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# Dimorphism of right-left asymmetry in the female genitalia of the brachypterous grasshopper *Parapodisma mikado* (Orthoptera, Acrididae)

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Running title: Asymmetric female genitalia of a grasshopper

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**Abstract**

Several insects exhibit morphological asymmetry in the mouthparts or genitalia. In a part of species with asymmetric genitalia, two mirror-image forms of the genitalia are reported to occur in a population. This dimorphism, called chiral dimorphism, is usually observed in male genitalia, but its examples in female genitalia are very limited. Here, we report that the females of the brachypterous grasshopper *Parapodisma mikado* are equipped with an asymmetric sclerite in the copulatory bursa, and that two mirror-image forms of the sclerite occur in local populations. This dimorphism was detected in all of seven populations examined, and the ratio of the two forms was approximately 2:1 for the right : left forms. In one population, the ratio of the two forms did not vary among three consecutive years. Thus, chiral dimorphism in the female genitalia is kept almost constant spatiotemporally. Mating experiments indicated that the direction of female genitalia did not affect the direction to which the males twist their abdomen (right or left side) during mating or the duration of mating. We propose two hypotheses, a neutral developmental mechanism and sexual conflict, for the maintenance of chiral dimorphism in the genitalia of *P. mikado* females.

**Key words**: bursa copulatrix, chiral dimorphism, female choice, laterality, mirror image, sexual conflict.

In insects, there are several examples of left–right asymmetry in genitalia (Walker 1922; Bohn 1987; Schilthuizen 2007, 2013; Huber 2010; Kamimura 2014). The majority of the reports refer to directional asymmetry, which represents the same side of a pair of morphological structures being always larger than the other. On the other hand, dimorphism of mirror-image asymmetry, called chiral dimorphism, has rarely been documented in male genitalia (Schilthuizen 2007); examples are known from praying mantids (Holwell & Herberstein 2010) and earwigs (Giles 1961; Kamimura 2006). In contrast, there have been no reports on chiral dimorphism in female genitalia except the migratory locust, *Locusta migratoria*, whose spermatheca exhibit chiral dimorphism in a population (Mika 1959). Since that report was published, however, no studies have clarified to what extent this phenomenon is common to grasshoppers or whether this dimorphism is related to female reproductive success.

In all species of the genus *Parapodisma* Mishchenko (Acrididae: Melanoplinae), brachypterous grasshoppers, the copulatory bursa in females is asymmetric in the shape (Ito 2015). During copulation, sperm from the male is ejaculated in the copulatory bursa and immediately transported to the spermatheca via the spermathecal duct. The copulatory bursa in female *Parapodisma* is partly or wholly sclerotized and clearly outlined, so that its shape and size are useful for discriminating closely related species (Ito 2015). Our preliminary study shows that the sclerite in the copulatory bursa of *Parapodisma mikado* Bolívar exhibits not only asymmetry but also chiral dimorphism in some populations. During copulation, the male always twists his abdomen to the right or left side on the female's back and connects his genitalia to the female's ones from below the female abdomen (that is, the false male-above position, Huber 2010). The sclerite in the copulatory bursa seems to contact directly with male genitalia.

Dimorphism in a population arises from genetic and/or environmental factors

63 (Zera & Denno 1997). However, few studies have elucidated the mechanism that
64 maintains chiral dimorphism in a population (but see Jesson & Barrett 2002;
65 Schilthuizen *et al.* 2007; Kamimura 2011). Thus, the present study first aims to examine
66 whether chiral dimorphism in the copulatory bursa is common in local populations of *P.*
67 *mikado*, and second whether chiral dimorphism in female genitalia affects mating
68 behavior or not.

69 To examine whether chiral dimorphism exists extensively, we collected adult
70 females of *P. mikado* from six localities of Hokkaido (the northern island of Japan) and
71 one locality of Honshu (the main island of Japan) from 2012 to 2014. The collection
72 localities are indicated in Figure 1, and sample sizes are shown in Table 1. Collection
73 was made in the last stage of the oviposition period to reduce collection impact on the
74 population as much as possible. For the morphological observation of the copulatory
75 bursa, the tip of the female abdomen, about 1 cm long, was excised with scissors. After
76 slitting open the abdominal tip longitudinally with forceps, we took out the copulatory
77 bursa by using an insect pin in physiological saline solution and under a stereoscopic
78 microscope. The sclerite in the copulatory bursa was mounted on glass slides with
79 Hoyer's medium, and the image of the sclerite was captured into a computer using a
80 microscope camera (E4500; Nikon, Tokyo, Japan).

81 We conducted mating experiments in the laboratory to examine whether: (i) the
82 direction of female genitalia affects the male's mating behavior; and (ii) matching or
83 mismatching between male mating behavior and the direction of female genitalia affects
84 the duration of copulation. To prepare virgin males and females for the experiments, we
85 collected *P. mikado* nymphs at Mt Teine, Sapporo, Hokkaido, in June 2013 using an
86 insect net. The nymphs were brought to the laboratory and maintained at 23°C and 16h
87 light:8h dark (LD16:8) in a climate chamber until adulthood. We fed them on leaves of

*Petasites japonicas giganteus*, *Fallopia sachalinensis* and *Rudbeckia laciniata*.

Prior to the experiment, we distinguished all individuals by marking on the legs with white paint. Ten virgin males and ten virgin females were placed into an arena (400×300×300mm) of a plastic container with food plants, and the containers were placed at 23°C and LD16:8 in an environmentally controlled laboratory at the Center for Genome Dynamics, Hokkaido University. We recorded all pairs that copulated and to which side males twisted their abdomen during copulation on the back of the mate. We stopped experiments either when five mating pairs had been made, or when four hours had passed after the onset of experiments. Mating pairs were separately isolated into a small cage (150 × 80 × 100 mm) in which they were maintained with food plants until the end of copulation to determine copulation duration. We monitored the mating behavior of the pairs by using video cameras (GZ-MS211; Victor, Tokyo, Japan) to determine mating duration. During observation, all the males mated once. After the observation, the females were kept frozen in a freezer, and then they were dissected to observe the shape of the copulatory bursa. In 2012 and 2013, we observed a total of 46 mating pairs.

We examined whether the ratio of the two female forms differs from randomness in each population or varies among populations by using log-likelihood ratio test. The relationship between the twisting direction of male abdomen and the direction of female genitalia was also tested by log-likelihood ratio test. The effect of the twisting direction of male abdomen and the direction of female genitalia on copulation duration was examined with a generalized linear model. In the model, the two explanatory variables were treated as fixed effects, and the interaction between the twisting direction of male abdomen and the direction of female genitalia were included, in addition to the experimental year (2012 or 2013).

A sclerite in the copulatory bursa was asymmetric in the shape (Fig. 2). Furthermore, there were two mirror-image forms in the sclerite among individuals; in the left form (Fig. 3a,c,e), the left side of the strongly pigmented area in the sclerite was larger than the right side with a gently curved margin, and the spermathecal duct is connected to the left side. On the other hand, the opposite is true in the right form (Figs 2, 3b,d,f). Laterality was obvious; of 706 females dissected, only two had copulatory bursas that were indistinct in laterality. There was a large variation among females in the shape and sclerotization of the sclerite as shown in Figure 3. The “standard” morphology (Fig. 3a,b) accounted for 72.1% of the females observed, followed by the morphology with a conspicuous constriction (18.1%; Fig. 3c,f). However, we were not able to observe marked differences among populations. On the other hand, asymmetry was not detected in male genitalia including the cerci (see also Ito 2015: fig. 1.31 K).

In all seven localities, chiral dimorphism was observed in female genitalia. In localities where the sample size is larger than 38, the frequencies of the two forms differed significantly from randomness (Table 1). In all the populations except Otaru in 2013, the right form was more numerous than the left form; on average, the ratio of the right to the left form was approximately 2:1. In the Iwamizawa population with sufficient numbers of samples, the ratio did not change among three consecutive years ($df = 2$, $\chi^2 = 0.723$, $P = 0.697$). In contrast, in the Otaru populations the ratio changed significantly between samples in 2013 and 2014 ($df = 1$, $\chi^2 = 9.47$, $P = 0.002$); however, in total, a ratio of approximately 2:1 was maintained. When samples collected in different years are pooled in each population, no significance was found among the

seven populations in the frequencies of the two forms (df = 6, $\chi^2$ = 3.98, $P$ = 0.680).

In the mating experiments, copulation lasted for on average 20.5 h ± 5.87 (SD). The twisting direction of the male abdomen was not significantly different from random (right-hand: 25, left-hand: 21, df = 1, $\chi^2$ = 0.348, $P$ = 0.555), and in this experiment, the ratio of the right to the left form in the females was 2.29:1. During copulation, there was no significant linkage between the direction of female genitalia to right or left (*R* or *L*, respectively) and the twisting direction of male abdomen to right or left (*r* or *l*, respectively) (frequencies of four combinations: *Rr* = 13, *Rl* = 19, *Lr* = 8, and *Ll* = 6; for additivity, df = 1, $\chi^2$ = 1.07, $P$ = 0.301). Copulation duration was not affected by the direction of female genitalia, direction of male abdomens, nor the interaction between the two directions (for the direction of male abdomen, df = 1, $F$ = 0.185, $P$ = 0.670; for the direction of female genitalia, df = 1, $F$ = 1.996, $P$ = 0.165; for the interaction, df = 1, $F$ = 0.333, $P$ = 0.569; for years, df = 1, $F$ = 3.98, $P$ = 0.053).

The present study indicates that in the female genitalia of *P. mikado*, chiral dimorphism is extensively maintained among local populations and that the ratio of the right to left form is approximately 2:1. This dimorphism is categorized as biased antisymmetry (Palmer 2005). Schilthuizen (2013) suggests that chiral dimorphism (i.e. antisymmetry) may be evolutionarily unstable because it is conceived as a transitional state from one type of directional asymmetry to the other. There has been no report on chiral dimorphism in female genitalia, except in spermatheca in *Locusta migratoria* (Mika 1959). Examples of chiral dimorphism have been known from the male genitalia of praying mantids *Ciulfina* spp. (Holwell & Herberstein 2010) and *Thespinae* sp. (Anisyutkin & Gorochov 2004).

Just prior to mating, males bring their genitalia near the female genitalia from

either the right- or left-hand side. Thus, male genitalia are inserted obliquely to female genitalia. The asymmetric sclerite of the copulatory bursa may have evolved in females to receive male genitalia entering obliquely, and it may mechanically correspond to the aedeagal valve in male genitalia (Ito 2015). However, it is not clear what kind of inconvenience arises when male genitalia are inserted from a wrong direction to the copulatory bursa if the direction to which male genitalia are inserted has any effect on male reproductive success. In the experiments, we did not observe that males dissociated their genitalia from the mate immediately after the start of copulation and tried to reinsert them from the opposite side. In addition, because males twisted their abdomen with an equal frequency to the right- or left- hand side, irrespective of the direction of female genitalia, mismatch between the directions of male and female genitalia appears not to hinder male mating behavior. This idea is further corroborated by the observation of copulation, the duration of which was not affected by the match or mismatch of the directions in male and female genitalia. We observed that in a series of copulations, each male bent his abdomen randomly to both sides (D Shimizu, unpubl. data, 2015).

In the case of the scale-eating cichlid, because the right or left forms are subject to frequency-dependent selection, the balance of them is kept at a ratio of approximately 1:1 (Hori 1993). If the two forms of *P. mikado* females have any genetic basis and if frequency-dependent selection is acting on the two forms, the ratio of them would be expected to converge to 1:1 (Schilthuizen 2007). However, the fact that the ratio of 2:1 is kept stable spatiotemporally for the two forms negates the hypothesis of frequency-dependent selection. There are no data on the genetic basis of this chiral dimorphism. Thus, we propose two working hypotheses accounting for the maintenance of chiral dimorphism in *P. mikado* females.

First, our experimental results and field data suggest that it may be difficult to interpret the chiral dimorphism in *P. mikado* from an adaptive point of view. Dimorphism in the copulatory bursa may be selectively neutral, with no reproductive impact on both of a mating pair. Thus, one of our hypotheses is that this dimorphism results from a developmental mechanism by which each mother produces the two forms randomly with a ratio of 2:1, without a genetic basis for either of the two forms. Kamimura and Iwase (2010) report that the direction of antisymmetric genital traits is randomly determined by non-genetic factors in all examples studied.

Second, sexual conflicts during mating may have promoted the evolution of the chiral dimorphism in females. In the melanopline species *Podisma sapporensis*, the females actively reject male courtship by jumping away from the approaching males and kicking the males with their hind legs (Sugano & Akimoto 2011). *Parapodisma mikado* males cannot sense the direction of a female's copulatory bursa before mating. Thus, given dimorphism in the copulatory bursa, *P. mikado* males must have time to detect the direction of female copulatory bursa just after insertion to fix their genitalia in the appropriate position in the copulatory bursa. Therefore, just after mating, females may have a chance to run away from the males by dissociating their genitalia from the mates' genitalia. For this reason, chirality in female genitalia increases the unpredictability for the status of female genitalia, resultantly increasing the chance of females running away from the mate.

Unfortunately, there is no evidence for these hypotheses at present. However, in a future study, it may be possible to explain the adaptive significance of chiral dimorphism in female genitalia by evaluating the relationship between matching between the directions of male and female genitalia and the amount of sperm transported to females.

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## Figure legends

Figure 1 Collection localities of Parapodisma mikado females (1, Teine; 2, Iwamizawa; 3, Otaru; 4, Niigata; 5, Matsumae; 6, Kuriyama; 7, Maruseppu).

Figure2 InternalgenitaliaoftheParapodismamikadofemale.A, spermathecal duct; B, copulatory bursa; C, sclerite.

Figure 3 Asymmetric sclerite in the copulatory bursa of *Parapodisma mikado* females. (a,c,e) left form and (b,d,f) right form. Females with these copulatory bursas were collected from Kuriyama (a), Teine (b,f), Niigata (c), Iwamizawa (d) and Otaru (e).

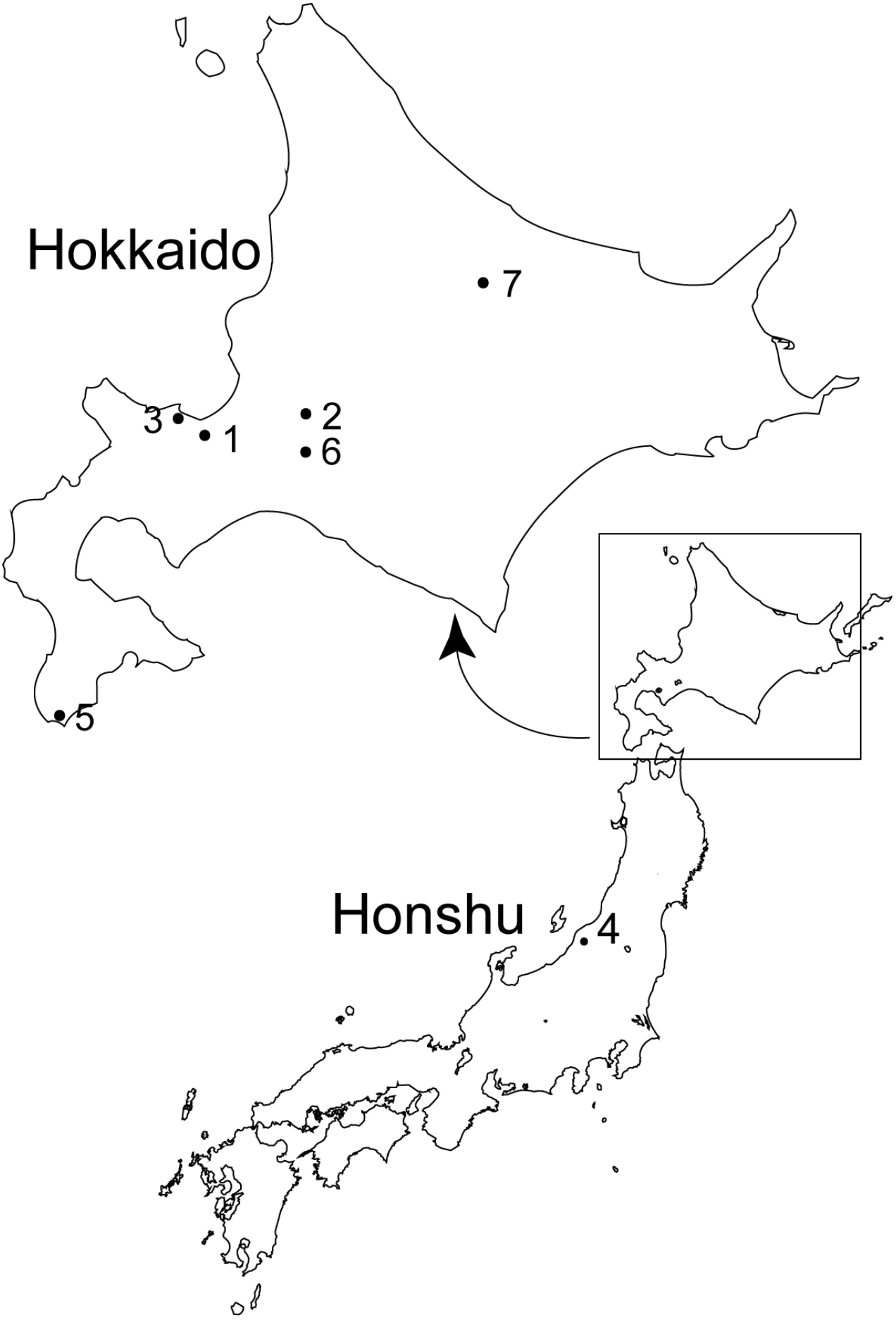
293 Table 1 Numbers of two forms of *Parapodisma mikado* females collected at each

294 locality

| Locality | Year | No. females | | $X^2$* | *P* |
|---|---|---|---|---|---|
| | | Left form | Right form | | |
| 1. Teine | 2012 | 2 | 7 | - | - |
| | 2013 | 28 | 51 | 6.794 | 0.009 |
| 2. Iwamizawa | 2012 | 13 | 21 | 1.900 | 0.168 |
| | 2013 | 25 | 55 | 11.530 | 0.001 |
| | 2014 | 37 | 65 | 7.786 | 0.005 |
| 3. Otaru | 2013 | 15 | 11 | 0.618 | 0.432 |
| | 2014 | 8 | 31 | 14.486 | <0.001 |
| 4. Niigata | 2013 | 5 | 7 | 0.335 | 0.563 |
| 5. Matsumae | 2013 | 1 | 8 | - | - |
| 6. Kuriyama | 2013 | 2 | 3 | - | - |
| 7. Maruseppu | 2013 | 5 | 5 | - | - |

295 *The results of tests for randomness (1:1) for the frequencies of two forms are

296 indicated. In case of the sample size being less than 11, test is omitted.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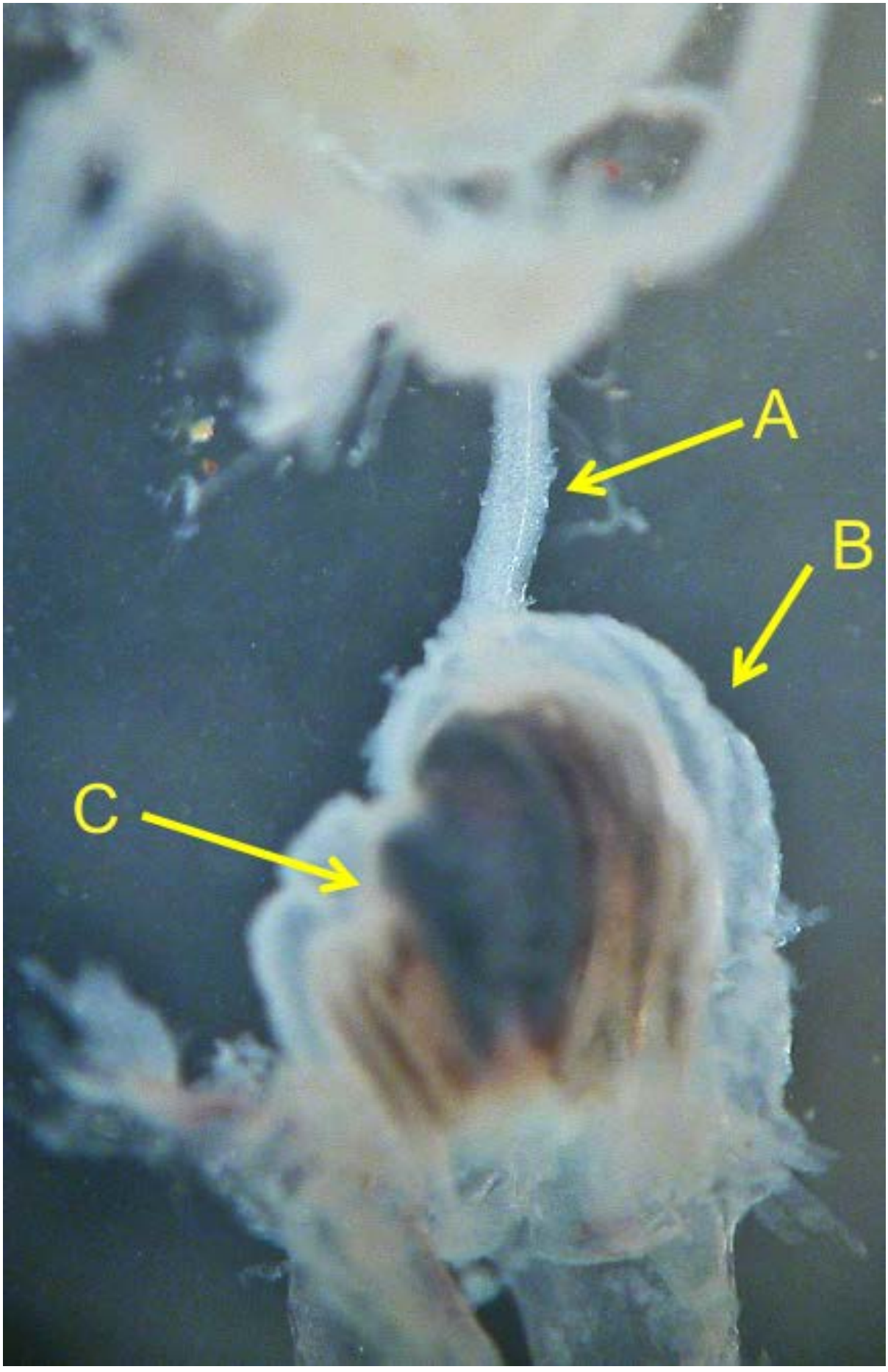
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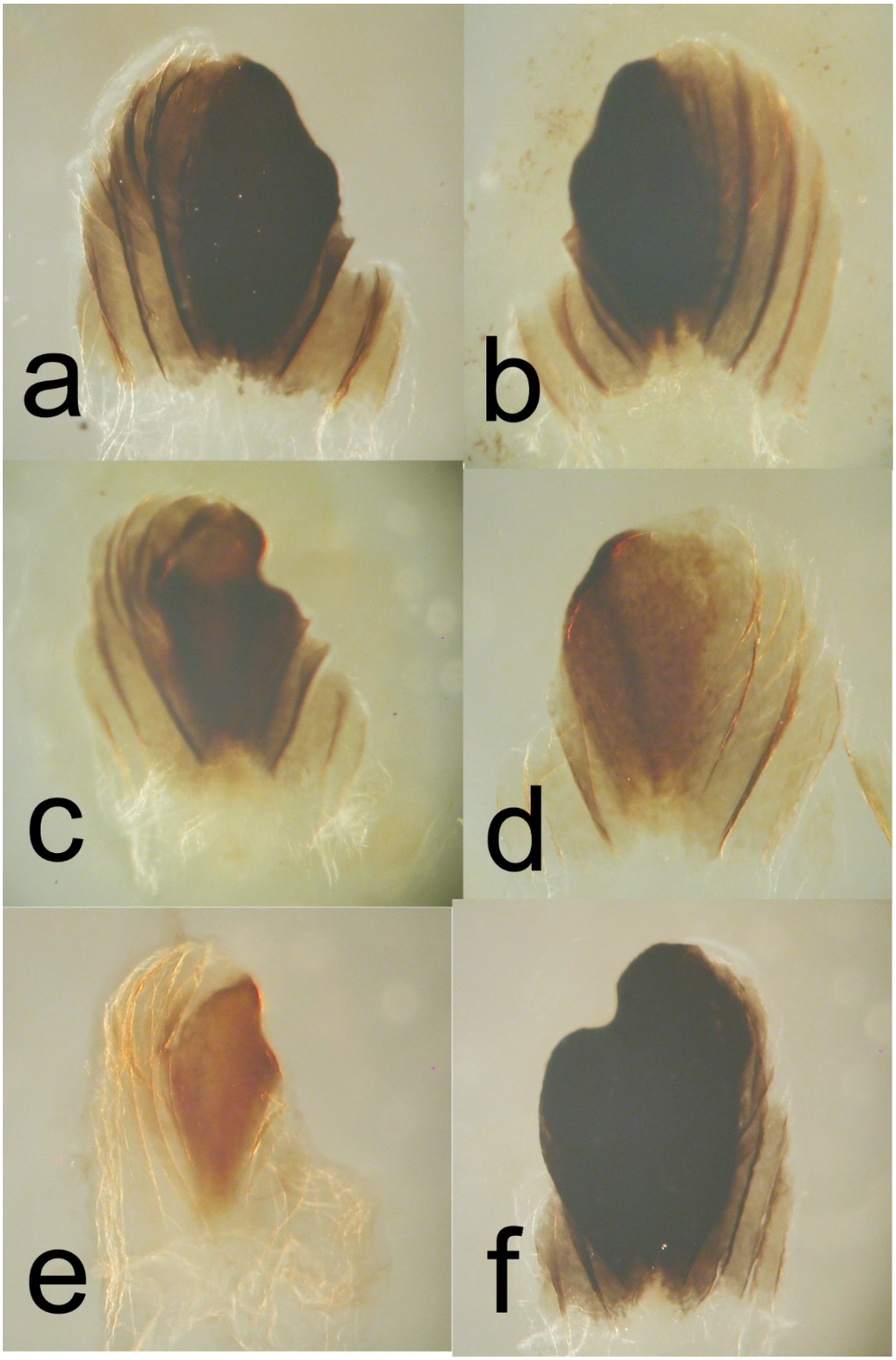
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