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RESEARCH ARTICLE

Dynamic digestive physiology of a female reproductive organ in a polyandrous butterfly

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ABSTRACT

Reproductive traits experience high levels of selection because of their direct ties to fitness, often resulting in rapid adaptive evolution. Much of the work in this area has focused on male reproductive traits. However, a more comprehensive understanding of female reproductive adaptations and their relationship to male characters is crucial to uncover the relative roles of sexual cooperation and conflict in driving co-evolutionary dynamics between the sexes. We focus on the physiology of a complex female reproductive adaptation in butterflies and moths: a stomach-like organ in the female reproductive tract called the bursa copulatrix that digests the male ejaculate (spermatophore). Little is known about how the bursa digests the spermatophore. We characterized bursa proteolytic capacity in relation to female state in the polyandrous butterfly *Pieris rapae*. We found that the virgin bursa exhibits extremely high levels of proteolytic activity. Furthermore, in virgin females, bursal proteolytic capacity increases with time since eclosion and ambient temperature, but is not sensitive to the pre-mating social environment. Post copulation, bursal proteolytic activity decreases rapidly before rebounding toward the end of a mating cycle, suggesting active female regulation of proteolysis and/or potential quenching of proteolysis by male ejaculate constituents. Using transcriptomic and proteomic approaches, we report identities for nine proteases actively transcribed by bursal tissue and/or expressed in the bursal lumen that may contribute to observed bursal proteolysis. We discuss how these dynamic physiological characteristics may function as female adaptations resulting from sexual conflict over female remating rate in this polyandrous butterfly.

KEY WORDS: Lepidoptera, Bursa copulatrix, *Pieris rapae*, Proteolysis, Sexual conflict, Spermatophore

INTRODUCTION

Reproductive characteristics are some of the most rapidly evolving traits (Clark et al., 2006; Torgerson et al., 2002). This rapid evolution is often thought to be the result of sexual co-evolution as each sex aims to increase their reproductive success. Such co-evolution may involve adaptive changes that increase the fitness of both sexes through reproductive cooperation. Alternatively, antagonistic co-evolution can occur when selection favors adaptations that increase the fitness of one sex at the expense of the opposite sex (Pitnick et al., 2003; Swanson and Vacquier, 2002). This latter situation, called sexual conflict, has been the subject of intensive research effort over the past two decades, with a particular focus on male reproductive traits that impose fitness costs on females during or following copulation (Arnqvist and Rowe, 1995; Rowe and Day, 2006). However, work on related female adaptations has lagged significantly behind, despite repeated calls for increased research attention to female reproductive traits (Ah-King et al., 2014; Méndez and Córdoba-Aguilar, 2004; Simmons, 2014). In fact, over the past decade, the male bias in the study of primary reproductive traits has worsened, rather than improved (Ah-King et al., 2014).

There are a number of compelling reasons to focus more attention on female reproductive adaptations. First, they should directly inform our understanding of how males and females interact upon reproduction. The implicit view provided by our male-biased knowledge base is that females are passive or less-active participants in key reproductive interactions. However, there is no clear argument for why this might always, or even often, be the case. Rather, female reproductive adaptations have been identified in all systems where females have been rigorously studied (Holman and Snook, 2006; Kelleher et al., 2007; Knowles and Markow, 2001; Simmons and Gwynne, 1991). However, more case studies are required to better inform how and why females influence reproductive outcomes using specific adaptations. An additional benefit to studying female reproductive traits is that this should enable us to identify reproductive interfaces subject to male–female co-evolution. Critical tests that parse between putative mechanisms of reproductive co-evolution (e.g. sexual conflict versus cooperative co-evolution) are much needed. However, in the absence of knowledge of both male and female traits, co-evolutionary explanations for reproductive diversity remain in the realm of speculation. Lastly, characterization of female reproductive adaptations should allow us to better understand reproductive incompatibilities that play a role in pre- and/or post-zygotic isolation during speciation (Orr, 2005; Swanson and Vacquier, 2002).

One promising interface for identifying female reproductive adaptations is the processing of male ejaculates by the female reproductive tract. During copulation, males often transfer complex mixtures of ejaculatory proteins to the female alongside their sperm (Perry et al., 2013; Vahed, 1998). The female reproductive system interacts with these diverse male proteins in ways that we are only just beginning to understand (Findlay et al., 2014; Ram and Wolfner, 2007; Wolfner, 2009). However, it is clear that these interactions form a key interface for both male and female reproductive fitness. Ejaculate proteins can directly influence male reproductive success via their role in both fertilization and sperm competition (Fiumera et al., 2005, 2006; Reinhart et al., 2015). For example, ejaculate proteins have been shown to provide energetic substrates and aid in sperm mobility, resulting in increased fertilization rates and male paternity share (Gillott, 2003). Ejaculate proteins have also been implicated in a wide range of effects on female post-copulatory phenotypes, including reduced female receptivity to subsequent mates, increased female reproductive output and reduced female

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lifespan (Perry et al., 2013; Ram and Wolfner, 2007). In addition, male ejaculates often form copulatory plugs that reduce the female remating rate (Baer et al., 2001; Bretman et al., 2010; Shine et al., 2000), although they can also be important for general fertility (Dean, 2013). Such effects may often benefit males at the expense of their female mates (Oberhauser, 1989; Wolfner, 1997), resulting in the potential for antagonistic co-evolution between manipulative compounds in male ejaculates and counter-adaptations in the female reproductive tract.

Identifying specific female adaptations to manipulative male ejaculate substances has proven to be challenging. This is, in part, due to the fact that many key interactions between male ejaculates and female reproductive adaptations occur within the main channel of the female reproductive tract [i.e. the vagina and oviducts (Kelleher et al., 2007; Knowles and Markow, 2001)]. Because a wide range of reproductive processes occur in this reproductive region, the specific function of focal female traits is often not clear. Nevertheless, researchers have begun identifying a number of female physiological traits that may serve as counter-adaptations to male ejaculatory substances. These include secreted proteases that may function to dislodge male copulatory plugs and/or de-activate manipulative ejaculate compounds (Kelleher et al., 2007, 2011), female receptors and associated hormonal processes that respond to male ejaculate constituents (Adams et al., 2005; Yapici et al., 2008) and morphological features of the female reproductive tract that could play a role in cryptic female choice via their effect on male fertilization success and sperm competition (Pitnick et al., 2003).

These female traits present promising leads for understanding reproductive co-evolution between male ejaculates and the female reproductive tract. However, with the exception of recent work on hormonal changes in the female reproductive tract following copulation (Heifetz et al., 2014), we know almost nothing about how these female traits change dynamically in response to female state or male ejaculatory secretions. For example, are female reproductive adaptations modulated by age or temperature? Are female reproductive adaptations sensitive to social cues such as the presence of conspecifics or potential mates? How do specific female adaptations respond to mating and how do they change post copulation? Research on this front is critical for understanding how these traits mediate male–female interactions as well as how they function across individual female life histories. However, with the exception of a few recent studies in *Drosophila melanogaster* (Bono et al., 2011; Kelleher and Pennington, 2009; Mack et al., 2006; McGraw et al., 2008), such information is largely lacking for even well-studied organisms.

We sought to answer these questions by focusing on a specific reproductive interaction in the polyandrous butterfly *Pieris rapae*: the digestion of the male ejaculate or spermatophore by a specialized organ in the female reproductive tract called the bursa copulatrix (hereafter bursa). In insects, bursae are common features of female reproductive tracts. Bursae that play a role in spermatophore processing have been described in the Coleoptera (van der Reijden et al., 1997), Trichoptera (Khalifa, 1949) and Lepidoptera (Arnqvist and Nilsson, 2000; Oberhauser, 1989; Vahed, 1998). In the Lepidoptera, the bursa copulatrix serves specifically to receive and break down the male spermatophore (Engelmann, 1970; Watanabe et al., 1998; Wiklund et al., 2001). Following spermatophore transfer, sperm migrate to a specialized sperm storage organ called the spermatheca (Rutowski and Gilchrist, 1986), leaving the bursa to process the remaining ejaculatory compounds.

Subsequent processing of the spermatophore by the bursa has important consequences for male and female fitness in the Lepidoptera. Females utilize ejaculate proteins for somatic maintenance and egg production (Boggs and Gilbert, 1979). Thus, in polyandrous lineages, females often gain fitness benefits from mating multiple times via increases in their lifespan and fecundity (Wiklund et al., 2001). However, the spermatophore can also function to reduce the female remating rate. Females typically do not remate until the spermatophore has been absorbed enough to allow space for another spermatophore (Oberhauser, 1989; Sugawara, 1979). Thus, male traits that delay spermatophore processing are likely to benefit male paternity share in polyandrous lineages (Sánchez et al., 2011). Researchers have begun to identify male and female traits that influence bursal processing of the spermatophore. These include tough outer spermatophore envelopes that delay female access to the softer material inside the spermatophore (Sánchez and Cordero, 2014) and toothed, muscularized devices attached to the bursal wall called signa, which serve as counter-adaptations via their role in mechanically abrading the spermatophore envelopes (Sánchez et al., 2011). The presence of tough envelopes and bursal signa both appear to be favored in more polyandrous lineages (Sánchez and Cordero, 2014; Sánchez et al., 2011).

Although researchers have often described bursal processing of the spermatophore as a digestive process, little is known about how this is accomplished physiologically or whether it involves enzymatic digestion at all. We therefore sought to first establish whether females produce protein-digesting enzymes in the bursa, or simply absorb male ejaculate proteins without enzymatic processing. We then asked whether female enzymatic activity was influenced by female state. More specifically, we evaluated changes in female bursal proteolysis related to female age, adult temperature and social experience. Dynamics of bursal proteolysis may help to provide insights into the control of bursal physiology, as well as the consequences of phenotypic plasticity for male–female reproductive interactions.

We first examined the effect of age. We expected to see either high levels of digestive activity at eclosion, implying that females eclose fully sexually mature and prepared to engage in spermatophore digestion immediately, or alternatively, that females eclose with low proteolytic capacity but increase their proteolytic capacity with age. This latter pattern would imply that adult females must produce and actively secrete proteolytic enzymes into the bursal lumen in advance of their first mating. Second, we tested the effect of the pre-mating social environment on digestive activity. It is well established that male traits, such as ejaculate composition, can vary in response to social cues (Cornwallis and Birkhead, 2007; Ramm and Stockley, 2009; Sirot et al., 2011; Smith and Ryan, 2011; Wigby et al., 2009). However, female responses to social cues remain largely unknown. Because digestive enzymes can present a physiological liability at high concentrations (Hirota et al., 2006; van Hoef et al., 2011), we expected that exposure to courting males might stimulate females to increase protease production in anticipation of imminent mating. However, if males are constantly present in a virgin female's environment or females rarely have the opportunity to realize a benefit from such phenotypic plasticity, we would expect little to no effect of male exposure on bursal physiology. Finally, we examined the effect of mating upon bursal enzymatic activity. We predicted that mating would stimulate increased bursal proteolytic activity, potentially leading to increased levels of proteolytic activity post copulation. Alternatively, a female's digestive activity might

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decrease, either as a result of adaptive regulation of bursal physiology by the female or as a result of enzymatic quenching by male ejaculate constituents (Dean et al., 2009).

We followed these physiological studies with work to preliminarily establish the identities of female proteases that underlie the proteolytic activity we observed in the bursal lumen. Using a combination of transcriptomic and proteomic approaches, we sought to identify proteases either highly transcribed in bursal tissue and/or expressed in detectable quantities in the bursal lumen of sexually mature females.

RESULTS

In all assays, the bursa exhibited high levels of protein digestive activity. Bursal proteolytic activity also appears to be influenced by the female's age, abiotic environment and mating status. Summary statistics for all studies are reported in Table 1.

Virgin age study

Digestive activity increased with increasing age of virgin females. In newly eclosed virgin females (N=10), digestive activity in the bursa was very low and not statistically different from that of our negative control tissue, the adult butterfly leg (N=10). However, as females aged, the mean amount of protein digesting activity in the bursa copulatrix increased at 1 day following eclosion (N=10) and 3 days post eclosion (N=9) to levels substantially higher than the leg, and equivalent to, if not higher than, the midgut of 4th instar larvae (N=10; F=11.440, d.f.=4, P<0.001) (Fig. 1).

Social environment study

The pre-mating social environment, namely exposure to males or other females, did not affect the level of digestive enzymatic activity within a female's bursa (F=0.363, d.f.=2, P=0.698). However, there was a significant difference in enzymatic activity between virgin females that were exposed to cooler temperatures (19°C) versus warmer temperatures (25°C) during the social environment experiment (F=19.777, d.f.=1, P<0.001) (Fig. 2). No interaction was observed between temperature and social environment (F=0.357, d.f.=2, P=0.702). The high interaction density in these constructed social environments combined with reduced female nectar foraging during trials led to some female mortality during the experiment, but mortality rates were not dependent on treatment (χ^2 d.f.=2, P=0.421).

Table 1. Summary statistics for proteolytic activity of all tissues and treatment groups

	Sample size	Proteolytic activity (mean units per organ±95% CI)
Leg	10	0.53±0.3
Caterpillar intestine (gut)	10	7.68±4.8
Virgin age study		
Virgin 0 day	10	2.21±1.4
Virgin 1 day	10	13.03±8.1
Virgin 3 day	9	19.98±13.1
Social environment study		
Alone, cool	6	20.76±5.0
Alone, warm	4	38.38±11.7
Same sex, cool	10	24.46±4.5
Same sex, warm	6	35.25±4.8
Both sexes, cool	9	25.82±4.6
Both sexes, warm	10	38.77±9.5
Mating study		
Unmated females	11	9.57±5.7
Mated 1 day	9	6.94±4.5
Mated 3 day	9	2.31±1.5
Mated 5 day	4	6.58±2.5

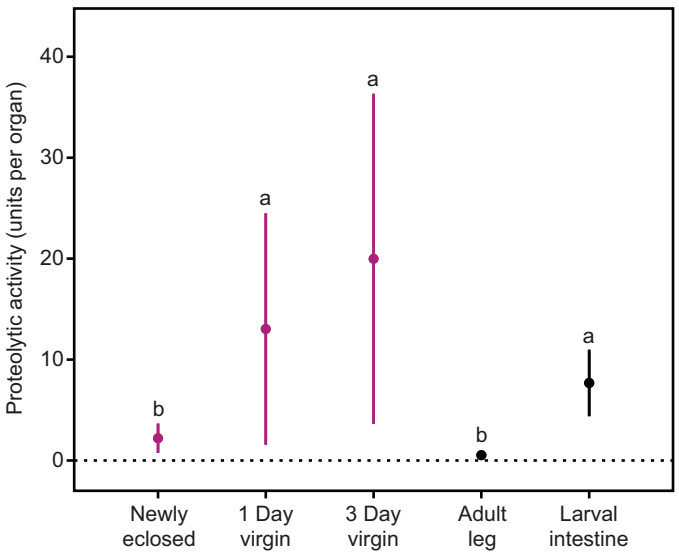

Fig. 1. Bursal proteolytic activity increases with female age in virgin *Pieris rapae* butterflies. Data are presented as means±95% confidence intervals. Lowercase letters indicate statistical groupings.

Mating study

We measured protein digestive activity in the bursa for unmated females (N=11), females 1 day after mating (N=9), females 3 days after mating (N=9) and females 5 days after mating (N=4) to determine the effect of mating on bursal proteolysis. Protein digestive activity in the virgin bursa begins at a significantly higher activity level than the butterfly leg (F=10.946, d.f.=5, P<0.001). Following mating, bursal proteolysis remains detectably higher than levels found in the adult butterfly leg. However, proteolysis shows a declining trend in both mean and variance during the first 3 days post copulation before increasing again on day 5 (Fig. 3), although differences between these time points were not statistically significant.

Protein identification

By extracting the proteins present in the bursal lumen, we were able to identify potential proteases that may act in the bursal digestive process. Mass spectrometry of the bursal lumen identified five unique proteases at protein identification probabilities exceeding 99% (Table 2). Using bursal transcriptomic data also collected from *P. rapae,* we identified an additional four proteases that are highly transcribed in the bursa. Their protein products contain secretion signals, making them also likely to act in the bursal lumen (Meslin et al., 2015). Thus, our transcriptomic and proteomic analyses identified a total of nine proteases with a putative role in bursal proteolysis (Table 2). These included two trypsin-like serine proteases, five papain family cysteine proteases and two proteases with poorly classified peptidase domains.

DISCUSSION

We found that the female bursa copulatrix is a dynamic and highly proteolytic organ system. Our study represents the first clear identification of active protein digestion by the lepidopteran bursa and also reveals the identities of nine proteases that are likely to contribute to bursal proteolysis. Although standard accounts of bursal function generally describe it as the site of spermatophore

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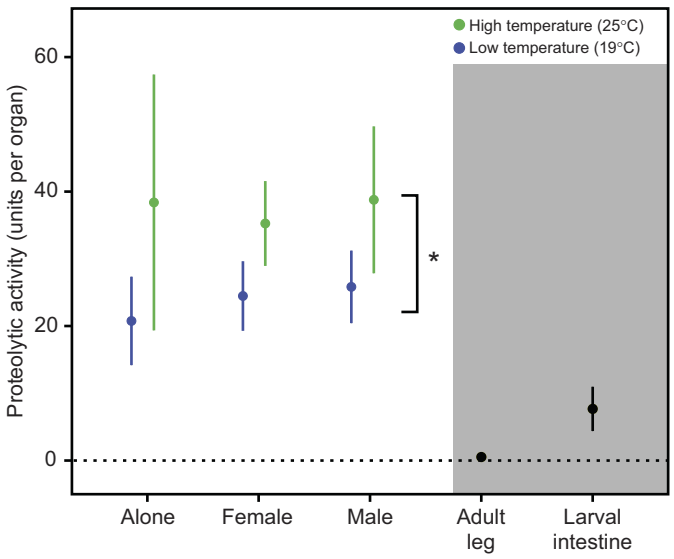

Fig. 2. Bursal proteolytic activity of virgins is not affected by exposure to courting males. Higher ambient temperatures (25°C, plotted in green) do significantly increase the digestive activity of the bursa as compared to lower ambient temperatures (19°C, plotted in blue). Data are presented as means± 95% confidence intervals. Proteolytic activity of adult leg and caterpillar intestine are presented for comparison, but were not included in the statistical analysis.

digestion (Engelmann, 1970), researchers have yet to directly quantify the proteolytic capacity of the bursa itself. Rather, studies of spermatophore processing in the silkworm *Bombyx mori* have suggested that male-donated enzymes are responsible for spermatophore breakdown (Osanai and Kasuga, 1990; Osanai et al., 1987). In contrast, we find that the bursa of *P. rapae* is extremely proteolytic, achieving levels of digestive activity equivalent to, if not higher than the midgut, which is the region of the larval gut responsible for protein digestion. While this finding is notable in absolute terms, it is even more impressive when

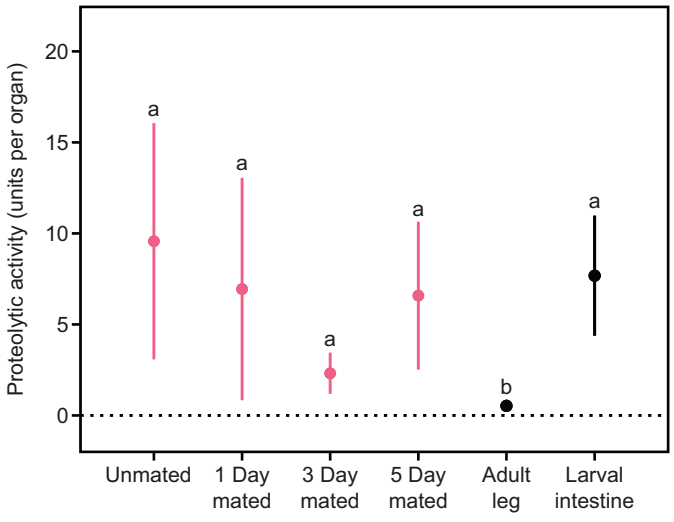

Fig. 3. After mating, bursal proteolytic activity decreases before rebounding at the end of a typical female refractory period. Data are presented as means±95% confidence intervals. Lowercase letters indicate statistical groupings.

considered in relation to the size of these two digestive organ systems. The larval midgut is roughly 20 mg, while the bursa is on average 1 mg. This means that an organ 1/20th the size of the larval midgut is able to produce equivalent amounts of proteolytic enzymes and implies that the bursal lumen exhibits substantially higher enzyme concentrations than the midgut. The extent to which this relatively extreme level of proteolysis is representative of bursal function across the Lepidoptera is unknown.

We also found that bursal proteolytic capacity is dynamic. Virgin females eclose with low levels of proteolytic activity in their bursae, but bursal proteolytic activity then increases steadily with age. This pattern is consistent with gradual secretion of proteolytic enzymes following adult eclosion, or alternatively, a gradual release of these enzymes from storage in the bursal tissue. However, we found no detectable levels of proteolytic activity in virgin bursal tissue following rinsing of the bursal lumen (data not shown). This implies that proteolytic enzymes are not stored in detectable quantities in bursal tissue and thus that active secretion of enzymes into the bursal lumen following synthesis is more likely. We do not know whether this process of gradual accrual of enzymes in the bursal lumen presents a liability for older unmated females as a result of autodigestion. Intestinal tissues and other digestive organs exhibit preservative or inhibitory mechanisms that reduce the risk of autophagy, such as storing enzymes in inactive forms or secreting specialized inhibitor-like proteins (Hirota et al., 2006; van Hoef et al., 2011). Whether such mechanisms are also present in the bursa is not known, but could present a fruitful avenue for further study. However, it is possible that females rarely experience such potential detrimental effects of high proteolytic activity in the wild, because nearly all females mate within the first several days of adult life (Watanabe and Ando, 1993).

In contrast to enzymatic changes associated with female age, we find no evidence that bursal proteolysis is modulated by the social experience of virgin females. We postulated that females might upregulate their proteolytic activity upon exposure to courting males as a ‘priming’ strategy prior to mating. This could be particularly relevant if bursal enzymes are costly, because of either enzyme synthesis costs or an increased risk of autophagy. However, we observed no effect of either the presence of conspecific females or conspecific males on female bursal proteolytic activity. This result may indicate that the rate at which wild females encounter prospective mates is sufficiently high that there is little fitness benefit to such phenotypic plasticity. However, we did observe that females experiencing higher ambient temperatures in these social exposure trials exhibit detectably higher levels of proteolytic activity. Again, this is consistent with our hypothesis above that females gradually synthesize and secrete enzymes into the bursal lumen. Increased ambient temperatures should raise the metabolic rate of these females, leading to increases in the rate of a number of biochemical processes, including the synthesis of bursal enzymes.

Following female mating, we found a trend indicating that bursal proteolytic activity declines over the first several days post mating. This decline may be the result of several processes. First, bursal proteases may be absorbed or incorporated into the male spermatophore during or following its deposition in the bursa, resulting in a decline in proteolytic activity in the bursal lumen. In addition, protease inhibitors are not uncommon in male ejaculate cocktails (Dean et al., 2009). Thus, male spermatophore constituents may act to directly reduce female proteolytic activity. Finally, females may themselves down-regulate bursal proteolysis following mating, a possibility supported by our data quantifying post-mating changes in bursal transcriptional profiles (Meslin et al., 2015).

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Table 2. Summary of putative proteases identified in the bursal lumen

Component number	Domain(s) found	Method of detection
comp93091_c0	CLIP, trypsin-like serine protease	RNA
comp94445_c1	Cathepsin propeptide inhibitor, papain family cysteine protease (Pept_C1)	RNA
comp95264_c1	Cystatin-like domain (CY), papain family cysteine protease (Pept_C1)	RNA
comp98020_c0	Peptidase_MA_2, ERAP1_C	RNA
comp97068_c0	Peptidase_S28	Proteomics
comp91676_c0	Cathepsin propeptide inhibitor domain, papain family cysteine protease (Pept_C1)	Proteomics
comp83824_c0	Trypsin-like serine protease	Proteomics
comp85455_c0	Papain family cysteine protease (Pept_C1)	Proteomics
comp83827_c1	Cathepsin propeptide inhibitor domain, papain family cysteine protease (Pept_C1)	Proteomics

Following mating, transcription of a number of bursal proteases decreases (Meslin et al., 2015). This rather counterintuitive result may indicate that females initially focus on enzymatically digesting the outside of the spermatophore, but then transition to mechanical digestion and subsequent absorption of the spermatophore contents. Interestingly, at 5 days post mating, we observe an increasing trend in proteolytic activity to approximately the levels seen 1 day post mating. This corresponds roughly to the length of the typical 'mating cycle' in this species [i.e. the length of the refractory period, beyond which females are willing to remate (Suzuki, 1979)]. Thus, this potential increase in proteolytic activity could indicate that females ramp up digestive enzyme production to prepare for the next spermatophore, or alternatively, that female protease production begins to exceed the rate of quenching by the male spermatophore at this stage.

The identities of the proteases further advance our knowledge of the potential modes of action involved in enzymatic digestion of spermatophore proteins. Three main protease classes were observed, including trypsin-like serine proteases and papain family cysteine proteases. While the value of this protease diversity to female reproduction is not clear, it is possible that employing proteases with different modes of action may increase the digestive efficiency of the bursa regardless of spermatophore substrate. Alternatively, these modes of action may reflect matching diversity in male ejaculate proteins, suggesting coevolution between female enzymes and male substrates. Whatever the case, information about female protease identities offers a critical first step in understanding both the function and evolution of female reproductive physiology in these animals. Future work should explore the evolutionary histories and current functions of these intriguing reproductive enzymes. In addition, the physiological dynamics we report here may be underlaid by changes in the titers of a specific subset of proteases. Evidence for independent regulation of these proteases across female reproductive state would provide additional clues into their reproductive role and evolutionary significance.

In conclusion, we found that the female bursa exhibits remarkably high proteolytic capacity and that bursal physiology is dynamic over a female's life experience. These results highlight the importance of attending to female reproductive adaptations and their responsiveness to female state. Future work should focus on the evolutionary importance of male–female interactions within the bursa, the mechanisms driving diversity in these physiological traits and their fitness consequences for both males and females. In addition, future research should explore how females dynamically regulate the suite of proteolytic enzymes we have identified to maximize their digestion of male ejaculate proteins. By expanding this work across a range of lepidopteran species with diverse mating strategies – from monandry to high levels of polyandry – we may begin to better understand the role of sexual conflict and reproductive cooperation in driving male–female reproductive interactions.

MATERIALS AND METHODS

Experimental animals

Pieris rapae rapae Linnaeus 1758 were raised in dedicated climate chambers that maintained a 16 h:8 h light:dark photoperiod at a constant 24°C and 60% relative humidity. Larvae were fed on a diet of kale leaves (*Brassica olercea*) grown on site, fertilized twice a week with Peter's Profession General Purpose 20-20-20. Individuals were all the descendants of wild-caught females collected at local agricultural sites in Rochester, PA (40°44′44.4″N 80°09′49.0″W) and Irwin, PA (40°26′34.4″N 79°74′78.3″W). For the social environment study, we used F1 generation individuals from field-caught females. For the remainder of the studies below, we used individuals from a continuous laboratory population established from wild-caught females in October 2012.

Enzyme collection

Enzyme solutions were collected from tissues of interest by micro-vivisection in ice-cold 10 mmol l^{-1} NaCl. After isolation, excess fluid was removed from the tissues and they were massed in 1.5 ml microcentrifuge tubes and placed on ice. 100 µl of the NaCl solution was added before the tissues were homogenized with 50 turns of a manual pestle. The homogenized solutions were then cooled in ice before centrifugation at 10,000 ***g*** for 15 min. Samples were stored at −20°C until assayed.

Bursal tissues were collected from virgins at specific time points post eclosion and from mated females at either 1, 3 or 5 days post mating. For bursa samples, extra fat bodies and extraneous tissues were removed. For bursae from mated females, the male's spermatophore was removed by peeling open the bursa and carefully pulling out the spermatophore to remove all male ejaculate contributions contained within the spermatophore mass. Bursae were not rinsed internally to avoid the loss of female enzymatic material from the lumen. For all experiments, the caterpillar intestine was used as a positive control because of its known protein-digesting properties and the butterfly leg was used as a negative control because of the low expected levels of proteolytic activity in this body part. For larval intestine sampling, the midgut of the 4th instar intestinal tract was used. Larval guts were isolated along with the contents in order to adequately capture the proteases within the lumen of the midgut (Broadway and Duffey, 1986). Leg tissue was collected from adult butterflies.

Proteolytic activity

We used a modified azocasein assay from previously described protocols (Ajamhassani et al., 2012). 10 µl of each enzyme solution was added to 100 µl of Tris-HCl buffer pH 7 (20 mmol l^{-1}) and 50 µl of 2% azocasein (Sigma-Aldrich, St Louis, MO). The solutions were incubated at 37°C for 60 min before 400 µl of 10% trichloroacetic acid was added. The solutions were then placed on ice for 5 min to allow precipitation of the excess protein–dye complex before centrifuging at 10,000 ***g*** for 10 min. 400 µl of the supernatant was added to an equal amount of 2 mol l^{-1} NaOH. Concurrently, a second set of enzymes were run in an identical fashion, with the exception of skipping the incubation step in order to record a standard time zero for enzymatic activity. The absorbance of the resulting solutions was measured in triplicate using an Epoch microplate absorbance spectrophotometer (BioTech, Winooski, VT, USA) at 450 nm with wells filled to 200 µl. Blanks were run in an identical manner, except instead of the enzyme, only the dissecting solution (10 mmol l^{-1} NaCl) was used.

Enzyme activity is reported in units, defined as the amount of enzyme required to result in a change of 0.01 absorbance per 60 min at 37°C. We confirmed linear responses of the azocasein assay to extracted bursal enzymes both across a range of incubation times (linear R^2=0.988, P<0.01) and enzymes concentrations (linear R^2=0.996, P<0.01). Incubations occurred at a pH of 7 based on previous work on pH-dependent enzymatic activity in the midgut. The enzymes of the late instar larval gut in Lepidoptera peak in activity at a pH of 7 and maintain this high level of activity through a pH of 9 (Ajamhassani et al., 2012; Berenbaum, 1980). Lepidopteran bursa have also been found to possess a pH of 7 (Khalifa, 1950).

Virgin age study

We evaluated changes in proteolytic activity in the bursae of virgin females following eclosion into adulthood. Individuals were collected immediately after eclosion, at 1 day post eclosion or at 3 days post eclosion. Bursae from these virgin females were dissected as described above and the proteolytic activity assayed.

Social environment study

We evaluated the effect of social environment on bursal physiology by measuring bursal proteolytic capacity following exposure to different social scenarios. Upon pupation, individual females were isolated to prevent exposure to other individuals prior to the social exposure treatments. All focal females used for trials had eclosed 2 days prior to the beginning of the treatment. Each trial consisted of three replicates of focal females for each treatment. Focal females were split between treatments designed to provide them with one of three different social environments: (1) isolated (i.e. no social exposure); (2) female-only social exposure; or (3) male and female social exposure. These social environments were accomplished by placing focal females in hanging cylindrical cages with proportional numbers of other butterflies. For the isolated treatment, focal females were housed alone in cages visually isolated from all other treatments (N=3 for each trial). For the female-only social exposure, three focal females were housed with three other virgin females. These non-focal females ranged in age but had never been exposed to males. The female and male social exposure treatment contained three focal females plus three experimentally castrated males. Male castration was accomplished by dripping unscented wax (Mainstays, Bentonville, AR) onto the male claspers and genitalia to prevent successful mating attempts while still permitting normal behavior by the male. To ensure that castrated males responded normally to virgin females and vice versa, we monitored male courtship behaviors, including approaches and copulation attempts. Castrated males courted females at statistically equivalent rates to those observed for non-castrated males (F=3.571, d.f.=1, P=0.132). We did not observe any other salient differences in male behavior or female responses to male courtship. Each trial was run for 24 h within a climate-controlled greenhouse. Temperature, humidity and light levels were monitored and treated as a random variable in statistical analyses. We conducted a total of 11 trials. An initial set of trials (N=7) was run early in the season (May and early June) when greenhouse temperatures were cooler (18.86±0.55°C). We ran a second set of trials (N=4) later in the summer (July), at which time greenhouse temperatures were notably higher despite climate control systems remaining operational (25.19±0.04°C). We consider this difference in temperature in our data analyses. Before being placed in trial enclosures, all individuals were fed 20% honey solution and cages were misted with water every 2 h during the daylight hours of the experiment in order to minimize death by dehydration. Following the 24 h trial period, females were removed, their bursae promptly vivisected, and bursal proteolysis assayed as described above.

Mating study

To analyze the effect of mating on bursal proteolytic activity, we collected bursae from unmated females and mated females 1 day post copulation and 3 days post copulation. Females were mated by housing them with males in a 60 cm×60 cm×90 cm insect mating enclosure in direct sunlight. Because matings typically last 30–45 min, mating enclosures were checked every 20 min to ensure no matings were missed. Males and females found in copula were removed to an individual cup until separation. For females that were analyzed at 1 day post mating, the females remained in this cup at 24°C until dissection. For females that were analyzed at 3 days post mating, the females were also kept at 24°C but provided with a cotton pad soaked with a 20% honey solution until dissection.

Statistical analyses

Statistical analyses were calculated using the statistical program IBM SPSS Statistics, Version 22.0. A one-way ANOVA was performed for all experiments, with the exception of the social environment experiment, where an ANCOVA was performed with ambient temperature as a co-variate. All datasets were evaluated for the assumptions of parametric statistics using Levene's test for normality and spread-versus-level plots for homoscedascity. Two datasets required natural log transformation to achieve data normality (virgin age study and mating study). These were evaluated statistically as transformed data, but are plotted in the manuscript as untransformed data to aid in cross comparisons between studies. Tukey's B test was used for all *post hoc* analyses to determine significance groupings. For the social exposure analysis, caterpillar intestine and leg were not included in the statistical analysis because they did not have an associated temperature value for the ANCOVA analysis.

Protein identification

Putative protein identities were determined using both RNA-sequencing techniques as well as proteomic analyses. RNA-sequencing methods and transcriptional quantification are described in detail in Meslin et al. (2015). In brief, bursas were dissected into 100 µl of RNAlater RNA Stabilizing Reagent (Qiagen, Valencia, CA, USA). RNA was then extracted using TRIzol (Life Technologies, Grand Island, NY, USA) and samples sent to the Genomics Resources Core Facility of Weill Cornell Medical College (New York, NY, USA) for sequencing. Assembly of transcriptomes was accomplished using the Trinity Software Package (v. r2013-02-25) (Haas et al., 2013). Genes coding putative bursa proteases were identified based on high quantitative levels of transcription in bursal tissue as well as the presence of secretion signals, which suggest a high likelihood of protease secretion into the bursal lumen.

For proteomics, bursas from 3-day-old virgin females were vivisected, removed from the abdominal cavity and their outsides rinsed with PBS. Bursas were then cut open and the contents of the lumen suspended in PBS. This lumen extract was then combined with loading buffer (8 mol l^{-1} Urea, 200 mmol l^{-1} Tris-HCl, pH 6.8, 0.1 mmol l^{-1} EDTA, pH 8.0, 100 mmol l^{-1} DTT, 100 mmol l^{-1}Tris base) and incubated at 37°C for 15 min. The solution was then run on a 12% SDS-PAGE gel until the band measured 0.25 cm^2. After staining with Coomassie Blue (Amresco, Solon, OH, USA), the protein band was excised and subsequently submitted to the Biomedical Mass Spectrometry Center at the University of Pittsburgh where liquid chromatography tandem mass spectrometry was used in conjunction with previously acquired transcriptomic sequences in order to determine protein identities within the bursal lumen (Granvogl et al., 2007a,b; Shevchenko et al., 2007). Tandem mass spectrometry data were visualized using Scaffold (Proteome Software, Portland, Oregon, USA), with subsequent annotation of highly abundant proteins using BLASTP.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceived and designed the experiments: M.S.P., N.I.M., A.B.D. and N.L.C. Performed the experiments: M.S.P., A.B.D. and C.M. Analyzed the data: M.S.P., N.I.M., C.M. and N.L.C. Interpreted the data and wrote the manuscript: M.S.P., N.I.M., A.B.D., C.M. and N.L.C.

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