact a complex of cryptic species or not. With a wide geographic distribution, this species were collected using traps or manual collection in tree regions that they were identified previously as *A. castro* (including the holotype): Ponta Grossa, Castro and Mauá da Serra (Paraná state) and Itatinga (São Paulo state) and in three new regions in Tibagi (Paraná state). Two characters were very conservative on the holotypes and paratypes: the subrostral process on proximal half well developed, high, broad, triangular, oriented downward and the third thoracic sternite that is tapered. Comparing this carachteristics with the other species, only *Aegla* sp. from Castro shared the same pattern indicating that there are more than one species identified as *A. castro* or they have varied in relation to this character.

Key words: cryptic species, taxonomy, endangered and threatened species, freshwater.

1 Introduction

It have been observed currently, however, in fact it was highlighted by the American naturalist Waldo Schmitt (1942) since he studied the Aeglidae, that they are similar in relation to taxonomic characters, i.e., it is very difficult to identify the species. In addition, recent studies involving *Aegla* spp. have dismembered one only species in several species revealing a cryptic diversity for this group (Bartholomei-Santos et al., 2012; Marchiori et al., 2015; Moraes et al., 2016; Crivellaro et al., 2017). Therefore, Aeglidae is a family that deserve attention since there are many variables involving it that should be considered and indeed investigated, i.e., if the simple wrong identification has occurred, if the species are in speciation process and they have some morphological differences but not genetics ones or if they are a complex of cryptic species.

The special attention in this group have increased not only because of the curiosity in solve the issues that have raised and mentioned above, however because this family is very particular endemic from the subtropical and temperate regions of continental South American (Schmitt, 1942). It is very restrict in relation to geographic distribution, it is not abundant with some rare species and are indicators of environmental quality (Bond-Buckup, 1994; Bond-Buckup et al., 2008, Bueno et al., 2016). In addition, *Aegla spp.* is the unique genera in Aeglidae family and the others two are fossils *Haumuriaegla* (Feldmann, 1984) and *Protaegla* (Feldmann *et al.*, 1998).

The main reason behind all particularities involving this species is that currently Aeglidae is the most threatened taxon among the South American freshwater decapods being 43 of the 78 species (55.13%, more than a half) in one of the three classifications established by the IUCN (2013) critically endangered (15.38%), endangered (28.21%) and vulnerable (11.54%) (Bueno et al., 2016). Concerned about the extinction risks of

aeglids species that research the efforts have increased in Brazil nowadays mainly related with diversity loss (Zimmerman et al., 2018).

One species that has a wide geographic distribution recognized and that has not been deeply studied is *Aegla castro* Schmitt, 1942. This species was identified previously and some studies were done about population biology for Ponta Grossa, Paraná state (Swiech-Ayoub & Masunari, 2001ab), Itatinga, São Paulo state (Fransozo et al., 2003) and Mauá da Serra, Paraná state (Marçal et al., 2017).

Aegla castro was described by Waldo Schmitt in the ‘s40 and at that time he noticed a palmar crest similarity with *A. obedrechtii* Muller, 1876 that the author thought at first it was the same species. Some doubts about its identification and real geographic distribution Bueno (personal communication) have been increased the need to study this species. Since many aeglids have been studied in relation to taxonomy, genetic and species complex and new discoveries have changed some classifications and researchers have described new species (Santos et al., 2009, 2012, 2013; Moraes et al., 2016, 2017; Bueno et al., 2017) is the reason why we decided to study *A. castro*. Therefore, in the present paper, *Aegla castro* sensu lato, type material and freshly specimens (topotypical), are compared based on morphology, and the hypothesis of more than one species is suggested.

2 Material & Methods

It was analyzed *Aegla castro* sensu lato species from museum collections and newly sampled materials from the type-locality and from additional locations. The additional collections comprised seven regions: Ponta Grossa, Castro, Tibagi (Pinheirinho Farm, São Damásio Farm and cachoeira Puxa Nervos) and Mauá da Serra in the state of Parana and Itatinga in the state of São Paulo. The collections were performed using plastic baited (dry cat food) traps and active collection.

We followed Moraes et al. (2016) for all morphological characters used and morphometric ratios. The authors performed a taxonomic revision for many species of Aeglidae and proposed new characters that can be included in species description and identification, beyond the characters described originally for each species and the characters proposed by Bond-Buckup & Buckup (1994).

The terminology used in the description of the chelipeds (first pereiopods) followed Martin & Abele (1988). A Zeiss Discovery V20 stereomicroscope with Zeiss Axiocam digital camera attached was used to take photographs.

Abbreviations used include: USNM (National Museum of Natural History), MZUSP (Museu de Zoologia, Universidade de São Paulo) and (CLE) Carapace length excluding rostrum.

3. Results

Taxonomic review of the *Aegla castro* Schmitt, 1942 holotype.

Type material. Holotype: male [22.99 mm - milimeters], ischium, merus and carpus of the fourth right pereiopod missing, Brazil, Paraná, Castro, Iapó river, collected in October1925, coordinates and altitude not available (USNM 80020) (Fig.1). **Paratypes:** 2 males [22.09 and 20.57 mm] Brazil, Paraná, Castro, Iapó river (USNM 169084). 2 males [21.45 and 19.10 mm] Brazil, Paraná, Castro, Iapó river (USNM 169086). 2 males [18.48 and 17.49 mm] Brazil, Paraná, Castro, rapids of Iapó river, Marumby Farm (USNM 169085). 1 male [18.92] Brazil, Paraná, Castro, Iapó river (USNM 169091). The date was October 1925 and the coordinates and altitude were not available for all paratypes.

Other material examined. 1 male [22.85 mm], Brazil, Paraná, Castro, Iapó river, collected in October1925, coordinates and altitude not available (USNM 169088).3 male [not measure] Brazil, Paraná, Castro, 1907, coordinates and altitude not available collected by E. Garbe (MZUSP 623).

Type-locality. Iapó river, Brazil, Castro, Paraná state.

Geographical distribution. South of São Paulo state and north of Paraná state.

Diagnosis. Rostrum triangular, base narrow, curved upward, extending beyond distal apex of compound eyes. Subrostral process on proximal half, well developed, high, broad, triangular, oriented downward. Orbital spines well developed. Epibranchial area with corneous scales on anterolateral angle and on lateral margin. Areola rectangular. Anteromesial region of third thoracic sternite tapered. Chelipeds large; palmar crest disciform with margin lobulate. Uropods wide. Posterolateral margin of telson slightly concave mesially.

Description of the male holotype. Carapace moderately convex, gastric region slighted inflated, dorsal surface scabrous, covered with punctations with small setae. Rostrum triangular, base narrow (value = 0.8), curved upward, extending beyond distal apex of compound eyes, with small corneous scales on lateral margins and tip (Figs 2 and 3). Rostral carina beginning at level of protogastric lobes, extending to the end of the apex, with small corneous scales. Subrostral process well developed, on proximal half of subrostral margin, high, broad, triangular, tip rounded without corneous scales and oriented downward (Fig. 2B).

Eyestalk and cornea well developed. Orbital sinus U-shaped, with plumose setae subventrally. Orbital spines well developed, pointed apically, with terminal small corneous scale. Anterolateral spines pointed apically with terminal pointed corneous scale on top and on lateral margin, straight, reaching basal margin of cornea.

Epigastric prominences and protogastric lobes pronounced (Fig. 2), with small corneous scales. Gastric area slightly inflated in relation to hepatic lobe and rostrum in lateral view Gastric pits inconspicuous. Only the distal hepatic lobe well demarked in relation to the second, lateral margins with small corneous scales and each lobe with one main corneous scale on top.

Transverse dorsal linea (TDL) slightly sinuous throughout its extension, sinuosity more pronounced laterally. Areola rectangular (value = 2.35). Cardiac area trapezoidal (value = 1.42) (Figs 1 and 3C).

Epibranchial area slightly elongated, triangular shaped, anterolateral angle blunt with small corneous scale and scattered small simple setae. Anterior branchial area with sulcus (Fig. 3D).

Anteromesial region of third thoracic sternite tapered with long simple setae over the surface. Fourth thoracic sternite with anterolateral angles moderately produced. There

is a carina between the third and fourth thoracic sternite (Fig. 3E). Chelipeds unequal in size (Fig. 5).

Major cheliped. Dactylus: dorsal margin and outer surface granulate; inner surface smooth; proximal lobe on dorsal margin blunt; cutting margin with lobular basal tooth well developed proximally, with flattened corneous scales, followed by row of wide corneous scales up to distal end; row of tufts of long, simple setae next to cutting margin. Propodus: outer surface granulate; palm high (value = 4); palmar crest disciform, margin markedly lobulate, outer surface excavated; cutting margin of fixed finger with lobular basal tooth well developed proximally, with flattened corneous scales, followed by row of wide corneous scales up to distal end; scattered tufts of long simple setae over inner surface and alongside inner and outer surfaces next to cutting margin. Carpus: dorsal margin with three spines in descending order of size (from distal to proximal) all of them with pointed terminal corneous scale; subterminal lobe well developed with terminal pointed corneous scale; inner surface with scattered simple long setae and one well developed tubercle with terminal pointed corneous scale; outer surface with carpal ridge high, formed by tubercles with small corneous scales and with two tubercle tipped by pointed corneous scales between the carpal ridge and dorsal margin. Merus: dorsal margin with one tubercle tipped by corneous scale on the dorsolateral edge as well on the ventrolateral edge; dorsolateral edge with eleven tubercles decreasing in size proximally, the proximal very small and naked; ventromesial edge with four spines tipped by corneous scales decreasing in size proximally; ventrolateral border with two tubercles being the distal much larger and pointed. Ischium: ventromesial border with four tubercles with corneous scales, the distal tubercle with much larger and pointed when compared with the others, the two tubercles on the middle very small and the proximal one larger than the middle ones, however smaller than the distal, ventrolateral border smooth.

Minor cheliped similar to major cheliped except as noted hereafter. Dactylus: cutting margin with a row of narrow corneous scales up to distal end however, with not a well developed basal tooth proximally. Propodus: palmar crest rectangular, margin less lobulated than the major cheliped; cutting margin with a row of narrow corneous scales up to distal end however, with not a well developed basal tooth proximally. Carpus: four spines on dorsal margin; distalmost spine tipped by one spiny, corneous scales; the next smaller and with a rounded corneous scale on top; the third bigger than the second one and tipped by one spiny, corneous scales and the proximal naked and smaller than the others; with one tubercle tipped by pointed corneous scales between the carpal ridge and dorsal margin. Merus: dorsolateral edge with ten tubercles decreasing in size proximally, only nine with corneous scales. Ischium: ventromesial border with only one tubercle between the proximal and the distal.

Second, third and fourth pereiopods morphologically similar, except where noted. They are covered by scattered, short, long setae and spiny corneous scales concentrated mainly along dorsal margin; dactylus small, flattened, forming setose minute chela with propodus; carpus with a spiny scale on distal portion of dorsal margin; meri with until 11 small tubercle tipped by pointed corneous scales on ventrolateral border; ischii naked on ventromesial border. Fifth pereiopod reduced, chelate. Sexual tube long, narrow, opening on coxa; coxa with setae (Figs 4 C, D).

Pleopods 2 and 5 absent, and 3 and 4 present as buds.

Anterolateral angle of second abdominal epimeron and ventral angles of third and fourth abdominal epimera well defined with spine corneous scale on top (Fig. 4B)

Uropods well developed, wide (Fig. 3F).

Telson with anterolateral and posterolateral margins well-differentiated; posterolateral margin slightly concave mesially (Fig. 3F).

Variations: some variations were observed mainly related with the areola format: rectangular in the male holotype 1 male paratype (169086) and subrectangular in the other specimens. The cardiac area was trapezoidal in the male holotype and varied from trapezoidal to rectangular in the paratypes. Excepting the holotype and one paratype (169085) (wide) all specimens had narrow uropod. The sexual tube had the same format however a variable tufts of setae number (2, 4 or 6 row of tufts of setae). The major cheliped was high (including the holotype) but the minor has some variation to low height.
Remarks: they were very similar in relation to the morphological characters, mainly in relation to the subrostral process and the third thoracic sternite. All specimens had at least one cheliped disciform (slightly). The initial doubt it was in relation to *Aegla schmitti* Hobbs III, 1979 because they have some similar characteristics as the disciform cheliped, however, *A. schmitti* have at least one of its chelipeds markably disciform.

Comparison among the "*A. castro*" species

Twenty six individuals were analized: *A. castro* from Ponta Grossa (2 males, only one measured 23.15 mm), *Aegla* sp. from Castro (2 males, 24.24 and 28.64 mm), *Aegla* sp. from Tibagi - Puxa Nervos (5 males, 22.87, 23.84, 19.44 and 22.81 mm), *Aegla* sp. from Tibagi - Pinheirinho (3 males, 17.24, 17.46 and 16.85 mm), *Aegla* sp. from Tibagi - São Damásio (7 males, 19.83, 11.68, 12.81, 12.92, 12.69, 17.72 and 11.27 mm), *Aegla castro* from Mauá da Serra (5 males 15.21, 15.72, 16.39, 17.36 and 18.26 mm), *A. castro* from Itatinga (5 males, 16.23, 16.62, 14.08, 15.90 and 19.39 mm).

The specimens collected were different from the holotype and paratypes at least in two main and evident characteristics: the third thoracic sternite and the subrostral process (Figs 8 and 7). The holotype and paratype subrostral process is tapered as the *Aegla* sp. from Castro and *Aegla* sp. from Tibagi - São Damásio (Figs 8A, B and H).

However, *Aegla* sp. from Tibagi - São Damásio has rostrum, subrostral process and cheliped very distinct of the holotype and paratype specimens. The other species are abrupt type of the third sternite, excepting *Aegla* sp. from Tibagi - Pinheirinho that is abrupt.

The subrostral process is a wide triangle, the tip is not acute or delicate and it is directed downward in the holotype and paratypes as well in the *Aegla* sp. from Castro that is the only species similar with what would be characteristic of *A. castro* (Fig. 7B). The other species have at least 3 different formats: 1) proximal half, well developed, high, acute triangular oriented downward - *Aegla castro* from Mauá da Serra (Fig. 7C); 2) proximal half, well developed, high, acute triangular and tip oriented anteriorly - *Aegla* sp. from Tibagi - Pinheirinho and *Aegla* sp. from Tibagi - São Damásio (Figs 7 E , F) and 3) the same as holotype but with the rostrum directed downward *A. castro* from Itatinga. The *A. castro* from Ponta Grossa seems to have the same pattern as the holotype however, it is shorter and delicate in relation to the tip (Fig. 7A).

Although the rostrum format have not been an easy characteristic to differentiate species, it is possible to observe distinct formats and the carina achievement, if it goes until the end of the rostrum tip: holotype, *Aegla* sp. from Castro, *A. castro* from Ponta Grossa, *Aegla* sp. from Tibagi - Puxa Nervos, *Aegla castro* from Mauá da Serra, *A. castro* from Itatinga or not *Aegla* sp. from Tibagi - Pinheirinho and *Aegla* sp. from Tibagi - São Damásio. The shape of the rostrum was narrow in all specimens, excepting one specimen from Tibagi - Pinheirinho, however they were shorter and wider in *Aegla castro* from Mauá da Serra, *A. castro* from Itatinga, *Aegla* sp. from Tibagi - Pinheirinho and *Aegla* sp. from Tibagi - São Damásio than the other species (Fig. 6).

The ischium is one of the taxonomic characteristics of *A. castro*, that is: one remarkable tubercle on the distal tip of the ventromesial edge, some smaller in the middle

and a medium size tubercle at the proximal position. *Aegla castro* from Ponta Grossa was very similar (Fig. 9A) however the second specimens was different and the tubercles were the same size (Fig. 9B). *Aegla* sp. from Castro was very similar with the holotype and *A. castro* from Mauá da Serra was different with not distinction in relation to the size of the tubercles (Figs 9C, D, E, F). All the other species, *A. castro* from Tibagi - Puxa Nervos, *A. castro* from Itatinga, *Aegla* sp. from Tibagi – Pinheirinho and *Aegla* sp. from Tibagi - São Damásio, have similar ischium with the holotype. Although *Aegla* sp. from Tibagi - São Damásio seems to be different, one of the specimens have the same patterns than the holotype (Fig. 10).

The chelipeds were in majority disciform (Fig. 11), however usually it was observed the variation in the same individual, i.e., two types of chelipeds. For example, *A. castro* from Ponta Grossa has a disciform and rectangular cheliped (Fig. 11C). That variation occurred in all species and the tendency was the occurrence of the disciform and rectangular formats.

Epibranchial area have always the same pattern in all species: triangle shape, slightly elongated, anterolateral angle blunt with small corneous scale, margins with scattered small simple setae.

The telson has a concave format and was variable in the holotype and paratypes (wide and narrow). It is not possible to stablish a pattern.

The presence of sulcus in the anterior branchial area in the holotype and paratypes could be useful in *A. castro* characterization. Only *Aegla* sp. from Castro, *A. castro* from Tibagi - Puxa Nervos, *Aegla* sp. from Tibagi – Pinheirinho and *Aegla* sp. from Tibagi - São Damásio presented sulcus in the anterior branchial area.

FIGURE 1. *Aegla castro*, male holotype, CLE 22.99 (USNM 80020). General view. Scale bar: 5 mm.

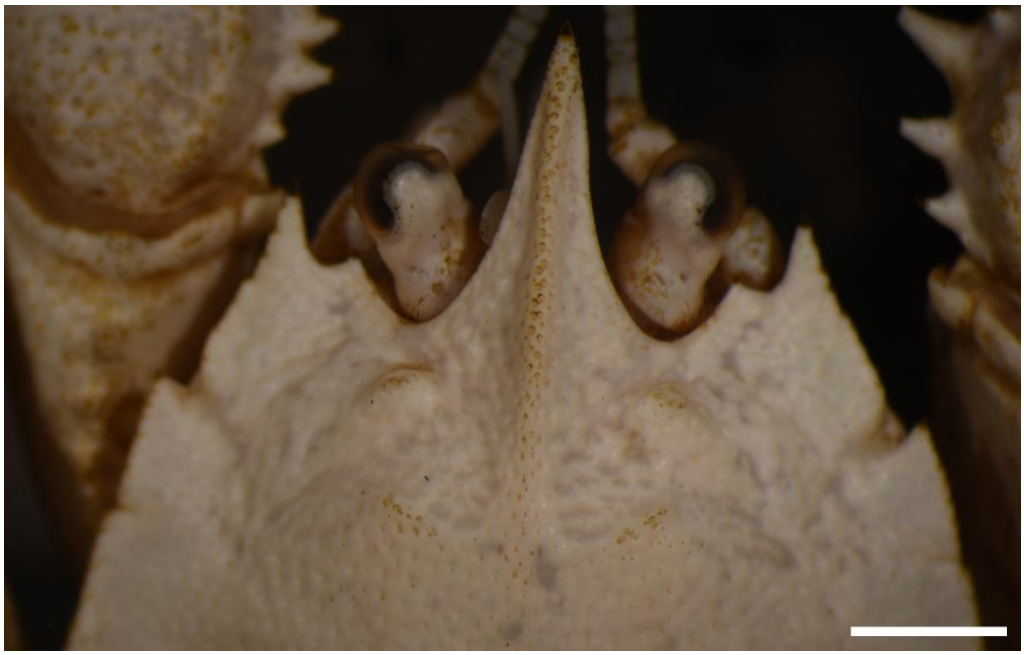


FIGURE 2. *Aegla castro*, male holotype, CLE 22.99 (USNM 80020). Dorsal view of the anterior region of the cephalothorax. Scale bar: 2.5 mm.

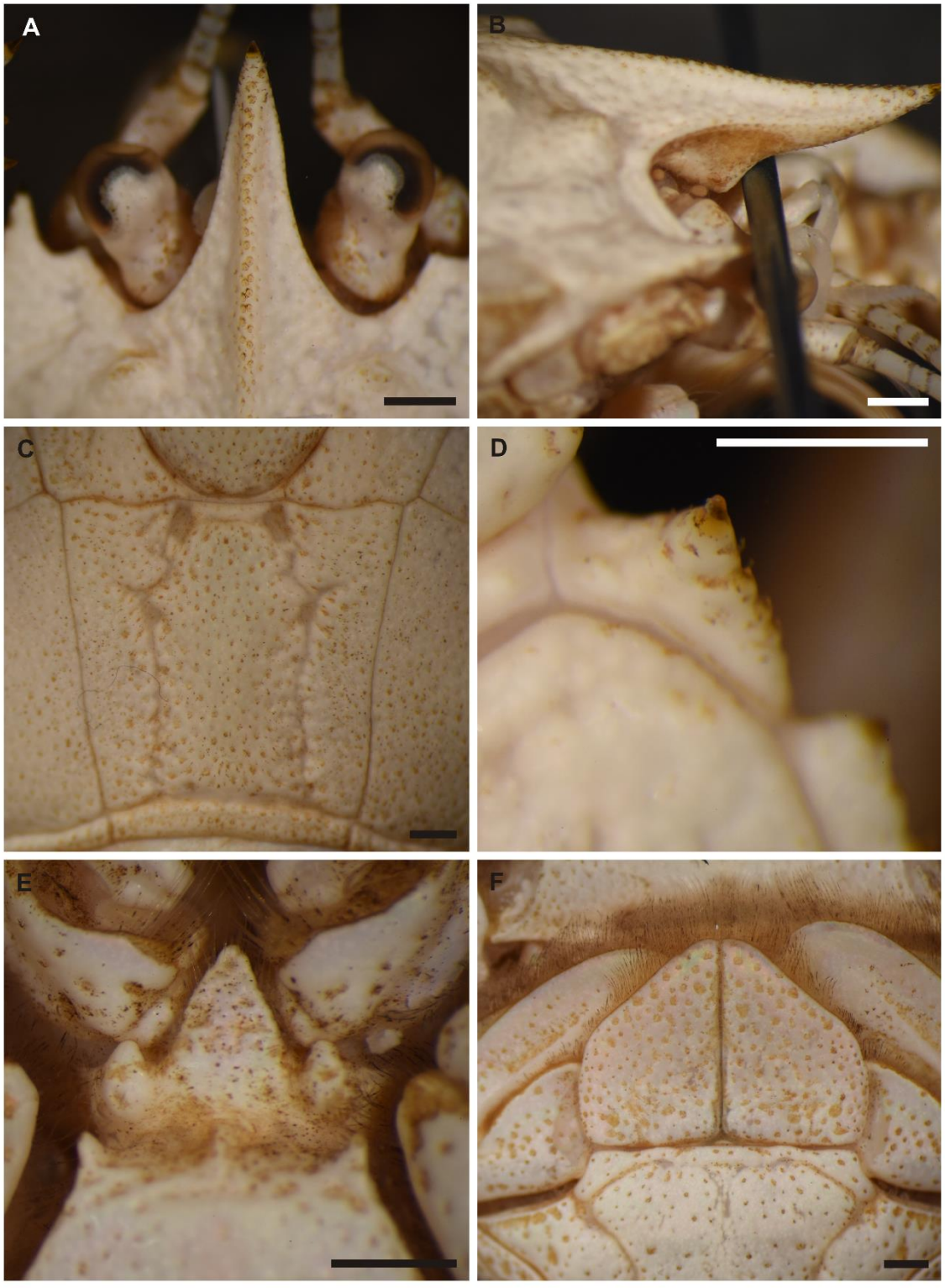


FIGURE 3. *Aegla castro*, male holotype, CLE 22.99 (USNM 80020). (A) Rostrum. (B) Subrostral process. (C) Areola. (D) Epibranchial area. (E) Third thoracic sternite that is tapered. (F) Telson. Scale bar: 2.5 mm.

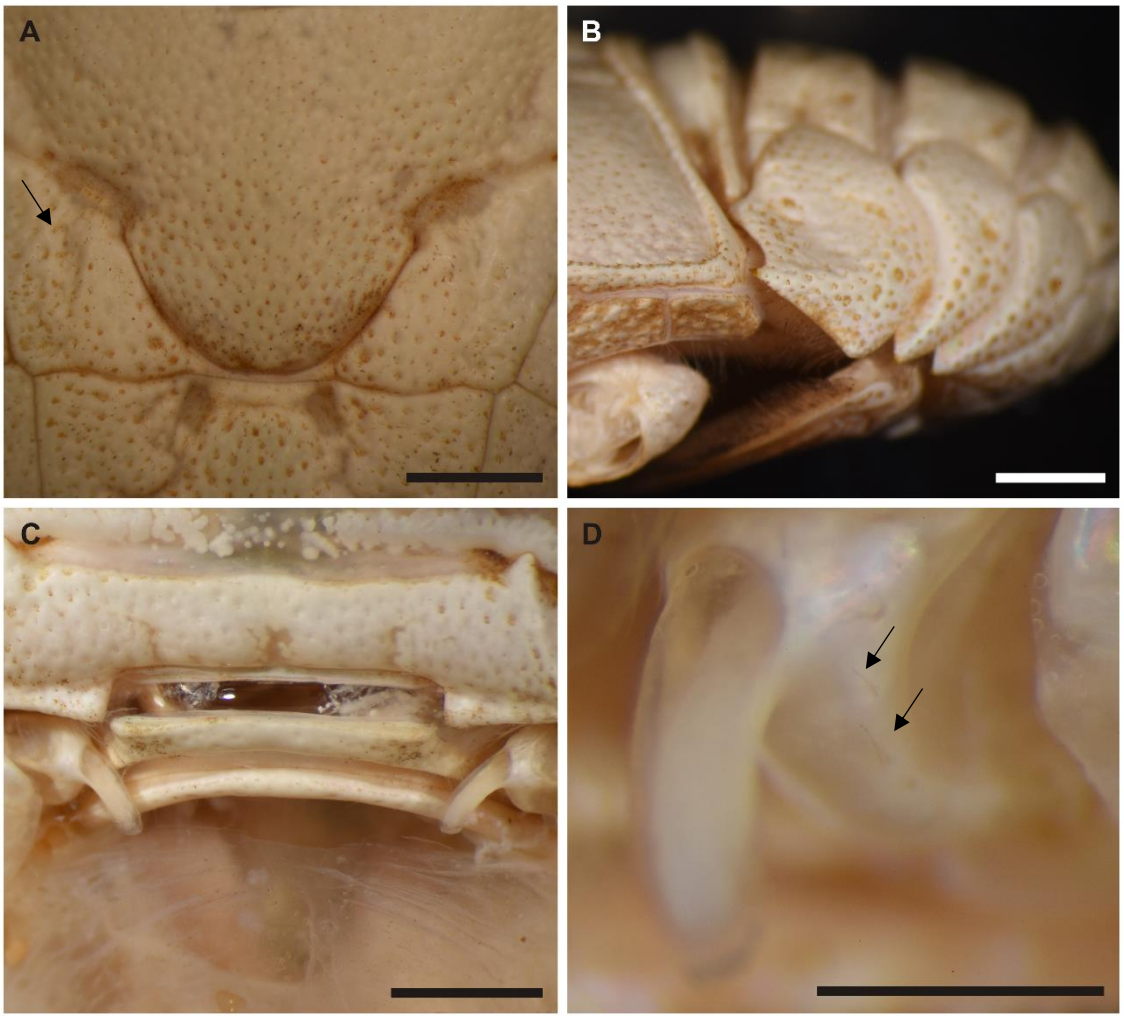


FIGURE 4. *Aegla castro*, male holotype, CLE 22.99 (USNM 80020). (A) Dorsal view of the cephalothorax and the anterior branchial area with sulcus (black arrow). (B) Lateral view of the abdomen and the three epimera with pointed scale on tip. (C) Sexual tubes. (D) Detail of the sexual tube showing the tufts of setae (black arrow). Scale bar: 2.5 mm.

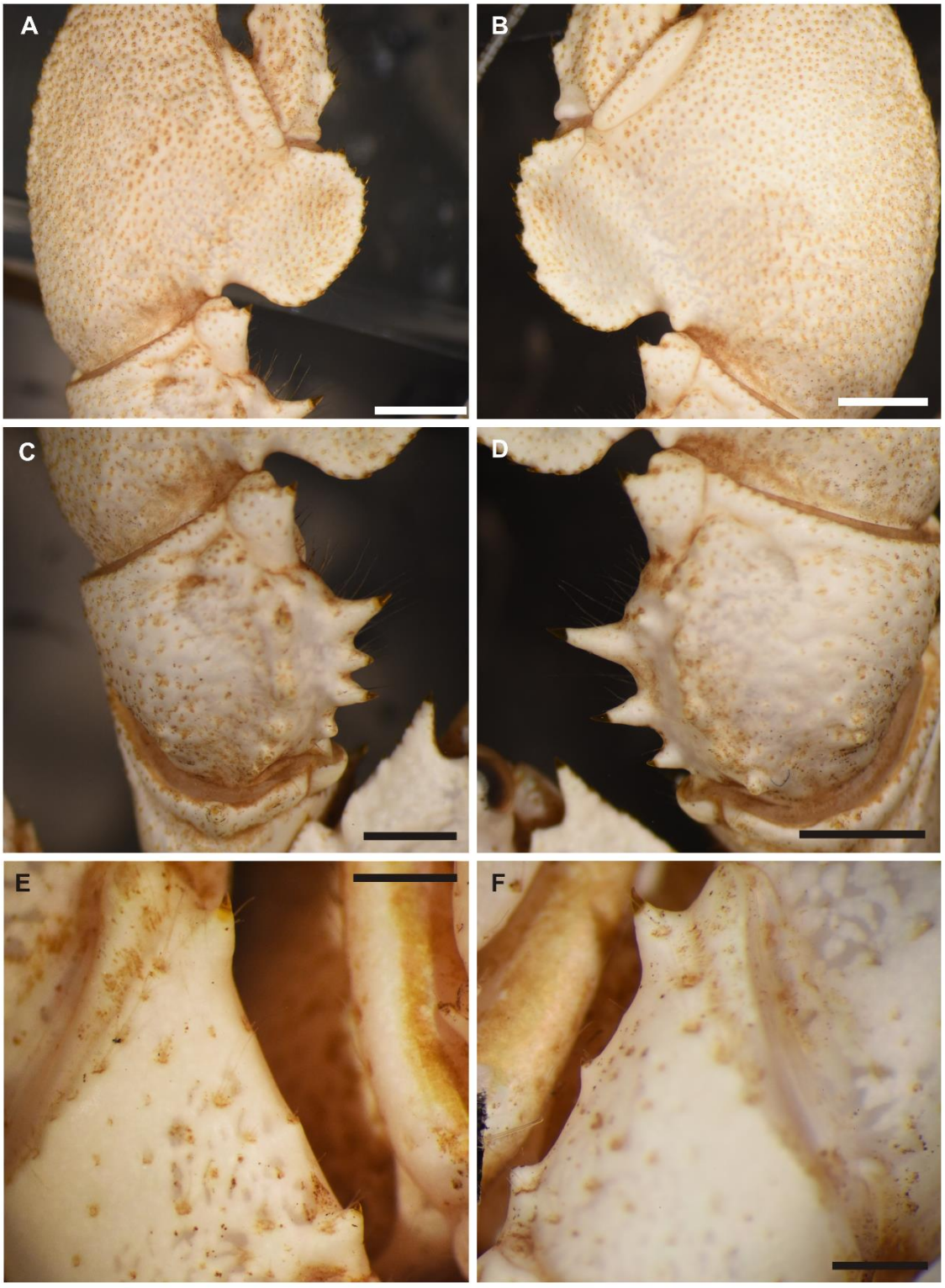


FIGURE 5. *Aegla castro*, male holotype, CLE 22.99 (USNM 80020). (A) Minor cheliped. (B) Major cheliped. (C) and (D) Carpus. (E) and (F) Ischium. Scale bar: 2.5 mm.

FIGURE 6. Rostrum. (A) *Aegla castro*, male holotype. (B) *Aegla* sp. from Castro. (C) *A. castro* from Ponta Grossa. (D) *Aegla* sp. from Tibagi - Puxa Nervos. (E) *Aegla castro*

from Mauá da Serra. (F) *A. castro* from Itatinga. (G) *Aegla* sp. from Tibagi – Pinheirinho. (H) *Aegla* sp. from Tibagi - Fazenda São Damásio. Scale bar: 1 mm.

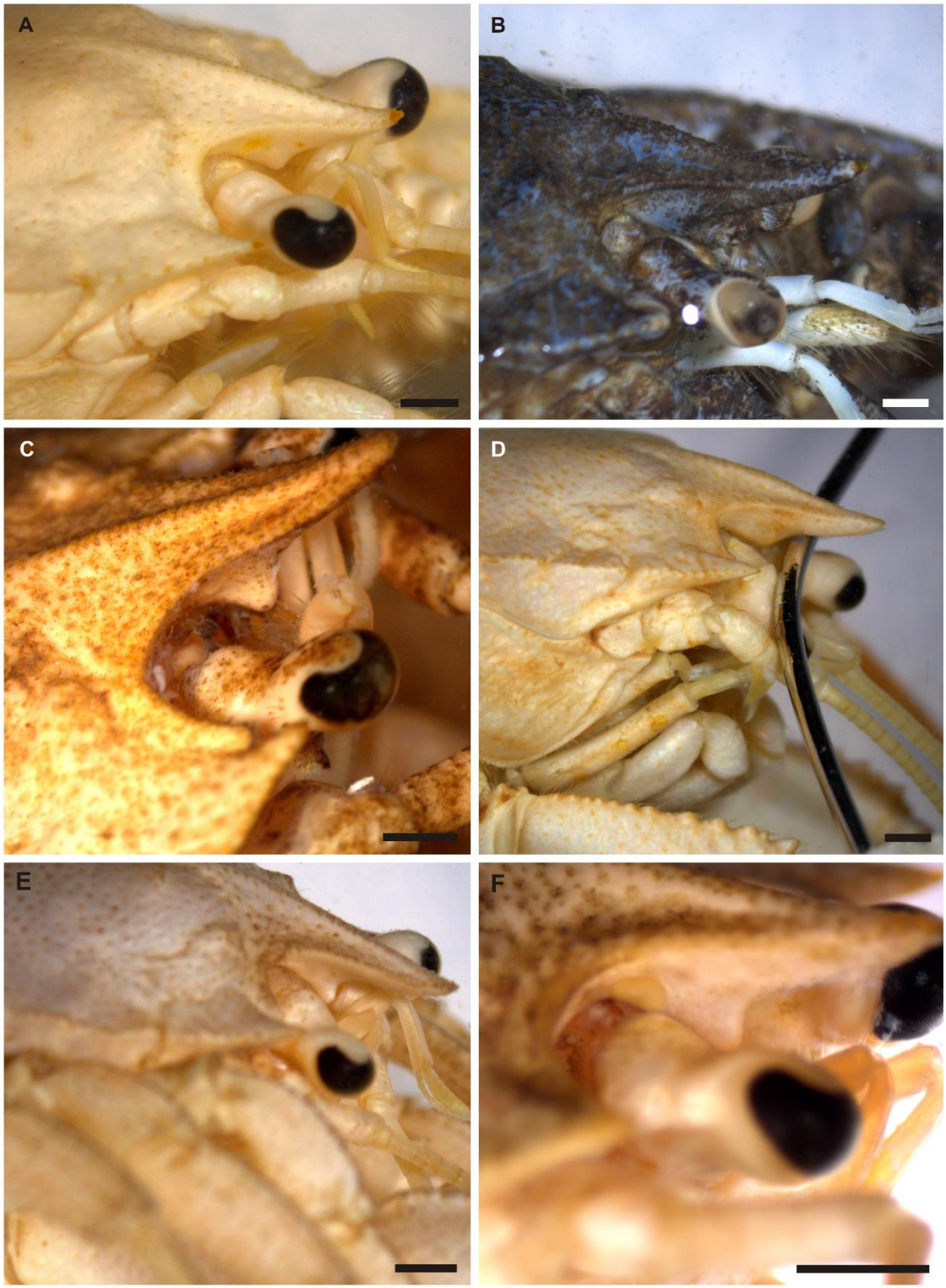


FIGURE 7. Rostrum. (A) *A. castro* from Ponta Grossa. (B) *Aegla* sp. from Castro. (C) *Aegla castro* from Mauá da Serra. (D) *A. castro* from Itatinga. (E) *Aegla* sp. from Tibagi - Pinheirinho. (F) *Aegla* sp. from Tibagi - São Damásio. Scale bar: 1 mm.

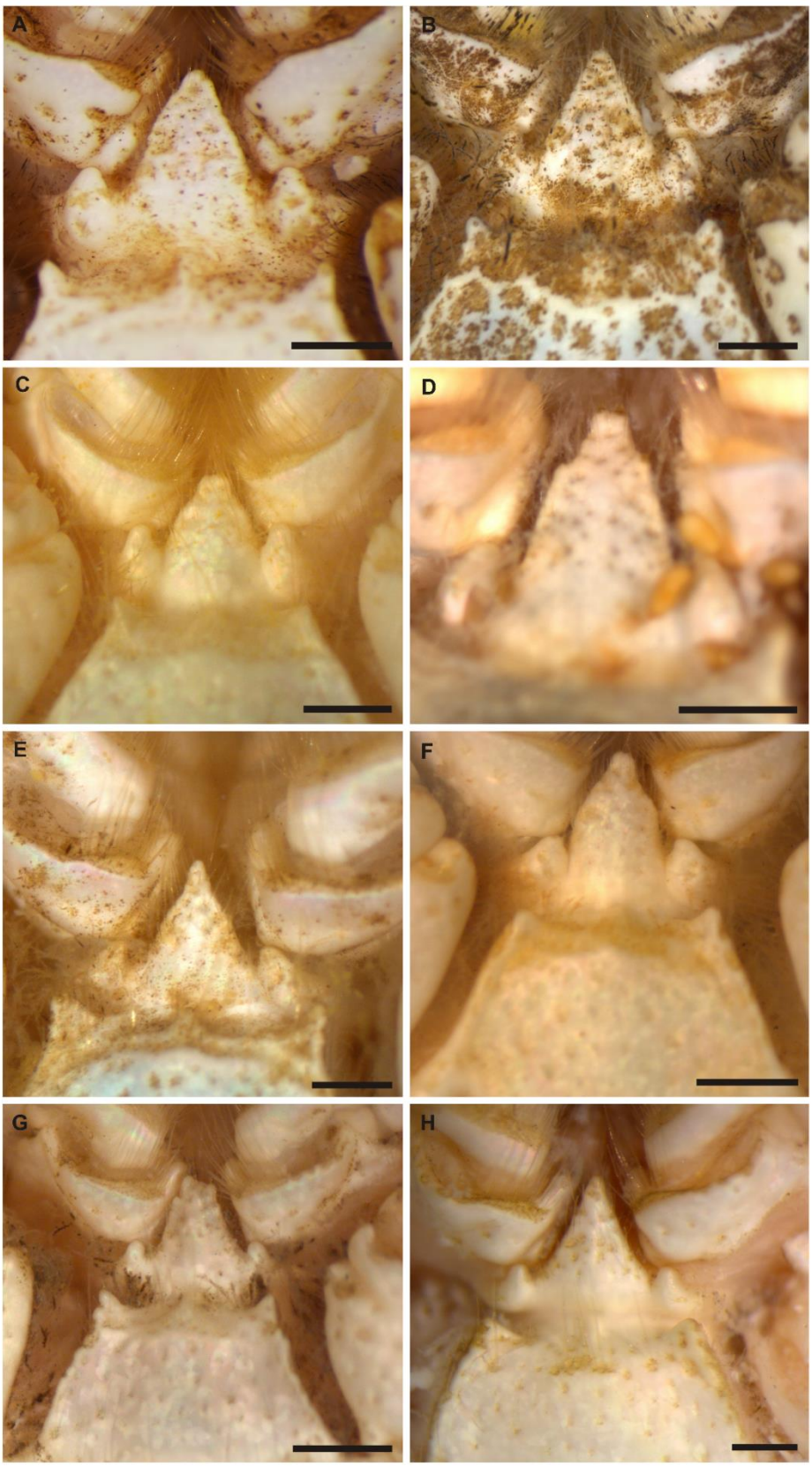


FIGURE 8. Third and fourth thoracic sternites. (A) *Aegla castro*, male holotype (tapered). (B) *Aegla* sp. from Castro (tapered). (C) *A. castro* from Ponta Grossa (abrupt). (D) *Aegla* sp. from Tibagi - Puxa Nervos (abrupt). (E) *Aegla castro* from Mauá da Serra (abrupt). (F) *A. castro* from Itatinga (abrupt). (G) *Aegla* sp. from Tibagi – Pinheirinho (truncate). (H) *Aegla* sp. from Tibagi - São Damásio (tapered). Scale bar: 1 mm.

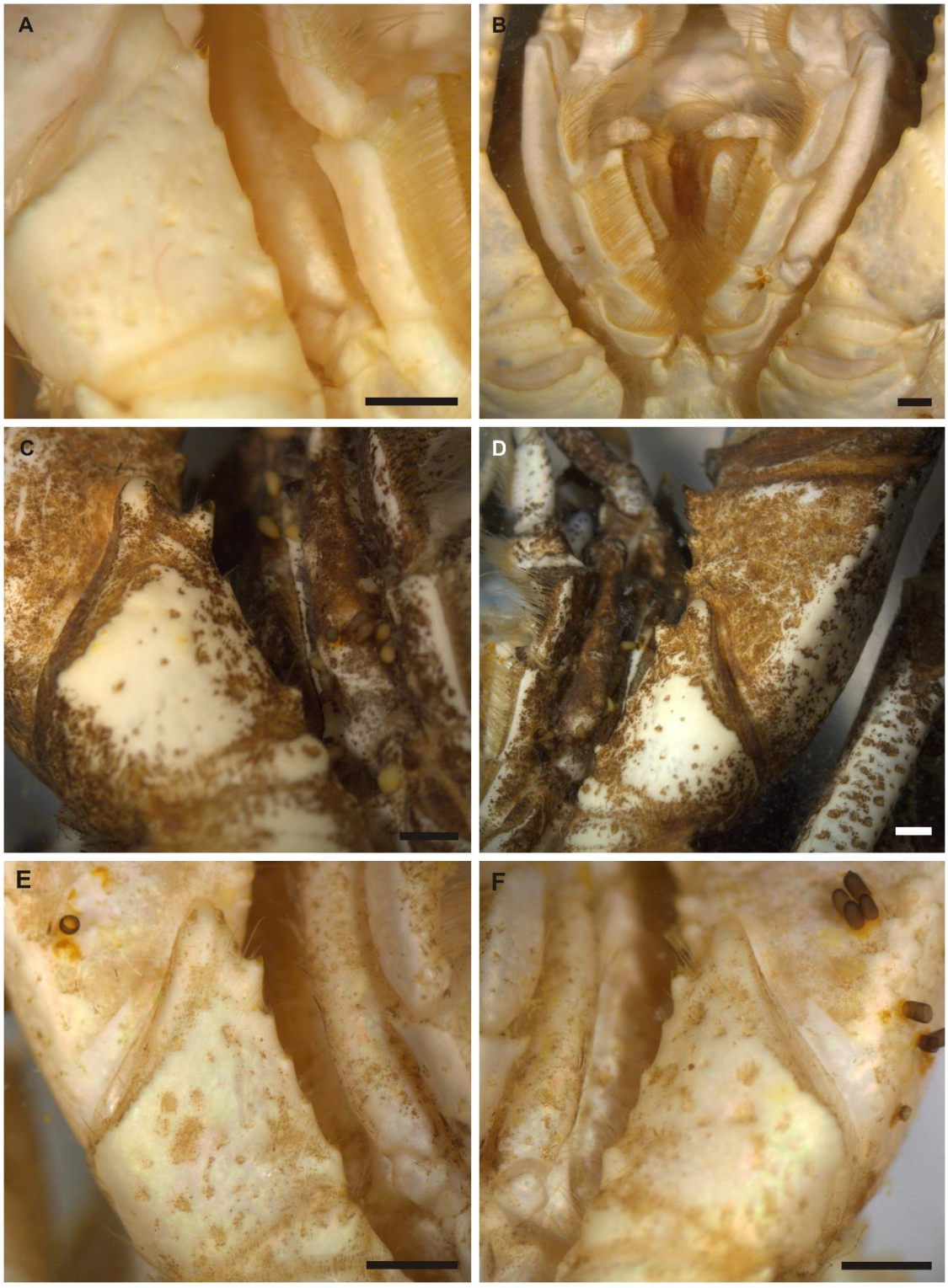


FIGURE 9. Ischium. (A) and (B) *A. castro* from Ponta Grossa (different specimens). (C) and (D) *Aegla* sp. from Castro. (E) and (F) *Aegla castro* from Mauá da Serra. Scale bar: 1 mm.

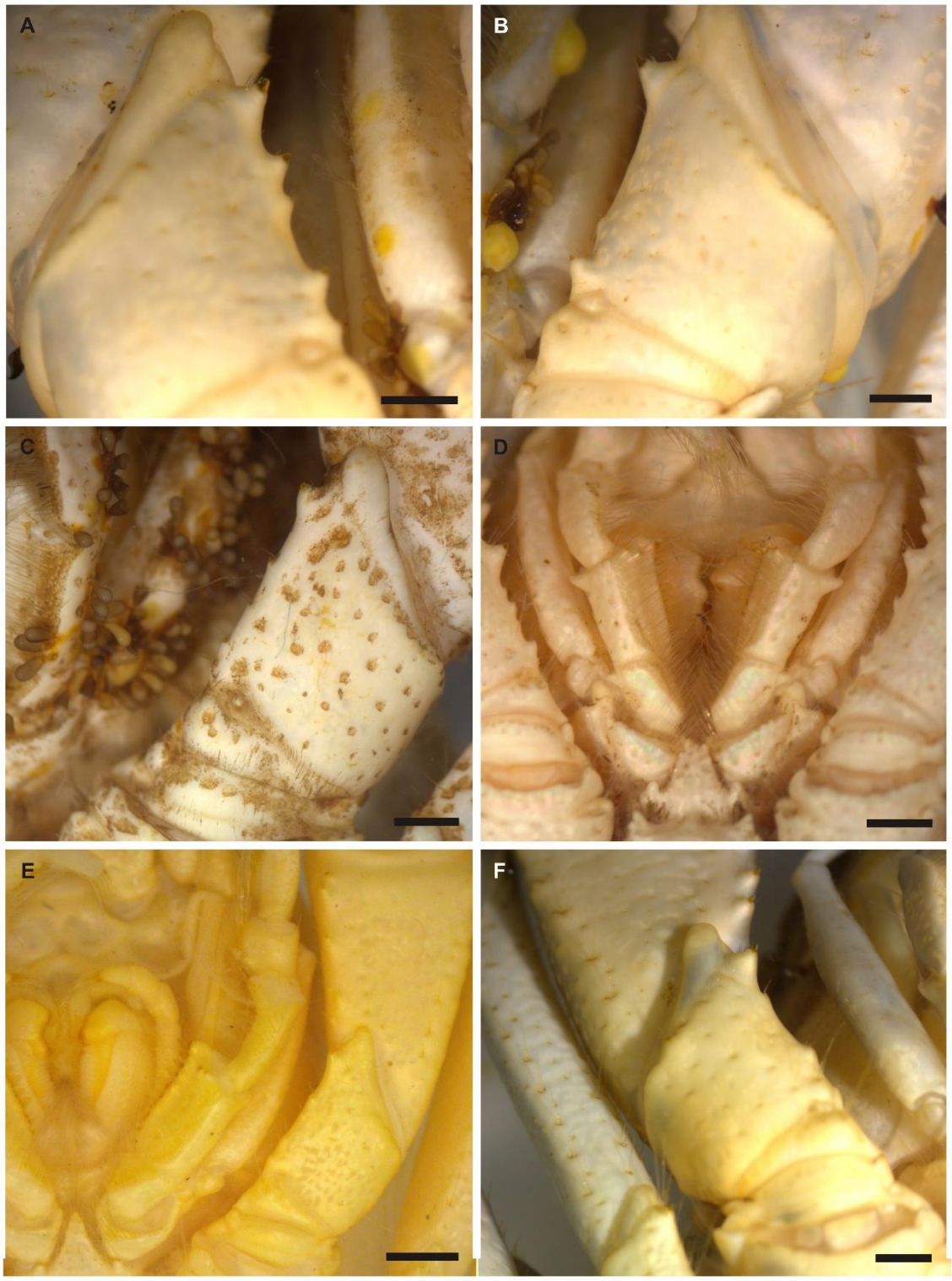
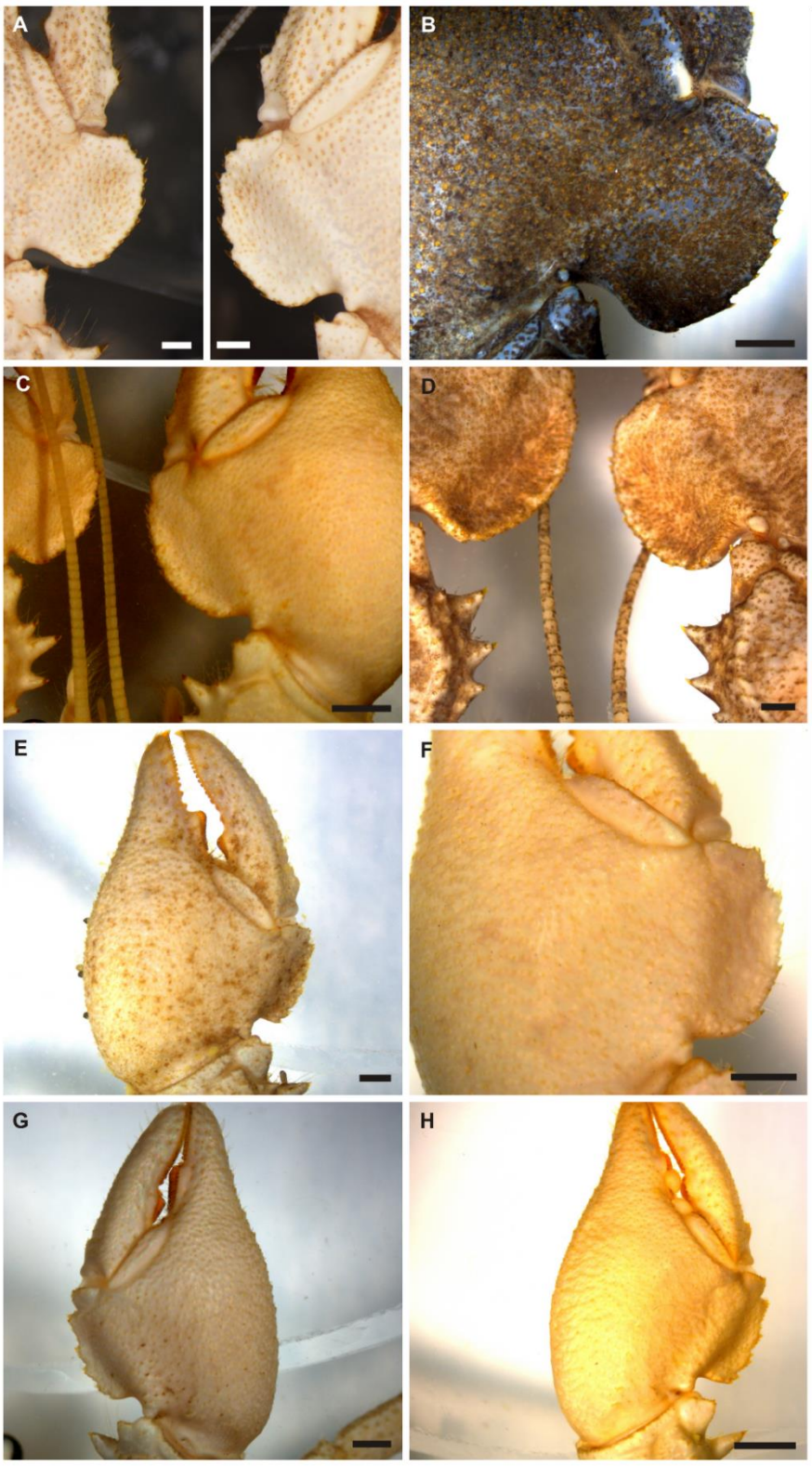


FIGURE 10. Ischium. (A) and (B) *A. castro* from Tibagi - Puxa Nervos. (C) *A. castro* from Itatinga. (D) *Aegla* sp. from Tibagi - Pinheirinho. (E) and (F) *Aegla* sp. from Tibagi - São Damásio. Scale bar: 1 mm.



F**IGURE 11**. Palmar crest of the cheliped (major cheliped or both). (A) *Aegla castro*, male holotype (disciform). (B) *Aegla* sp. from Castro (disciform). (C) *A. castro* from Ponta Grossa (disciform and rectangular). (D) *Aegla* sp. from Tibagi - Puxa Nervos (disciform) (E) *Aegla castro* from Mauá da Serra. (F) *A. castro* from Itatinga (rectangular). (G) *Aegla* sp. from Tibagi – Pinheirinho (rectangular). (H) *Aegla* sp. from Tibagi - São Damásio (rectangular). Scale bar: 1 mm.

4. Discussion

The remarkable result found in the present manuscript is the potential division of *A. castro* in more than one species and the potential new species. That simple idea synthetized in only one phrase has a bigger meaning that implicates in the real diversity of this group that is so peculiar, however has not received the deserved attention, mainly in relation to the Brazilian Environmental Policy.

The main issue reflected by this result is that Aeglidae diversity is not well known yet, however more than a half of species from Brazil (53 of 78) (Bueno et al., 2016) are included in the IUCN (2013) list of threatened species. In this scenario, the efforts in discovery the real diversity should be prioritized (Bickfort et al., 2007). It should be known by people and it should be protected by law.

The way to protect this species from extinction is to protect their environment. Freshwater have been one of the most destructed environment once human have taken advantage of the natural sources. The original forest for example in Brazil has changed, and most of the gallery forests that protected freshwater streams from siltation were removed, pollution and dam constructions are other examples of environmental changes (Bond-Bockup et al., 2010). This has become so severe, that freshwater ecology nowadays is mainly interesting in investigate the losses of biodiversity and the reasons related to it (Dudgeon, 2006; Strayer and Dudgeon, 2010; Cumberlidge & Kawai, 2016).

Efforts in re-describe species and investigate species complex have been performed in relation to Aeglidae and new species have been described. For example, Moraes et al., (2016) after investigate *Aegla paulensis* Schmitt, 1942 complex described four new species. They results changed all the conception of "wide" distribution range for *A. paulensi*s and consequently its conservation status. This species was not classified by the IUCN (2013) due to the lack of data, however it was recognized previously as

Least Concern (LC) by Pérez-Losada et al. (2009), because it is distributed through three hydrographic basins Ribeira de Iguape, Upper Tietê (Paraná Basin), and Paraíba do Sul. After Moraes et al., (2016), the scenario changed from an only one largely distributed species to six species, *Aegla paulensis* (s. str.), *A. rosanae*, *A. vanini* n. sp., *A. jaragua* n. sp., *A. japi* n. sp. and *Aegla jundiai* n. sp., highly endemic and restrict.

Aegla castro had it distribution expanded based on Rio Grande Bacin in the Parana and São Paulo state (Bond-Buckup & Buckup, 1994). However, the real distribution of this species can change in the same way as was observed in Moraes et al., (2016). It is possible to observe that only *Aegla* from Castro has similar characteristics compared to the holotype. The subrostral process format and the tapered third thoracic sternite are the characteristics that remained stable in the *A. castro* holotype and paratypes. Moraes et al., (2016, 2017) used the subrostral process as a good character to include on species diagnosis. This character seems to be conservative in the species and variable between species, combined with other characteristics it is a useful character. The presence of sulcus in the third thoracic sternite on the holotype and paratypes, as well *Aegla* sp. from Castro could be an additional character.

The potential *A. castro* (Ponta Grossa, Tibagi - Puxa Nervos, Mauá da Serra, Itatinga) different from the holotype shared a truncate format in the third thoracic sternite, while the holotype is tapered. They differed among them in relation to the subrostral process, but those differences are not clear, also it is not clear yet if these differences are only some population variation in the phenotype. Terossi et al., (2010) for example, found genetic and morphological variations in populations of *Hyppolite obliquimanus* Dana, 1852 that did not support their separation in different species. It should be used other tool to help in answer with more accuracy this question as genetics.

The two species from Tibagi, *Aegla* sp.- Pinheirinho and *Aegla* sp. - São Damásio, are very different from the others and are potential new species, once they are located in very isolated freshwater streams. However, although they are very close distributed, they are very different in relation to the third thoracic sternite: truncate in *Aegla* sp.- Pinheirinho and tapered in *Aegla* sp. - São Damásio.

It is very clear that there is morphological differences in relation to the species collected during this research that certainly, there is more than one species; as well, some species are similar and it would be the complex. To be more confident about these results it will be essential the performance of genetics. As it can be observed in Crivellaro et al., (2017) with genetic analysis, from seventeen populations studied of *Aegla longirostris* Bond-Buckup & Buckup, 1994 there were at least fourteen new species. Otherwise, only one species was recognized to be different from other sixteen populations of *Aegla platensis* studied by Zimmerman et al., (2018) though genetic analysis. So, these results reflected how *Aegla* spp. are an controversial group, very interesting though. The importance to continue more deeply studies in relation to *A. castro* is necessary to answer the questions that were started here, mainly because it does not know the real conservation status for this species.

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CAPÍTULO 2

Ultrastructure of spermatozoa of *Aegla sp*. (Crustacea, Decapoda, Aeglidae): description of morphological patterns, comparison of the potential cryptic taxon *Aegla castro* Schmitt, 1942 and information for conservation

Abstract

The diversity of the endemic South American genera *Aegla* have been increased since the currently taxonomy revisions and the advance of the genetics analysis. The existence of a hidden diversity reflected as cryptic complex for taxon with wide geographic distribution is investigated here for *A. castro.* For this, it was described the ultrastructure of spermatozoa for three supposed "*A. castro*" species, for one potential new species of *Aegla* spp. and for two external group *A. parana* and *A. quilombola*. Two general spermatozoa formats were identified: the spherical and the irregular with the two hemispheres (cytoplasma and nucleus) separated by a membrane. We did not find different spermatozoa morphology for each species; however, we can determine at least three patterns of spermatozoa morphology 1) spherical with short acrosome 2) irregular with long and lobulated acrosome and 3) irregular with short acrosome. This means that even we could have different species they can share the same pattern of spermatozoa morphology. Our results support the exclusion of Aeglidae from the superfamily Galatheoidea and agree with the previous information of no evidence of spermatophore in this group. It is remarkable the scant spermatozoa production in all the species studied here and it is the same information from literature, which leads us in thinking if there is some implication for future population recovery. These species are very sensitive to environmental changes and currently at least 31 species are threatened.

Key words: threatened, freshwater, conservation, sperm transfer.

1 Introduction

The occurrence of cryptic species, i.e., very morphologically similar species from the same genera (Mayr, 1963) has been described for the family Aeglidae Dana, 1852 by (Bartholomei-Santos et al., 2011, Marchiori et al., 2015, Moraes et al., 2016, Crivellaro et al., 2017) and although the efforts of different research groups in Brazil many gaps need to be solved, mainly related to the morphology. In the current scenario, 84 species of *Aegla* Leach, 1820 were already registered for the Southern South America (Bueno et al., 2017), however, in the compilation performed by Bueno et al. (2016) for the 78 species described until 2016, more than half i.e., 43 (55.13%) were classified as critically endangered, endangered or vulnerable at that time, based on the IUCN criteria (IUCN, 2013).

This mean that the extinction of some species could be occurring without them being studied or at least their registration as a new species, e.g., from 2016 to 2017, at least 6 new species of aeglids were described for Brazil and these numbers have been increasing (Moraes et al., 2016, 2017, Bueno et al., 2017). Based on the information considered above, the true diversity of aeglids is a challenge for researchers, because, it have not been well known, i.e., the number of species would be greater than the current number (hidden diversity). This occurs mainly because speciation could occur not necessarily thought the phenotypic expression (Bickford et al., 2017) what make species identification very difficult and because many areas have not been exploited yet.

Besides *Aegla spp.* being the unique genera in Aeglidae family, once the others two are fossils *Haumuriaegla* (Feldmann, 1984) and *Protaegla* (Feldmann *et al.*, 1998), the high endemism to temperate and subtropical regions of South American and the restrict distribution to freshwater streams (Schmitt, 1942) have contributed for these species to be endangered. Habitat destruction, e.g., deforestation and consequent drainage

sedimentation, pollutants and the construction of reservoirs (Bond-Buckup et al., 2010) are other factors responsible to the high percentage of the endangered species cited above.

Some aeglid species have restrict distribution and others a wide one, what it is an important issue once wide distributed species are not considered a priority concern by IUCN, because they "would be" larger and stables populations, unlike the restrict ones (Moraes et al., 2017). However, species with a wide distribution may be a complex of cryptic species (as mentioned above). In this case, their distribution would be narrower than the distribution registered and consequently a different conservation status should be applicable (Moraes et al., 2017; Santos et al., 2017). Moraes et al. (2016) discovered that although *A. paulensis* Schmitt, 1942 being considered to have a wide distribution, its contention in several isolated streams contributed for their speciation and the authors found at least seven species of which four are new. Interesting in this issue, we chose to investigate *A. castro* Schmitt, 1942 because has a wide geographic distribution (from the northeastern region of state of Parana to the southern region of state of São Paulo) (Bond-Buckup and Buckup, 1994) and some doubts about its validation as only one species have increased (personal observation).

For these reasons, we have tried to use additional tools to see morphological differences among species as well to bring useful information about this not well-known group. Since it has been used in phylogenetic relationships studies of several crustaceans decapod species (Jamieson, 1991; Tudge, 1997; Medina, 1995; Camargo et al., 2016; Assugeni et al., 2017), and in general has a diverse morphology in this group (Jamieson 1991), the ultrastructure of spermatozoa is considered a successful source of taxonomic and phylogenetic information (Tudge, 2009). Only Tudge and Scheltinga (2002) and Tudge (2003) described the ultrastructure of spermatozoa for the species *A. longirostris*

(Bond-Buckup, 1994). Their results were an important argument to put *Aegla spp.* out of Galatheoidea family and in their own family.

Therefore, we described the ultrastructure of spermatozoa for some species of *Aegla* and for *A. castro* from different regions. Our hypothesis is based on the possibility to observe differences and at least more than one pattern in the ultrastructure of spermatozoa for the taxon *Aegla spp*. additionally, if *A. castro* is in fact more than one species, could be these differences detected?

2 Material & Methods

2.1 Sampling

Specimens were collected in seven regions: União da Vitória, Ponta Grossa, Castro, Tibagi (Pinheirinho Farm, São Damásio Farm and cachoeira Puxa Nervos) and Mauá da Serra in the state of Parana and Itatinga and Intervales in the state of São Paulo. The collections were performed in two periods based on *A. castro* reproductive period from Ponta Grossa (Swiech-Ayoub and Masunari, 2001) 1) July, August and September of 2016 and 2) March and May of 2017. It was performed through plastic baited (dry cat food) traps arranged randomly and distributed in the stream and when necessary through active collection (by hand after being visualized) or with the invertebrate D-net.

2.2 Transmission Electron Microscopy (TEM)

Samples of the reproductive system were taken from adult males and were fixed for 4 hours in 3% glutaraldehyde in 0.1 mol cacodylate buffer (ph 7.2) (*A. castro* - Ponta Grossa and *A. parana*) or in the less toxic fixative 2.5% glutaraldehyde and H2O in PHEM buffer (PIPES, HEPES, EGTA) (ph 7.2), details of the PHEM buffer see Montanaro et al. (2016) (*Aegla sp.* Pinheirinho and São Damásio, *A. castro* – Mauá, Itatinga and Ponta Grossa, *A. quilombola* and *A. parana*). After 4 hours, the samples were post-fixated in 1% osmium tetroxide in the same buffer for 2 hours and then they were washed three times in the same buffer. Samples were stained “en bloc” with 1% uranyl acetate for 12 hours, and then they were dehydrated in an acetone series (from 50% to 100%) and embedded in Epon-Araldite resin. The subsequent step was to cut the resin blocks on a Leica UC7 ultramicrotome in ultrathin sections (50 nm) that were collected on cooper grids and contrasted in 5% uranyl acetate in water (45 min) and 0.5 % lead citrate in 0.1 N NaOH (10 min) (Reynolds, 1963; Venable and Coggeshall, 1965). The

photos were obtained with a transmission electron microscope JEOL J1010 using an 80 KV electron beam.

3 Results

In general, the spermatozoa ultrastructure of *Aegla* spp. studied here are composed of two parts, the upper or apical hemisphere with the acrosome vesicle and the cytoplasm and the lower composed by the nucleus. The acrosome is "embedded" on the cytoplasm region and we did not notice the presence of distinct encapsulated spermatophores on the vas deferens. We observed in all specimens analyzed a reduced number of spermatozoa, regardless of the reproductive system portion, i.e., vas deferens or testis, and including specimens in which for some reason (e.g. plane of section) the material was not used in this manuscript (personal observation). In addition, it was observed some large cells (not identified yet) at the lumen of the testis and vas deferens together with the spermatozoa cells, which could be cell debris (see Figures 1a, 2a, 4a, 6a, 7ab, 9a, 11a) for a general view).

3.1 Spermatozoa of *Aegla castro* Ponta Grossa (state of Paraná)

The spermatozoa of *A. castro* is observed at the testis have an irregular format and in longitudinal section the two hemispheres described above are observed. The acrosome vesicle is electron dense with an external thin membrane. It seems to does not achieve the top of the perforatorial chamber (center region), only the acrosome membrane achieve it although it is difficult to see its continuity through the top once something seems to have damaged this region (Figure 1b). More internally, it is observed a less electron dense region with a granular content, mainly on the top of the perforatorial chamber, which we considered the operculum, characterized by a wide opening. It is important to say that the coverage of these structures could be different and it depends on the section that we obtained, therefore we chose the Figure 1b as the best section of the spermatozoon for this species, however we used others to confirm the details. At the center of the acrosome,

the perforatorial chamber has an electron-pale spherical format with granular content. It is not possible to observe a clear subacrosomal region. The cytoplasm is composed of many non-functional mitochondria (acristate or poorly cristate) and occupies most of the apical hemisphere. It is possible to observe singlets microtubules embedded in this granular matrix, however the centriole is not observed. Between the cytoplasm and the nucleus there is a membrane, not totally continue however very distinctive (Figures 1bc, 2bc and 3abdf). The nucleus occupies at least 50% of the cell in some cases (Figure 2bc and 3abdf) and less than 50% (Figure 1bc). It has a more electron-dense material when compared with the cytoplasm but it is also granular with a double membrane (corresponding to the nucleus and plasma membrane) externally that it is not easy to observe (Figure 2f). The microtubules are seeing at the nucleus matrix probably the continuum microtubules from de cytoplasm. There are a set of membranes around the spermatozoa (Figures 1, 2 and 3) and the arms are clearly observed at the Figures (1c and 3b). Two spermatozoa are observed to be in acrosome reaction (Figures 3ab).

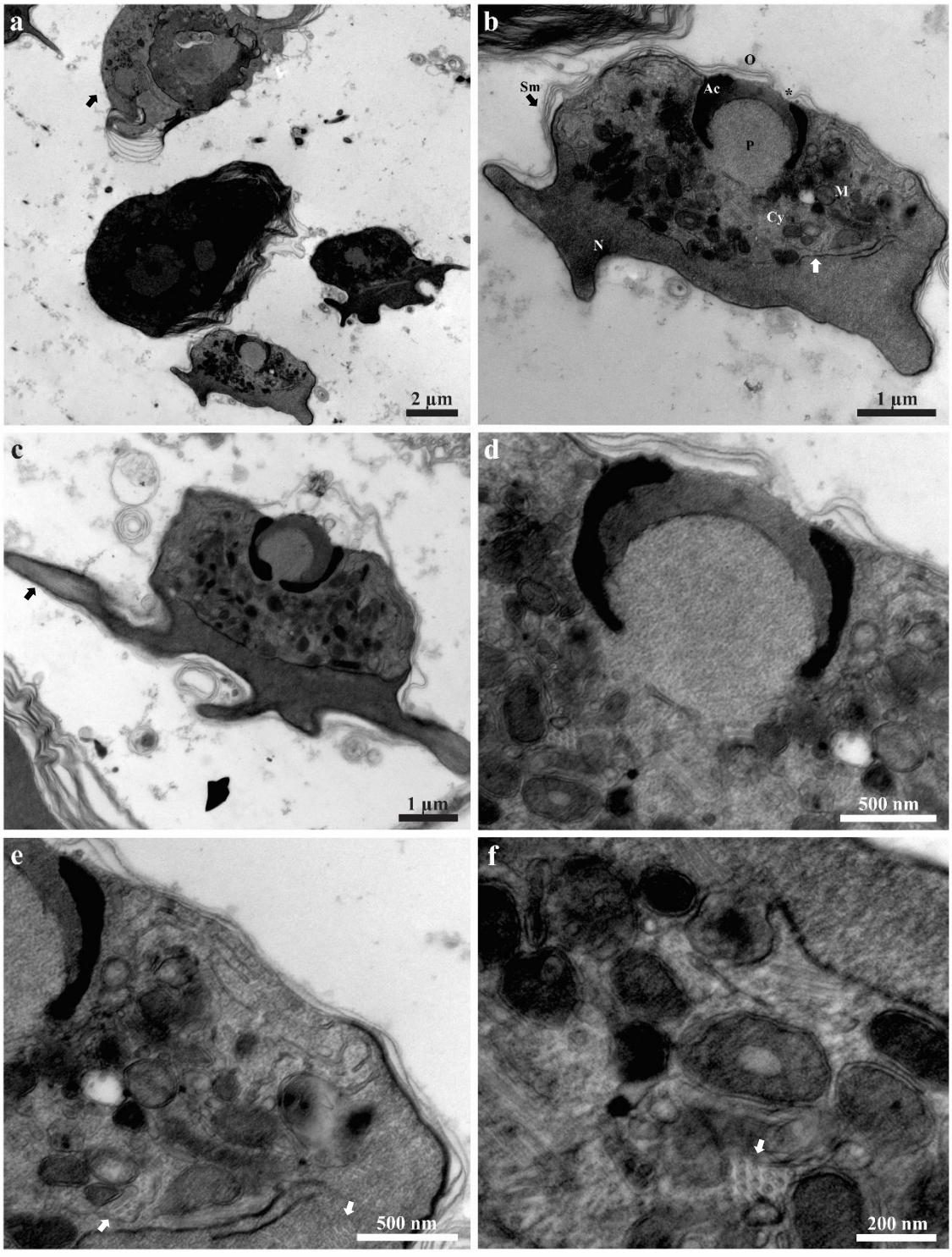

Figure 1 *Aegla castro* from Ponta Grossa (state of Paraná) region. a. general view of the testis lumen with spermatozoa and some non-identified cells (black arrow). b. First spermatozoon in longitudinal section with an indicative of damage at the membrane region (black asterisk). c. Second spermatozoon in longitudinal section showing at least two arms (black arrow). First spermatozoon in details: d. Detail of the acrosome vesicle. e. Detail of the cytoplasm with the microtubules (white arrow). f. Detail of the nucleus with the microtubules (white arrow). Ac = acrosome; Cy = cytoplasm; M = mitochondria; N = nucleus; O = operculum; P = perforatorial chamber; Sm = set of membranes.

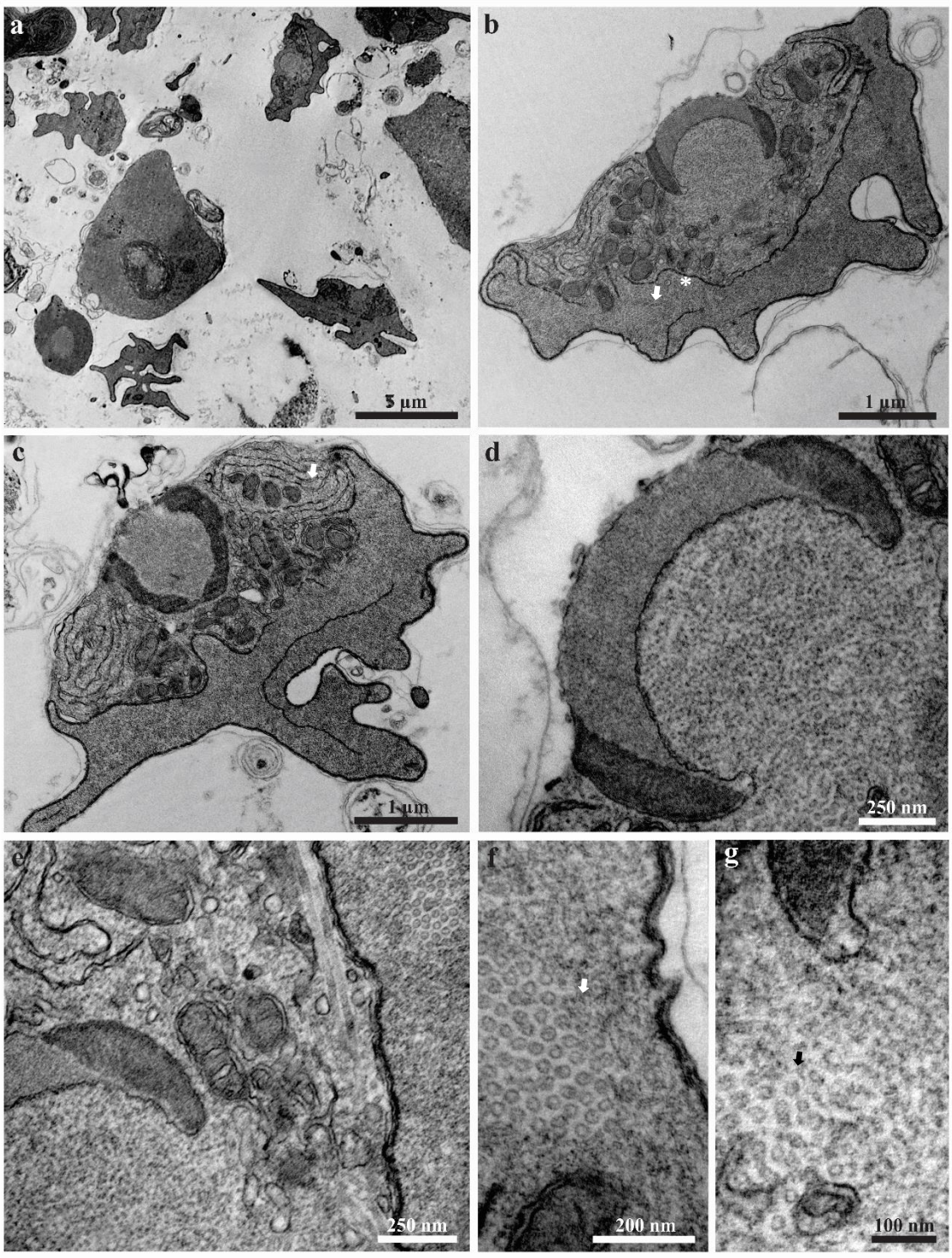

Figure 2 *Aegla castro* from Ponta Grossa (state of Paraná) region. a. general view of the testis lumen with spermatozoa and some non-identified cells. b. First spermatozoon in longitudinal section with an indicative of the membrane between the cytoplasm and the nucleus (white asterisk) and the microtubules in the cytoplasm matrix (white arrow). c. Second spermatozoon in longitudinal section showing a set of intern membranes at the cytoplasm matrix (white arrow). First spermatozoon in details: d. Detail of the acrosome vesicle. e. Detail of the cytoplasm with the mitochondrias. f. Detail of the nucleus with the microtubules (white arrow). g. Detail of the cytoplasm with the microtubules (black arrow).

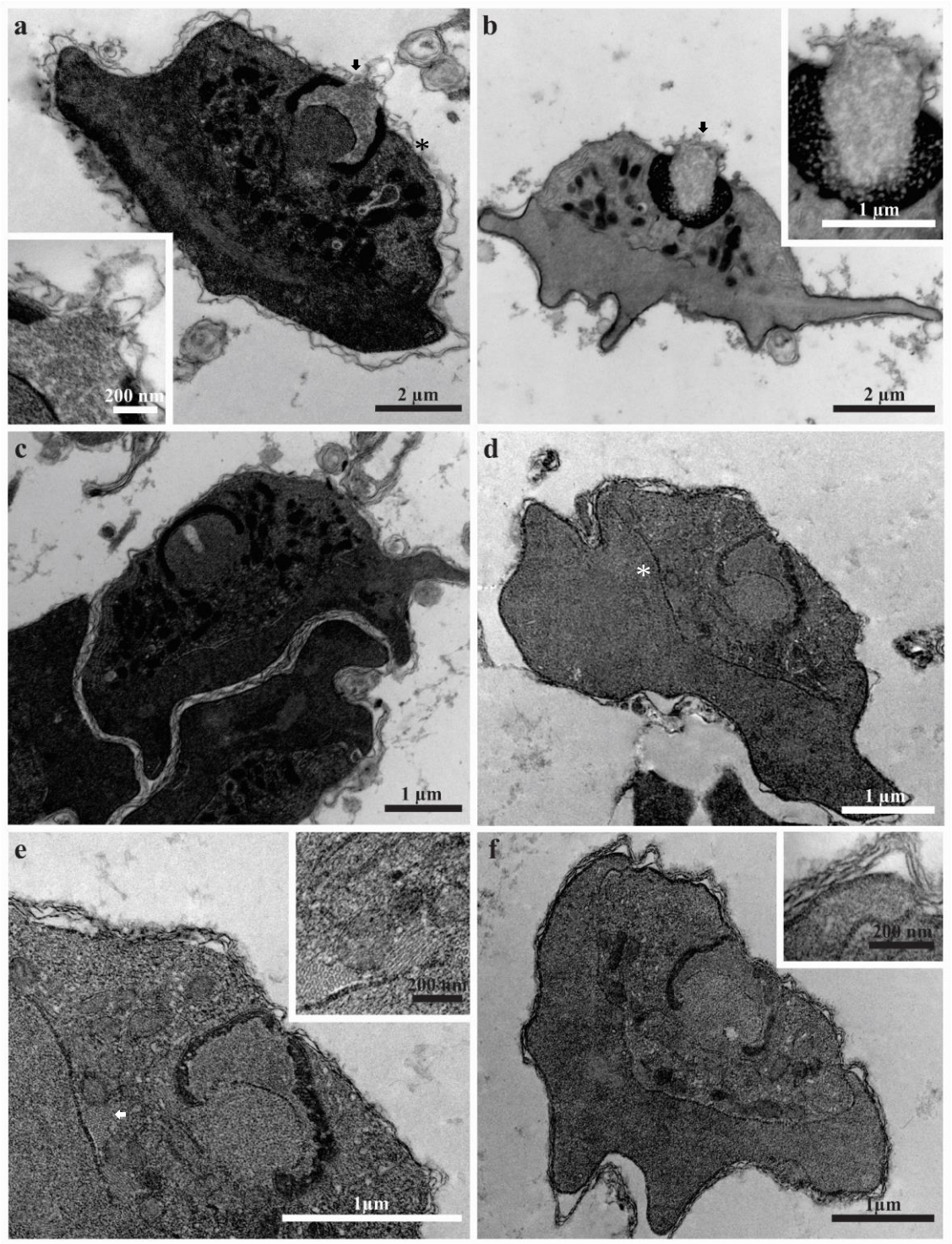

Figure 3 *Aegla castro* from Ponta Grossa (Paraná state) region. a. First spermatozoon in longitudinal section with an indicative of the acrosome reaction (black arrow) and the external set of membranes (black asterisk). b. Second spermatozoon in longitudinal section with an indicative of the acrosome reaction (black arrow). c. Third spermatozoon in longitudinal section, however with a more superficial section. d. Fourth spermatozoon in longitudinal section with a distinctive membrane between the cytoplasm and nucleus (black asterisk). e. Detail of the fifth spermatozoon showing the microtubules (white arrow). f. Sixth spermatozoon in longitudinal section.

3.2 Spermatozoa of *Aegla castro* Mauá da Serra (state of Paraná)

In a general view of the testis, a large number of spermatozoa are observed in the lumen when compared with *A. castro* from Ponta Grossa (Figure 4a). However, it is very difficult to find a spermatozoon in a longitudinal section in which the structures are possible to be seen. The spermatozoon has an irregular format and the two hemispheres are observed (Figure 4b), however although it is in the lumen, maybe the cell is in maturation. The acrosome vesicle is in a rounded format that covers the perforatorial chamber and it is not like a continuum matrix pattern, i.e., patches of more electron dense material in a less electron dense and more granular material. In addition, the acrosome vesicle has an external membrane and it is surrounded by a granular matrix including the top that could be the cytoplasm (Figure 4b). If the cell is in maturation, this could be an extra material that subsequently will be reabsorbed or discarded. The perforatorial chamber is clearly observed and it is smaller when in comparison with the acrosome vesicle. The operculum region is more difficult to be differentiated. The cytoplasm is granular containing many mitochondria and laterally a striated area that seems to be the microtubules in a parallel position (Figures 4be). In Figure 4f is possible to see clearly that the mitochondria do not have cristae. The nucleus corresponds around 50% of the cell and it has a more electron dense matrix with some electron-lucent spaces. There is not membrane between the nucleus and cytoplasm. The set of membranes observed externally in *A. castro* from Ponta Grossa spermatozoon is not observed in this species.

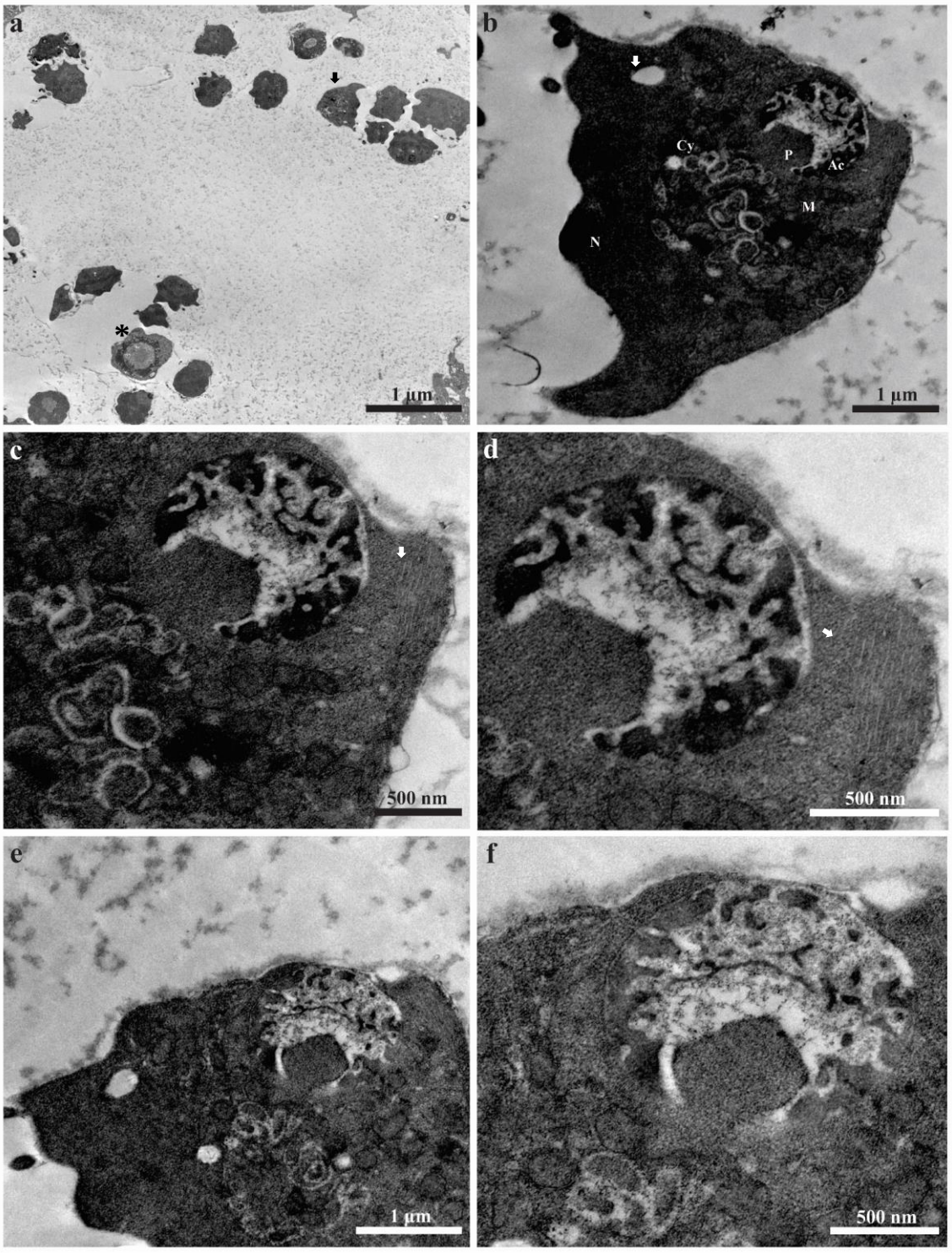

Figure 4 *Aegla castro* from Maua da Serra (state of Paraná) region. a. general view of the testis lumen with spermatozoa (black arrow) and some non-identified cells (black asterisk). b. Spermatozoon in longitudinal section with an indicative of the lucent-spaces (white arrow). c and d. Detail of the spermatozoon indicating the microtubules (white arrow). e and f. The same spermatozoon in a more superficial different section. Abbreviations see figure 1.

3.3 Spermatozoa of *Aegla castro* Itatinga (state of São Paulo)

The spermatozoon of this species has an irregular format, the two hemispheres and the set of external membranes (Figure 5ab). The acrosome vesicle has an external membrane (Figure 5c) and it is very electron-dense except the top where possibly the operculum is located (Figure 5b), maybe in a deeper section will be possible to see better the operculum region. The perforatorial chamber has a granular matrix and a rounded format (Figure 5c). The cytoplasm has a less granular matrix compared with the perforatorial chamber and it has some mitochondria, microtubules (Figure 5d) and other organelles that are more difficult to identify. Between the cytoplasm and the nucleus, it is possible to see a membrane (Figure 5f). The nucleus occupies 50% of the cell, with some microtubules in it. There is one arm visible laterally and it is possible to see the microtubules (Figure 5ae).

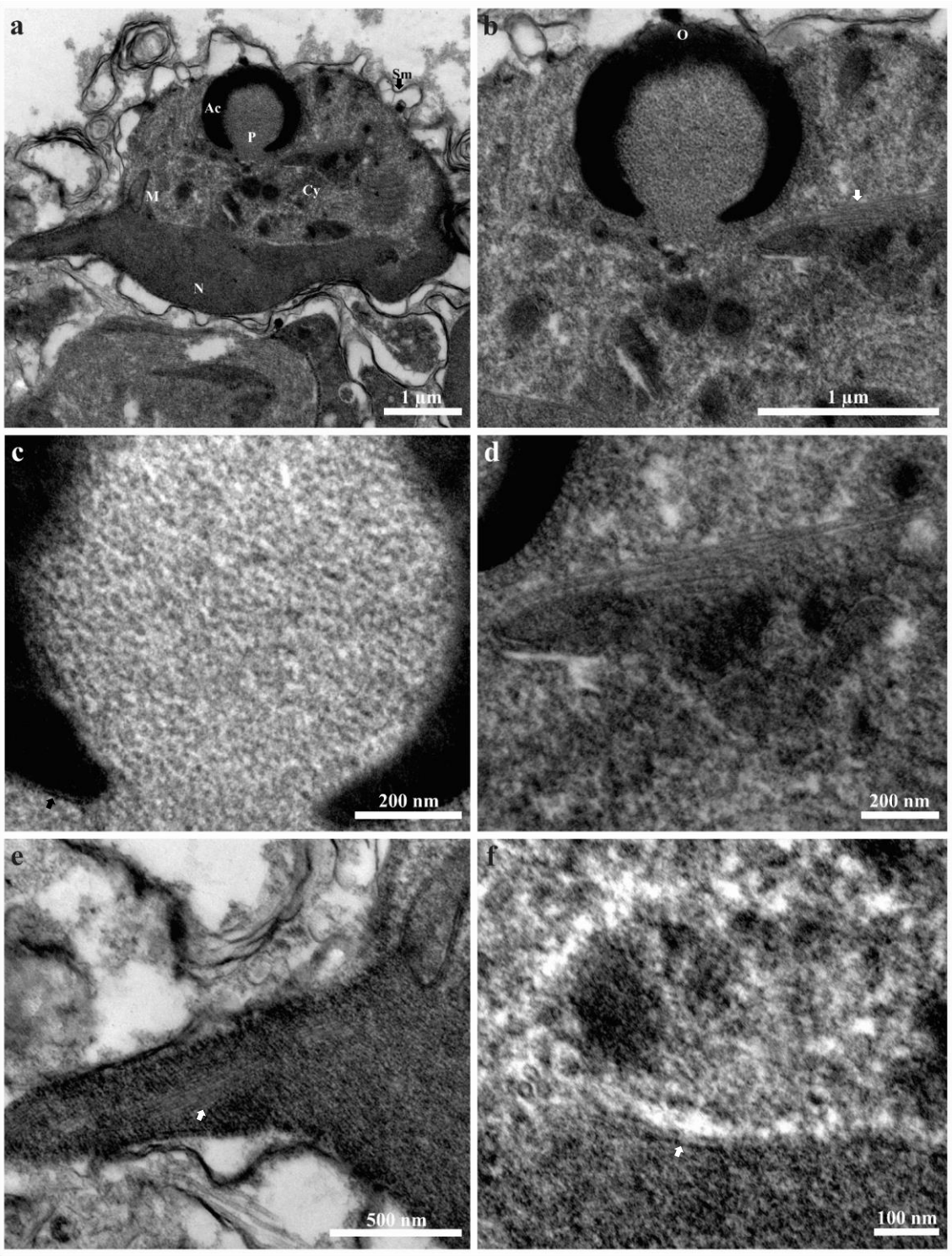

Figure 5 *Aegla castro* from Itatinga (state of São Paulo) region. a. Spermatozoon in longitudinal section with an indicative of the external set of membranes (black arrow). b. Detail of the acrosome vesicle, operculum region and the microtubules in the cytoplasm (white arrow). c. Detail of the perforatorium and acrosome vesicle membrane (black arrow). d. Detail of the microtubules. e. Detail of the arm and microtubules (white arrow). f. Detail of the nuclear membrane (white arrow). Abbreviations see figure 1.

3.4 Spermatozoa of *Aegla parana* Schmitt, 1942 União da Vitória (state of Paraná)

In *A. parana* testis we can also observe that there are few spermatozoa in the lumen with some cell debris and that this lumen is filled with a secretion usually observed in other specimens during observation of grids in the TEM (personal observation) (Figure 7abd). The spermatozoon also is divided in two hemispheres and has an irregular format (Figure 6a). The acrosome vesicle has two parts, one external thin electron dense but granular matrix with irregular boundaries and other internally less electron dense granular matrix. The acrosome membrane goes until the top where the operculum is located. The perforatorial chamber is short (Figure 6b and 7ef). The cytoplasm has acristate mitochondrias, microtubules and a very distinctive set of intern membranes (Figure 6bf). The nucleus is electron dense with a set of microtubules (Figure 6e) and corresponds as 50% of the cell body. It is observed a membrane between the cytoplasm and nucleus (Figure 6ad).

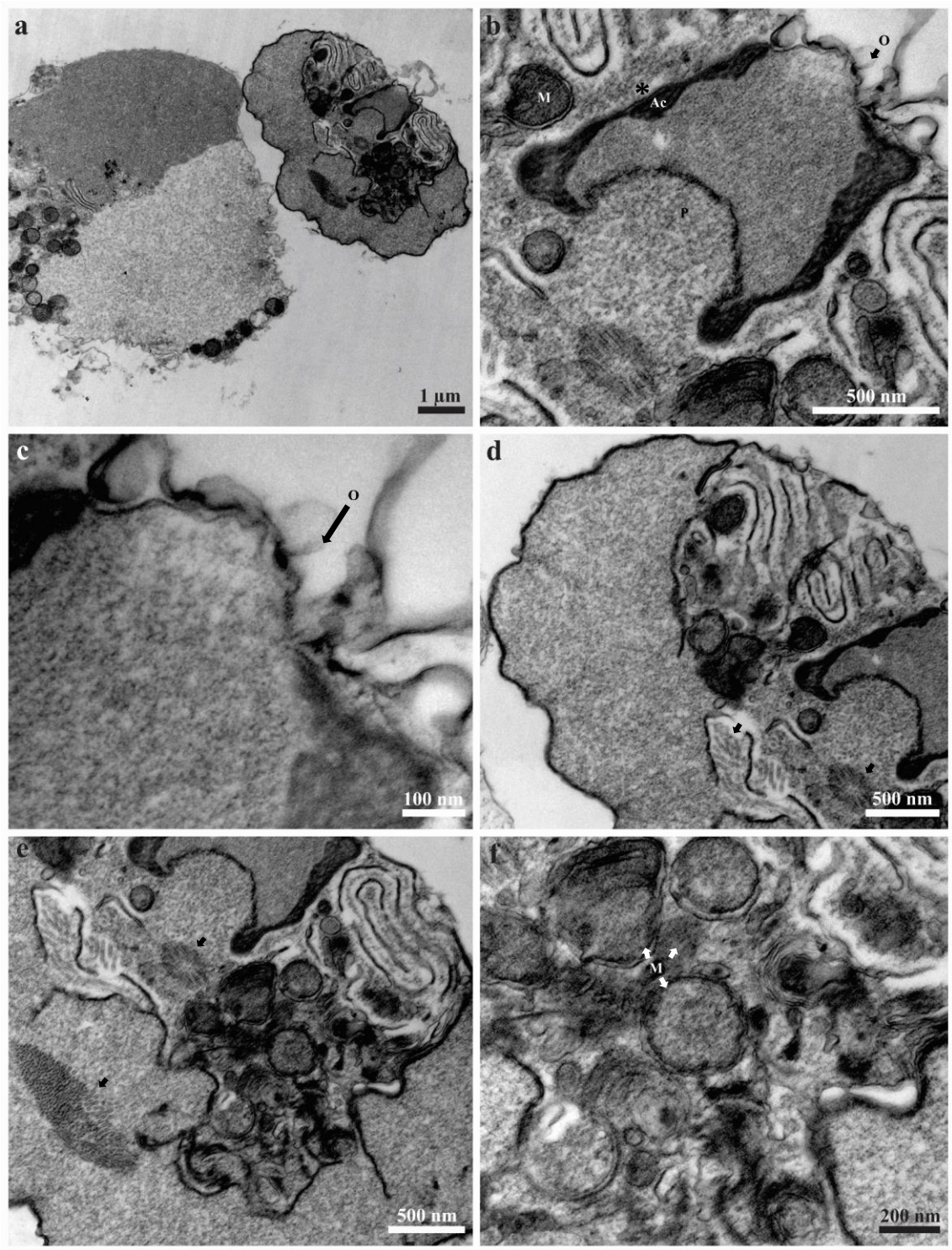

Figure 6 *Aegla parana* from União da Vitória (state of Paraná) region. a. Spermatozoon in longitudinal section. b. Detail of the acrosome vesicle, operculum region (black arrow), perforatorium and part of the cytoplasm (asterisk indicate acrosome membrane). c. Detail of the acrosome vesicle (black arrow). d. Detail of the cytoplasm and the microtubules (black arrow). e. Cytoplasm and nucleus with mitochondria and microtubules (black arrow). f. Mitochondria in the cytoplasm (white arrow). Abbreviations see figure 1.

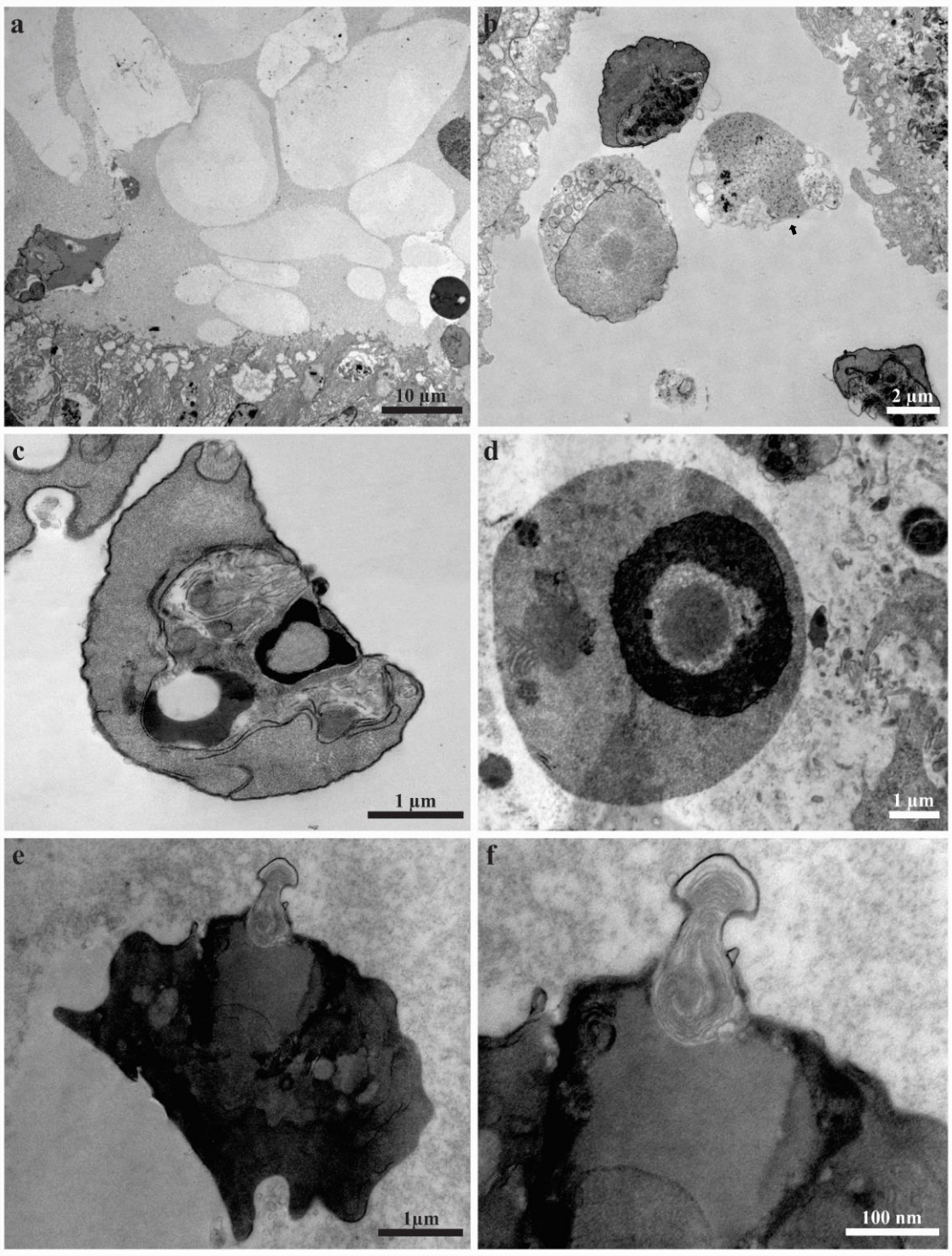

Figure 7 *Aegla parana* from União da Vitória (state of Paraná) region. a and b. General view of the testis lumen with secretion and spermatozoon and cell debris (black arrow). b. Spermatozoon in transversal section. c. Cell debris in the lumen. e and f. Spermatozoon in longitudinal section and a possible acrosome reaction.

3.4 Spermatozoa of *Aegla quilombola* Moraes, Bueno and Tavares, 2017 Intervales (state of São Paulo)

The spermatozoon of *A. quilombola* is very scarce and distributed in a similar secretion to *A. parana* along with other unknown cells (Figure 9a) in the anterior vas deferens. It seems to have the most spherical spermatozoon observed, however other spermatozoa observed in different sections could generate doubts about the format (Figure 8ab). In longitudinal section, the spermatozoa has the upper and lower parts (Figure 8a) and the acrosome vesicle is more electron dense in the outer layer and less in the inner one, which has a granular matrix. An evident membrane can be observed externally in the outer layer of the acrosome vesicle (Figure 8bc). There is a translucent area between the perforatorial chamber and the acrosome vesicle (Figure 8b). The cytoplasm is granular with many vesicles embedded in it and a large evident homogeny structure is observed (Figure 8d and 9df). Many vesicular structures are present in the cytoplasm, including mitochondria, however it was not observed microtubules (Figure 8fd and 9bdf). There is a membrane between the nucleus and the cytoplasm. The nucleus has approximately 80% of the cell area and we also do observed microtubules.

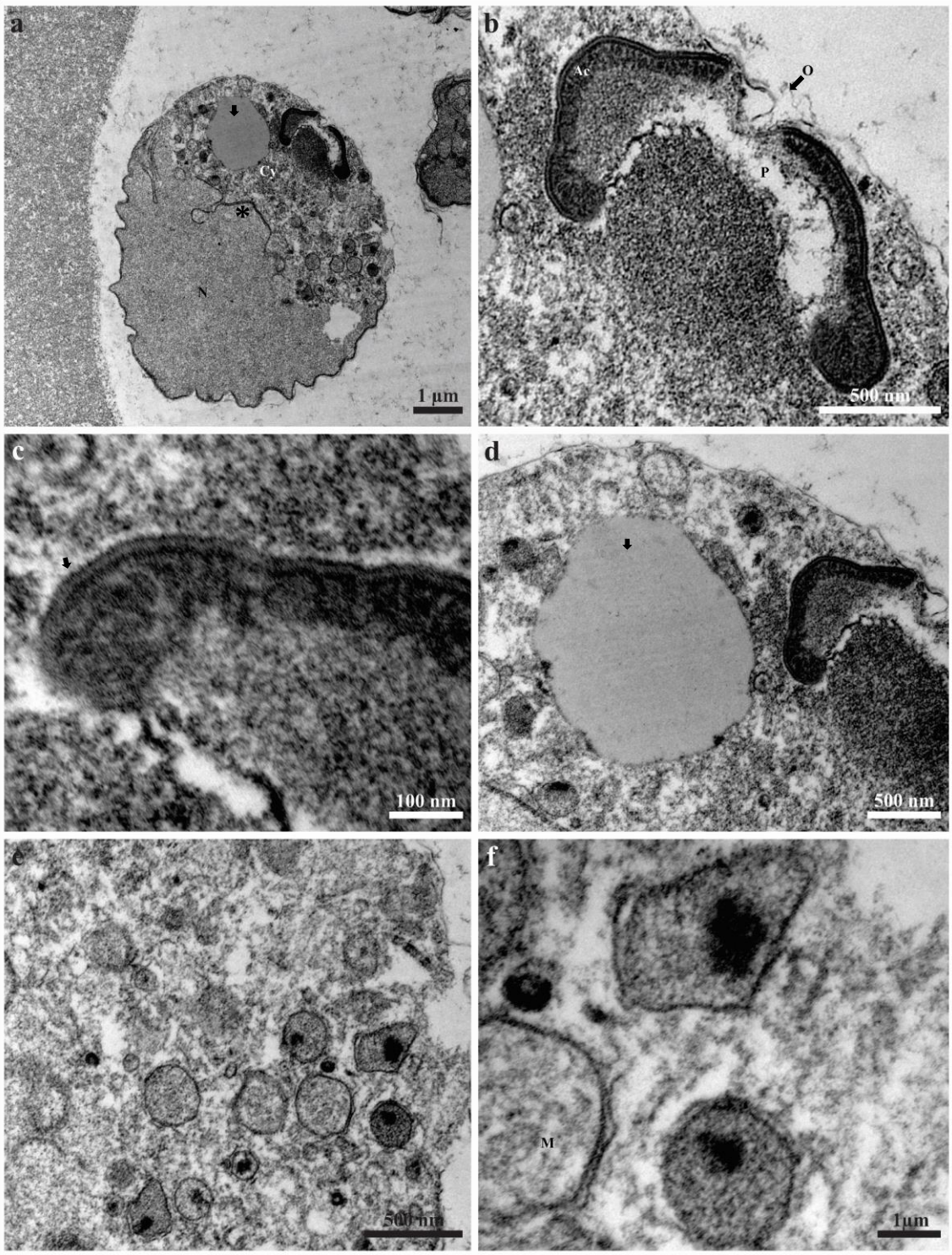

Figure 8 *Aegla quilombola* from Intervales (of São Paulo) region. a. Spermatozoon in longitudinal section, the black arrow indicate the homogeny structure and the black asterix the membrane between the cytoplasm and nucleus. b and c. Detail of the acrosome vesicle with the external membrane (black arrow). d. Detail of the homogeny structure. e and f. Detail of the cytoplasm with mitochondria and other organelles. Abbreviations see figure 1.

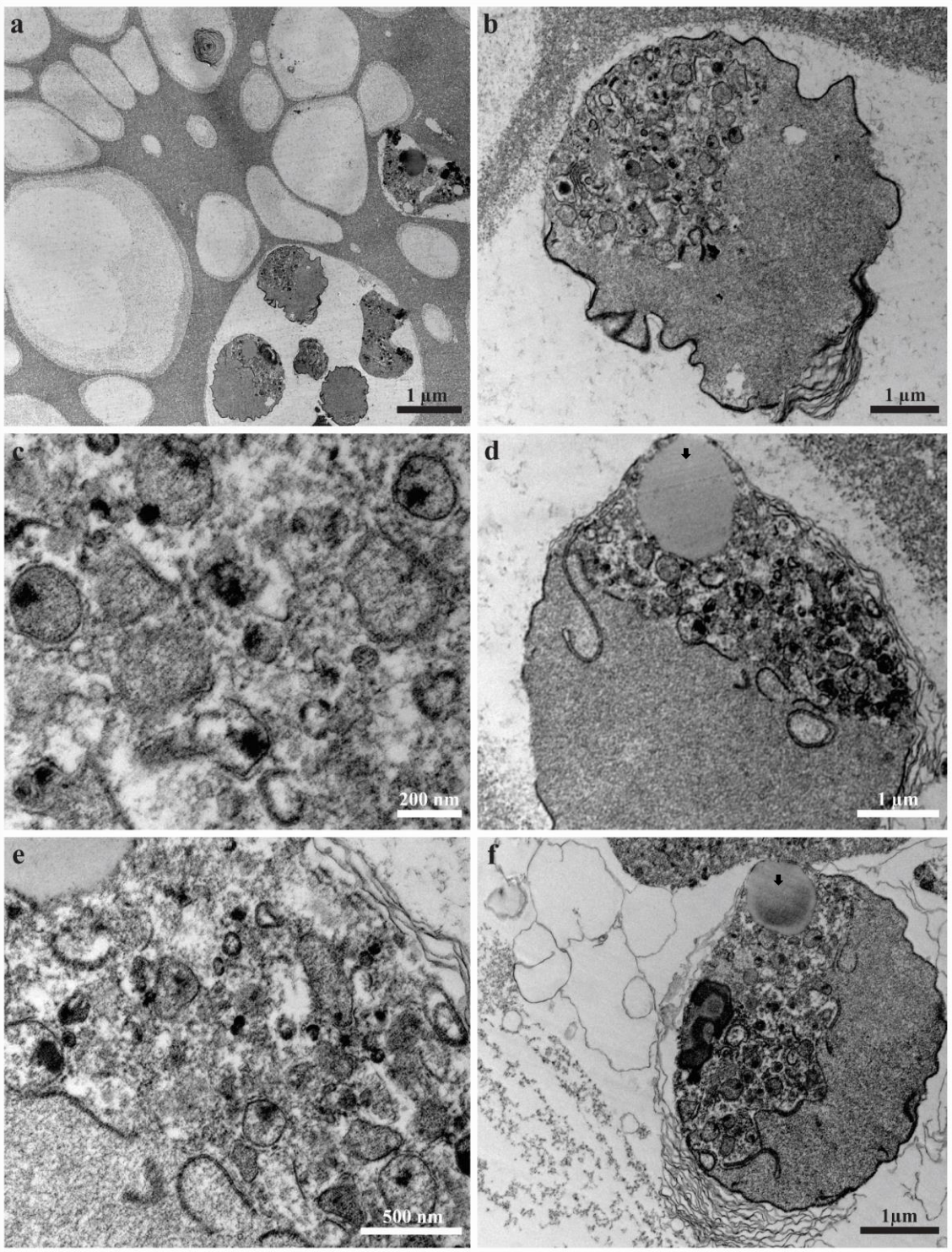

Figure 9 *Aegla quilombola* from Intervales (state of São Paulo) region. a. b. General view of the anterior vas deferent with secretion and spermatozoon. b. First Spermatozoa with a section showing the cytoplasm and nucleus and c. detail of the cytoplasm. d. Second Spermatozoa with the homogeny structure (black arrow) and e. detail of the cytoplasm. f.. Third Spermatozoa with the homogeny structure (black arrow).

3.5 Spermatozoa of *Aegla sp.* Tibagi (state of Paraná)

The spermatozoa of *Aegla* sp. from Tibagi "Fazenda São Damásio" has an irregular format and is divided in two hemispheres (Figure 10a). The acrosome vesicle is long, more electron dense and irregular in format (vesicular appearance), surrounded by a membrane (Figure 10b). In the center, we can observed the perforatorial chamber as a long granular column. The cytoplasm is granular and has acristate mitochondria, microtubules and a set of intern membranes laterally to the acrosome vesicle (Figure 10de). The nucleus is smaller than the cytoplasm, which can be a section effect. It is more electron dense than the cytoplasm and it is not possible to observe microtubules in it (Figure 10a). There is a membrane between the cytoplasm and nucleus and a set of external membranes surrounding the spermatozoa. The arm is observed laterally with the microtubules (Figure 10f).

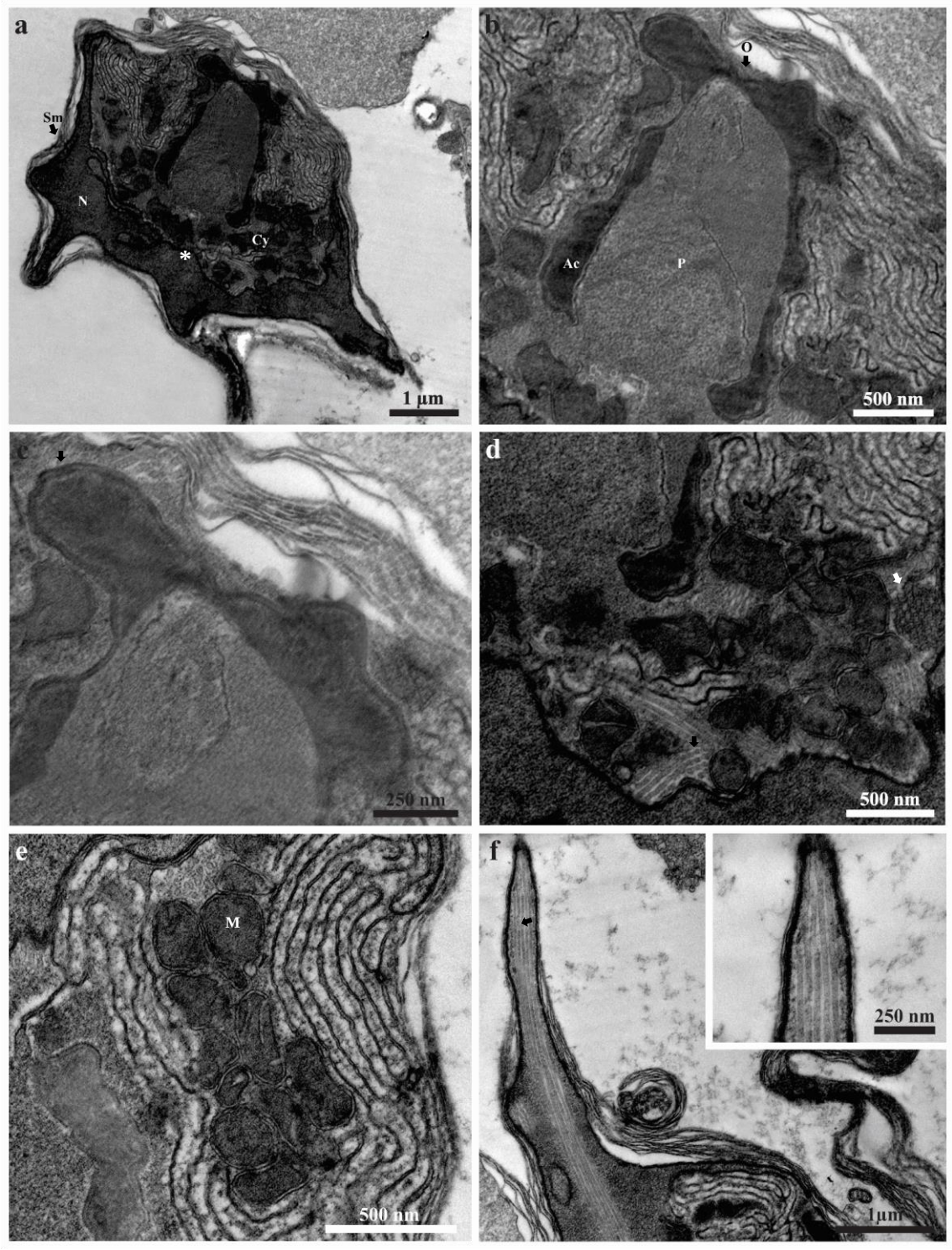

Figure 10 *Aegla sp.* from Tibagi – Fazenda São Damásio (state of Parana) region. a. Spermatozoon in longitudinal section with the membrane between the cytoplasm and nucleus (black arrow). b and c. Detail of the acrosome vesicle with the external membrane (black arrow). d. Detail of the cytoplasm and the microtubules (white and black arrow). e. Detail of the cytoplasm with the mitochondria and the intern set of membranes. f. Detail of the cytoplasm and the microtubules (black arrow). Abbreviations see figure 1.

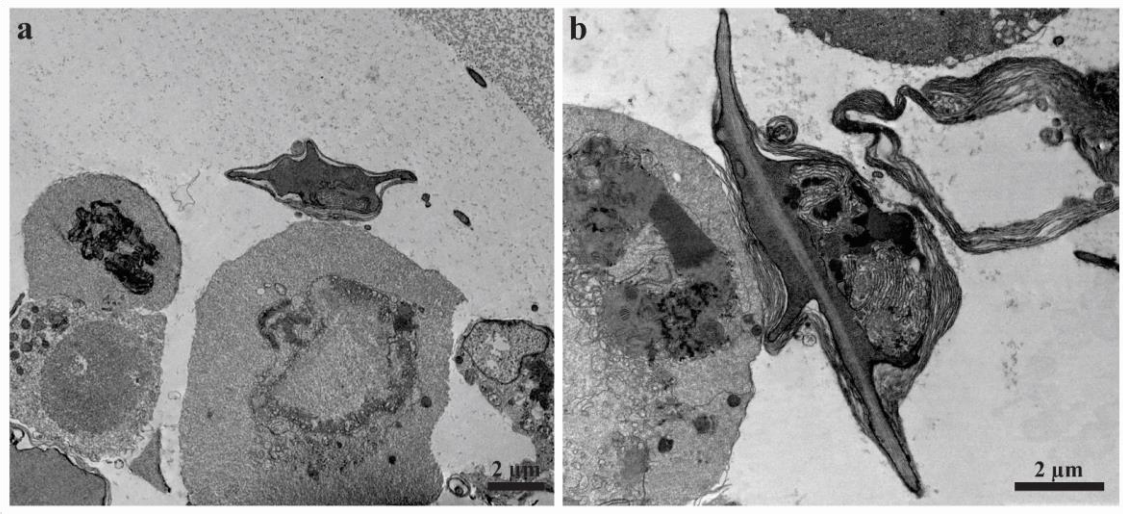

Figure 11 *Aegla sp.* from Tibagi – Fazenda São Damásio (state of Parana) region. a. General view of the anterior vas deferent with a spermatozoon and other cells at the vas deferens. b. Spermatozoa in the vas deferens, it is possible to notice the presence of arms.

3.6 Spermatozoa of *Aegla sp.* Tibagi– Fazenda Pinheirinho (state of Paraná)

The spermatozoon of *Aegla sp.* from Tibagi, Fazenda Pinehirinho is at the lumen of testis, it has an irregular format and two hemispheres. It is not totally in a transversal section, but it is possible to observe the nucleus, the cytoplasm, the acrosome vesicle and the set of external membranes (Figure 12ab). The acrosome vesicle is electron dense and occupied by the central granular column, i.e., the perforatorial chamber. It is possible to see a transitory matrix between the electron dense layer of the acrosome vesicle and the perforatorial chamber that could be section effect or the operculum region. The cytoplasm is a granular matrix with many rounded organelles, probably mitochondria (Figure 12c). The nucleus is more electron dense than the cytoplasm and the membrane between both is observed (Figure 12ab).

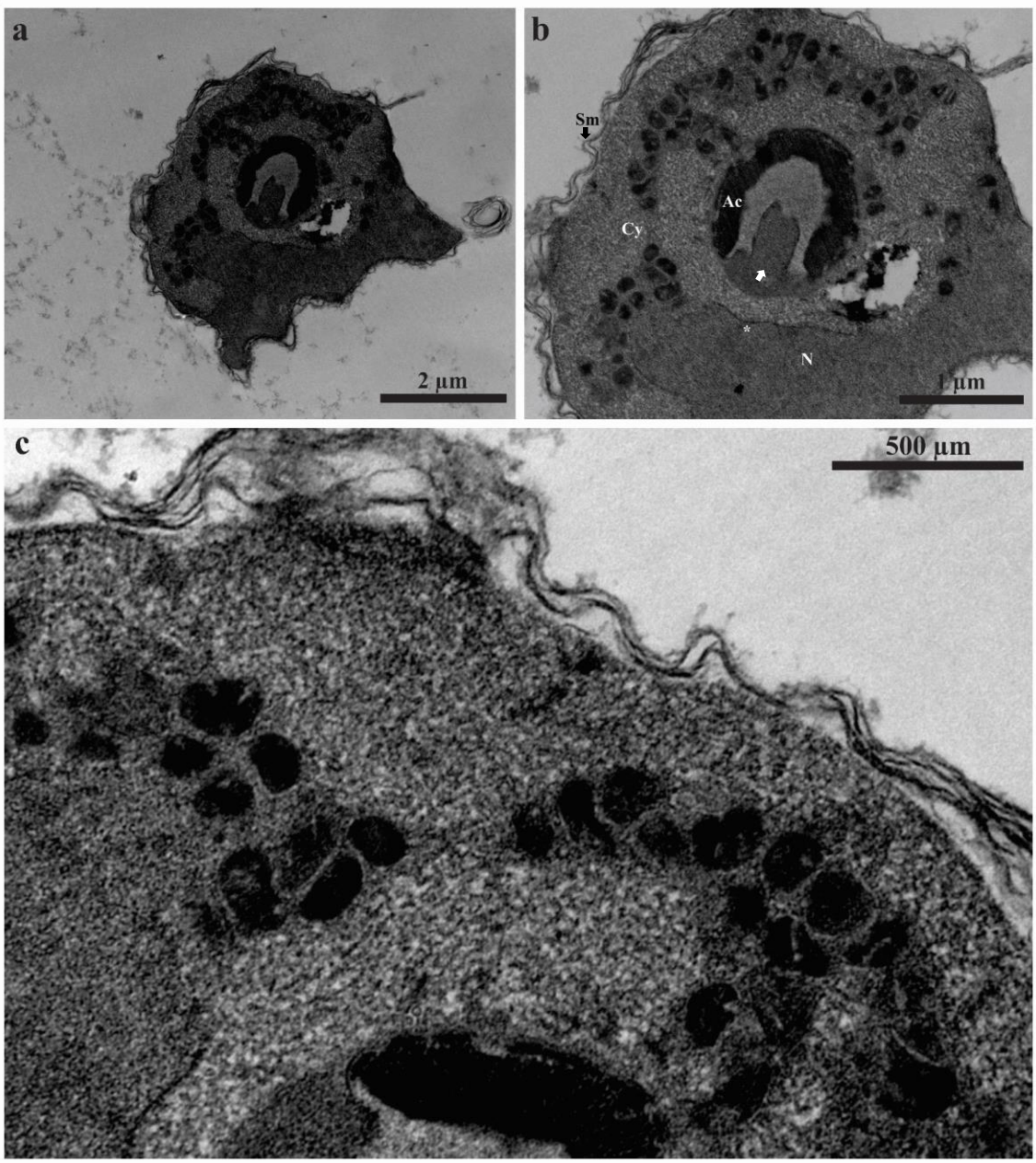

Figure 12 *Aegla sp*. from Tibagi – Fazenda Pinheirinho (state of Parana) region. a. Spermatozoon at the lumen of testis b. Detail of the spermatozoon with details of the membrane between the cytoplasm and the nucleus (white asterisk), the set of external membranes (black arrow) and the transitory matrix between the acrosome vesicle and the perforatorial chamber (white arrow). c. Detail of the cytoplasm and set of membranes.

3.7 Spermatozoa of *Aegla sp.* Tibagi – Puxa Nervos (state of Paraná)

The two hemisphere spermatozoa (nucleus and cytoplasm) is observed in *Aegla sp.* from Tibagi, Puxa Nervos waterfall (state of Parana) at the lumen between a transition region that includes the testis and the anterior vas deferent. The acrosome vesicle is electron dense at the external layer and less at the internal one and a clear membrane can be observed externally that goes until the top of the acrosome however is interrupted probably for an acrosome reaction. The cytoplasm have many mitochondria and some microtubules. The nucleus is more electron dense than the cytoplasm and have a structure space-like less electron dense. A membrane separates cytoplasm and nucleus. It is not observed a clear membrane or set of membranes externally the spermatozoa.

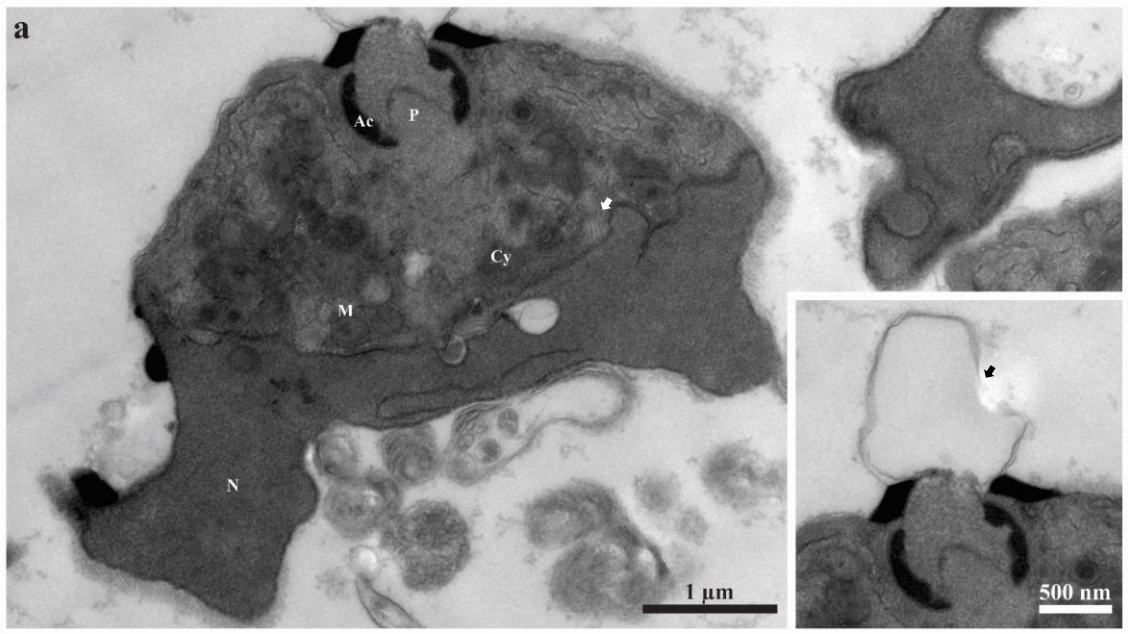

Figure 13 *Aegla sp.* from Tibagi – Puxa Nervos (state of Parana) region. a. Spermatozoa at the lumen of testis, the white arrow indicates the microtubules. Detail of the spermatozoa with a possible acrosome reaction (black arrow).

4 Discussion

Our results are the second general idea about how the *Aegla spp.* spermatozoa looks like and because we used more than one species, it is possible to observe that at least there are two morphology patterns in this group: the irregular and the spherical. When we compare the pattern found by Tudge (2003), Tudge, and Schetinga (2002) that was spherical, only *A. quilombola* and *A. parana* seems to present this format. In general, *Aegla spp.* spermatozoa has two hemispheres (cytoplasma and the nucleus) and both parts have approximately the same size and were separated by a membrane. Definitely two layers are observed in the acrosome (internal less electron dense and external more electron dense) with the external layer rounded by a membrane that can be very distinct or not, this is a very consistent characteristic for all spermatozoon observed.

The spermatozoon of all the species from this study are different when compared to the Galatheoidea, agreeing with the comparison performed by Tudge and Scheltinga (2002) and with McLaughlin et al. (2007) proposal to take out Aeglidae from the superfamily Galatheoidea and put it in a separated superfamily: Aegloidea. In addition, the spermatozoon observed have more similarities with the endemic Australian Lomidae *Lomis hirta* (Lamarck, 1812) (Tudge, 1997) than the other Anomura species (Tudge and Scheltinga 2002) mainly *A. quilombola* that has a spherical format.

Three different species and three different spermatozoon were observed, for *A. parana* the spherical spermatozoon with a long, lobulated acrosome and without the multiple membranes externally; *A. quilombola* the most spherical spermatozoa, without the set of membranes externally, with a large homogeny structure present in the cytoplasm and a short acrosome; *Aegla sp*. (Fazenda São Damásio) has an irregular format, long and lobulated acrosome and the set of membranes externally. These three spermatozoon are different between each other and from the "*A. castro*" spermatozoa morphology.

Aegla quilombola were so distinctive when compared to the others due to the large homogeny structure present in the cytoplasm. We suggest that this structure could be a vesicle of fatty acids that are insoluble and form spherical droplets (lipid) that are not membrane bounded and seems to be homogeny (Hodgson et al., 1990). Similar structures were observed and called lipid-like droplets, for the shrimp *Pleoticus muelleri* (Spence Bate, 1888) and *Parapenaeus longirostris* (Lucas, 1846), that are not as marked as the ones we observe here and are immersed at the nucleoplasm instead of the cytoplasm (Medina, 1994; Medina et al., 2006). *Penaeus kerathurus* (Forskal, 1775) and the anthozoan *Actinia equina* (Linnaeus, 1758) presented these lipid-like droplets in the nucleus region that are more similar to *A. quilombola,* however, the justifications are different, *P. kerathurus* droplets derived from degenerating cytoplasmic membranes (Medina et al., 1994) and *A. equina* seems to have the function of high-energy reserve and stability during the locomotion (Larkman and Carter, 1980).

We did not find these lipid-like droplets in all *Aegla* species studied, that could be an sample effect or a simple variation of presence or absence in the same genera, as observed for *P. japonicus* Spence Bate, 1888 (absent) and *P. kerathurus* (present) (Medina et al., 1994). The function for the lipid-like droplets are unclear yet for eglids, it could result from some organelles degenerating or for some energy reserve. However, it makes sense the energy reserve for the anthozoan spermatozoa once it is motile, what is a remarkable difference from crustaceans and therefore it goes against the idea of the same function in eglids.

Although internalized microtubules arms are observed, we could not verify and answer how many arms the *Aegla spp*. studied here have. Tudge (2003) and Tudge and Scheltinga (2002) indicated the presence of three arms through the light microscopy but mentioned that it was not possible to see under the TEM level. The presence of three (

apomorphic condition) (except Porcelanids, Thalassinidae and Hippoidea) instead of four arms (plesiomorphic) is an important characteristic that separated the clade Anomura using this most derived characteristic based on spermatozoa morphology. Therefore, more studies have to be done for corroborate the first information about the number of arms in Aeglidae.

Would be the membrane that englobe the spermatozoa of *A. castro* (São Paulo), *A. castro* (Ponta Grossa), *Aegla spp.* (Tibagi - São Damásio), *Aegla spp.* (Tibagi - Pinheirinho) a spermatophore-like? That question needs some more investigation once we did not see the spermatophore like-lobes that Tudge (2003) described in any of our material either during the dissection and the light microscopy observations or in all of the sections performed to electron microscopy. Mouchet (1932), Sokolowicz et al., (2007) and Viau et al., (2006) results indicated that *Aegla spp.* do not have spermatophore, Tudge and Scheltinga (2002) did not observed, however, Lopretto (1978) noticed small capsules. Therefore, there is a controversial idea about presence or not of some structure containing spermatozoon, even they are not considered complex spermatophores.

Our suggestion based on personal observations is that the lobes observed by Tudge (2003) could be in fact the lobes of the testis, as the author had limited material to work on; it becomes more difficult to have comparisons and other sections that could help. However, the set of membranes that we observed around the spermatozoon here could be some structure like spermatophore. Nonetheless, it is not present in all spermatozoon (only *A. castro* – Itatinga, *A. castro* Ponta Grossa, *Aegla sp.* São Damásio, *Aegla sp.* Pinheirinho). Not much is known about reproductive behavior of *Aegla*. spp. and sperm transference, Almerão et al., (2010) observed a white mass into the female abdominal chamber after the copula that they supposed to be the mass of sperm, however they did not prepared fresh slides and observed under light microscopy to have assurance.

Although anomurans in general have simple storage, i.e., modification of female body to receive spermatophore imperceptible, and sperm transfer (Bauer, 1986) they have a complex and very characteristic spermatophore (in majority: pedunculate with an ampullae) (Tudge, 1991; Subramonian, 1993). Most of Crustacea and all Decapoda spermatozoon are aflagellate and non-motile (Felgenhauer and Abele, 1991) and these are the reasons why spermatophore is so important in sperm transfer (Subramonian, 1993). The fact is why Aeglidae is an exception in Anomura and how is the evolutionary history that led to these differences? Once, due to the external fertilization and the small amount of spermatozoon we would expect that they would be packed into spermatophores.

It is not clear yet if there are differences between the species in relation to the spermatozoa morphology. It seems as the spermatozoa of *A. castro* - Itatinga, Maua and Ponta Grossa are similar based mainly when we compared them with the very distinct species *A. quilombola*, *A. parana* and *Aegla sp.* (Fazenda São Damásio). Major differences between *A. castro* Ponta Grossa and Itatinga in relation to Mauá are the acrosome vesicle morphology, the size of the perfatorial chamber in relation to the acrosome vesicle, and the non-evidence of membrane between the nucleus and cytoplasm and externally in *Aegla castro* from Mauá. However, we are not sure if the spermatozoa from Mauá species is totally mature and more collections should be performed to confirm if the mature cell has this morphology or not. It is really possible to find out more than one morphology according to the group and they have been very useful to give information about taxonomy and phylogeny of the group as which species are primitive and which one are derived (Tudge, 1997; Camargo et al., 2016; Assugeni et al., 2017).

As we observed, it is possible to have more than one pattern of spermatozoa morphology in a taxonomic group however, this does not mean that we will have one

different spermatozoa morphology for each species, which means that even *A. castro* complex could have different species they can share the same pattern of spermatozoa morphology. However, we can determine at least three patterns of spermatozoa morphology in a very general view in Aeglidae: 1) spherical with short acrosome (*A. quilombola* and *A. longirostris*), 2) irregular with long and lobulated acrosome (*A. parana* and *Aegla sp.* Fazenda São Damásio) and 3) irregular with short acrosome (*A. castro* complex).

Our results agree with Pérez-Losada et al., (2004) phylogenetic tree specifically in the group C where the Brazilian species were clustered and *A. parana* and *A. castro* were separated, as our results in spermatozoa morphology. *A. marginata* type-locality was recently redescribed by Bertacini et al., (2017) and a new species identified before as *A. marginata* was described as the new species *A. quilombola*, therefore we know that in Pérez-Losada et al., (2004) tree the "*A. marginata*" is different from *A. parana* and *A. castro*, however we do not know if it is really *A. marginata*.

Because the limited number of spermatozoon and the hardship in find them in a longitudinal section is that to describe the *Aegla spp*. spermatozoa morphology have been so difficult. During the collections, we observed differences in the field from the first period (July - September 2016) with the presence of ovigerous females and the second one (March - May 2017) with reproductive females in Stage 4 (orange ovaries) (Bueno et al., 2008) (personal observation). However, there was not significant differences in the males in relation to the amount of spermatozoon. Tudge and Scheltinga (2002), Tudge (2003), Sokolowicz et al. (2006) and Viau et al. (2006) had exactly the same results what it is an evidence that the small amount of spermatozoon is probably a pattern in Aeglidae. Viau et al. (2006) also did not find spermatozoon through the entire vas deferens, only at the anterior portion near to the testis lumen, which was observed in the present study (or

testis lumen or anterior vas deferens). Sokolowicz et al. (2006) said that probably the spermatozoon are retained in this portion until the reproduction "protected" by some secretion.

The freshwater decapods usually have less amount of spermatozoon by spermatophore than the marine ones (e.g. Guinot at el., 1997; López Greco et al., 2007; Klaus et al., 2009), however they have many more spermatozoon than *Aegla spp.* We could speculate about if the scarce number of spermatozoon in Aeglidae is related with its evolutionary history and have some implication in species reproduction and consequent capacity of population recovery or if it has some environmental influence.

The spermatozoon production can be affected by environmental changes (Reuter et al., 2007; Lewis and Ford, 2012). As freshwater ecosystems has been degraded by human impacts, e.g., deforestation and consequently siltation, contamination by pesticides, construction of dams, etc (Dudgeon et al., 2006; Bueno et al., 2016; Santos et al., 2017), it is possible that this could have influence in freshwater species reproduction. Yang et al., (2008) observed that the sperm production in the amphipod *Echinogammarus marinus* (Leach, 1815) diminished 20% in sites contaminated by polychlorinated biphenyl (PbC), metals and hydrocarbons. As aeglids are perhaps the most threatened freshwater decapods in the Neotropical Region (Bueno et al., 2016) and for example of our study many issues have not been answered yet, the efforts to study this group should be prioritized and more information regarding spermatozoa morphology, phylogeny and reproductive aspects (e.g., spermatozoa production and transfer) are necessary.

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CONCLUSÃO

Foi possível observar a presença de mais de uma espécie em toda a extensão geográfica que procuramos abranger durante esse trabalho e que seria equivalente a distribuição de *Aegla castro* Schmitt, 1942. Duas características não variaram no holótipo e parátipos de *A. castro*: processo subrostral e o terceiro esternito torácico como tapered. Essas duas características nos auxiliaram na separação do que seria *A. castro* de fato das demais espécies que coletamos.

A ultraestrutura do espermatozoide foi tão distinta das demais espécies de Anomura que suporta sua exclusão de *Aegla* spp. de Galatheidae e sua inclusão dentro de da sua própria família Aeglidae. Apesar de não acharmos diferenças na ultraestrutura do espermatozoide para cada espécie de *Aegla* que estudamos, isso não significa que elas pertençam a mesma espécie e sim que o taxon pode ter apenas dois padrões morfológicos de espermatozoides.