Immunological female role tested on artificial plugs in three scorpion species

Short Title: Artificial genital plugs trigger immune response in scorpions

Mariela A. Oviedo-Diego[12]*, Camilo I. Mattoni[12], Alfredo V. Peretti[12]

[1]Universidad Nacional de Córdoba. Facultad de Ciencias Exactas, Físicas y Naturales. Departamento de Diversidad Biológica y Ecología. Córdoba Capital, Córdoba, Argentina.

[2]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). Instituto de Diversidad y Ecología Animal (IDEA). Laboratorio de Biología Reproductiva y Evolución. Córdoba Capital, Cordoba, Argentina.

*Corresponding author

E-mail address: marie27oviedo@gmail.com (MO)

Abstract

Within arachnids, genital plugs are morphologically diverse, and they can be formed by male, female or be a contribution of both sexes. Although several species of scorpions with genital plugs are known, the physiological effects on the female after being plugged have not been well studied yet. This work compares three scorpion species, two with genital plugs and one without. We first describe the genital plugs morphology of two *Urophonius* species. Second, through the placement of artificial genital plugs in the female genital atrium, we tested 1) whether there are interspecific differences in the immune encapsulation response on the artificial genital plug, 2) if there are an effect in the hemocyte load in the hemolymph, and 3) if individual's immunological parameters and body weight are correlated. Additionally, we describe and quantify the hemocytes in these species. In both species of *Urophonius*, genital plugs were found covering the female genital aperture and blocking the genital atrium. The plugs consist of three zones that are distinct in morphology and coloration. We found different patterns of encapsulation and melanization on the artificial plug according to the species, with a greater and more specific response in females of plug producing species. Also, these species showed a decrease in the hemocyte load one month after the placement of the artificial plug, possibly due to the recirculation of the hemocytes into the genital area. In addition, correlations were found between the body weight and the immunological parameters, as well as between different immunological parameters. Our results suggest that females contribute to the formation of genital plugs by adding material and generating the darkening of the genital plugs in certain zones. This comparative study can help to provide a wider framework of different physiological consequences related to a particular postcopulatory mechanism such as the genital plugs.

Keywords: Sexual Selection – Genital plug – Immune response – Encapsulation – Scorpions

Introduction

Among the many reproductive strategies that organisms exhibit, there are some that involve males' adaptations favored by sperm competition to increase their reproductive success [1-3]. Males would compete for the monopolization of females toward preventing, reducing or avoiding sperm competition [2,4]. Genital plugs are structures that block or cover some portion of the female genitalia after mating, and consequently can prevent sperm competition, acting as mechanical or visual impairments [5-6] or by a physiological or behavioral alteration, such as decreased female receptivity [7-10]. The plugging of females is a widespread phenomenon in the animal kingdom, including insects and arachnids [4, 11-14]. Within arachnids, genital plugs are morphologically diverse, varying according to the taxa. In general, genital plugs can be formed by the coagulation of the male's ejaculate or glandular substances [12, 15-21], portions of the spermatophores [5,9,11] or even parts of the male's body or genitalia [22-24]. However, because the plugs could represent the result of a sexual conflict in the domain of fertilization [25-26], sexually antagonistic coevolution would favor counter-adaptations of the females. For example, females can prevent the placement of a plug [27-29], controlling the duration of the mating by means of the formation of a plug [30-31] or actively removing it or degrading it [32-37]. Female control of the fate of the genital plug has been proposed as a mechanism of cryptic female choice [38-39]. This would imply their cooperation (or not) in forming the genital plug, depending on such characteristics of males (e.g., copulatory courtship) [40-41] or characteristics of the genital

plug (e.g., the quality of nutritive substances) [42-45] or as a mechanical obstruction to potentially harmful or unnecessary new copulas [46].

The post-mating physiological consequences that are triggered in the female after the deposition of the male's genital plug have not been well studied. Some studies have described secretions of the epithelium of female genitalia that adhere material to the plug and may help to anchor or consolidate the genital plug, or conversely, this material may degrade the male's plug [15, 47-48]. In *Drosophila nasuta*, the male transfers a substance within the ejaculate that would activate the phenoloxidase pathway, a humoral component of the immune system on arthropods, and lead to the formation of a large, opaque mass in the female's uterus [49-50]. In other cases, females produce proteases that degrade the genital plug and, in turn, the male's plug has ejaculatory proteins with specific inhibitors for these proteases, evidencing antagonistic coevolution between males and females on the effectiveness of the genital plug [48]. Several studies have found changes in the immune system after mating [51]. For example, it has been found that, after mating, several immunological parameters may be improved [52] or weakened [53-55]. Also, mating may cause the activation of immune system molecules in reproductive tissues [56-57] or changes in the expression of immunity genes [58].

Arthropods have a relatively simpler immune system than vertebrates, since they lack acquired immunity [59, but see 60], although this does not mean that the immune system is less specific [61-63]. Immune responses comprise cellular-like responses mediated by the hemocytes -granulocytes (GRs) and plasmatocytes (PLs)- (e.g., coagulation, phagocytosis, nodule formation, encapsulation), and humoral-mediated responses (e.g., complement-like proteins, antimicrobial peptides, products generated by the phenoloxidase pathway) [64-66]. In particular, the encapsulation response (i.e., hemocytes' adhesion in tight layers around an extrinsic factor) involves the action of GRs that recognize the extrinsic factor and release granules (with chemical

84 signals of recruitment of PLs, enzymes and precursors for melanin synthesis and 'encapsulation-
85 promoting factors') [64,67]. Therefore, the capsule formation is associated with melanization
86 produced by the prophenoloxidase (proPO) cascade (activation of the phenoloxidase enzyme)
87 with reactive oxygen and nitrogen species emitted and targeted against the extrinsic factor [68].
88 In chelicerates, some studies investigate hemocyte ultrastructure [69-70] and the presence of
89 antimicrobial molecules in the hemolymph [71-73]. But we still need studies that evaluate the
90 relationship between postcopulatory mechanisms and immune response parameters. In the
91 framework of the theory of immunocompetence, higher quality individuals are better able to meet
92 the costs of maintaining good sexual characters and good immunological defense, and will
93 therefore be preferred as couples [74-75].

94 The reproductive biology of scorpions has certain characteristics that make it a potentially
95 useful model for the study of these topics. Their courtship is complex and ritualized, after which
96 males adhere a sclerotized spermatophore to the soil, from which the female receives the sperm
97 [76-78]. The sperm penetrates the genital aperture of female and advances through the genital
98 atrium towards the seminal receptacles [79]. After fertilization, the viviparous embryos develop
99 within the ovariuterus until the time of parturition [80-81]. In many species of scorpions, females
100 present a genital plug after the sperm transfer [12], although their morphology is very diverse and
101 the function is discussed [11]. The efficacy in preventing sperm competition is in many cases
102 linked to the morphology of the genital plug. It has been observed that in *Mesomexovis punctatus*
103 (Karsch, 1879) inseminated females experience a decrease in sexual receptivity, that coincides
104 with the presence of plugs with strong anchoring mechanisms to the genital atrium and
105 obstruction of the genital aperture [9,19]. In contrast, in *Bothriurus bonariensis* (C.L Koch, 1842)
106 this fall in receptivity after mating is not observed, coinciding with a small and membranous plug
107 that does not block the female genital aperture [82]. However, a strict relationship between plug

size and efficiency in the sperm competition avoidance should be reviewed in depth in scorpions, since other factors might affect the effectiveness of the plug (e.g., accompanying chemical substances, anchoring and blocking mechanisms, hormonal activator-deactivators, triggering factors of the immune response). In some cases, the formation of the genital plug is almost completely attributed to the male [9,12,19], although the participation of the female has been suggested [11-12,19,83-84]. In ultrastructural studies of the atrial epithelium of the female, pores and glandular cells and high secretory activity have been described, so the possibility of female participation is strongly expected [18,84-85].

In this work, females of three scorpion species are compared. *Urophonius brachycentrus* (Thorell, 1877) and *U. achalensis* (Ábalos and Hominal, 1974) [11,86] two species of the family Bothriuridae that have genital plugs were compred with *Zabius fuscus* (Thorell 1877), a buthid species with no genital plug [12]. A wide variety of genital plugs are known in Bothriuridae, which may be 'gel-like' (formed by sperm or accessory gland substances) [11,18,82] or 'sclerotized' plugs (derived from cuticular portions that detach from the spermatophore) [11,18]. Within sclerotized plugs, there are two subtypes: simple (filamental, membranous) and complex plugs (cone-shaped, mixed) [12]. It has been proposed that *Urophonius* has a 'mixed' plug since it presents a combination of detachable portions of the spermatophore and glandular substances [11]. It is known that males of *Urophonius brachycentrus* and *U. achalensis* transfer an 'initial plug' formed by two hemi-mating plugs (one per hemispermatophore) that join in the formation of the spermatophore during sperm transfer. This 'initial plug' presents a translucent coloration when it has just been transferred to the female and shows a progressive darkening during the reproductive season (see below the results of this study). Certain changes in the size and coloration of the plugs of *Urophonius*, and other species [9,11,19], might be linked to immunological responses such as encapsulation and melanization. These reactions strongly

132 resemble the immune response that is activated on artificial implants (e.g., nylon filaments) that
133 are inserted into the hemocoel of individuals and that, after a time, present areas with dark
134 encapsulations [87-90].

135 To elucidate these questions about the female role in the formation of genital plugs, we
136 first described the genital plugs and their positioning within the female genital atrium in both
137 *Urophonius* species. In second place we evaluated the encapsulation immune response to a
138 mechanical stimulus, similar to that of the genital plug, in the female genital atrium. We expected
139 to find larger, dark-colored (melanotic) encapsulations in the artificial genital plugs of females of
140 species that have a genital plug, resembling those observed in the true genital plugs of these
141 species. We used this experimental approach due to the impossibility of replacing extracted plugs
142 in the females since these are extremely fragile and strongly anchored within the female genital
143 atrium (Oviedo-Diego, Mattoni and Peretti, unpublished data). Thirdly, we surveyed the types of
144 hemocytes present in the hemolymph and total hemocyte load (THL) of the females of each
145 species. It was observed if there were changes in the THL before and after the artificial plug
146 placement. We expected recirculation of the hemocytes toward the genital area if these are
147 involved in the changes observed in the artificial plugs. Finally, we looked for relations between
148 the immunological parameters (encapsulation areas/coloration as a proxy of melanization and
149 THL) and between the parameters and the body weight of individuals, since heavier females
150 could have greater THL and be more competent to face an immune challenge such as the
151 artificial genital plug.

152

Materials and methods

Studied species, collection and rearing

Two sister species of the family Bothriuridae were studied. *Urophonius brachycentrus* and *U. achalensis* present winter surface activity [91] and were collected at the beginning of the season (from May to June). The time of collection was determined to ensure that the females were virgins and did not have a genital plug, as when the females are inseminated they always present a genital plug, and their distal portion is visible below the genital operculum (Fig 1A). In contrast, *Zabius fuscus* individuals are active in summer (November to March) [92] and inseminated females do not present genital plug [12,18]. Individuals were collected during the day by turning rocks over in the Sierras Grandes at altitudes from 800 to 1900 MASL. Although *Zabius fuscus* (Fam. Buthidae) is phylogenetically distant from the two species of Bothriuridae, it was chosen for this study because as far as we know there are no bothriurid species that do not have a genital plug [11-12,18,82,93-94]. In the laboratory, each specimen was weighed with a digital balance (Ohaus Pioneer PA114). The scorpions were conditioned in individual plastic containers (9 cm x 6 cm) and were kept with moistened cotton as a water supply, and fed once a week with larvae of *Tenebrio molitor* (Coleoptera, Tenebrionidae) or adults of *Shelfordella tartara* (Blattodea, Blattidae). The specimens were maintained at constant temperatures (10°C in winter, 25°C in summer). Voucher specimens were deposited in the collection of Laboratorio de Biología Reproductiva y Evolución, Universidad Nacional de Córdoba, Argentina.

Morphology of genital plugs and positioning within female

To observe the positioning of the plug within inseminated females (*U. brachycentrus* N=20; *U. achalensis* N=20) dissections were performed. The specimens were sacrificed in a freezer at -20 ° C for fifteen minutes and then dissected under a stereoscopic microscope (Nikon SMZ 1500). After the dissection, the genital plug was removed from the female's atrium with straight tweezers. The dissected specimens and genital plugs were photographed with a digital camera (Nikon Digital Sight DS-FI1-U2) coupled to the stereoscopic microscope. Some genital plugs (N=10 per species) were kept in a 1 mL microcentrifuge tube exposed to the air and were photographed every week for a month. In this way, we observed if there were changes in the coloration or morphology of the genital plugs outside the female (e.g., by oxidation of the plug material). We also evaluated changes in the coloration and morphology of the plugs inside the females throughout the reproductive season (N=10 per species) by examining the external portion of the plugs below the genital operculum.

Encapsulation response on the artificial genital plug

Placement and removal of artificial plug

A piece of sterile nylon monofilament (3 mm x 0.1 mm) was placed in the genital atrium of each female, resembling in size and positioning to the genital plug present in females of the species of *Urophonius* (Fig 1). The specimens were immobilized on a microscope slide with Parafilm®. A small hole was made in the parafilm to access the genital operculum of the individual, and the artificial plug was gently inserted up to the end of the atrium by lifting the operculum with straight tweezers (Fig 1B, D, F). The surface of the artificial plug was slightly

roughened with sandpaper to reach a rough surface and enhance the adhesion of hemocytes to the artificial genital plugs [95-96]. This procedure does not cause any damage to the female genitalia. The artificial plug was left in the genital atrium of the female for a month, since it has been observed that the genital plug of *Urophonius* takes approximately this time to present some darkening (Oviedo-Diego, Mattoni, Peretti personal observations). After this period, the artificial plugs were carefully removed, and the tissue remnants were cleaned. Each artificial plug was photographed from two perspectives (front and back), rotating 180º [97] with a digital camera (Nikon Digital Sight DS-FI1-U2) coupled to a stereomicroscope (Nikon SMZ1500). A photographic protocol was used that kept light exposure and magnification constant. Then the artificial genital plugs were preserved in ethanol 80%.

Fig 1. Genital plugs and artificial genital plugs of the study species. (A) 'Distal' zone of genital plug below the female genital operculum of *Urophonius achalensis*. (B) Protruding distal portion of the artificial genital plug positioned within the female genital atrium of *Urophonius achalensis*. (C) Genital plug extracted of an inseminated female of *Urophonius brachycentrus*. (D) Artificial genital plug before being placed on a female. (E) Scheme of a genital plug (*Urophonius*) and its positioning within the female genital atrium. (F) Scheme of an artificial genital plug and its positioning within the female genital atrium. Abbreviations: ag, genital atrium; agp, artificial genital plug; al, lumen of the genital atrium; bp, basal piece; Dz, distal zone; ga, genital aperture; gp, genital plug; ls, lumen of the seminal receptacle; ME, melanotic encapsulation; Mz, middle zone; NME, non-melanotic encapsulation; og, genital operculum; p, pectine; Pz, proximal zone; sr, seminal receptacle. Scale bars: 1 mm.

Area and coloration of the encapsulations on the artificial plug

The encapsulations on each artificial plug were measured by processing the images with ImageJ 1.45 software [98]. For statistical analysis, the artificial plugs were divided into three zones, since it is known that the genital plugs of the *Urophonius* species studied also have three zones ('distal', 'middle' and 'proximal' to the body of the individual) (See Results). The zones of the artificial plugs were defined by dividing the total length of the filament (3 mm) into three parts of equal length so that each zone was 1 mm long. The area that remained within the body of the female contacting the end of the genital atrium was the 'proximal' zone of the artificial plug, while the zone more distal to the body of female was the 'distal' zone of the artificial plug. The areas of the encapsulations were compared between the zones of the artificial plugs and between species. The encapsulations were classified as melanotic (ME) or non-melanotic (NME) according to their coloration (Fig 1F). The coloration was calculated with the average grayscale value from the pixels of the different areas of the artificial plug encapsulations. The 0 value represents black and 255, white. The classification of encapsulations coloration was carried out using a threshold value of 50 in the average grayscale, being ME if the color was lower than the threshold value and NME if it was higher than this value.

Extraction, characterization and quantification of hemocytes

The second left leg of individuals was completely excised (between tarsus and tibia) to allow a considerable drop of hemolymph to flow from the wound. A sample of 0.75 µl of hemolymph was taken with a glass capillary from the wound [99]. This sample was mixed with 9.25 µl of Spider Saline Solution [100] in a microcentrifuge tube. A five-second pulse of vortex was carried out three times to the solution to homogenize the sample. Immediately after, the

sample was placed in a Neubauer chamber for counting under a light microscope with a phase contrast objective 100x (Nikon Eclipse 50i) [101] at 400X. All hemocytes from virgin females of the different species were identified and counted [102]. The characterization of hemocytes was performed by observing and photographing their characteristics with a digital camera coupled to the microscope (Nikon Digital Sight DS-FI1-U2). The total hemocyte load (THL) (number of hemocytes per milliliter of hemolymph) was compared in two stages: before the placement, and after extraction of the artificial genital plug (through a second cut of the same leg).

Statistical analyses

We analyze the data with generalized linear mixed models (GLMM). In the analysis of the artificial plugs encapsulations the variables response were the ME and NME areas (mm^2) and the average grayscale value of each type of encapsulation. The zone of the artificial plug ('distal', 'middle' and 'proximal'), the species and the body weight of the individuals were the fixed effects. The body weight of the individuals was measured before and after the placement of the artificial plugs and since there were no significant differences between both instances (Mann–Whitney U test; $Z = 0.409$, $p = 0.683$) the average weight value for statistical analyzes was considered. In the quantification of hemocytes the variable response was the THL, and the fixed factors were the species, the stage of quantification (before and after the placement of the artificial plug) and the body weight of the individuals. We also evaluated the possible interactions between the fixed factors analyzed. The individuals' identity was included in all the models as a random effect. If the random effect variance was small, the effect of the random variable was discarded. Normality and homogeneity of variances of the variables were assessed graphically and analytically. If the assumptions were not met, the variable according to the best distribution

was modeled. The coloration of ME and the THL presented a normal distribution. The areas of ME and NME and the coloration of NME presented a gamma distribution, so they were modeled using the glmmadmb function [103]. We used the package lme4 [104] and lsmeans [105] for a posteriori tests in R v. 3.3.3 64 bit [106]. Also, multiple correlations with the Spearman's method were performed between the different immunological parameters including the three species of scorpions together, and between these parameters and the individuals' body weight. A significance level α of 0.05 was considered.

Results

Morphology of genital plugs and positioning within female

In both species, the genital plug adjusted exactly to the female's atrium and blocked the lumen and the genital aperture. The genital plugs presented three double-shaped zones (Fig 1C and 2). The 'distal zone' to the individual's body was visible from the outside, and extended below the genital operculum covering the genital aperture (Fig 1A). It was always sclerotized, brittle and darkly colored. In *U. brachycentrus* this zone resembled two thin 'wings'. In contrast, in *U. achalensis*, this zone was wider with concave platform shape towards the genital aperture. Next to the 'distal' zone was the 'middle' zone, also sclerotized and dark, formed by two fused structures running along the lumen of the female atrium. While in *U. brachycentrus* this zone was thin and long, in *U. achalensis* it was shorter and it was sometimes more difficult to distinguish the fused structures. Finally, the 'proximal' zone consisted of one or more sacciform globular structures, with a flexible gelatinous consistency and a white-yellowish coloration. Projections ascended from the end of the genital atrium to the duct of one of the spermathecae, sometimes occluding the duct (Fig 1E). In *U. brachycentrus* two projections were always found in the

286 'proximal' zone, while in *U. achalensis* the number was variable from one to four proximal
287 projections (Fig 2). We found that the plug undergoes changes in coloration and morphology
288 over time in the genitalia of the female. After mating the plugs presented the 'distal' zone (visible
289 below the operculum) with translucent coloration and a thin, fragile consistency. As the
290 reproductive season progressed, the plug darkened and acquired a sclerotized consistency (June
291 to August). Towards the end of the season before parturition (November to December), a
292 decrease in the size of this zone of the plug was observed. Conversely, no changes were observed
293 in the coloration or morphology of the plugs extracted from the females and exposed to the air.
294
295 **Fig 2. Genital plugs of *Urophonius* species.** (A) Genital plug of *Urophonius brachycentrus*. (B)
296 Genital plug of *Urophonius achalensis*, note that the plug has been excised below the distal zone
297 by handling during removal. Abbreviations: Dz, distal zone; Mz, middle zone; Pz, proximal zone.
298 Scale bars: 1 mm.
299

300 Encapsulation on artificial genital plugs

301 Regarding the encapsulation response of the females, different characteristics and
302 magnitudes of this type of immune response were observed, depending on the species and the
303 zone of the artificial genital plug (Fig 3A-F). Occasionally, a non-melanotic encapsulation
304 response was observed, generally with excrescences of larger surfaces and almost continuously
305 surrounding the genital plug. This type of encapsulation was white-yellowish, translucent or
306 opaque. Melanotic encapsulation presented more specific arrangements, generally in the form of
307 isolated granules in different zones. It was classified according to its color tones from brown to
308 reddish. In all the artificial plugs some type of encapsulation response was found, although in

some cases the encapsulations were not present in all the zones of the artificial plug (Fig 3A-F). Hemocytes could be observed on the artificial plugs and in their surroundings under an optical microscope (Fig 4A-B). Sometimes it was possible to see the deposition of a substance around the entrance of the artificial plug by the genital aperture, and even the formation of projections (Fig 4C-D).

Fig 3. Different encapsulation responses on artificial genital plugs and comparison with *Urophonius* genital plugs. (A) Artificial genital plug with encapsulation response of *Urophonius achalensis*. (B) Artificial genital plug with encapsulation response of *Zabius fuscus*. (C) 'Distal' zone of artificial genital plug of *U. achalensis*. (D) 'Distal' zone of artificial genital plug of *U. brachycentrus*. (E) 'Proximal' zone of artificial genital plug of *U. achalensis*. (F) 'Proximal' zone of artificial genital plug of *U. brachycentrus*. (G) 'Distal' zone of the genital plug of *U. achalensis*. (H) 'Proximal' zone of the genital plug of *U. brachycentrus*. Abbreviations: agp, artificial genital plug; dp, distal projections; Dz, distal zone; gp, genital plug; ME, melanotic encapsulation; Mz, middle zone; NME, non-melanotic encapsulation; ps, proximal sacciform projections; Pz, proximal zone. Scale bars: A, B, G, G= 0.5 mm; C, D, E, F= 0.2 mm.

Fig 4. Hemocytes cells and distal substances found on the artificial genital plugs of females of *Urophonius*. (A)(B) Surface of the artificial genital plug of *Urophonius brachycentrus* under an optical microscope, note hemocyte cells. (C)(D) External female genitalia of *Urophonius achalensis*, note artificial genital plug and surrounding substance in the area of the genital aperture. Abbreviations: agp, artificial genital plug; bp, basal piece; ds, distal substance produced by the female; GR, granulocyte; ME, melanotic encapsulation; og, genital operculum; p, pectine; PL, plasmatocyte. Scale bars: A= 0.2 mm; B= 40 μm; C, D=1 mm.

Areas of encapsulation

We found an effect of the interaction between the species and the encapsulated zone of the artificial plug (ME: Df=2, χ^2=14.827, p=0.005; NME: Df=2, χ^2=20.411, p=4.144E-04) (Fig 5A-B and S1 Table). ME values for *U. achalensis* were higher, although they were not significantly different from those of *U. brachycentrus*. In contrast, the two species of *Urophonius* did show differences with the ME values of *Z. fuscus* that were the lowest (Fig 5A). Differences were also found in the areas covered by encapsulations in the different zones of the artificial plug. All species had higher ME in the 'distal' zone, although *U. brachycentrus* had fewer differences between zones. As for NME, all species presented similar areas of encapsulations. *Z. fuscus* showed a homogenous, low response in all zones, and *Urophonius* species showed a greater response in the 'distal' and 'proximal' zone (Fig 5B). The interaction of body weight with the artificial plug area for NME was significant (Df=2, χ^2=18.454, p=9.835E-05).

Fig 5. Graphs of immunological parameters of the encapsulation response in females of three scorpion species. Top boxplots showing distribution of data set and differences between species, below heat maps charts in which average values are represented by colors (scale of reference of each variable to the side of the graph) according to the zones of the artificial genital plug. (A) Area of melanotic encapsulation (ME) (mm^2). (B) Area of non-melanotic encapsulation (NME) (mm^2). (C) Color of NME encapsulation (average grayscale value). (D) Coloration of ME encapsulation (average grayscale value). Numbers indicate significant differences ($p < 0.05$) between species above the boxplots. Letters on heat maps charts indicate significant differences

355 ($p < 0.05$) between zones of the artificial genital plug within each species (i.e. intraspecific
356 comparison: reading vertically, not horizontally).
357
358 ## Coloration of the encapsulated zones

359 Since *Z. fuscus* females rarely presented ME in the 'middle' and 'proximal' zone of the
360 artificial plugs, only the coloration of these encapsulations was analysed for the females of two
361 *Urophonius* species (Fig 5C and S1 Table). We found a significant interaction between the
362 species and the encapsulated zone (Df=2, χ^2=29.698, p=3.558E-07). *Urophonius achalensis*
363 females showed lower values on the mean grayscale than *U. brachycentrus,* i.e. darker coloration
364 of ME and there was also variation in the artificial plug zone. *Urophonius achalensis* presented
365 'distal' zones significantly darker than the rest of the zones. In contrast, *U. brachycentrus*
366 presented darker 'middle' zones, followed by the 'distal' zones and significantly clearer
367 'proximal' zones. For the coloration of NME, we found an effect of the interaction between the
368 species factor and the artificial plug zones (Df=4, χ^2= 23.463, p=1.023E-04). *Z. fuscus* presented
369 in general clearer encapsulations although they were not significantly different from those of
370 *Urophonius* spp. (Fig 5D and S1 Table). In this species, the 'distal' zone was darker than the
371 others. For the *Urophonius* species, all the zones presented NME encapsulations of equal
372 coloration. There was an interaction between body weight and the artificial plug zone for NME
373 (Df=2, χ^2=10.976, p=0.004).
374
375 # Characterization of hemocytes of females of the studied species

376 We identified different types of hemocyte cells in the hemolymph of the females in the
377 species studied. Hemocytes presented different morphology and size (Fig 6). Granulocytes (GRs)

were cells with spherical and isodiametric shapes and regular contours. GRs had cytoplasmic extensions variables in shape, although the extensions were in general short and acute. The cytoplasm of the GRs was dense and always presented abundant refractive oval shaped granules (Fig 6B-C). Plasmatocytes (PLs) were highly variable in shape, generally spindle-shaped with multiple large, rounded cytoplasmic extensions radiating in ameboid form from the central zone. The cytoplasm was hyaline and homogenous, with vacuoles and small or no inclusions (Fig 6E-F). Sometimes the vacuoles occupied a large portion of the cytoplasm of the cell, pushing the nucleus to an eccentric position, giving it a signet-ring appearance with sharp projections (Fig 6F). Also, other types of hemocytes were observed, although they were not found in all the samples. All these hemocytes had a rounded and rather an isodiametric shape, and did not expand cytoplasmic extensions such as PLs. They presented granules in the cytoplasm of different shapes and nature. Cystocytes generally presented a cytoplasm with small granules, a large vacuole and eccentric nucleus. Spherulocytes possessed large, dark granules or spherules of a homogeneous size, which completely obscured the nucleus of the cell. Adipohemocytes presented an eccentric nucleus with typical fat lipid droplets in their cytoplasm. Free cells were observed in the hemolymph and also grouped in agglomerates and, although in these cases it was difficult to identify the clustered cells, we were able to determine that on occasions the clusters may have cells of a different type (Fig 6A, D).

Fig 6. Hemocytes present in the hemolymph of females of three scorpions' species. (A) Plasmatocytes (PLs) and granulocytes (GRs) cluster of *Urophonius brachycentrus*. (B) GRs of *Urophonius brachycentrus*, note isodiametric morphology, granules in the cytoplasm and short and acute cytoplasmic extensions. (C) GRs of *Zabius fuscus*, note granules in the cytoplasm and short cytoplasmic extensions. (D) PL cluster of *Urophonius achalensis*. (E) PLs of *Zabius fuscus*

expanding their cytoplasmic extensions with small inclusions in their cytoplasm. (F) Signet-ring PL of *Urophonius brachycentrus*, note vacuole occupied a large portion of the cytoplasm of the cell. Abbreviations: ce, cytoplasmatic extensions; GR, granulocyte; gs, granules in the cytoplasm; PL, Plasmatocyte; sg, signet-ring plasmatocyte; va, vacuole. Scale bars: A, D= 50 μm; B, C= 20μm; E, F=10 μm.

Quantification of hemocytes

We found a significant interaction between the fixed factors: 'species' and 'stage of quantification' (Df=2, χ^2=35.364, p = 2.093E-08) (Fig 7 and S1 Table). *Zabius fuscus* females showed the highest THL values both before and after the placement of the artificial genital plug with respect to *Urophonius* females. The *Urophonius* spp. showed similar values of THL before the placement of the artificial genital plug. *Zabius fuscus* females showed no difference in THL between the two stages of quantification, while in both species of *Urophonius* females presented a decrease in THL after one month of the placement of the artificial plug, with a greater decrease of *U. brachycentrus* than that of *U. achalensis* (Df=1, F=7.261, p=0.015). Body weight had an effect on THL (Df=1, χ^2=7.708, p =0.006).

Fig 7. Total Hemocyte Load of females of the three scorpion species. Differences in total hemocyte load (THL) (number of hemocytes per milliliter of hemolymph) according to the stage of quantification (pre-implantation of the artificial genital plug and post-implantation of the artificial genital plug) of three scorpion species. Letters indicate significant differences ($p<0.05$) between species. Values are shown in scientific E-notation where 'E' represents the exponential to 10.

425

426 Correlations between immunological parameters

427 We noted that the immunological parameters, in general, were highly correlated (Fig 8).
428 A positive correlation was found between the female body weight and the THL but a negative
429 relationship was found between the body weight and the area of both types of encapsulations.
430 Females with a higher THL also presented smaller ME and NME areas on the artificial plugs.
431 However, the THL were positively correlated with the coloration of the encapsulated (i.e. clearer
432 encapsulations). We noted a negative correlation between the area of ME and the coloration of
433 the encapsulated zones, as well as between the NME area and the coloration of the ME
434 encapsulations. A positive correlation was observed between the areas of both types of
435 encapsulation.

436

437 **Fig 8. Correlation matrix between the individual's immunological parameters and body**
438 **weight.** In the diagonal portion of the matrix are found the parameters that were correlated with
439 their corresponding frequency histogram. In the upper portion of the matrix are the Spearman
440 correlation coefficients for each pair of variables and below the correlation p-value. In the lower
441 portion of the matrix are graphs of dispersion of the variables, the colors of points represent the
442 different species of scorpions (Black dots: *Urophonius achalensis*; Grey dots: *Urophonius*
443 *brachycentrus*; White dots: *Zabius fuscus*). Abbreviations: ME, melanotic encapsulation; NME,
444 non- melanotic encapsulation. Some values are shown in scientific E-notation where 'E'
445 represents the exponential to 10.

446

Discussion

The description of the morphology of the *U. brachycentrus* and *U. achalensis* genital plugs allowed us to perceive a complex three zoned structure anchored to the female genital atrium. We also observed changes in the plugs' coloration over time, probably attributable to a female's role. Given the need for experimental approaches to answer questions about the function or origin of genital plugs [11-13], artificial genital plugs were used in this study to evaluate the female's contribution to their morphology and coloration. We found that females deposited secretions on the artificial plugs and that some of its zones had darker encapsulations. We confirmed that in plug producing species, the presence of an artificial plug caused a decrease in the hemocyte load. This could mean consequences on the plugged female's immune system. These results could help to clarify a possible female role in the plugs' formation and may help to provide a wider framework of different physiological consequences related to this post-copulatory mechanism.

Urophonius' genital plugs and the female's possible role in its formation

We described inseminated females' genital plugs of *U. achalensis* and *U. brachycentrus*. In all cases, the plugs blocked completely the lumen of the atrium, the genital aperture, and, in some cases one of the spermathecae' ducts. Both the female and the male could be involved in the plug's formation. The double conformation of the plug clearly indicates a male' s contribution in that, the 'initial plug' transferred by the male is formed from the 'hemi-plugs' in each hemispermatophore when fused in the spermatophore (involving portions of the ejaculate and

469 glandular products). The changes of the plug over time in morphology, size and coloration could
470 indicate a female's role. We could discard the hypothesis that these changes of the genital plugs
471 were due to air contact or O_2 influence. The *Urophonius'* genital plugs present three different
472 zones. Females of *U. achalensis* showed darker melanotic encapsulation in the 'distal' zone, and
473 females of *U. brachycentrus* in the 'middle' zone. The 'proximal' zone of the genital plugs does
474 not present dark coloration, and coincidentally, this was the area in the artificial genital plugs
475 with smaller melanotic encapsulations and clearer coloration. It was also found that the 'distal'
476 and 'proximal' zones presented the larger non-melanotic encapsulations areas. The projections
477 formed in the 'distal' zone of the artificial plugs extending below the genital operculum were
478 similar in shape, size and consistency to those found in the genital plugs. This also adds evidence
479 to the female's role in the formation of this zone.

480 In a sexual conflict framework, genital plugs would be strongly associated with the male's
481 monopolization of females [25-26], and could imply some cost for the females such as a
482 limitation of their possibilities of remating [107]. The plugs that trigger processes, such as those
483 described in this work, could mean a physiological challenge for females, which could in time
484 affect some parameters of fitness or life history. This would be an interesting topic to explore in
485 future experimental studies because the evaluation of these costs could shed light on the
486 discussion of female's role to the plug formation or degradation.

487 Another non-exclusive possibility is the occurrence of cryptic female choice [38-39]. This
488 can be manifested as a modulation of the female's immune response regarding characteristics of
489 the plug (e.g., mechanical effectiveness, chemical composition, size) as well as those of the male
490 (e.g., male's quality, duration of pre-copulatory and copulatory courtship) [29,40-41,46,108-110].
491 A comparative and phylogenetic study of the plugs in the Bothriuridae Family could provide
492 information about the evolution of male and female strategies in terms of the plugging

493 phenomenon, and whether this strategy, for example, is related in any way to other traits of the
494 immune system or genital characters.
495

496 An artificial genital plug compromises the immune system of plug
497 producing species

498 A change in the THL after an immunological challenge has been previously described in
499 species of insects and crabs [111-117]. This change could be explained by the recirculation of
500 free hemocytes in the hemolymph towards affected areas, where the phagocytic or encapsulating
501 action of the hemocytes is necessary. Both the formation of encapsulations on artificial genital
502 plugs and the presence of hemocyte cells in their surroundings evidence a direct action of the
503 hemocytes against this immunological challenge [118]. We found that the encapsulation response
504 varied regarding the analyzed species and the zone of the artificial genital plug. The females of *Z.*
505 *fuscus* did not show a depletion of hemocytes' number after the placement of the artificial plug.
506 Besides, the artificial plugs of *Z. fuscus* presented a minimal and homogeneous encapsulation. In
507 contrast, females of plug producing species showed a decrease in the number of circulating
508 hemocytes, accompanied by greater encapsulation on certain zones of the artificial plugs. These
509 findings suggest that species with genital plugs (*Urophonius* spp.) have developed a more
510 sensitive immune system to specific challenges like the one used in this study. In scorpions, the
511 cuticle thickness and histological complexity of the female's genital atrium have been related to
512 mechanical damage caused by the capsular eversion of the spermatophore or by the introduction
513 of genital plugs [12,18,84]. For instance, *Z. fuscus,* a non-plug producing species, presents simple
514 spermatophores and a thin walled genital atrium, whereas plug producing bothriurid species have
515 more complex spermatophores, and the genital atrium with folded epithelium and thick cuticular

walls [18]. In addition, in several bothriurids, including *U. brachycentrus*, there have been seen regions in the atrium's apical zone which contain epithelial cells with microvilli and pores connected to ducts [18,84]. We also found differences in the encapsulation response in the different zones of the artificial genital plug, especially in the plug producing species. There is evidence that female's genitalia is complex and may present a modularized immune response (specificity) [47,119-121]. This specificity can be explained because the female's tract has evolved by making contact with sperm, with male's ejaculate substances and with infectious agents [120,122]. Preliminary data on *U. brachycentrus* suggests that the immune response triggered by artificial implants in females' genitalia may be much more specific and intense than that triggered in other non-genital parts of their bodies. For example, there has been observed a weaker encapsulation response in implants placed in the dorsal pleural membrane compared to that triggered on artificial plugs in the genital area (Oviedo-Diego, Mattoni, Peretti personal observations).

Types of identified hemocytes and quantified hemocyte load

We have described, for the first time, the types of hemocytes found in the female's hemolymph in *Urophonius achalensis, U. brachycentrus* and *Z. fuscus*. Two main types of cells were found: plasmatocytes (PLs) and granulocytes (GRs), in agreement with the findings of existing works on the subject [69,102,123-126]. Subtypes of hemocytes, Cystocytes (CYs), Spherulocytes (SPs) and Adipohemocytes (ADs), previously cited for scorpions [69,102,123-124] were also identified. The existence of several types of hemocytes in the hemolymph would be an ancient character [127], which could have been retained in scorpions, one of the oldest arthropod groups [128-131, but see 132]. There were not found prohemocytes, described as stem

539 cells with embryonic nature [69,102,123,126], probably due to their rapid conversion to other cell
540 types [133]. Even though other subtypes of hemocytes, like oenocytoids or coagulocytes, were
541 observed in scorpion species [69,102,123-124) there was not found any evidence of their
542 presence in the species studied herein. It would be very useful to include novel techniques such as
543 genetic markers or antibodies in future studies, as well as other microscopy techniques such as
544 scanning or electronic transmission, for the purpose of a precise classification, quantification and
545 elucidation of the action mechanisms of these cells [124,134-137]. *Zabius fuscus* had the highest
546 values of THL compared those of plug producing species. The causes of this difference could
547 respond to the evolutionary history of each species and the sexual and ecological context in
548 which they have evolved [138-139]. Although the characteristics of the habitats are similar, the
549 species present contrasting characteristics regarding patterns of surface activity at different times
550 of the year [91-92]. In addition, these species could exhibit differences in microhabitats (Oviedo-
551 Diego, Mattoni, Peretti personal observations), or in other parameters such as diet or potential
552 parasites [83].
553

554 ## Correlations between immune parameters

555 Since multiple immunological parameters can be costly to maintain [74] trade-offs may
556 exist between parameters within the same system. This would indicate an overlap in the resources
557 used by different defense mechanisms, a cross-regulation between them or a common underlying
558 mechanism [64,61,138]. We found a negative correlation between the areas and the coloration of
559 the encapsulations. This would suggest a trade-off between the encapsulation response per se
560 (aggregation of layers of hemocytes) [140] and the melanization that occurs in these
561 encapsulations (products generated by the phenoloxidase pathway) [66]. There was a close

562 interrelationship between the humoral and cellular components [141], and some studies have
563 reported antagonisms between the parameters of these systems, without investigating the
564 underlying physiological mechanisms [97,142-143]. On the other hand, we found a positive
565 correlation between body weight and THL. Variation in immunological parameters between
566 individuals and species is expected [138,144]) since the management of trade-offs between the
567 costs of immune defense and other life history traits that overlap in the use of resources, can vary
568 (75,145]. Heavier individuals may be more immunocompetent since they would have more
569 circulating hemocytes [146, but see 147]. However, it was also found that higher body weight
570 individuals (*Z. fuscus* females) presented smaller areas of melanotic encapsulations on the
571 artificial plug and clearer encapsulations. These results were expected since a higher THL value
572 would indicate a higher concentration of free hemocytes in hemolymph, and a lower number of
573 hemocytes in the genital area, resulting in less encapsulated and melanizated artificial plugs. It
574 has also been reported that individuals with large numbers of hemocytes have a lower proportion
575 of phagocytic hemocytes [148].
576

577 Conclusions

578 The morphology of genital plugs of two scorpion species (*U. achalensis* and *U.*
579 *brachycentrus*) was described, being complex structures with three different zones, strongly
580 anchored to the female genital atrium, with a coloration that considerably resembles a type of
581 immune response. The encapsulation and melanization patterns on the artificial plugs may
582 indicate greater and more specific response in females of species that have a genital plug. In turn,
583 these species presented a depletion in the number of hemocytes in hemolymph, indicating
584 possible recruitment of these cells into the genital area. Body weight was correlated positively

585 with THL but negatively with the encapsulation area on the artificial genital plug. These results
586 and the negative correlations between different immunological parameters may indicate complex
587 interrelations within the immune system that remains to be investigated. These results suggest
588 that females of *U. achalensis* and *U. brachycentrus* contribute to the formation of the genital plug
589 by attaching encapsulations to it and darkening the plug in certain zones by melanization of these
590 encapsulations. Further studies would include analysis of changes in THL comparing virgin and
591 inseminated females (with genital plug) to elucidate whether the observed results with artificial
592 plugs actually reflect what happens when females are plugged. A comparison of the immune
593 response triggered by implantation in the genital area with respect to other body regions and
594 comparisons with the immunological parameters of the male would provide information on the
595 specificity of the female's genital immune response. Finally, modulation of female's immune
596 response according to male's characteristics would provide information about female cryptic
597 choice mechanisms, or about the female's influence on the effectiveness of the plug to avoid
598 sperm competition.
599

600 # Acknowledgments

601 We would like to thank J. Heywood for the revision of the language of the manuscript, to
602 G. Gonzalez for his help with statistical questions, to D. Fink for collaborating in the artwork.
603 Also, we are grateful to L.E. Costa-Schmidt and all members of LAB.R.E, especially P. Olivero
604 and F. Bollatti for their useful suggestions on a previous version of the manuscript.
605

References

1. Parker GA. Sperm competition and its evolutionary consequences in the insects. Biol. Rev. 1970;45(4):525-567. doi: 10.1111/j.1469-185X.1970.tb01176.x .
2. Wigby S, Chapman T. Sperm competition. Curr. Biol. 2004;14(3):100-103. doi: 10.1016/j.cub.2004.01.013.
3. Parker GA, Birkhead TR. Polyandry: the history of a revolution. Phil. Trans. R. Soc. B. 2013;368(1613):20120335. doi: 10.1098/rstb.2012.0335.
4. Simmons LW. Sperm competition and its evolutionary consequences in the insects. Princenton: Princeton University Press; 2001.
5. Drummond BA. Multiple mating and sperm competition in the Lepidoptera. In: Smith RL, editor. Sperm competition and the evolution of animal mating systems. New York: Academic Press; 1984. pp. 291-370.
6. Orr AG, Rutowski RL. The function of the sphragis in *Cressida cressida* (Fab.) (Lepidoptera, Papilionidae): a visual deterrent to copulation attempts. J. Nat. Hist. 1991;25(3):703-710. doi: 10.1080/00222939100770461.
7. Dickinson JL, Rutowski RL. The function of the mating plug in the chalcedon checkerspot butterfly. Anim. Behav. 1989;38(1):154-162. doi: /10.1016/S0003-3472(89)80074-0.
8. Baer B, Morgan ED, Schmid-Hempel P. A nonspecific fatty acid within the bumblebee mating plug prevents females from remating. P. Natl. Acad. Sci. 2001;98(7):3926-3928. doi: 10.1073/pnas.061027998.
9. Contreras-Garduño J, Peretti AV, Córdoba-Aguilar A. Evidence that mating plug is related to null female mating activity in the scorpion *Vaejovis punctatus*. Ethology. 2006;12(2): 152-163. doi: 10.1111/j.1439-0310.2006.01149.x.

629 10. Bretman A, Lawniczak MK, Boone J, Chapman T. A mating plug protein reduces early
630 female remating in *Drosophila melanogaster*. J. Insect Physiol. 201056(1):107-113. doi:
631 10.1016/j.jinsphys.2009.09.010.
632 11. Mattoni CI, Peretti AV. The giant and complex genital plug of the asper group of
633 *Bothriurus* (Scorpiones, Bothriuridae): morphology and comparison with other genital
634 plugs in scorpions. Zool. Anz. 2004;243(1-2):75-84. doi: 10.1016/j.jcz.2004.07.002.
635 12. Peretti AV. An ancient indirect sex model: Single and mixed patterns in the evolution of
636 scorpion genitalia. In: Leonard J, Córdoba-Aguilar A, editors. The Evolution of Primary
637 Sexual Characters in Animals. Oxford: Oxford University Press; 2010. pp. 218-248.
638 13. Uhl G, Nessler SH, Schneider JM. Securing paternity in spiders? A review on occurrence
639 and effects of mating plugs and male genital mutilation. Genetica. 2010;138(1):75. doi:
640 10.1007/s10709-009-9388-5.
641 14. Carvalho APS, Orr AG, Kawahara AY. A review of the occurrence and diversity of the
642 sphragis in butterflies (Lepidoptera, Papilionoidea). ZooKeys. 2017;(694):41–70. doi:
643 10.3897/zookeys.694.13097.
644 15. Giglioli MEC. The Female Reproductive System of *Anopheles gambiae* mêlas. I. The
645 Structure and Function of the Genital Ducts and Associated Organs. Riv. di Malariologia.
646 1963;42(4/6):149-76.
647 16. Duvoisin N, Baer B, Schmid-Hempel P. Sperm transfer and male competition in the
648 bumblebee *Bombus terrestris*. Anim. Behav. 1999;58(4):743–749. doi:
649 10.1006/anbe.1999.1196.
650 17. Sauter A, Brown MJ, Baer B, Schmid-Hempel P. Males of social insects can prevent
651 queens from multiple mating. Proc. R. Soc. Ser. B-Biol. 2001;268(1475):1449-1454. doi:
652 10.1098/rspb.2001.1680.

18. Peretti AV. Functional morphology of spermatophores and female genitalia in bothriurid scorpions: genital courtship, coercion and other possible mechanisms. J. Zool. 2003;261(2): 135 153. doi: 10.1017/S095283690300400X.

19. Althaus S, Jacob A, Graber W, Hofer D, Nentwig W, Kropf C. A double role of sperm in scorpions: The mating plug of *Euscorpius italicus* (Scorpiones: Euscorpiidae) consists of sperm. J. Morphol. 2010;271(4):383-393. doi: 10.1002/jmor.10804.

20. Avila FW, Sirot LK, LaFlamme BA, Rubinstein CD, Wolfner MF. Insect seminal fluid proteins: identification and function. Annu. Rev. Entomol. 2011;56:21-40. doi: 10.1146/annurev-ento-120709-144823.

21. Uhl G, Kunz K, Vöcking O, Lipke E. A spider mating plug: origin and constraints of production. Biol. J. Linn. Soc. 2014;113(2):345-354. doi: 10.1111/bij.12359.

22. Fromhage L, Schneider JM. Emasculation to plug up females: the significance of pedipalp damage in *Nephila fenestrata*. Behav. Ecol. 2006;17(3):353-357. doi: 10.1016/j.zool.2007.07.003.

23. Uhl G, Nessler SH, Schneider J. Copulatory mechanism in a sexually cannibalistic spider with genital mutilation (Araneae: Araneidae: *Argiope bruennichi*). Zoology. 2007;110(5): 398-408. doi: 10.1016/j.zool.2007.07.003.

24. Kralj-Fišer S, Gregorič M, Zhang S, Li D, Kuntner M. Eunuchs are better fighters. Anim. Behav. 2011;81(5):933-939. doi: 10.1016/j.anbehav.2011.02.010.

25. Parker GA. Sperm competition and the evolution of animal mating systems. In: Smith RL, editor. Sperm Competition and the Evolution of Animal Mating Systems. New York: Academic Press; 1984. pp. 1-60.

26. Arnqvist G, Rowe L. Sexual conflict. Princeton: University Press Princeton; 2005.

676 27. Snow LS, Andrade MC. Multiple sperm storage organs facilitate female control of
677 paternity. Proc. R. Soc. London B. 2005;272(1568):1139-1144. doi:
678 10.1098/rspb.2005.3088.
679 28. Schneider JM. Sexual cannibalism as a manifestation of sexual conflict. C. S. H. Perspect.
680 Biol. 2014;6:a017731. doi: 10.1101/cshperspect.a017731.
681 29. Andrade MC, MacLeod EC. Potential for CFC in black widows (genus *Latrodectus*):
682 mechanisms and social context. In: Peretti AV, Aisenberg A, editors. Cryptic Female
683 Choice in Arthropods. New York: Springer International Publishing; 2015. pp. 27-53.
684 30. Aisenberg A, Barrantes G. Sexual behavior, cannibalism, and mating plugs as sticky traps
685 in the orb weaver spider *Leucauge argyra* (Tetragnathidae). Naturwissenschaften. 2011;
686 98(7):605-613. doi: 10.1007/s00114-011-0807-y.
687 31. Herberstein ME, Wignall AE, Nessler SH, Harmer AMT, Schneider JM. How effective and
688 persistent are fragments of male genitalia as mating plugs?. Behav. Ecol. 2012;23(5):1140-
689 1145. doi: 10.1093/beheco/ars088.
690 32. Giglioli MEC, Mason GF. The mating plug in anopheline mosquitoes. Physiol.
691 Entomol. 1966;41(7-9):123-129. doi: 10.1111/j.1365-3032.1966.tb00355.x.
692 33. Hartmann R, Loher W. Control of sexual behaviour pattern 'secondary defence' in the
693 female grass-hopper, *Chorthippus curtipennis*. J. Insect Physiol. 1974;20(9):17131721-
694 17191728. doi: 10.1016/0022-1910(74)90201-7.
695 34. Diesel R. Structure and function of the reproductive system of the symbiotic spider crab
696 *Inachus phalangium* (Decapoda: Majidae): observations on sperm transfer, sperm storage,
697 and spawning. J. Crustacean Biol. 1989;9(2):266-277. doi: 10.1163/193724089X00089.

35. Lorch PD, Wilkinson GS, Reillo, PR. Copulation duration and sperm precedence in the stalk-eyed fly *Cyrtodiopsis whitei* (Diptera: Diopsidae). Behav. Ecol. Sociobiol. 1993;32(5):303-311. doi: 10.1007/BF00183785.

36. Knoflach B, van Harten A. *Tidarren argo* sp. nov. (Araneae: Theridiidae) and its exceptional copulatory behaviour: emasculation, male palpal organ as a mating plug and sexual cannibalism. J. Zool. 2001;254(4):449-459. doi: 10.1017/S0952836901000954.

37. Lee KM, Daubnerova I, Isaac RE, Zhang C, Choi S, Chung J, et al. A neuronal pathway that controls sperm ejection and storage in female *Drosophila*. Curr. Biol. 2015;25(6):790-797.doi: 10.2307/1382026.

38. Eberhard WG. Female control: sexual selection by cryptic female choice. Princeton: University Press; 1996.

39. Peretti AV, Aisenberg A. Cryptic female choice in arthropods. New York: Springer International Publishing; 2015.

40. Aisenberg A, Eberhard WG. Female cooperation in plug formation in a spider: effects of male copulatory courtship. Behav. Ecol. 2009;20(6):1236-1241. doi: 10.1093/beheco/arp117.

41. Sentenská L, Pekár S, Lipke E, Michalik P, Uhl G. Female control of mate plugging in a female-cannibalistic spider (*Micaria sociabilis*). BMC Evol. Biol. 2015;15(1):18. doi: 10.1186/s12862-014-0278-9.

42. Koprowski JL. Removal of copulatory plugs by female tree squirrels. J. Mammal. 1992;73(3):572-576. doi: 10.2307/1382026.

43. Subramoniam T. Spermatophores and sperm transfer in marine crustaceans. Adv. Mar. Biol. 1993;29:129-214. doi: 10.1016/S0065-2881(08)60131-5.

721 44. Timmermeyer N, Gerlach T, Guempel C, Knoche J, Pfann JF, Schliessmann D, et al. The
722 function of copulatory plugs in *Caenorhabditis remanei*: hints for female benefits. Front.
723 Zool. 2010;7(1):28. doi: 10.1186/1742-9994-7-28.
724 45. Dean MD. Genetic disruption of the copulatory plug in mice leads to severely reduced
725 fertility. Plos genetics. 2013;9(1):e1003185. doi: 10.1371/journal.pgen.1003185.
726 46. Kuntner M, Gregorič M, Zhang S, Kralj-Fišer S, Li D. Mating plugs in polyandrous giants:
727 which sex produces them, when, how and why?. Plos One. 2012;7(7):e40939. doi:
728 10.1371/journal.pone.0040939.
729 47. Alonso-Pimentel H, Tolbert LP, Heed WB, Ultrastructural examination of the insemination
730 reaction in *Drosophila*. Cell Tissue Res. 1994;275(3):467-479. doi: 10.1007/BF00318816.
731 48. Dean MD, Findlay GD, Hoopmann MR, Wu CC, MacCoss MJ, Swanson WJ, et al.
732 Identification of ejaculated proteins in the house mouse (*Mus domesticus*) via isotopic
733 labeling. BMC Genomics. 2011;12(1):306. doi: 10.1186/1471-2164-12-306.
734 49. Markow TA, Ankney PF. Insemination reaction in *Drosophila*: found in species whose
735 males contribute material to oocytes before fertilization. Evolution. 1988;42(5):1097-1101.
736 doi: 10.1111/j.1558-5646.1988.tb02529.x.
737 50. Polak M, Wolf LL, Starmer WT, Barker JSF. Function of the mating plug in *Drosophila*
738 *hibisci* Bock. Behav. Ecol. Sociobiol. 2001;49(2-3):196-205. doi: 10.1007/s002650000281.
739 51. Lawniczak MK, Barnes AI, Linklater JR, Boone JM, Wigby S, Chapman T. Mating and
740 immunity in invertebrates. Trends Ecol. Evol. 2007;22(1):48-55. doi:
741 10.1016/j.tree.2006.09.012.
742 52. Shoemaker KL, Parsons NM, Adamo SA. Mating enhances parasite resistance in the
743 cricket *Gryllus texensis*. Anim. Behav. 2006;71(2):371-380. doi:
744 10.1016/j.anbehav.2005.05.007.

745 53. McKean KA, Nunney L. Increased sexual activity reduces male immune function in
746 *Drosophila melanogaster*. P. Natl. Acad. Sci. 2001;98(14):7904-7909. doi:
747 10.1073/pnas.131216398.
748 54. Rolff J, Siva-Jothy MT. Copulation corrupts immunity: a mechanism for a cost of mating in
749 insects. P. Natl. Acad. Sci. 2002;99(15):9916-9918. doi: 10.1073/pnas.152271999.
750 55. Fedorka KM, Zuk M. Mousseau TA. Immune suppression and the cost of reproduction in
751 the ground cricket, *Allonemobius socius*. Evolution. 2004;58(11):2478-2485. doi:
752 10.1111/j.0014-3820.2004.tb00877.x.
753 56. Tzou P, Ohresser S, Ferrandon D, Capovilla M, Reichhart JM, Lemaitre B, et al. Tissue-
754 specific inducible expression of antimicrobial peptide genes in *Drosophila* surface
755 epithelia. Immunity. 2000;13(5):737-748. doi: 10.1016/S1074-7613(00)00072-8.
756 57. Peng J, Zipperlen P, Kubli E. *Drosophila* sex-peptide stimulates female innate immune
757 system after mating via the Toll and Imd pathways. Curr. Biol. 2005;15(18):1690-1694.
758 doi: 10.1016/j.cub.2005.08.048.
759 58. Lawniczak MK, Begun DJ. A genome-wide analysis of courting and mating responses in
760 *Drosophila melanogaster* females. Genome. 2004;47(5):900-910. doi: 10.1139/g04-050.
761 59. Gillespie JP, Kanost MR, Trenczek T. Biological mediators of insect immunity. Annu. Rev.
762 Entomol. 1997;42(1):611-643. doi: 10.1146/annurev.ento.42.1.611.
763 60. Moret Y, Siva-Jothy MT. Adaptive innate immunity? Responsive-mode prophylaxis in the
764 mealworm beetle, *Tenebrio molitor*. Proc. R. Soc. Ser. B-Biol. 2003;270(1532):2475-2480.
765 doi: 10.1098/rspb.2003.2511.
766 61. Schmid-Hempel P, Ebert D. On the evolutionary ecology of specific immune
767 defence. Trends Ecol. Evol. 2003;181(1):27-32. doi: 10.1016/S0169-5347(02)00013-7.

768 62. Sadd BM, Schmid-Hempel P. Insect immunity shows specificity in protection upon
769 secondary pathogen exposure. Curr. Biol. 2006;16(12):1206-1210. doi:
770 10.1016/j.cub.2006.04.047.
771 63. Schulenburg H, Boehnisch C, Michiels NK. How do invertebrates generate a highly
772 specific innate immune response?. Mol. Immunol. 2007;44(13):3338-3344. doi:
773 10.1016/j.molimm.2007.02.019.
774 64. Ratner S, Vinson SB. Phagocytosis and encapsulation: cellular immune responses in
775 arthropoda. Am. Zool. 1983;23(1):185-194. doi: 10.1093/icb/23.1.185.
776 65. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. Insect Biochem.
777 Molec. 2002;32(10):1295-1309. doi: 10.1016/S0965-1748(02)00092-9.
778 66. Cerenius L, Söderhäll K. The prophenoloxidase-activating system in invertebrates.
779 Immunol. Rev. 2004:198(1):116-126. doi:10.1111/j.0105-2896.2004.00116.x.
780 67. Schmit AR, Ratcliffe NA. The encapsulation of Araldite implants and recognition of
781 foreignness in *Clitumnus extradentatus*. J. Insect Physiol. 1978;24(6-7):511–521. doi:
782 10.1016/0022-1910(78)90052-5.
783 68. Dubovskiy IM, Kryukova NA, Glupov VV, Ratcliffe NA. Encapsulation and nodulation in
784 insects. Invert. Surviv. J. 2016;13.
785 69. Patil AE, Shah UH. Types of hemocytes in scorpion *Mesobuthus tamulus*
786 *tamulus*. Bioscan. 2011;6(40):597-599.
787 70. Kuhn-Nentwig L, Kopp LS, Nentwig W, Haenni B, Streitberger K, Schürch S, et al.
788 Functional differentiation of spider hemocytes by light and transmission electron
789 microscopy, and MALDI-MS-imaging. Dev. Comp. Immunol. 2014;43(1):59-67. doi:
790 10.1016/j.dci.2013.10.008.

71. Cociancich S, Goyffon M, Bontems F, Bulet P, Bouet F, Menez A, et al. Purification and characterization of a scorpion defensin, a 4kDa antibacterial peptide presenting structural similarities with insect defensins and scorpion toxins. Biochem. Bioph. Res. Co. 1993;194(1):17-22. doi: 10.1006/bbrc.1993.1778.

72. Silva PI, Daffre S, Bulet P. Isolation and characterization of gomesin, an 18-residue cysteine-rich defense peptide from the spider *Acanthoscurria gomesiana* hemocytes with sequence similarities to horseshoe crab antimicrobial peptides of the tachyplesin family. J. Biol. Chem. 2000;275(43):33464-33470. doi: 10.1074/jbc.M001491200.

73. De La Vega RR, Garcia BI, D'Ambrosio C, Diego-Garcia E. Scaloni A, Possani LD. Antimicrobial peptide induction in the hemolymph of the Mexican scorpion *Centruroides limpidus limpidus* in response to septic injury. Cell. Mol. Life Sci. 2004;61(12):1507-1519. doi: 10.1007/s00018-004-4096-x.

74. Hamilton WD, Zuk M. Heritable true fitness and bright birds: a role for parasites?. Science. 1982;218(4570):384-387. doi: 10.1126/science.7123238.

75. Folstad I, Karter AJ. Parasites, bright males, and the immunocompetence handicap. Am. Nat. 1992;139(3):603-622. doi: 10.1086/285346.

76. Polis GA, Sissom WD. Life history. In: Polis GA, editor. The Biology of Scorpions. Stanford: University Press; 1990. pp. 161–223.

77. Weygoldt P. Arthropoda–Chelicerata: sperm transfer. In: Adiyodi KG, Adiyodi, RG, editors. Reproductive Biology of Invertebrates, Vol. IV, Part B. New Dehli: Oxford and IBH Publishing Co.; 1990; pp. 77–119.

78. Proctor HC. Indirect sperm transfer in arthropods: behavioral and evolutionary trends. Annu. Rev. Entomol. 1998;43(1):153-174. doi: 10.1146/annurev.ento.43.1.153.

814 79. Farley RD. Structure, reproduction and development. In: Brownell P, Polis GA, editors.
815 Scorpion biology and research. Oxford: University Press; 2001. pp. 13–78.
816 80. Varela JC. Gestacion, nacimiento y eclosion de *Bothriurus bonariensis* var *bonariensis*
817 (Koch, 1842)(Bothriuridae, Scorpiones). Rev. Fac. Humanit. Cien. Montevideo.
818 1961;19:225-244.
819 81. Francke OF. Parturition in scorpions (Arachnida, Scorpiones). Rev. Arachnol. 1982;4:27-
820 37.
821 82. Castelvetri S, Peretti AV. Receptividad sexual y presencia de tapón genital en hembras de
822 *Bothriurus bonariensis* (C.L. Koch)(Scorpiones: Bothriuridae). Revue Arachnol.
823 1999;13,:15–23.
824 83. Polis GA. The biology of scorpions. Stanford: University Press; 1990.
825 84. Carrera P. Estudio comparado de los patrones de selección sexual en escorpiones y ácaros
826 acuáticos: evaluación de hipótesis de conflicto sexual y cooperación intersexual. In: Ph.D.
827 Thesis, Universidad Nacional de Córdoba, Córdoba. 2008.
828 85. Peretti AV, Battán-Horenstein M. Comparative analysis of the male reproductive system in
829 Bothriuridae scorpions: structures associated with the paraxial organs and the presence of
830 sperm packages (Chelicerata, Scorpiones). Zool. Anz. 2003;242(1):21-31. doi:
831 10.1078/0044-5231-00085.
832 86. Ojanguren-Affilastro AA. Estudio monográfico de los escorpiones de la República
833 Argentina. Rev. Iber. Arachnol. 2005;11:75-247. ISSN 1576-9518.
834 87. Aisenberg A, Peretti AV. Sexual dimorphism in immune response, fat reserves and muscle
835 mass in a sex role reversed spider. Zoology. 2011;114(5):272-275. doi:
836 10.1016/j.zool.2011.05.003.

837 88. Calbacho-Rosa L, Moreno-García MA, Lanz-Mendoza H. Peretti AV, Córdoba-Aguilar A.
838 Reproductive activities impair immunocompetence in *Physocyclus dugesi* (Araneae:
839 Pholcidae). J. Arachnol. 2012;40(1):18-22. doi: 10.1636/Hi11-13.1.
840 89. González M, Costa FG, Peretti AV. Funnel-web construction and estimated immune costs
841 in *Aglaoctenus lagotis* (Araneae: Lycosidae). J. Arachnol. 2015;43(2):158-167. doi:
842 10.1636/M14-65.
843 90. Gilbert R, Karp RD, Uetz GW. Effects of juvenile infection on adult immunity and
844 secondary sexual characters in a wolf spider. Behav. Ecol. 2016. 2016;27(3):946-954. doi:
845 10.1093/beheco/arv241.
846 91. Maury EA. Escorpiofauna patagónica. II. *Urophonius granulatus* Pocock 1898
847 (Bothriuridae). Physis Sec. C. 1979;38:57-68.
848 92. Peretti AV. Estudio de la biología reproductiva en escorpiones argentinos (Arachnida,
849 Scorpiones): un enfoque etológico. Ph.D. Thesis, Universidad Nacional de Córdoba.
850 Córdoba. 1993.
851 93. Ojanguren-Affilastro AA, Ochoa JA, Mattoni CI, Prendini L. Systematic Revision of the
852 granulatus Group of *Urophonius* (Scorpiones, Bothriuridae), with Description of a New
853 Species from Central Chile. Am. Mus. Nov. 2010;(3695):1-40. doi: 10.1206/3695.2.
854 94. Ojanguren-Affilastro AA, Pizarro-Araya J, Prendini L. New data on Chilean *Urophonius*
855 Pocock, 1893 (Scorpiones, Bothriuridae), with description of a new species. Am. Mus.
856 Nov. 2011;(3725):1-44. doi: 10.1206/3725.2.
857 95. Simmons LW, Zuk M, Rotenberry JT. Immune function reflected in calling song
858 characteristics in a natural population of the cricket *Teleogryllus commodus*. Anim. Behav.
859 2005;69(6):1235-1241. doi: 10.1016/j.anbehav.2004.09.011.

860 96. Chemnitz J, Bagrii N, Ayasse M, Steiger S. Variation in sex pheromone emission does not
861 reflect immunocompetence but affects attractiveness of male burying beetles—a
862 combination of laboratory and field experiments. Sci. Nat. 2017;104(7-8):53. doi:
863 10.1007/s00114-017-1473-5.
864 97. Rantala MJ, Kortet R. Courtship song and immune function in the field cricket *Gryllus*
865 *bimaculatus*. Biol. J. Linn. Soc. 2003;79(3):503-510. doi: 10.1046/j.1095-
866 8312.2003.00202.x.
867 98. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image
868 analysis. Nat. Methods. 2012;9(7):671-675.
869 99. Ryder JJ. Male calling song provides a reliable signal of immune function in a cricket. P.
870 Roy. Soc. Lond. B. Bio. 2000;267(1449):1171-1175. doi: 10.1098/rspb.2000.1125.
871 100. Snow LS, Andrade MC. Pattern of sperm transfer in redback spiders: implications for
872 sperm competition and male sacrifice. Behav. Ecol. 2004;15(5):785-792. doi:
873 10.1093/beheco/arh080.
874 101. Bastidas O. Cell counting with neubauer chamber, basic hemocytometer usage. Celeromics,
875 Tech. Rep. 2013.
876 102. Ravindranath MH. The hemocytes of a scorpion *Palamnaeus swammerdami*. J.
877 Morphol. 1974;144(1):1-9. doi: 10.1002/jmor.1051440102.
878 103. Fournier DA, Skaug HJ, Ancheta J, Ianelli J, Magnusson A, Maunder MN, et al. AD
879 Model Builder: using automatic differentiation for statistical inference of highly
880 parameterized complex nonlinear models. Optim. Method. Soft. 2012;27(2):233-249. doi:
881 10.1080/10556788.2011.597854.
882 104. Bates D, Maechler M, Bolker B, Walker S. lme4: Linear mixed-effects models using Eigen
883 and S4. R package version. 2014;1(7):1-23- http://CRAN.R-project.org/package=lme4.

884 105. Lenth RV. Least-squares means: the R package lsmeans. J. Stat. Softw. 2016;69(1):1-33.
885 doi: 10.18637/jss.v069.i01.
886 106. R Core Team. R: A language and environment for statistical computing. R Foundation for
887 Statistical Computing, Vienna, Austria. 2017. URL https://www.R-project.org/.
888 107. Jennions MD, Petrie M. Why do females mate multiply? A review of the genetic
889 benefits. Biol. Rev. 2000;75(1):21-64. doi: 10.1111/j.1469-185X.1999.tb00040.x.
890 108. Matsumoto K, Suzuki N. Effectiveness of the mating plug in *Atrophaneura alcinous*
891 (Lepidoptera: Papilionidae). Behav. Ecol. Sociobiol. 1992;30(3-4):157-163. doi:
892 10.1007/BF00166698.
893 109. Masumoto T. The effect of the copulatory plug in the funnel-web spider, *Agelena limbata*
894 (Araneae: Agelenidae). J. Arachnol. 1993;21(1):55-59.
895 110. Kunz K, Witthuhn M, Uhl G. Do the size and age of mating plugs alter their efficacy in
896 protecting paternity?. Behav. Ecol. Sociobiol. 2014;68(8):1321-1328. doi: 10.1007/s00265-
897 014-1742-7.
898 111. Gagen SJ, Ratcliffe NA. Studies on the in vivo cellular reactions and fate of injected
899 bacteria in *Galleria mellonella* and *Pieris brassicae* larvae. J. Invertebrate Pathol.
900 1976;28(1):17-24. doi: 10.1016/0022-2011(76)90066-5.
901 112. Chain BM, Anderson RS. Selective depletion of the plasmatocytes in *Galleria mellonella*
902 following injection of bacteria. J. Insect Physiol. 1982;28(4):377-384. doi: 10.1016/0022-
903 1910(82)90051-8.
904 113. Söderhäll K, Smith VJ. Separation of the haemocyte populations of *Carcinus maenas* and
905 other marine decapods, and prophenoloxidase distribution. Dev. Comp.
906 Immunol. 1983;7(2):229-239. doi: 10.1016/0145-305X(83)90004-6.

907 114. Geng C, Dunn PE. Plasmatocyte depletion in larvae of *Manduca sexta* following injection
908 of bacteria. Dev. Comp. Immunol. 1989;13(1):17-23. doi: 10.1016/0145-305X(89)90012-8.
909 115. Lorenzon S, De Guarrini S, Smith VJ, Ferrero EA. Effects of LPS injection on circulating
910 haemocytes in crustaceans in vivo. Fish shellfish Immun. 1999;9(1):31-50. doi:
911 10.1006/fsim.1998.0168.
912 116. Lorenzon S, Pasqual P, Ferrero EA. Different bacterial lipopolysaccharides as toxicants and
913 stressors in the shrimp *Palaemon elegans*. Fish Shellfish Immun. 2002;13(1):27-45. doi:
914 10.1006/fsim.2001.0379.
915 117. Lv S, Xu J, Zhao J, Yin N, Lu B, Li S, et al. Classification and phagocytosis of circulating
916 haemocytes in Chinese mitten crab (*Eriocheir sinensis*) and the effect of extrinsic
917 stimulation on circulating haemocytes in vivo. Fish shellfish Immun. 2014;39(2):415-422.
918 doi: 10.1016/j.fsi.2014.05.036.
919 118. Moreno-García M, Córdoba-Aguilar A, Condé R, Lanz-Mendoza H. Current immunity
920 markers in insect ecological immunology: assumed trade-offs and methodological
921 issues. B. Entomol. Res. 2013;103(2):127-139. doi: 10.1017/S000748531200048X.
922 119. Quayle AJ. The innate and early immune response to pathogen challenge in the female
923 genital tract and the pivotal role of epithelial cells. J. Reprod. Immunol. 2002;57(1-2):61-
924 79. doi: 10.1016/S0165-0378(02)00019-0.
925 120. Wira CR, Fahey JV, Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in
926 female genital tract: cellular responses and interactions. Immunol. Rev. 2005;206(1):306-
927 335. doi: 10.1111/j.0105-2896.2005.00287.x.
928 121. Schuberth HJ, Taylor U, Zerbe H, Waberski D, Hunter R, Rath D. Immunological
929 responses to semen in the female genital tract. Theriogenology. 2008;70(8):1174-1181. doi:
930 10.1016/j.theriogenology.2008.07.020.

122. Chapman T. Seminal fluid-mediated fitness traits in *Drosophila*. Heredity. 2001;87(5):511-521. doi: 10.1046/j.1365-2540.2001.00961.x.

123. Patil A, Shah U. Hemocytes in scorpion–*Heterometrus xanthopus*. Bioscan. 2012;7(1):139-141.

124. Patil A, Shah U. Ultrastructural studies of hemocytes in scorpion Heterometrus xanthopus. Bioscan. 2012;7:491-493.

125. Patil A. Shah U. Hemocytes in scorpion–*Heterometrus phipsoni*. Bioscan. 2013;8:33-35.

126. Gonjari GR, Shah UH. Scorpion Prohemocytes: A Comparative Study. Indian Streams Res. J. 2013;3:7.

127. Gupta AP. Cellular elements in the hemolymph. Compr. Insect Physiol. Biochem Pharm. 1985;3:401-451.

128. Weygoldt P, Paulus HF. Untersuchungen zur Morphologie, Taxonomie und Phylogenie der Chelicerata I. Morphologische Untersuchungen. J. Zool. Syst. Evol. Rese. 1979;17:85-116. doi: 10.1111/j.1439-0469.1979.tb00694.x.

129. Shultz JW. A phylogenetic analysis of the arachnid orders based on morphological characters. Zool. J. Linn. Soc. 2007;150(2):221-265. doi: 10.1111/j.1096-3642.2007.00284.x.

130. Dunlop JA. Geological history and phylogeny of Chelicerata. Arthropod Struct. Dev. 2010;39(2-3):124-142. doi: 10.1016/j.asd.2010.01.003.

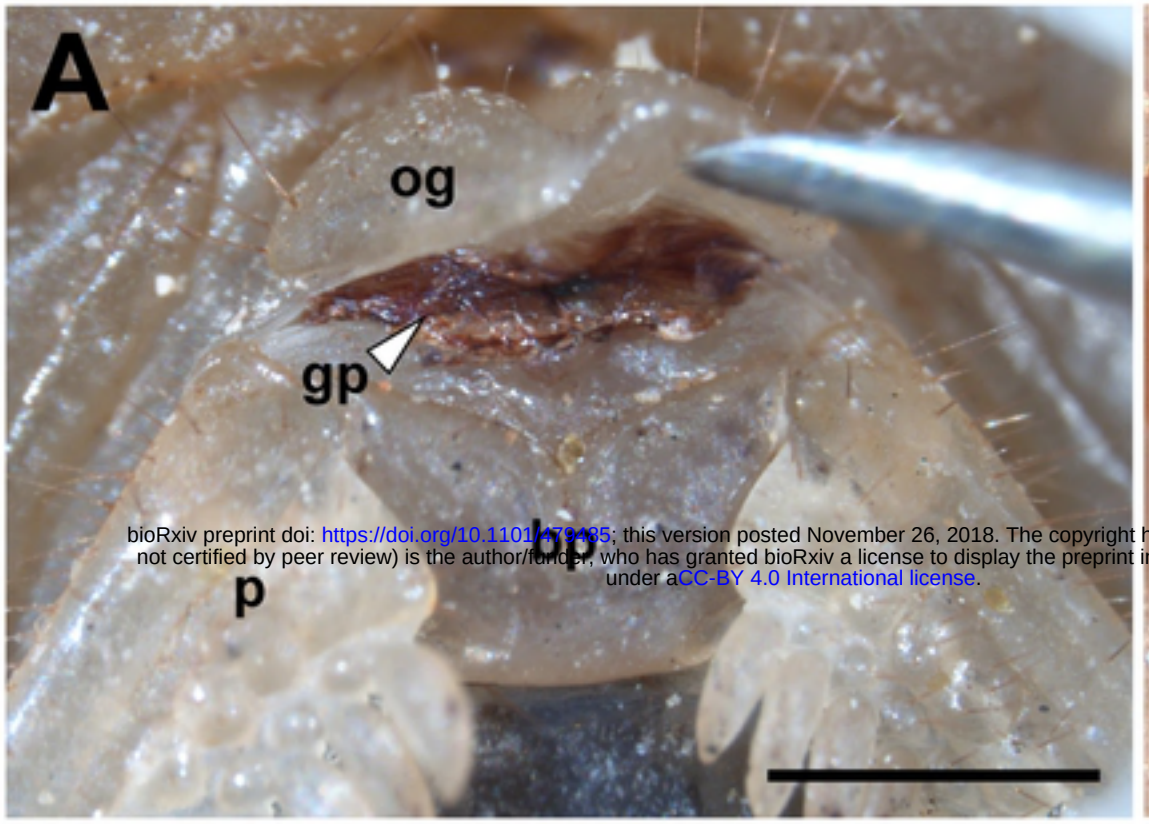
131. Sharma PP, Baker CM, Cosgrove JG, Johnson JE, Oberski JT, Raven RJ, et al. A revised dated phylogeny of scorpions: Phylogenomic support for ancient divergence of the temperate Gondwanan family Bothriuridae. Mol. Biol. Evol. 2018;122:37-45. doi: 10.1016/j.ympev.2018.01.003.

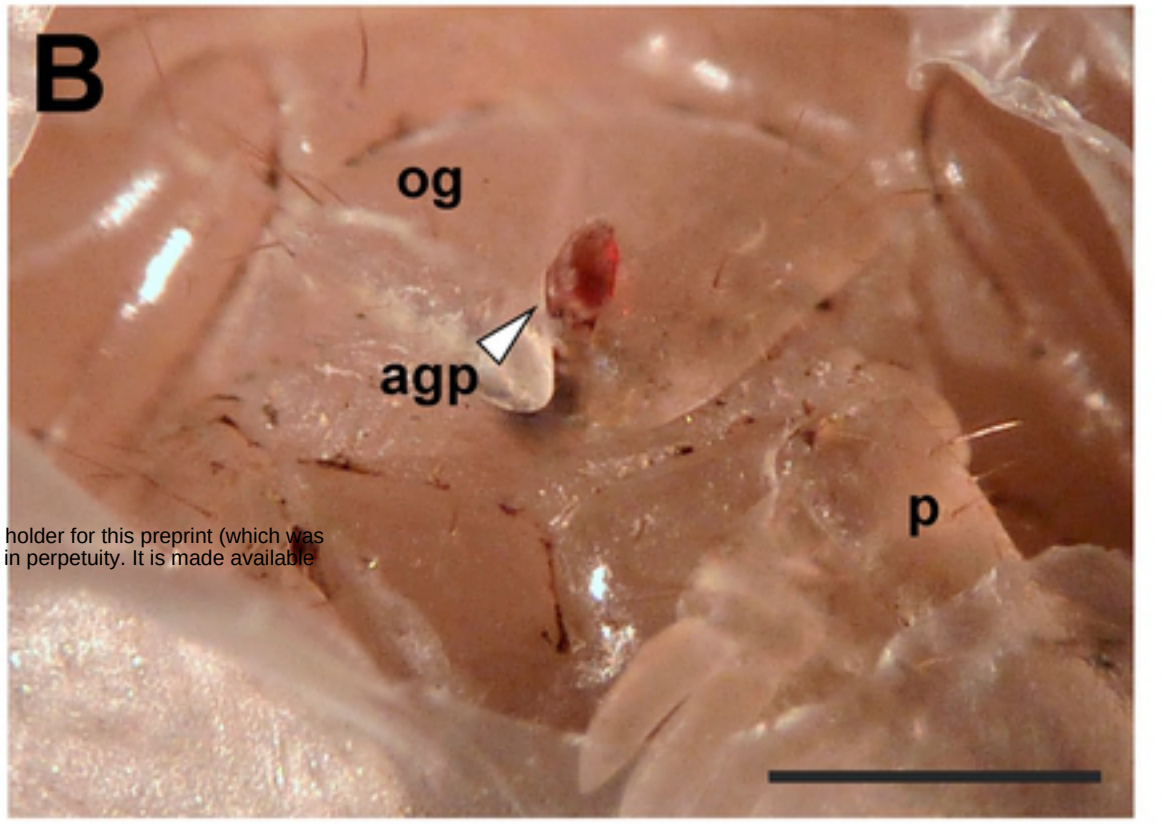
954 132. Sharma PP, Kaluziak ST, Pérez-Porro AR, González VL, Hormiga G, Wheeler WC, et al.
955 Phylogenomic interrogation of Arachnida reveals systemic conflicts in phylogenetic signal.
956 Mol. Biol. Evol. 2014;31(11):2963-2984. doi: 10.1093/molbev/msu235.
957 133. Yamashita M, Iwabuchi K. *Bombyx mori* prohemocyte division and differentiation in
958 individual microcultures. J. Insect Physiol. 2001;47(4-5):325-331. doi: 10.1016/S0022-
959 1910(00)00144-X.
960 134. Lebestky T, Chang T, Hartenstein V, Banerjee U. Specification of *Drosophila*
961 hematopoietic lineage by conserved transcription factors. Science. 2000;288(5463):146-
962 149. doi: 0.1126/science.288.5463.146.
963 135. Xylander WER. Hemocytes in Myriapoda (Arthropoda): a review. ISJ-Invert. Surviv. J.
964 2009;6(2):114-124.
965 136. King JG, Hillyer JF. Spatial and temporal in vivo analysis of circulating and sessile
966 immune cells in mosquitoes: hemocyte mitosis following infection. BMC Biol.
967 2013;11(1):55. doi: 10.1186/1741-7007-11-55.
968 137. Cueto JA, Rodriguez C, Vega IA, Castro-Vazquez A. Immune defenses of the invasive
969 apple snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae): phagocytic
970 hemocytes in the circulation and the kidney. Plos One. 2015;10(4):e0123964. doi:
971 10.1371/journal.pone.0123964.
972 138. Lee KA. Linking immune defenses and life history at the levels of the individual and the
973 species. Integ. Comp. Biol. 2006;46(6):1000-1015. doi: 10.1093/icb/icl049.
974 139. Oliver JD, Loy JD, Parikh G, Bartholomay L. Comparative analysis of hemocyte
975 phagocytosis between six species of arthropods as measured by flow cytometry. J.
976 Invertebr. Pathol. 2011;108(2):126-130. doi: 10.1016/j.jip.2011.07.004.

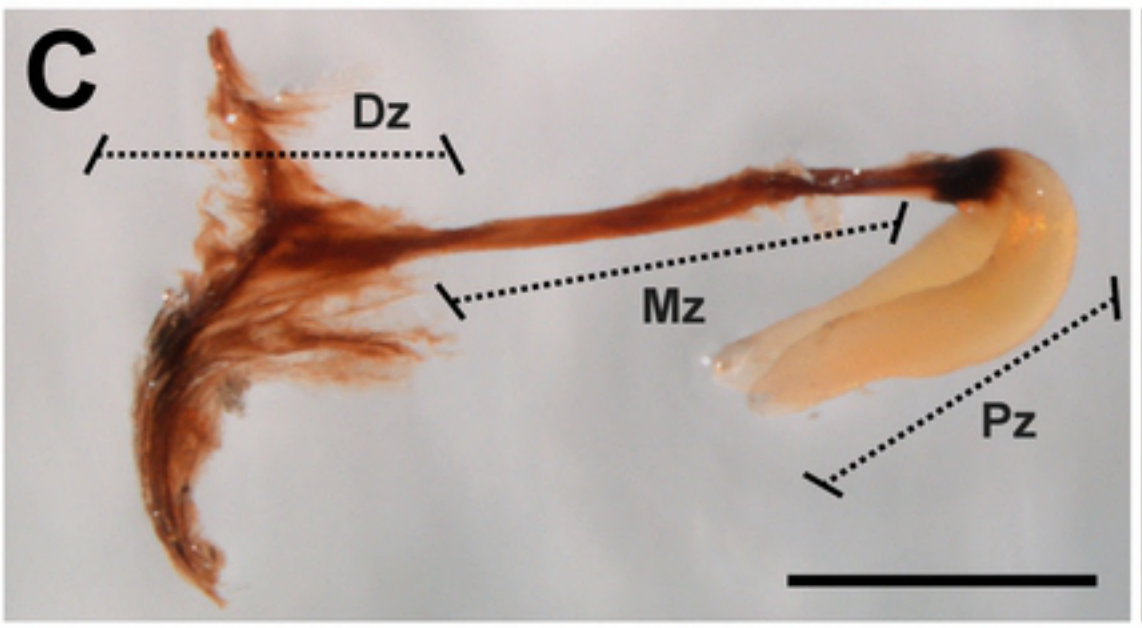
977 140. Strand MR, Pech LL. Immunological basis for compatibility in parasitoid-host
978 relationships. Ann. Rev. Entomol. 1995;40(1):31-56. doi:
979 10.1146/annurev.en.40.010195.000335.
980 141. Elrod-Erickson M, Mishra S, Schneider D. Interactions between the cellular and humoral
981 immune responses in *Drosophila*. Curr. Biol. 2000;10(13):781-784. doi: 10.1016/S0960-
982 9822(00)00569-8.
983 142. Moret Y, Schmid-Hempel P. Entomology: immune defence in bumble-bee
984 offspring. Nature. 2001;414(6863):506. doi: 10.1038/35107138.
985 143. Cotter SC, Kruuk LEB, Wilson K. Costs of resistance: genetic correlations and potential
986 trade-offs in an insect immune System. J. Evol. Biol. 2004;17(2):421-429. doi:
987 10.1046/j.1420-9101.2003.00655.x.
988 144. Schmid-Hempel P. Variation in immune defence as a question of evolutionary
989 ecology. Proc. R. Soc. Ser. B-Biol. 2003;270(1513):357-366. doi: 10.1098/rspb.2002.2265.
990 145. Sheldon BC, Verhulst S. Ecological immunology: costly parasite defences and trade-offs in
991 evolutionary ecology. Trends Ecol. Evol. 1996;11(8):317-321. doi: 10.1016/0169-
992 5347(96)10039-2.
993 146. Kraaijeveld AR, Limentani EC, Godfray HCJ. Basis of the trade–off between parasitoid
994 resistance and larval competitive ability in *Drosophila melanogaster*. Proc. R. Soc. Ser. B-
995 Biol. 2001;268(1464):259-261. doi: 10.1098/rspb.2000.1354.
996 147. Doums C, Moret Y, Benelli E, Schmid-Hempel P. Senescence of immune defence in
997 *Bombus* workers. Ecol. Entomol. 2002;27(2):138-144. doi: 10.1046/j.1365-
998 2311.2002.00388.x.
999 148. Kanost MR, Jiang H, Yu XQ. Innate immune responses of a lepidopteran insect, *Manduca*
1000 *sexta*. Immunol. Rev. 2004;198(1):97-105. doi: 10.1111/j.0105-2896.2004.0121.x.

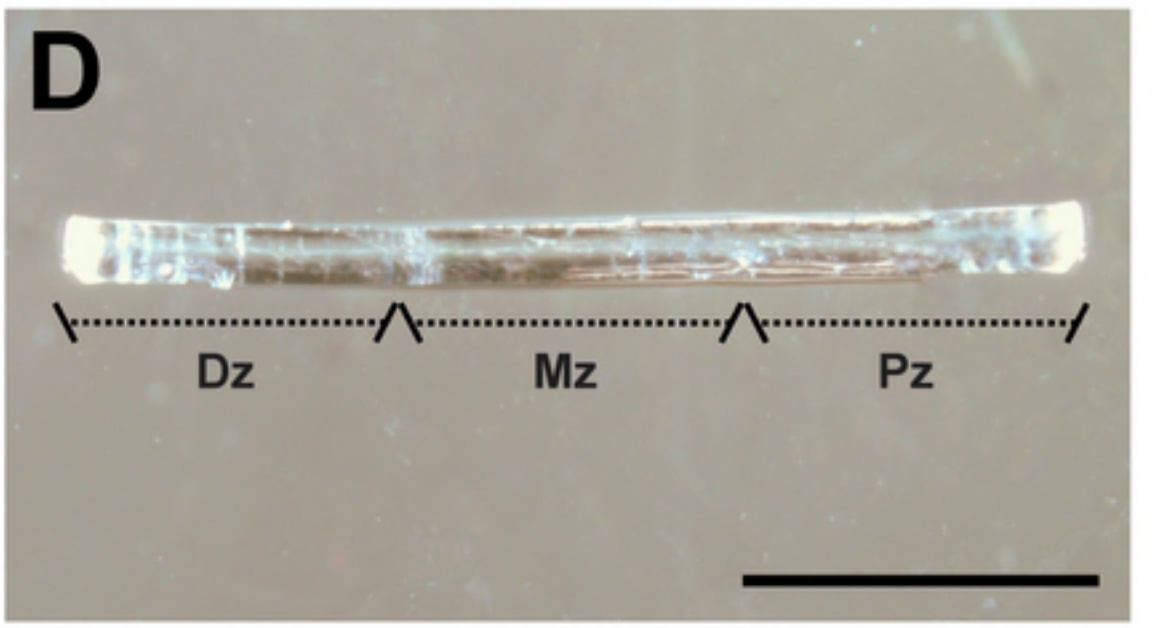
Supporting information

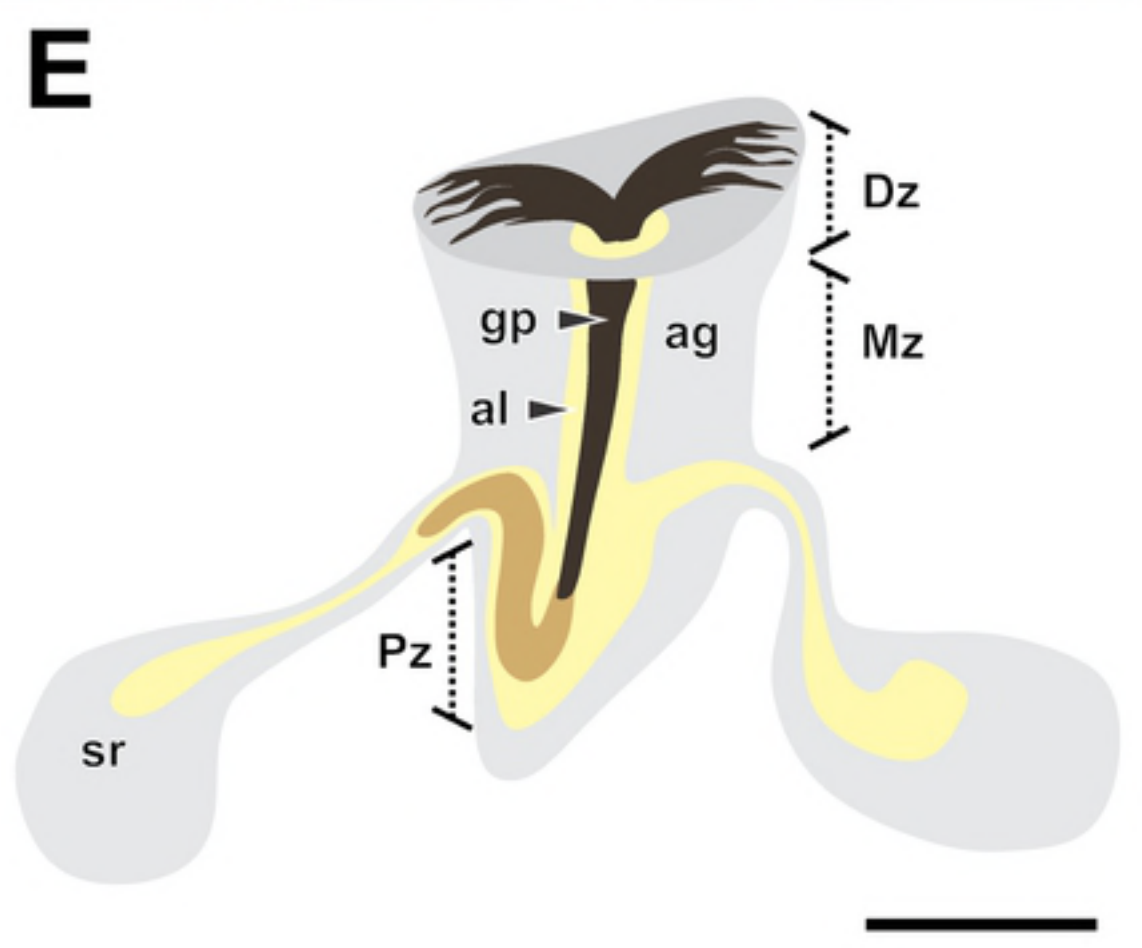
S1 Table. Mean values and standard deviations of different immunological parameters of *Urophonius brachycentrus*, *U. achalensis* and *Z. fuscus*. The total hemocyte load (THL) before and after implantation of the artificial genital plug, and the decrease in hemocyte concentration between both stages are presented. The melanotic (ME) and non-melanotic (NME) encapsulation response was measured in all three zones of artificial genital plugs. a, b, and c indicate the grouping and separation between stages and zones ($p < 0.05$) of the artificial genital plug. Capital letters (A,B) indicate the grouping and separation between species ($p < 0.05$).











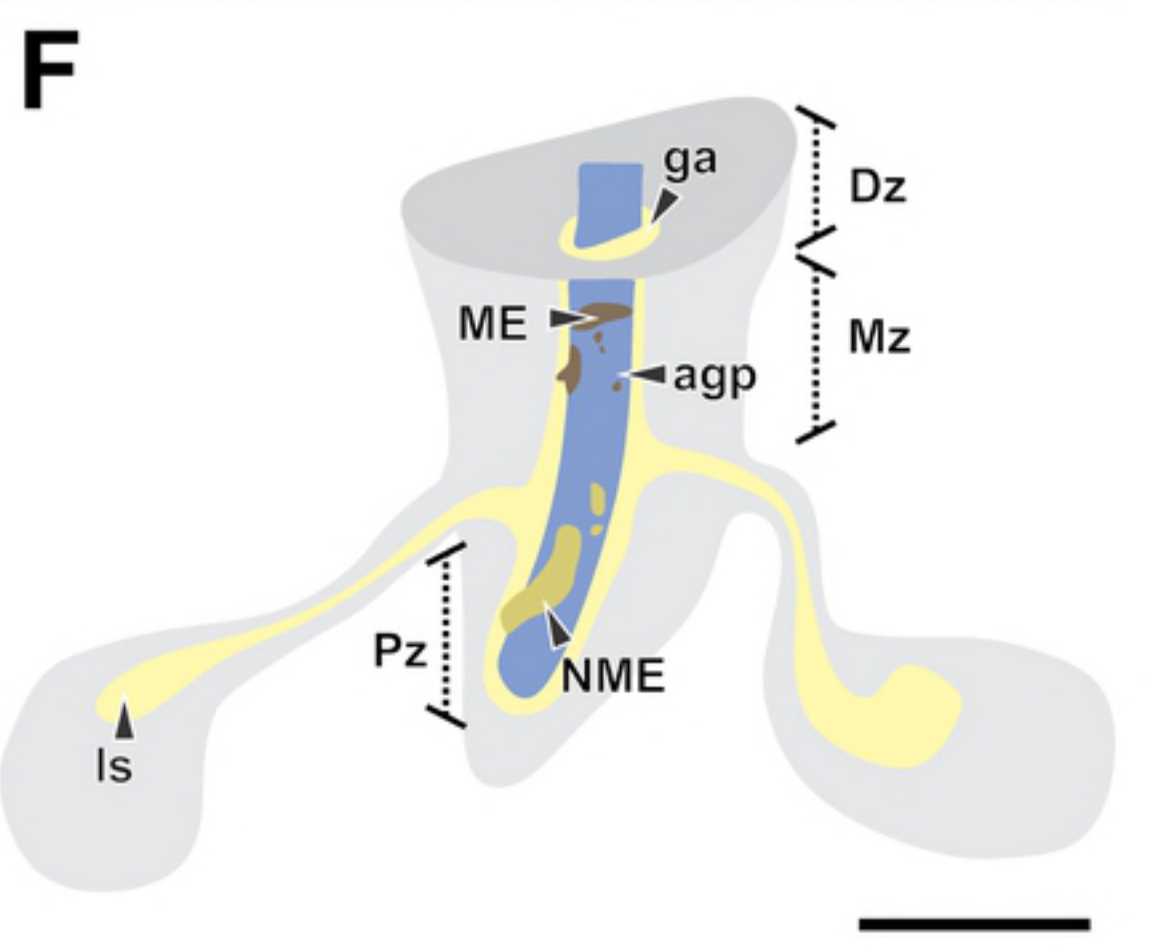


Fig 1

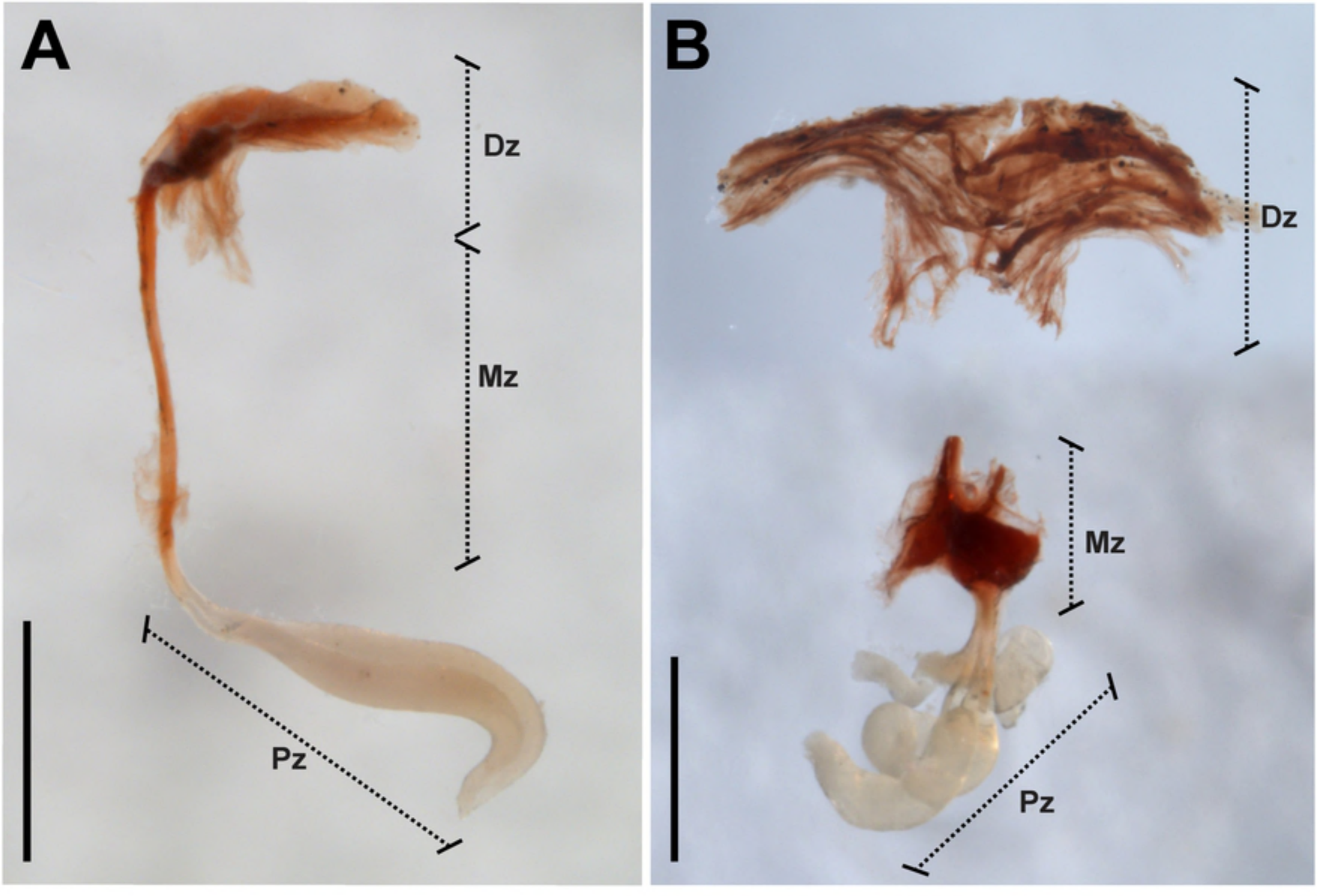
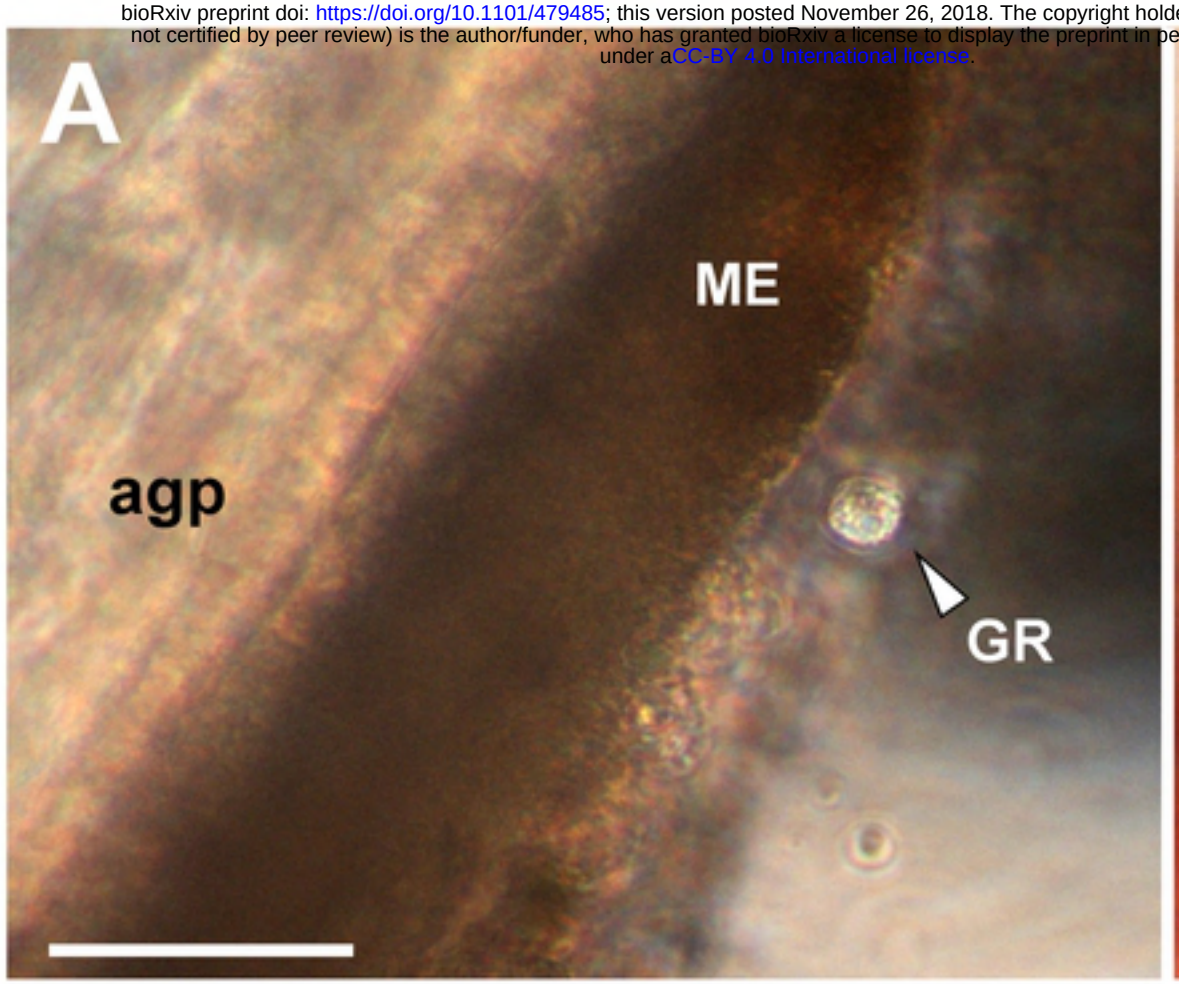
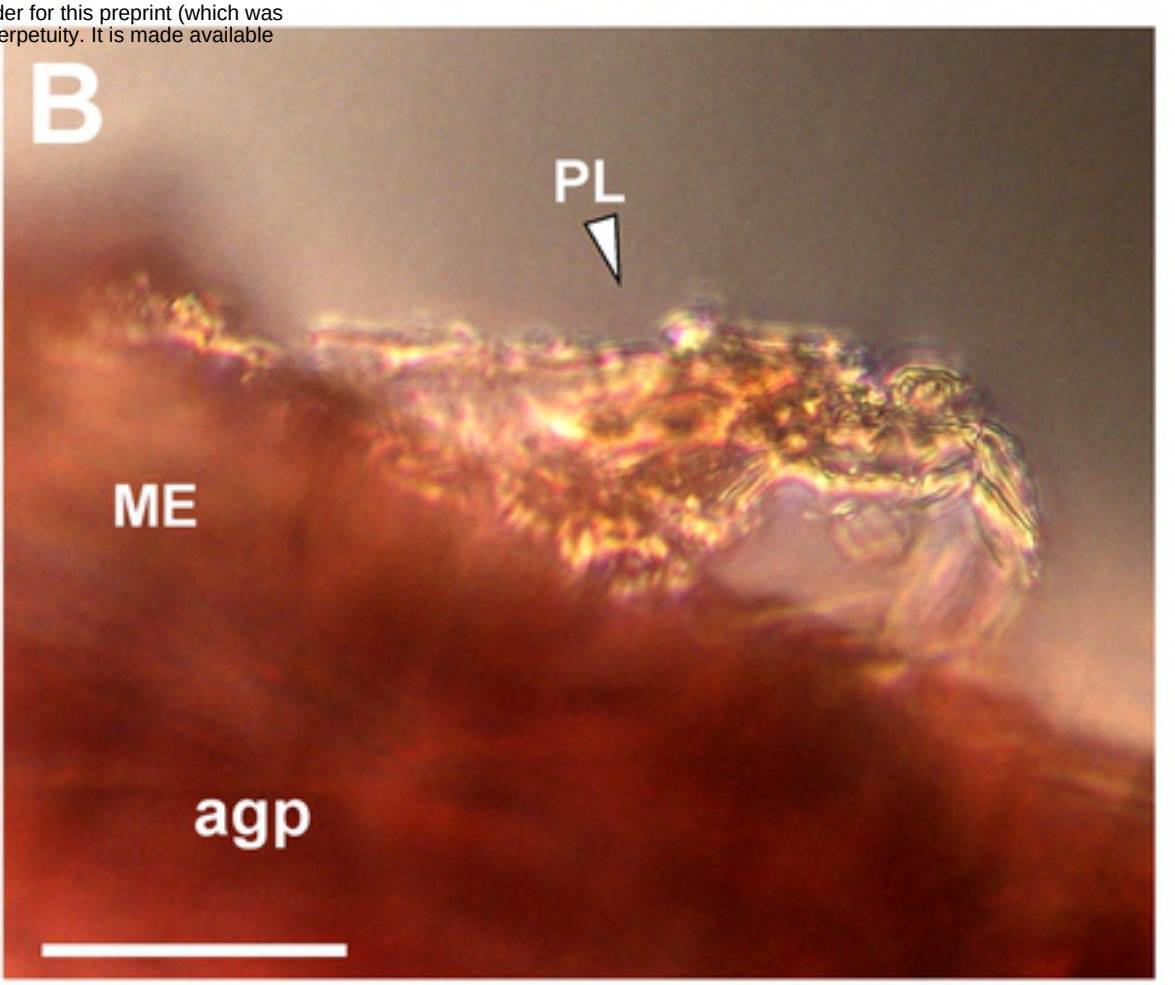
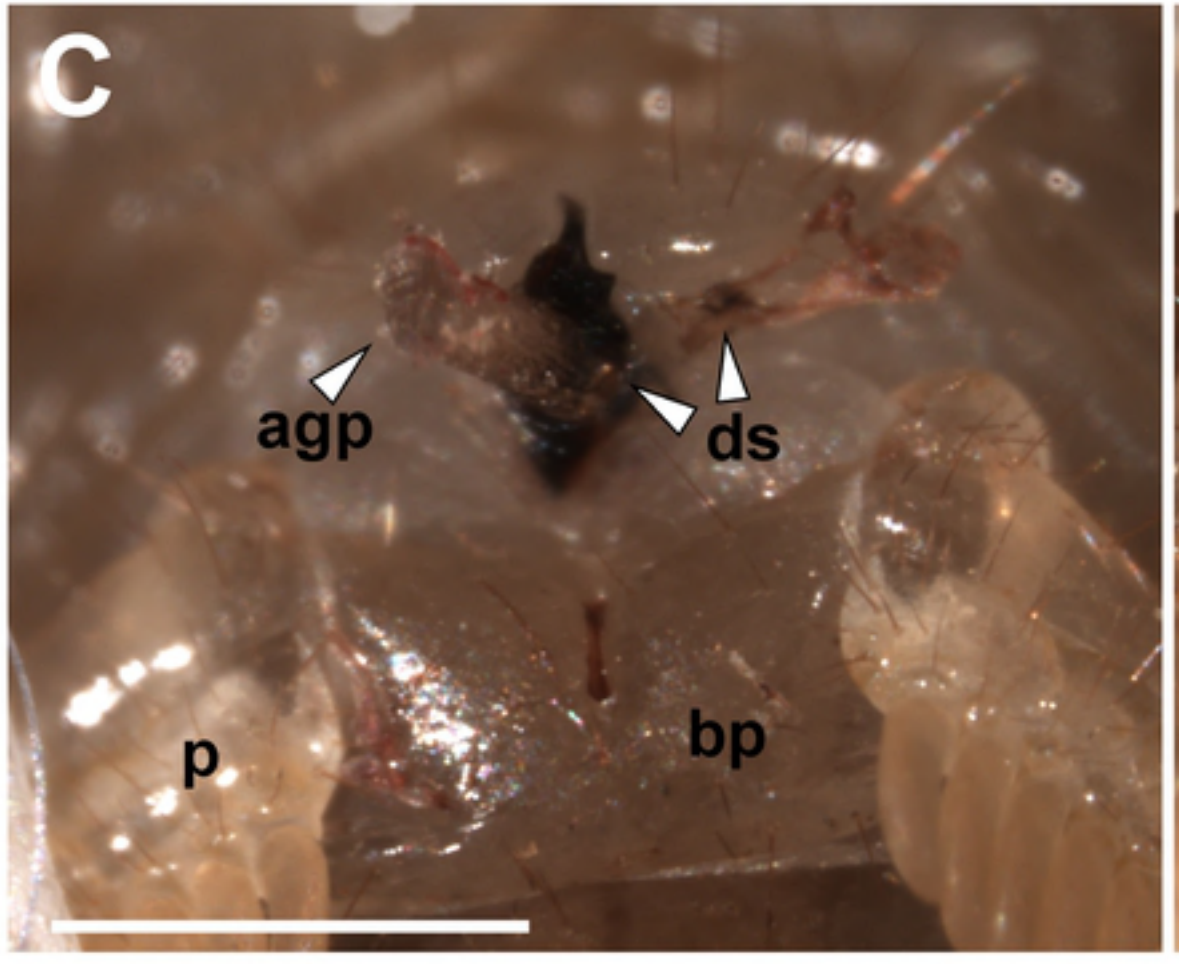


Fig 2

Fig 3







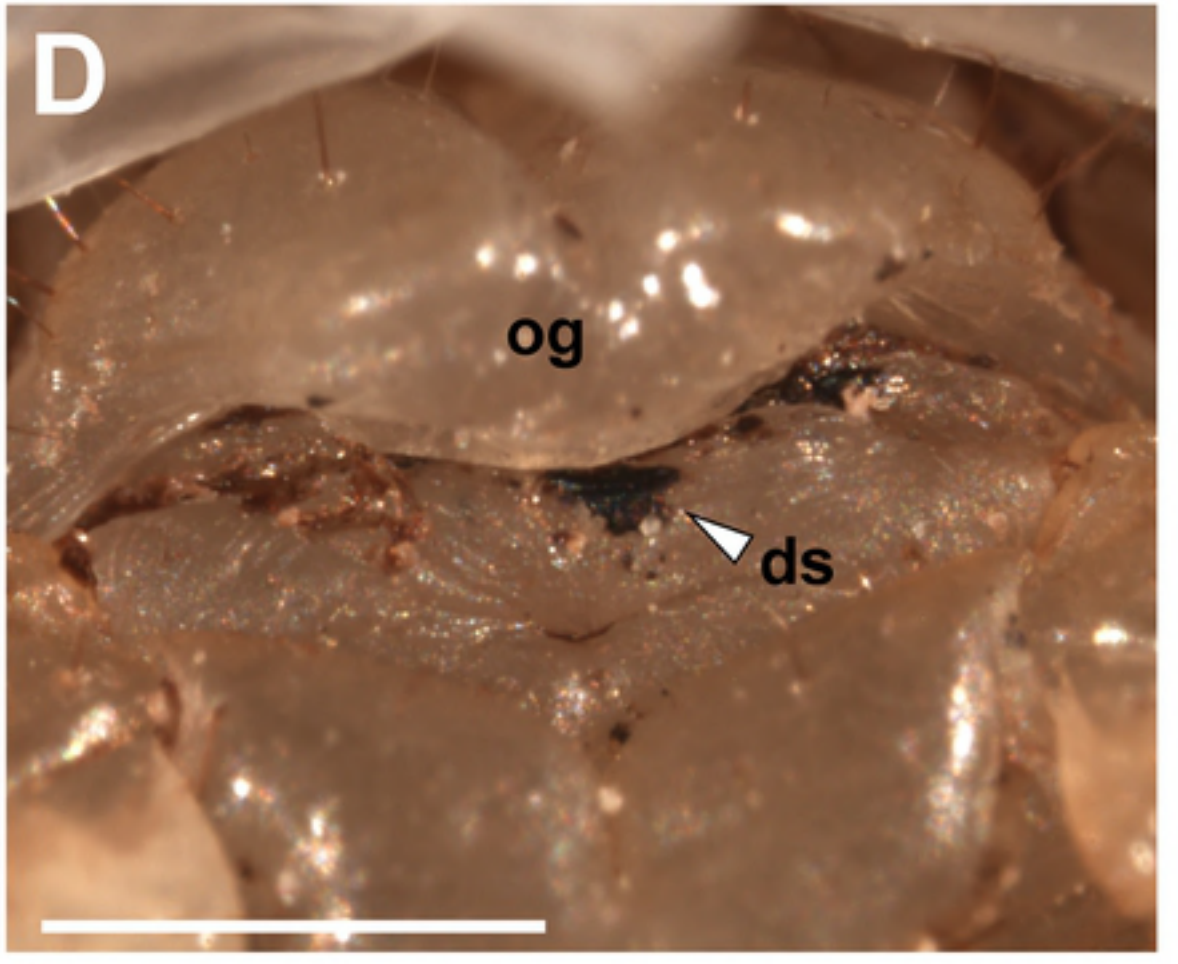
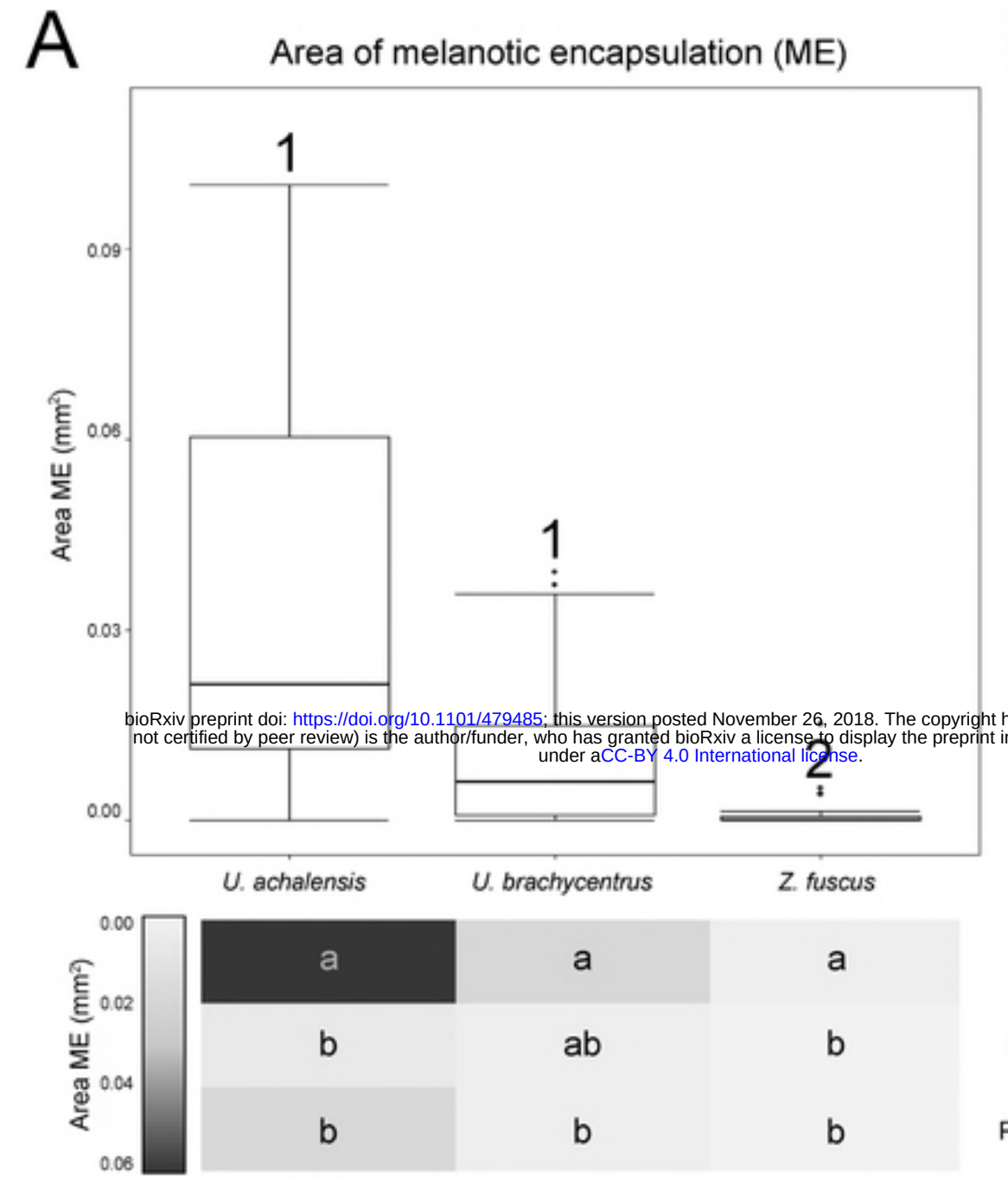
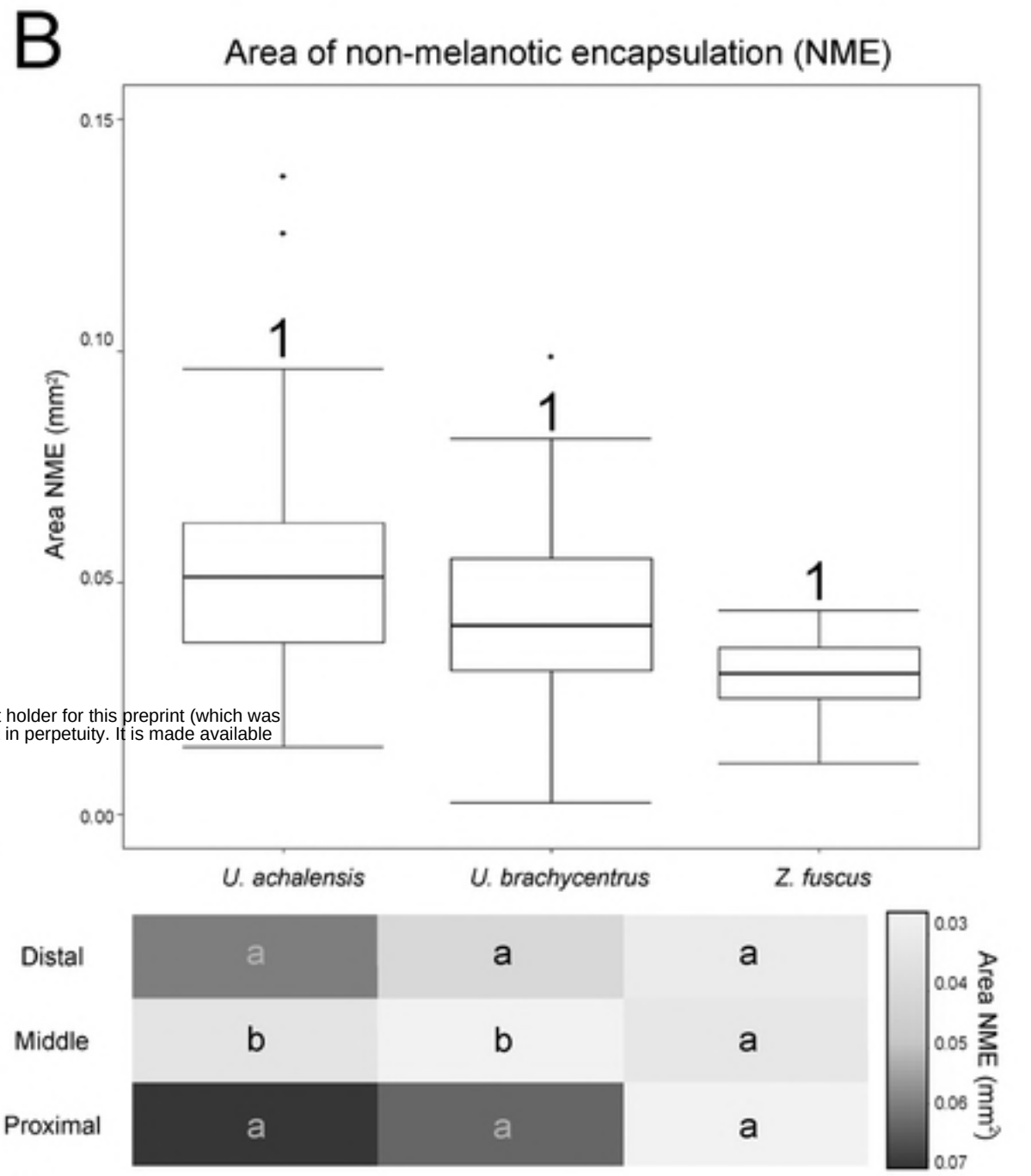
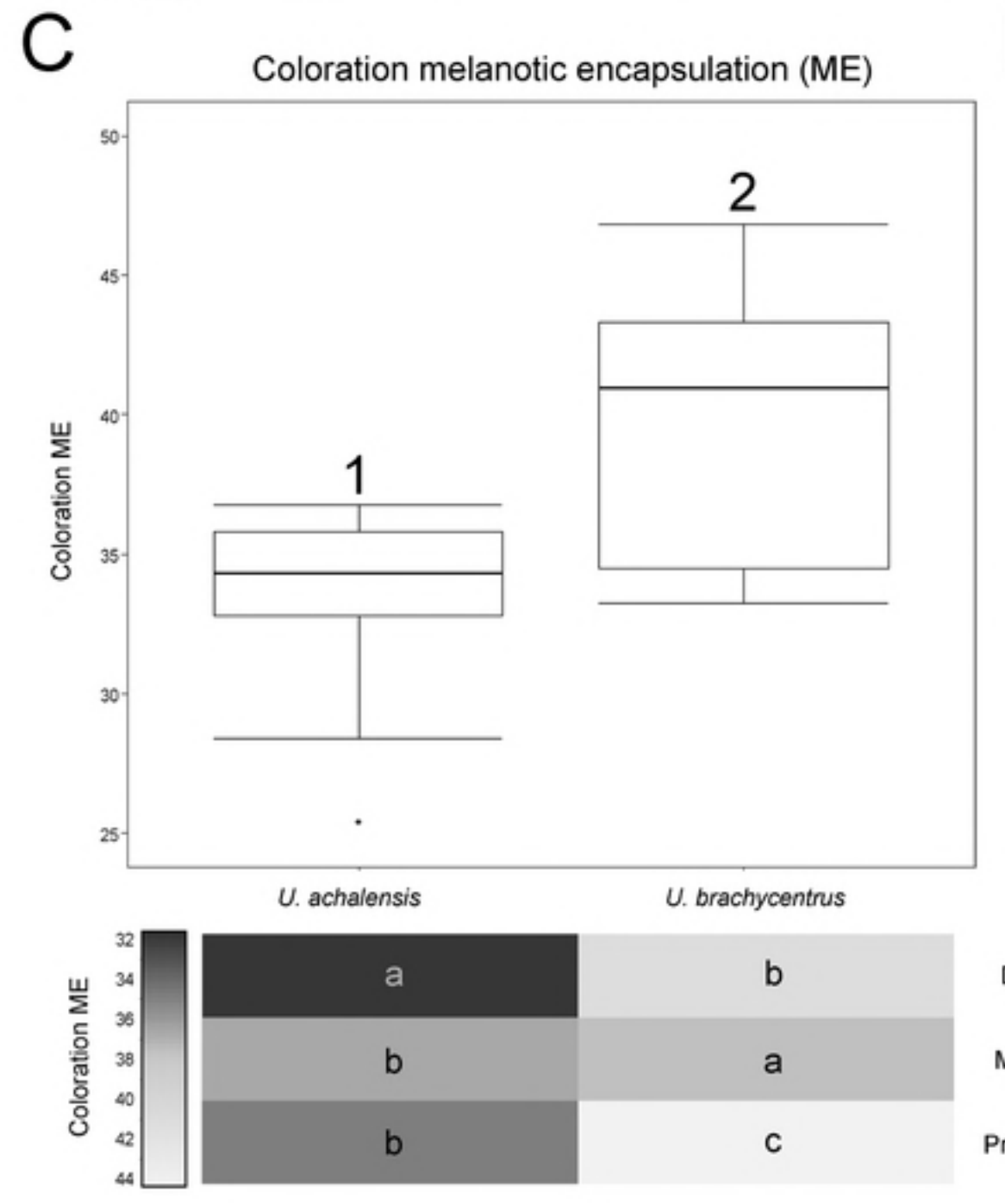


Fig 4







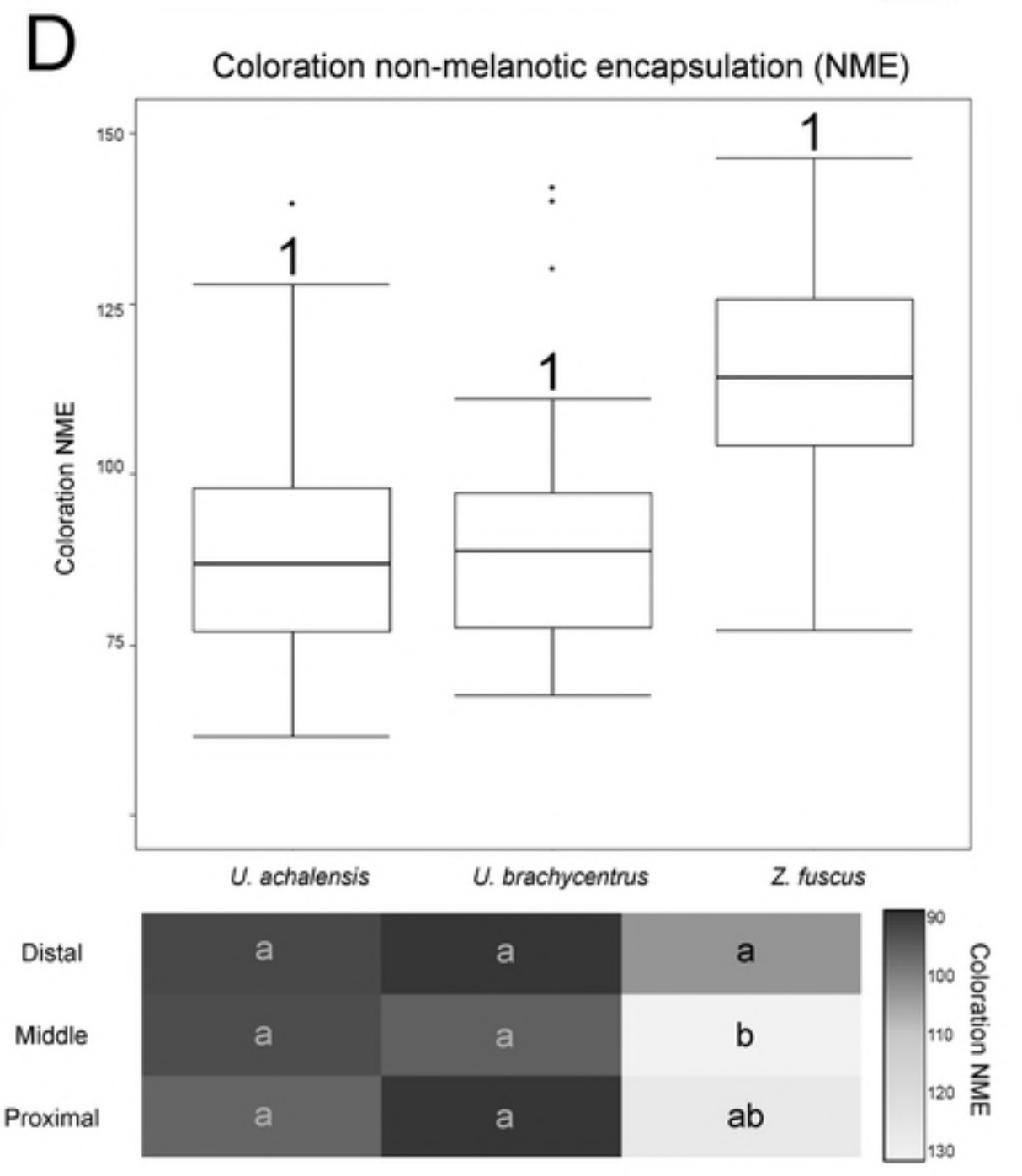
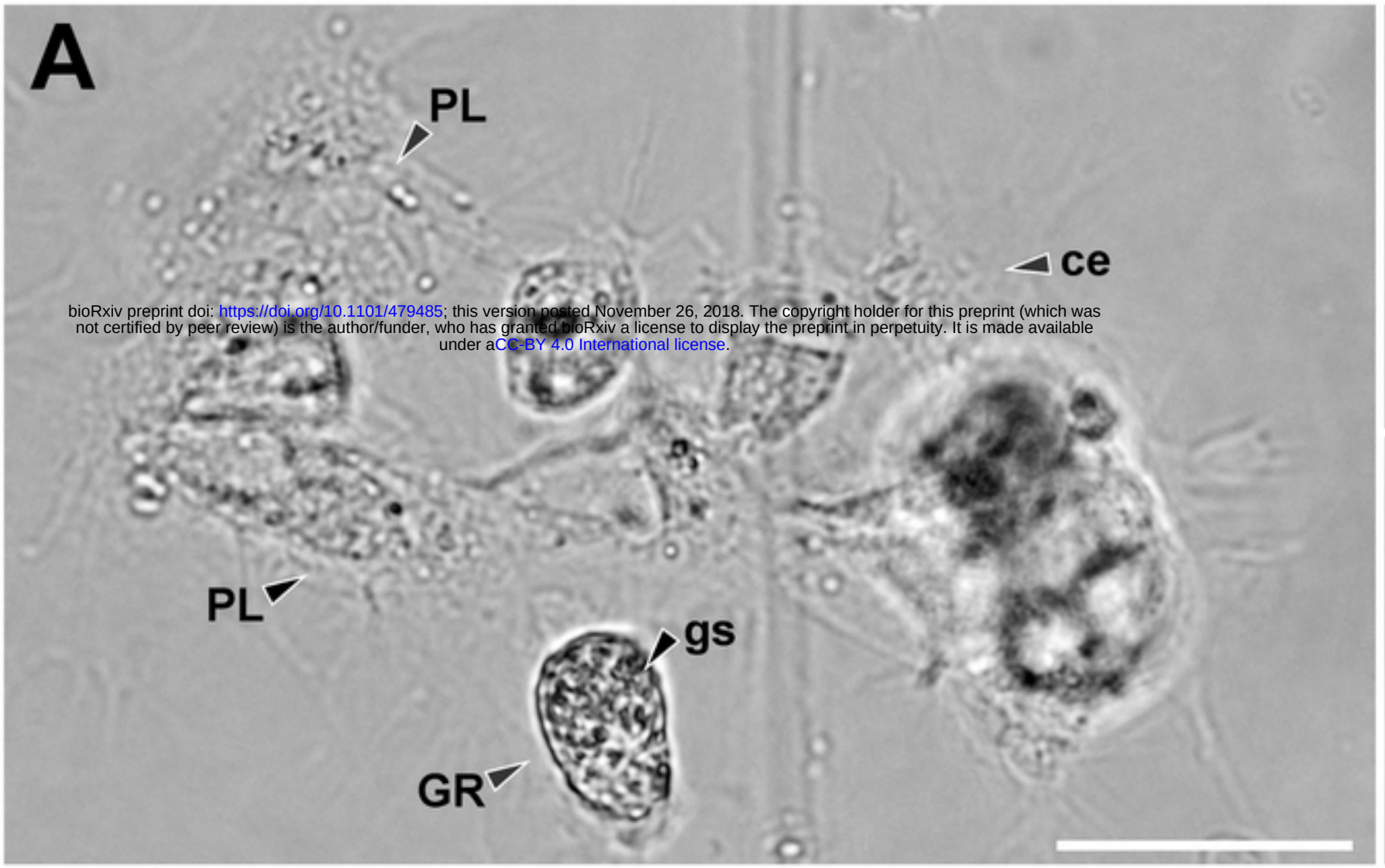
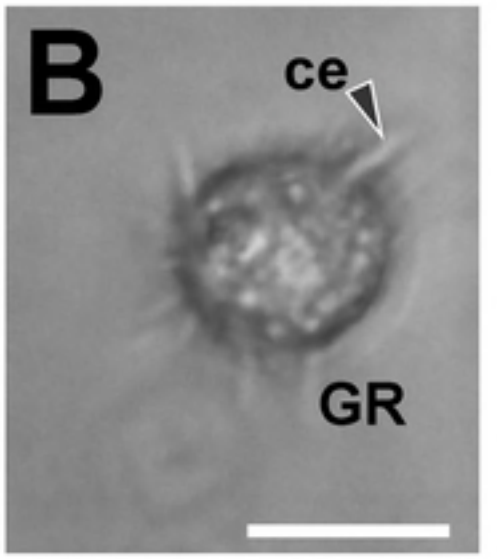
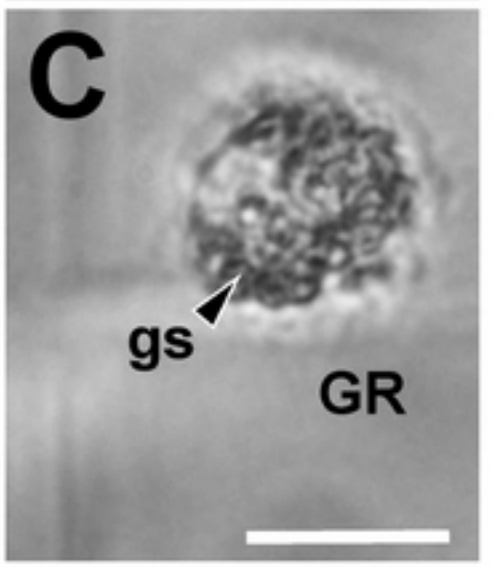
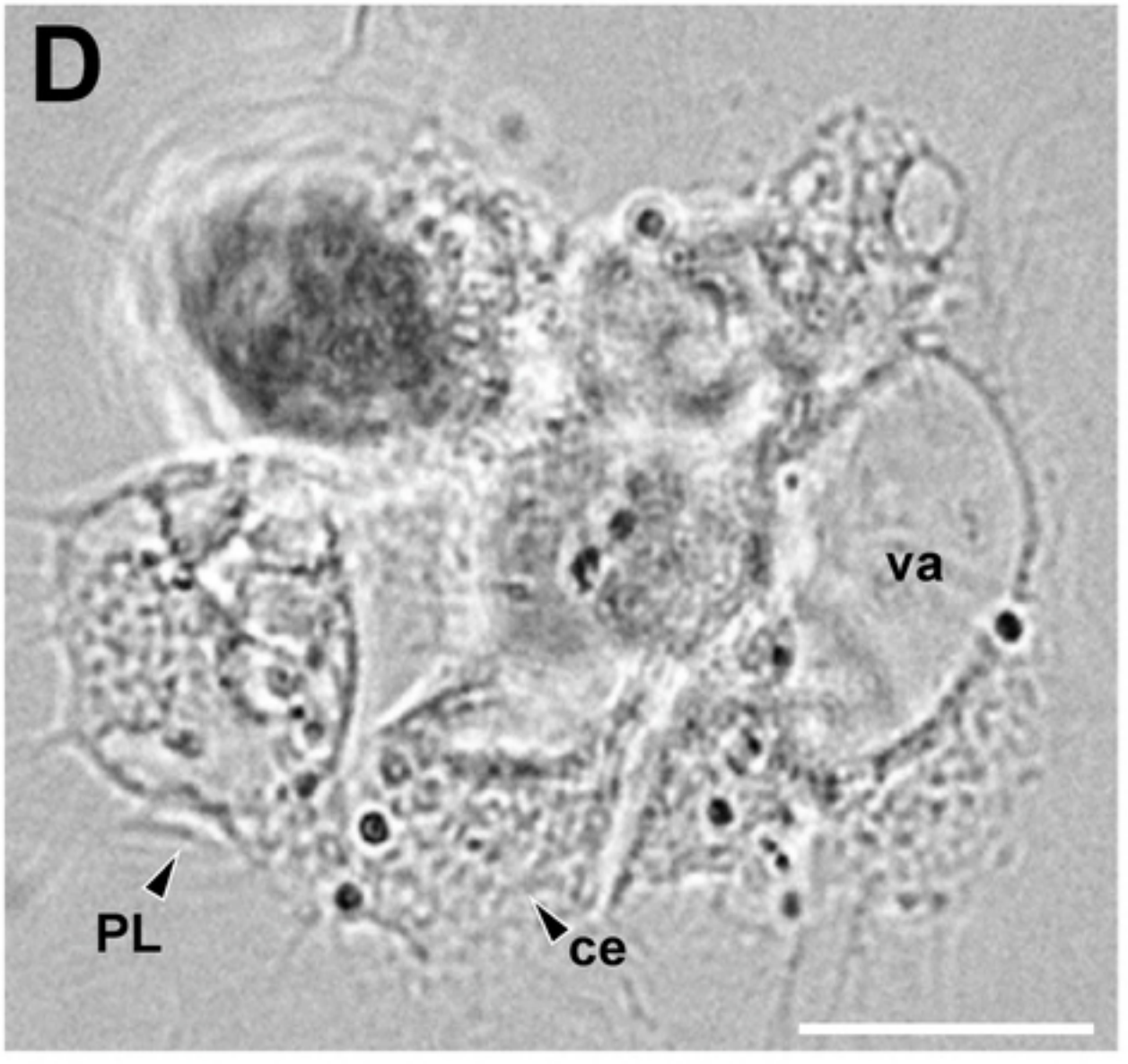


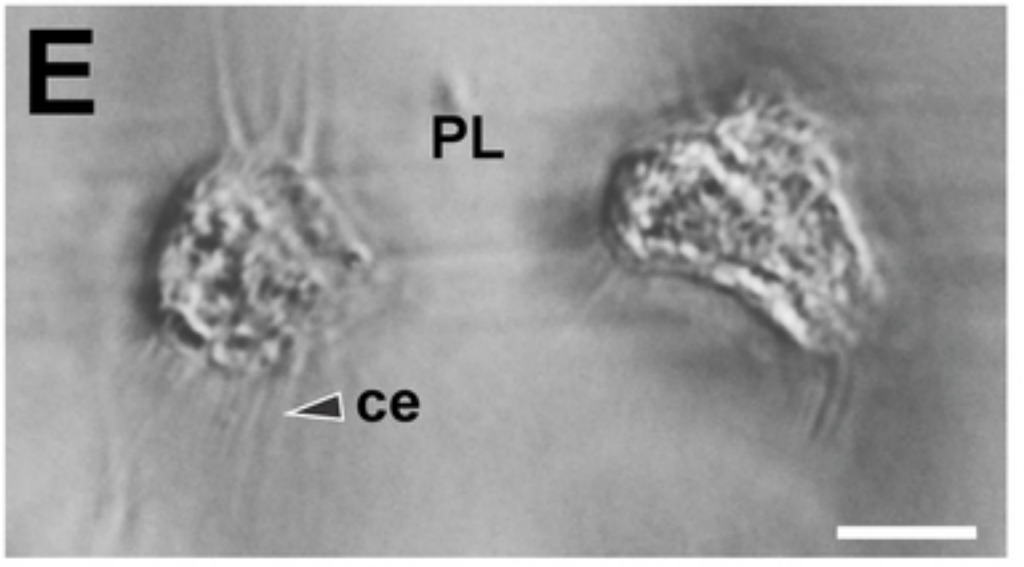
Fig 5











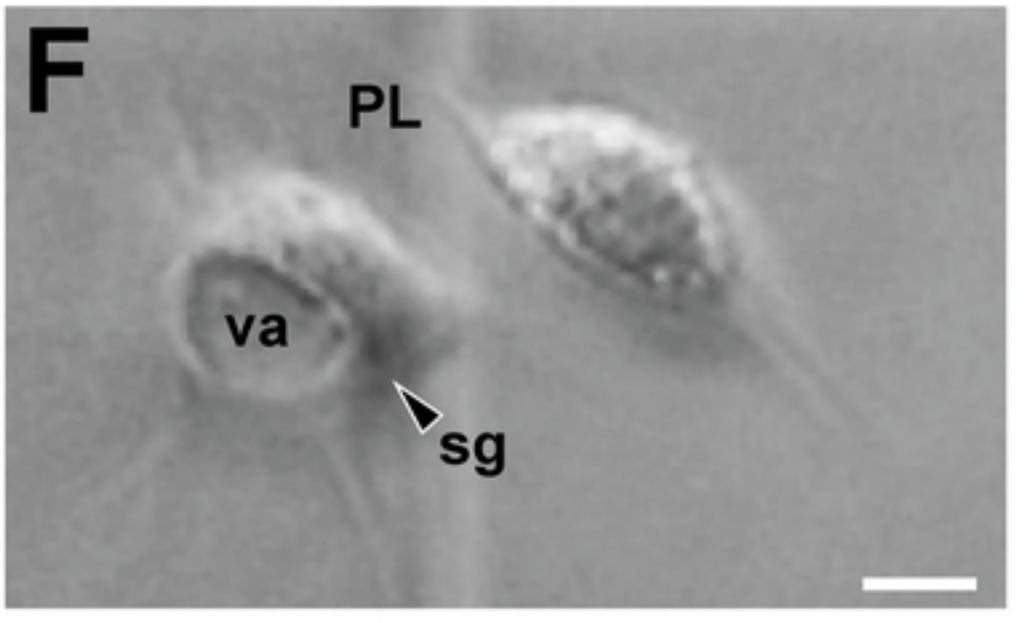


Fig 6

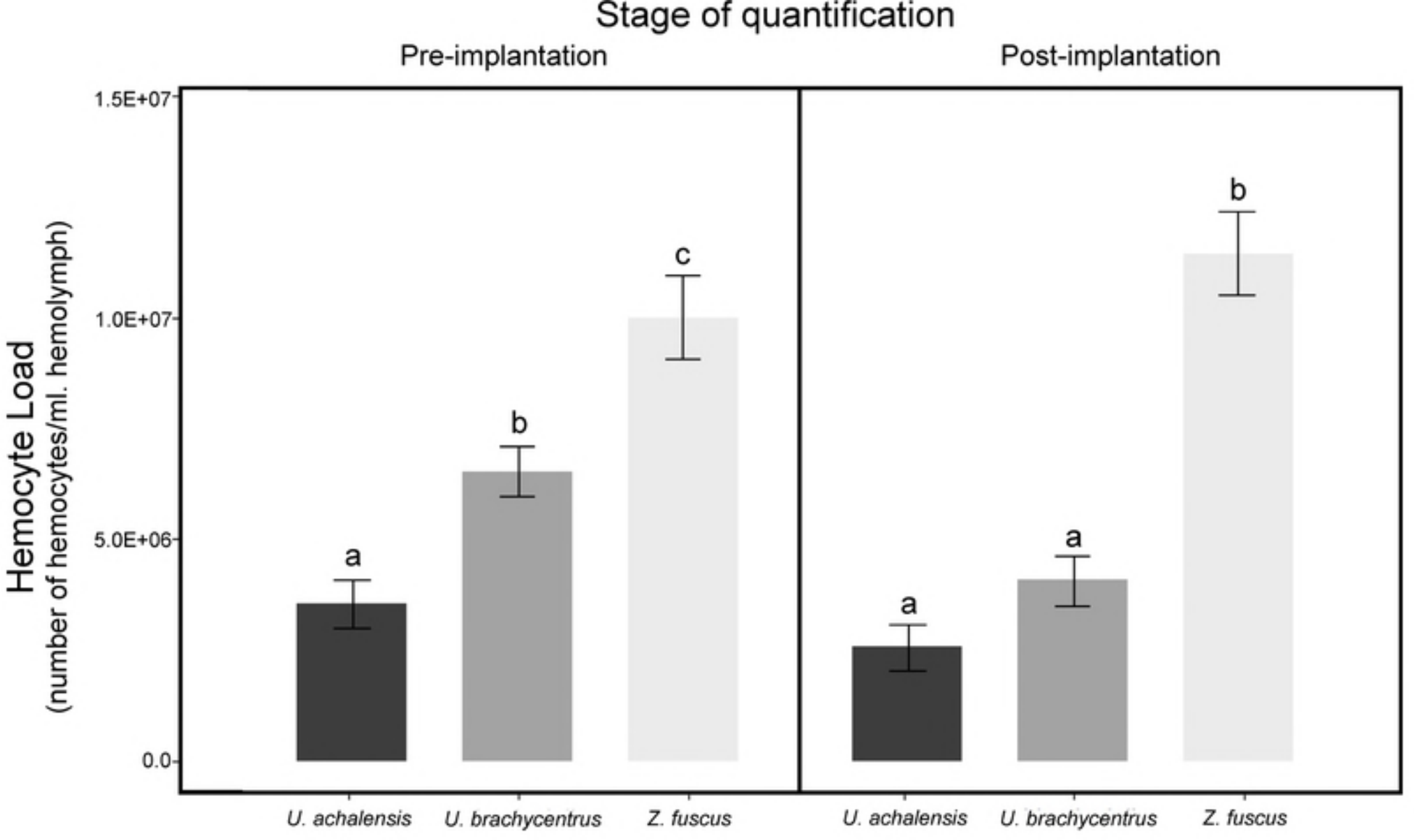

Stage of quantification
Pre-implantation
Post-implantation
Hemocyte Load
(number of hemocytes/ml. hemolymph)
1.5E+07
1.0E+07
5.0E+06
0.0
a
b
c
a
a
b
U. achalensis
U. brachycentrus
Z. fuscus
U. achalensis
U. brachycentrus
Z. fuscus

Fig 7

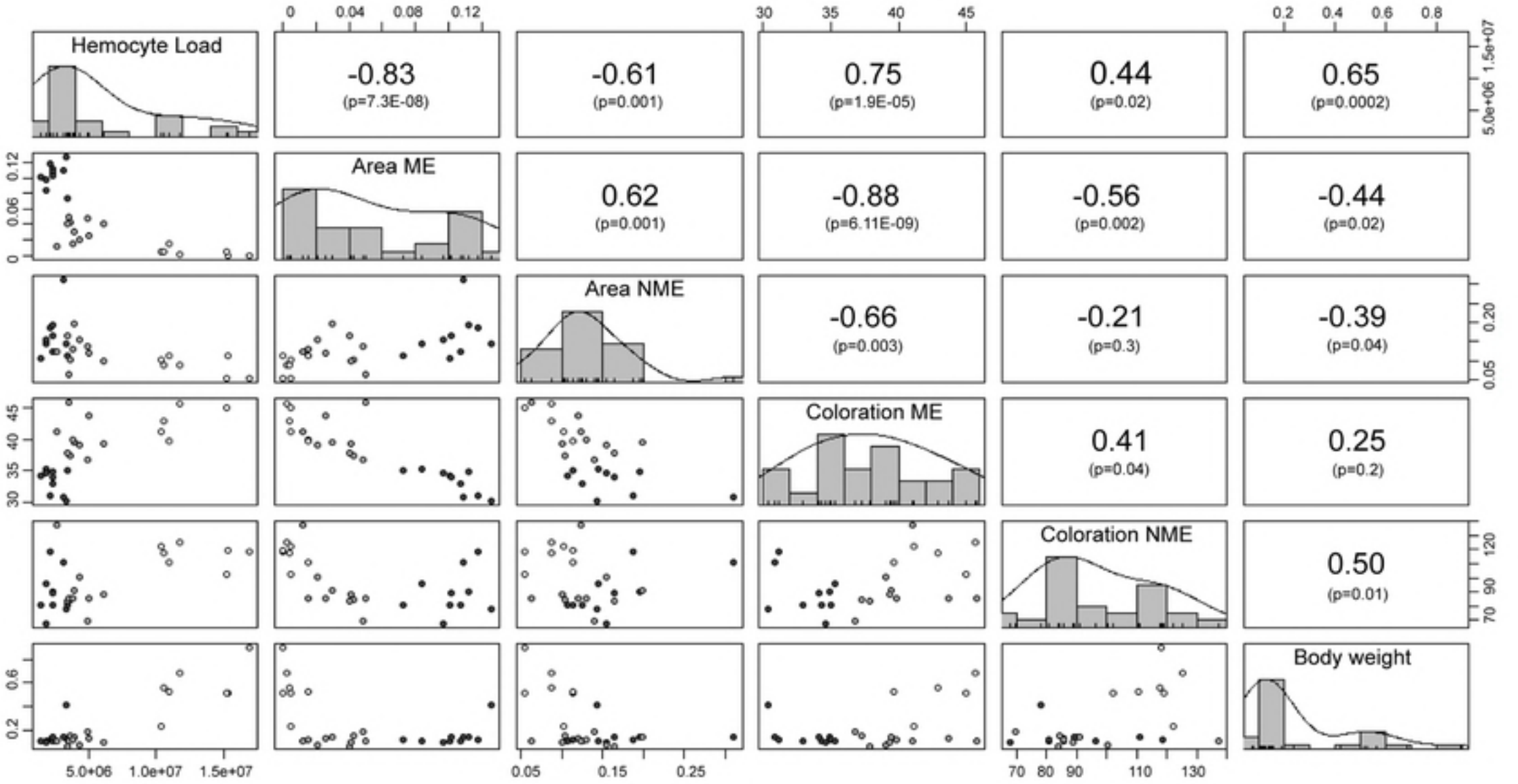

Hemocyte Load
-0.83
(p=7.3E-08)
-0.61
(p=0.001)
0.75
(p=1.9E-05)
0.44
(p=0.02)
0.65
(p=0.0002)
Area ME
0.62
(p=0.001)
-0.88
(p=6.11E-09)
-0.56
(p=0.002)
-0.44
(p=0.02)
Area NME
-0.66
(p=0.003)
-0.21
(p=0.3)
-0.39
(p=0.04)
Coloration ME
0.41
(p=0.04)
0.25
(p=0.2)
Coloration NME
0.50
(p=0.01)
Body weight
0
0.04
0.08
0.12
30
35
40
45
0.2
0.4
0.6
0.8
5.0e+06
1.5e+07
0.05
0.20
70
90
120
5.0+06
1.0e+07
1.5e+07
0.05
0.15
0.25
70
80
90
110
130

Fig 8