JOURNAL OF
UNDERGRADUATE RESEARCH

Morphology of male and female genitalia across *Acraea* (Nymphalidae) butterfly species with and without a mating plug

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Abstract

Male mating plugs have been used in many species to prevent female re-mating and sperm competition. One of the most extreme examples of a mating plug is the sphragis, which is a large, complex and externalized plug found only in butterflies. This structure is found in many species in the genus *Acraea* (Nymphalidae) and provides an opportunity for investigation of the effects of the sphragis on the morphology of the genitalia. This study aims to understand morphological interspecific variation in the genitalia of *Acraea* butterflies. Using museum collection specimens, abdomen dissections were conducted on 19 species of *Acraea*: 9 sphragis-bearing and 10 non-sphragis-bearing species. Genitalia were photographed and then measured using ImageJ software. Some distinguishing morphological features in the females were found. The most obvious difference is the larger and more externalized copulatory opening in sphragis-bearing species, with varying degrees of external projections. Females of the sphragis-bearing species also tend to have a shorter ductus (the structure that connects the copulatory opening with the sperm storage organ) than those without the sphragis. These differences may be due to a sexually antagonistic coevolution between the males and females, where the females evolve larger copulatory openings that are more difficult to plug, while males attempt to prevent re-mating with larger plugs (sphragis).

Keywords: Lepidoptera, mate conflict, mating plug, sexual coevolution

Introduction

Sexual conflict occurs when there are different selection pressures on males and females. (Parker, 1979; Rutowski, 1997). These differing selection pressures may drive antagonistic coevolution between the sexes (Bergsten et al., 2001; Carvalho et al., 2019; Clutton-Brock & Parker, 1995; Rice, 1996); in which adaptations by one sex reduce the fitness of the other sex (Bergsten et al., 2001; Rice, 1996) and may lead to elaboration of genitalia and other secondary structures (Arnqvist & Rowe, 1995). Sexual conflict in butterflies also stems from the different levels of investment by males and females in the offspring (Hammerstein & Parker, 1987;

Parker, 1979, 1984; Thornhill & Alcock, 1983). Males typically increase their mating success each time they copulate as they increase their probability of having more offspring, whereas females do not necessarily increase their fitness with more mating events. (Arnqvist & Nilson, 2000; Drummond, 1984; Ehrlich & Ehrlich, 1978; Parker, 1970, 1984;). However, in some cases, females may mate more than once to gain nutritional benefits, increase their offspring's genetic variation, add to their sperm stores, or reduce conflicts arising from forced copulations (Arnqvist & Nilsson, 2000; Thornhill & Alcock, 1983; Wedell, 2005).

Another contributing factor of this conflict is male-male competition. Female butterflies can store sperm from multiple mating events, but often the last male is the one that fertilizes the eggs (Drummond, 1984; Ehrlich & Ehrlich, 1978; Labine, 1966; Parker, 1970). Males, then, attempt to block females' future mating attempts and restrict sperm competition (Arnqvist & Rowe, 1995; Orr, 1988). One strategy that males of some species employ is the production of a mating plug that blocks the copulatory opening and prevents remating (Drummond, 1984; Orr, 1995; Orr & Rutowski, 1991; Parker, 1970). In butterflies this mating plug can be permanent as it does not prevent oviposition. The ostium, or copulatory opening, is located ventrally within the sinus vaginalis and separate from the ovipositor. Thus, these mating plugs can be large and complex and are often elaborate externalized structures covering part of the female's abdomen, but not the ovipositor (Carvalho et al., 2017; Carvalho et al., 2019; Orr, 1988, 1995). The externalized and species-specific plug found in some butterflies is termed a sphragis (Carvalho et al., 2017). The production of the sphragis by males is part of an ongoing evolutionary arms race between the sexes in some groups of butterflies (Carvalho et al., 2019).

It is not fully understood how the sphragis contributes to evolution of male and female traits (Matsumoto et al., 2018; Orr, 1988). However, in sphragis-bearing species (SBS), it is predicted that the morphology of the genitalia will reflect the antagonistic coevolution between the sexes. In almost all SBS, the shape of female genitalia makes small plugs ineffective (Matsumoto et al., 2018; Orr, 1988, 1995).

This study aims to detail the morphological differences of the genitalia in *Acraea* Fabricius, 1807 (Nymphalidae: Heliconiinae) species with and without a sphragis. For this study, it was hypothesized that the ductus bursae, the connecting duct between the ostium and sperm storage organ, would be shorter and that females of SBS are likely to have a more heavily sclerotized and externalized genital plate, or sterigma, to help deflect plugging by the males (Orr, 1988). Orr

(1988) outlines potential types of genitalia found in SB females. Type 0 is the ancestral form that has a shallow sinus and no sclerotization. Type I has either a flat, convex, or projecting structure surrounding an exposed ostium. Type II has a deep sinus but reduced sclerotization and projections. Finally, type III has a medium to deep sinus with complex genital sclerotization and projections. It is expected that females of SBS will have a higher frequency of type I and type III genitalia and non-SBS females will have a higher frequency of type 0 genitalia. Analyzing the structure of the genitalia may reveal morphological traits found only in SBS, which could illuminate the impacts of the sphragis on genital morphology and the ongoing arms race between the sexes.

Methods

A total of 19 species of *Acraea* were dissected, 9 species with a sphragis and 10 without a sphragis, and their genitalia examined and photographed. For each species, 3 females and 3 males were dissected, except for a non-SBS, *A. bonasia*, in which only males were dissected. All of the dissected genitalia came from specimens deposited in the McGuire Center for Lepidoptera and Biodiversity (MGCL), Florida Museum of Natural History, Gainesville, FL. Abdomens were removed from the dried museum specimens, labeled, and placed in 1.5ml tubes. They were then macerated in 10% KOH at 80^{o}C for 15-20 minutes depending on the size of the abdomen. Abdomens were rinsed in distilled water and stored in a 1.5ml tube with ethanol. Following the dissection protocol described in Lafontaine (2004) a paintbrush was used to gently remove the scales and the abdomen pleuron ripped until the eighth segment. Male abdomens were cut, and the genitalia removed as one capsule. Female genitalia were carefully removed from the eighth segment to keep the sterigma and ovipositor connected and intact.

Genitalia were examined using a dissecting microscope and pictures of every prepared genitalia were shot using a Canon EOS 6D camera with an infinity Model K2 LongDistance Microscope with a CF-4 objective. For males, the genitalia were orientated laterally and dorsally, and for females, they were taken laterally and ventrally. For each image, 20 shots were taken across a series of close-spaced focal planes using the Automated Macro Rail for Focus Stacking StackShot (Cognisys Inc., Traverse City, MI). These were later stacked using the software Helicon Focus (Helicon Soft, Ukraine). These images were examined and used as reference for the morphological descriptions.

Genitalia measurements were conducted using the ImageJ software (Schneider et al. 2012). The analysis was focused on the aedeagus, valvae and claspers of males and the ostium and ductus bursae of females (terminology follows Klots, 1970). Measurements were taken from three individuals of each sex in each species and then averaged resulting in an average for males and females for each species. For males, the length of the uncus and tegumen were taken together and the aedeagus was measured from the lateral view. Due to the curvature of the aedeagus, it was measured using the line segment tool in ImageJ. The ratio between the length of the aedeagus and length of the tegumen plus uncus was calculated as a measure for the aedeagus that could be compared across species. For females, the length of the ductus and area of the ostium was measured from the ventral view. Due to the short length of the ductus and difficulty in measuring it, a threshold was established in which a ductus is considered "short" if it is less than 0.3 mm in length, "medium" if its length is between 0.3 and 0.5 mm and "long" of it is longer than 0.5mm. The area of the ostium that was measured included the copulatory opening and the surrounding sclerotization, using the polygon feature in ImageJ. The entire area of genitalia from ventral view was also measured. The ratio between the ostium area and entire area of genitalia was calculated as measure of the area of the ostium. An average of those ratios was then calculated.

For all measurements the standard deviation and 95% confidence interval was calculated. A two-sample t-test (two-tailed, equal variance assumed) was conducted to test for differences in aedeagus length and ostium area between species with and without a sphragis. The ductus length was categorized based on the established thresholds and the average category length across species was calculated.

Results

After the analyses of 115 specimens, clear differences were found in male and female genitalia between SB and non-SBS. These differences are detailed below.

Females

sphragis-bearing species.

These species lacked a sinus vaginalis, had an exposed ostium, and had a heavily sclerotized sterigma with a range of projections. Orr (1988) outlines a variety of these structures in his system of genitalia types and this system was modified for the purposes of this study. These classifications are subjective and do not account for all possible genitalia structures but provide a framework for comparing morphologies that are important to the sphragis and may represent traits adapted by the females to prevent plugging.

A total of four species were classified as having type I genitalia, two species as type II, and two species as type III, and one species that could not be placed in the classification scheme. Type I, the most common type, is where the pre and post vaginalis (Orr, 1988) are heavily sclerotized and protrude out past the ostium, creating a disk shape around the large ostium opening. *Acraea issoria* (Figure 1) is one such example. In some cases, only the pre vaginalis will protrude past the ostium but both the pre and post vaginalis are heavily sclerotized. In most species, there are also some form of armaments.

In type II the armaments are lacking and the sterigma is sclerotized around the ostium opening, forming a flat disk without any protrusions. *Acraea anacreon* provides an excellent example of this type as the ostium lacks projections but is heavily sclerotized around the large opening (Figure 2).

Figure 1. *Acraea issoria*, ventral view. Shown with 1 mm scale bar and arrow pointing to ostium. An example of type I genitalia.

Figure 2. *Acraea anacreaon*, ventral view. Shown with 1 mm scale bar and arrow pointing to ostium. An example of type II genitalia.

Finally, in type III genitalia, the sterigma forms a heavily sclerotized convex plate. The ostium opening is often small or tucked into the sinus vaginalis. *Acraea alciope* provides an example of this type as the genitalia has a very sclerotized convex plate (Figure 3).

There is one notable exception in the females, *Acraea penelope,* which could not be placed into the classification scheme. The sinus vaginalis is present and the ostium opening is tucked in.

It also lacks the heavy sclerotization of the sterigma found in most species. The total average area of the ostium for SBS females was 0.164mm^2 (Table 1).

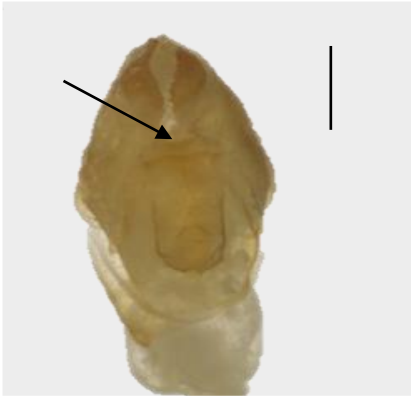

Figure 3. *Acraea alciope*, ventral view. Shown with 1 mm scale bar and arrow pointing to ostium. Example of type III genitalia.

non-sphragis-bearing species.

There appeared to be two main types of genital plates in species without a sphragis. One type has a highly sclerotized sterigma with no protrusions and a small ostium opening as in type III genitalia. The other type has very little sclerotization and the ostium opening is small and tucked into the sinus vaginalis, like type 0 described by Orr (1988). There are some notable exceptions to these however, including *A. acerata* and *A. johnstoni*, as well as *A. natalica* which is vastly different from all the other *Acraea* examined in this study. *Acraea natalica* is especially noteworthy because the females have a very long ductus (on average, 16mm) with a small bursae and the ostium is sclerotized with a bifurcated protrusion. *Acraea acerata* has a very large, sclerotized ostium with the pre vaginalis protruding out, as in type II described earlier. *Acraea johnstoni* also has an externalized ostium with a fairly large ostium opening.

The total average across all species was 0.208 mm^2 which is larger than the average of the SBS (Table 1).

measurement data.

Measurements of the area of the ostium were not found to be significant, with no difference between the ostium areas of SBS and non-SBS females (t= -0.58174, df=16, P=0.5688; Figure

4). Based on the threshold and established categories, the SBS do appear to have a shorter ductus on average.

Table 1. Average ostium area, ductus length and genitalia type for each SBS and non-SBS

SBS	Average Area of ostium(mm^2)	Ductus length(mm)	Ductus category (short/medium/long)	Genitalia Type
A. alciope	0.181	< 0.5	medium	3
A. anacreon	0.24	< 0.5	medium	2
A. issoria	0.189	< 0.3	short	1
A. penelope	0.036	≤ 0.5	medium	N/A
A. pharsalus	0.217	≤ 0.3	short	1
A. polis	0.21	< 0.3	short	1
A. rahira	0.155	< 0.3	short	1
A. semivitrea	0.158	> 0.5	long	3
A. serena	0.087	< 0.3	short	2
Total Average	0.164	< 0.3	short	
Non-SBS	**Average Area of ostium(mm^2)**	**Ductus length(mm)**	**Ductus category (short/medium/long)**	**Genitalia Type**
A. acerata	0.179	< 0.5	medium	2
A. bonasia	0.083	< 0.3	short	0
A. circeis	0.029	< 0.5	medium	0
A. encedon	0.069	> 0.5	long	0
A. esebria	0.337	≤ 0.5	medium	3
A. jodutta	0.133	< 0.3	short	3
A. johnstoni	0.2	> 0.5	long	2
A. natalica	0.955	> 0.5	long	N/A
A. parrhasia	0.035	< 0.5	medium	3
A. peneleos	0.056	> 0.5	long	0
Total Average	0.208	0.5	medium/long	

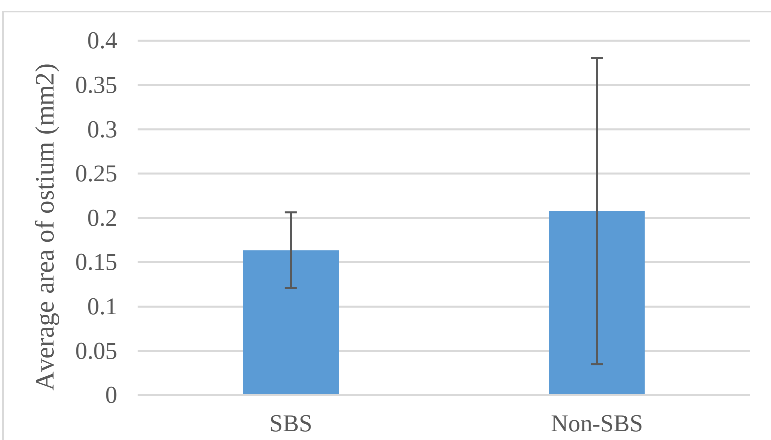

Figure 4. Average area (in mm^2) of the ostium compared between SBS and non-SBS with 95% CI error bars.

Males

sphragis-bearing species.

In SBS, the aedeagus is very sclerotized and the uncus has a variety of shapes, but the majority seem to be long, thin and pointed. The presence and absence of claspers on the posterior end of the valvae was also examined.

In SB males, the aedeagus does tend to be shorter but this varies a lot by species. The average length of the aedeagus of all the species, was 1.383 mm (Table 2).

Figure 5. *Acraea alciope*, dorsal view. Shown with 1 mm scale bar. Arrows point to an example of claspers found on valvae and the aedeagus.

Acraea p*enelope and A. alciope* once again provide an interesting study. In *Acraea penelope* the uncus is short and the valvae have claspers terminally. *Acraea alciope* also has claspers and the aedeagus appears bifurcated terminally (Figure 5). These two species are in the minority as only one other SBS (*Acraea semivitrea*) have the clasper structures at the end of the valvae.

non-sphragis-bearing species.

There were no obvious morphological trends in these species and the average size of the aedeagus was 1.776 mm, which is slightly larger than that of SBS (Table 2). There does seem to be a higher percentage of males that have some form of claspers on their valvae. Of the nine species dissected, seven have the appearance of claspers or clasper-like structures on the valvae (77.8%, see Figure 6). *Acraea natalica* are noticeably distinct from the other *Acraea* species;

males have a very long aedeagus (approximately 8 mm) and saccus, and the uncus is large and points down almost 90 degrees.

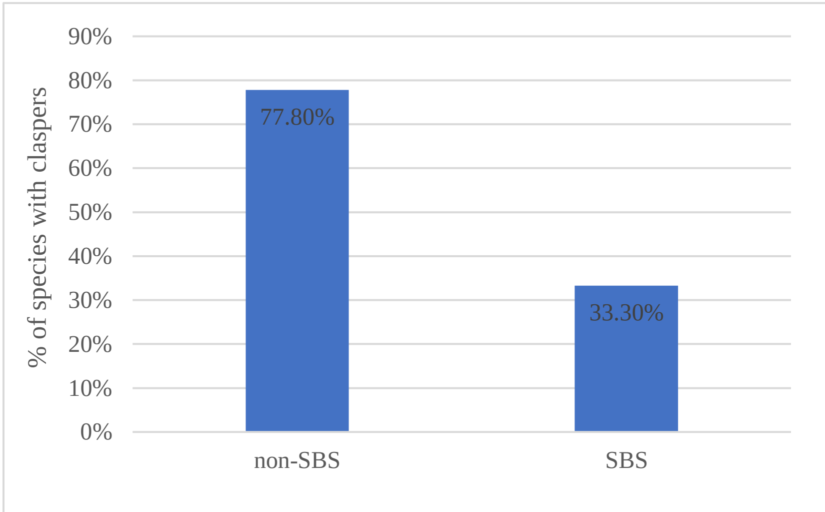

Figure 6. Percent of clasper structures found on non-SB and SB *Acraea* species

measurement data.

The average aedeagus lengths were not significantly different between SBS and non-SBS ($t = -1.874$, $df = 16$, $P = 0.079$; Figure 7).

Table 2. Average length of the aedagus in mm of each of the SBS and non-SBS

SBS	Average aedagus size(mm)	non-SBS	Average aedagus size(mm)
A. alciope	1.678	*A. acerata*	1.563
A. anacreon	1.3	*A. circeis*	1.671
A. issoria	1.198	*A. encedon*	1.579
A. penelope	1.224	*A. esebria*	1.628
A. pharsalus	1.354	*A. jodutta*	1.943
A, polis	1.354	*A. johnstoni*	1.506
A. rahira	1.226	*A. natalica*	3.301
A. semivitrea	1.459	*A. parrhasia*	1.602
A. serena	1.653	*A. peneleos*	1.194
Total Average	1.383	Total Average	1.776

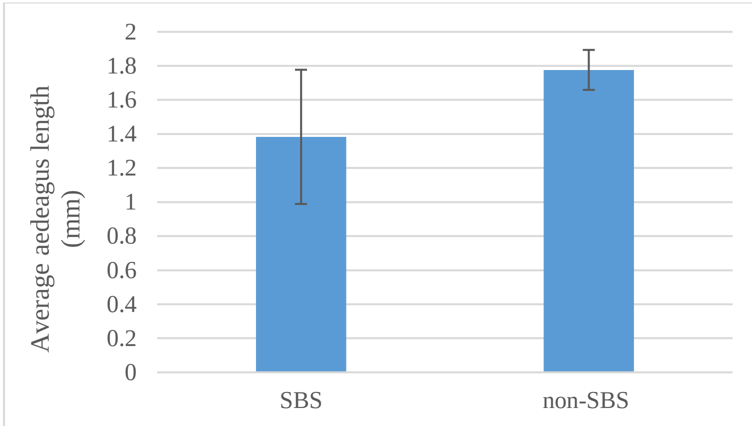

Figure 7. Average aedeagus length (mm) of SBS vs non-SBS.

Discussion

Female SBS typically had similar morphological characters among species. Most females have heavily sclerotized and external copulatory openings (ostium). The ostium and the surrounding genitalia plate (sterigma) are sclerotized, but this varies by species. The bursae also tend to be small and the ductus very reduced, but this varies by species and non-SBS also may have a short ductus. Type I genitalia was the most common in SBS and in non-SBS type 0 genitalia was most common. This supports the original hypothesis and shows that the genitalia type with heavy sclerotization (Type I) is more common in SBS.

This description of morphological characters has helped to illuminate a possible pattern in sexually antagonistic traits in *Acraea* butterflies. There were certain specific characteristics that appeared to be found more prominently in *Acraea* species with a sphragis than those without. This is illustrated in the types of genitalia found in the SB females, type I being more common. SB females have much more sclerotization around the ostium and projections than females of species that do not produce a sphragis. In females of species where plugging by males is common, this could be an adaptation by the females to resist male plugging. The reasons for the morphology of the sterigma and sinus vaginalis, as Orr (1988) points out, have three possible causes; to ensure copulation, to better retain sperm, and to give the females control over how long and how often they mate. The present study was concerned with this final reasoning and helped to describe the possible adaptations females could have developed in response to the sphragis plugging by males. Three types of sclerotization in female genitalia were documented and could illustrate the degree to which sphragis plugging is detrimental to the females of that species.

Within the genus *Acraea,* there is little variation in measurements between sphragis and non-SBS. The measurements of genitalia structures in males and females were not significantly different. This could be due the relatively small sample size, as only a total of 19 species were dissected. Genitalia measurements may also be ineffective for comparing between species. However, there are clear structural differences in the genitalia, such as the level of sclerotization, between the females of each group, and these differences may provide a clearer distinction between SBS and non-SBS within the genus. Future studies could investigate which types of genitalia, if any, are present in species that have higher frequencies of plugging by males.

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