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RESEARCH ARTICLE

Mating for male-derived prostaglandin: a functional explanation for the increased fecundity of mated female crickets?

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ABSTRACT

Direct benefits are considered to be the driving force of high female mating rates, yet species in which females do not receive material resources from males still experience increased fitness from mating frequently. One hypothesis suggests that substances within the ejaculate may boost survival or offspring production. If these materials are limiting to females, they will require continual renewal via mating and could provide a functional understanding of how high mating rates lead to increased female fitness. Using the Texas field cricket, *Gryllus texensis*, we investigated the sexual transfer of prostaglandin E_2, an important mediator of invertebrate reproduction. We determined that like other gryllid species, males include significant quantities of prostaglandin E_2 (PGE_2) and its precursor molecule, arachidonic acid (AA), within the spermatophore. These components are passed to females during copulation and then stored within the spermatheca. We then tested the novel hypothesis that PGE_2 is ephemerally available after mating and that females must frequently mate to maintain access to this limiting compound. We found that PGE_2 within the spermatheca is indeed depleted through time, with only a small amount remaining 1 week after mating, but that its presence can be maintained at high quantities and for prolonged periods of time by remating. Our results support the hypothesis that high female mating rates increase the amount and availability of PGE_2 throughout the breeding season, which could explain the positive relationship between female mating rate and fecundity.

KEY WORDS: *Gryllus texensis*, PGE2, Arachidonic acid, Invertebrate reproduction, Sperm, Spermatophore

INTRODUCTION

Direct fitness benefits obtained by females from mating, such as greater fecundity or longevity, have been widely acknowledged as a driving force of the high mating rates observed in nature (Andersson, 1994; Arnqvist and Nilsson, 2000; Reynolds, 1996). Unlike indirect benefits (e.g. genetic diversity of offspring) that often require mating with multiple males, in many cases females can obtain direct benefits regardless of whether they mate repeatedly with the same male or polyandrously with different males. Numerous studies of direct benefits have focused on species in which females receive nutritious food gifts from males that are used to increase reproductive investment after mating (Gwynne, 1984; Simmons, 1990; Vahed, 1998); yet, a positive correlation between offspring production and mating rate is also pervasive in species that receive no obvious material benefits (Dunn et al., 2005; House et al., 2009; Taylor et al., 2008; reviewed in Arnqvist and Nilsson, 2000; South and Lewis, 2011).

Although fertilization assurance via sperm replenishment is one direct benefit of mating several times (Barbosa et al., 2012; Drnevich et al., 2001; Torok et al., 2003; Uller and Olsson, 2005; Wang and Davis, 2006), many studies have demonstrated the influence that ejaculatory compounds have on reproductive fitness as well. In addition to sperm, ejaculates are composed of a multitude of substances including seminal fluid proteins, sex peptides, salts, sugars, defensive compounds and lipids (Perry et al., 2013), some of which have profound effects on female fitness (Gioti et al., 2012). For example, seminal fluid proteins in *Drosophila melanogaster* are known to increase egg production (Soller et al., 1999), mediate ovulation and oviposition (Heifetz et al., 2000; Taylor et al., 2008), and elevate sperm storage and utilization (Tram and Wolfner, 1999). Additionally, essential nutrients in the ejaculate such as salts, sugars and lipids may act to increase overall female fitness (Boggs, 1990; Gwynne, 2008; Ursprung et al., 2009; Vahed, 1998). If substances comprising the ejaculate significantly increase reproductive output, then females might copulate more frequently to gain access to these fitness-enhancing compounds.

Like *D. melanogaster*, female gryllid crickets do not obtain additional material resources from the male during mating. Despite the lack of nutritional gifts, however, female gryllid crickets that mate multiple times have increased lifetime fecundity relative to females that mate only once (Simmons, 1988; Wagner et al., 2001) and egg output is positively correlated with the number of ejaculates received (Gershman, 2007, 2010). Indirect benefits do affect hatching success (Tregenza and Wedell, 1998) and offspring quality (Simmons, 1988) and should therefore not be overlooked; however, a functional explanation of how females receive direct benefits when only an ejaculate is acquired remains an outstanding question.

That mating multiple times results in increased fecundity in gryllid crickets might be a function of prostaglandins contained within the spermatophore. Prostaglandins are oxygenated metabolites of C_{20} polyunsaturated fatty acids that are fundamental to many aspects of animal physiology (Craig, 1975; Harris et al., 2002; Wolfe and Coceani, 1979) and are found in the ejaculates of various animals (Kennedy et al., 2003; Kobayashi et al., 2013; Loher et al., 1981; Templeton et al., 1978). The biological significance of prostaglandins in mediating physiological actions essential for reproduction is well understood in mammals (Didolkar and Roychowdhury, 1980; Herrero et al., 1997; Kurzrok and Lieb, 1930; Marey et al., 2013), and is considered to be important in invertebrate reproduction as well. Prostaglandins are found in cricket seminal fluid (Loher et al., 1981) and have noted effects on the reproductive physiology of two species, *Teleogryllus commodus* and *Acheta domesticus*, in mediating egg production and oviposition in mated females (Loher and Edson, 1973; Murtaugh and Denlinger, 1985). Changes in reproductive behavior as

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The Journal of Experimental Biology (2015) 218, 2720-2727 doi:10.1242/jeb.121327

a consequence of mating are similar to the effects exhibited by females that have been experimentally treated with prostaglandin. Injection of prostaglandin into virgin females induces oviposition in a dose-dependent fashion (Destephano and Brady, 1977; Loher, 1979; Loher et al., 1981; Tanaka, 2014), increasing both the rate (Destephano and Brady, 1977) and duration (Destephano et al., 1982) of egg laying. Specifically, prostaglandin E_2 (PGE_2) is successful at effecting these physiological changes (Stanley-Samuelson et al., 1986a) by stimulating nerves located within the female genital chamber (Sugawara, 1986).

Seminal fluids from *A. domesticus* and *T. commodus* contain approximately 20 pg of preformed PGE_2 (Loher et al., 1981) that originates from the testes (Murtaugh and Denlinger, 1982). Male crickets pass this PGE_2 to females with the rest of the ejaculate (testicular and accessory gland material) in a small package called a spermatophore. During copulation, the spermatophore is placed into the female's genitalia so that sperm and seminal fluids can be transferred to and stored in the female's internal sperm storage organ, the spermatheca. Although PGE_2 is absent in the spermathecae of virgin females, mated females have an average of 500 pg of PGE_2 (Loher et al., 1981; Stanley-Samuelson and Loher, 1983), far more PGE_2 than contained in a single spermatophore. This suggests not only that preformed PGE_2 is transferred to females at the time of mating but also that females synthesize additional PGE_2 after copulation. Post-copulatory synthesis of PGE_2 is possible because males transfer the precursors of PGE_2 in the seminal fluid (Destephano et al., 1974; Loher et al., 1981; Stanley-Samuelson et al., 1987), which include the enzyme complex prostaglandin synthetase derived from the testes (Tobe and Loher, 1983) and the substrate arachidonic acid (AA; 20:4n-6) derived from both the testes and accessory glands. Stanley-Samuelson et al. (1987) traced the transfer of radioactive arachidonic acid from males to females and demonstrated that females indeed use the substrate obtained from mating to synthesize additional PGE_2 post-copula. Interestingly, most insects must obtain AA from the diet as few are capable of synthesizing it *de novo* (Blomquist et al., 1991); however, some crickets retain this ability. Male *T. commodus*, for example, can synthesize AA (Jurenka et al., 1988) as well as prostaglandins (Stanley-Samuelson et al., 1986b) *de novo* within the reproductive organs, thereby providing males with a non-dietary source of these essential compounds. Because prostaglandin is quickly metabolized in the hemolymph and excreted from the body (Stanley-Samuelson and Loher, 1985), its storage in the spermatheca is relatively ephemeral (Destephano and Brady, 1977) and females likely need to mate frequently to maintain their supply of PGE_2.

Our current understanding of the reproductive effects of prostaglandin in crickets is derived from two species, *T. commodus* and *A. domesticus*. Here, we extend our knowledge by investigating the sexual transfer of PGE_2 and its precursor AA in the Texas field cricket, *Gryllus texensis* (Cade and Otte 2000), a species that has not yet been used to study the reproductive effects of prostaglandin. *Gryllus* spp. are frequently used in studies demonstrating the benefits of mating, where females that mate multiple times indeed have increased lifetime fecundity relative to once-mated females (Gershman, 2010; Wagner et al., 2001; A.M.W. and C.D.K., unpublished), but females must remate throughout the breeding season to obtain this reproductive benefit as mating multiple times in a single bout does not increase fecundity (Simmons, 1988; A.M.W. and C.D.K., unpublished). We hypothesized that the functional mechanism underlying the positive relationship between mating rate and fecundity in *G. texensis* is the prostaglandin received from the male, such that mating more frequently increases the amount of prostaglandin available to females, and mating throughout the breeding season allows females to replenish this important and ephemeral egg-laying stimulator.

Our goal in this paper was to provide a detailed explanation of the manufacture and transfer of PGE_2 by males and its use by females in *G. texensis*. From the male perspective, we first tested the hypothesis that like other gryllids, male *G. texensis* package PGE_2 into the spermatophore. We accomplished this by directly measuring the quantity of PGE_2 and AA in individual spermatophores. Second, we examined the origin of the preformed PGE_2 that is in the spermatophore. We predicted that PGE_2 is manufactured in the testes and tested this prediction by measuring the amount of PGE_2 in the spermatophores of males that have had their testes experimentally removed (i.e. castrated). If the preformed PGE_2 within the seminal fluid is indeed derived from the testes and is responsible for the physiological changes we see in females after mating, then PGE_2 will be absent from the spermatophores of castrated males and these spermatophore contents will not stimulate oviposition in females.

From the female perspective, we tested the hypothesis that male-derived PGE_2 is transferred to females during mating and that mating more frequently increases the amount of PGE_2 acquired. We tested this hypothesis by comparing the quantity of PGE_2 in the spermathecae of virgin and mated females. We predicted that virgin females would have negligible amounts of PGE_2 in the spermatheca, that spermatheca from once-mated females would contain the same quantity of PGE_2 as is present in a single spermatophore, and that females mated four times would have significantly more PGE_2 than both virgin and once-mated females. We then tested whether females synthesize additional PGE_2 after mating by analyzing spermathecae immediately or 24 h after copulation. We predicted that the quantity of PGE_2 within the spermatheca would increase within 24 h of copulation as a result of female synthesis of the compound, thereby exceeding the amount of PGE_2 that can be found within a single spermatophore. Next, we tested the hypothesis that PGE_2 found within the spermatheca is only ephemerally available by measuring its quantity 3, 24 and 168 h after copulation. We predicted that after the initial increase at 24 h, the quantity of PGE_2 would decrease as the time since last mating increased. Finally, whether frequent mating throughout the breeding season increases the quantity of PGE_2 available to the female is unknown. We tested this novel hypothesis by comparing the quantity of PGE_2 in the spermathecae of females that mated with a male once or *ad libitum* and were then allowed to oviposit for 168 h. We predicted that the spermathecae of females mated *ad libitum* would have more PGE_2 relative to the spermathecae of females that mated only once.

According to previous studies, the prostaglandin that males transfer to females during copulation initiates oviposition, and greater quantities of prostaglandin (via injections) increase the number of eggs a female lays. Therefore, if females can accumulate and maintain continual access to mating-derived PGE_2 by mating multiple times, then prostaglandins may provide a functional mechanism linking higher female mating rates with increased fecundity.

RESULTS

AA and prostaglandin in spermatophores

The GC/MS results indicated that spermatophores contain 43±19 ng of AA (20:4n-6) (Fig. 1A), in addition to other common fatty acids. Based on retention time and mass spectra comparison to known standards (Christie, 2014), we found 156± 87 ng of 5,11,14-20:3, a structural analog of AA. The importance of this analog in the seminal fluid has yet to be determined, but apparently it cannot be

The Journal of Experimental Biology (2015) 218, 2720-2727 doi:10.1242/jeb.121327

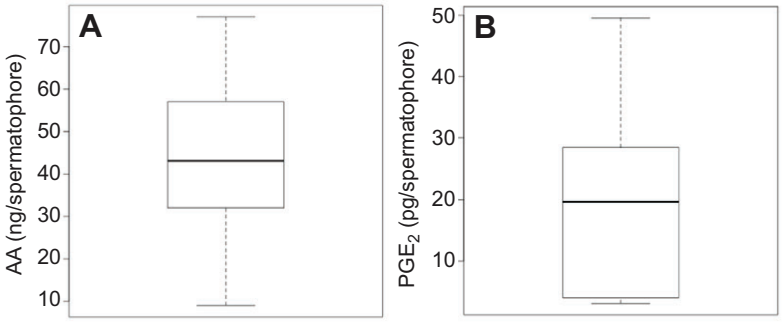

Fig. 1. Average quantity of arachidonic acid and prostaglandin E_2 in the spermatophores of males. Boxplots show (A) arachidonic acid (AA) and (B) prostaglandin E_2 (PGE_2). Boxes represent the lower (25%) and upper (75%) quartiles, the solid dark horizontal line is the median, and the whiskers indicate 1.5 times the interquartile range.

converted into PGE_2 (Berger et al., 2002). The fluorometric-HPLC results confirmed the presence of prostaglandin within the spermatophore and identified the most common form as PGE_2. The PGE_2-enzyme immunoassay (EIA) detected an average of 17.2±14.3 pg of PGE_2 per spermatophore (Fig. 1B). There was no correlation between the amount of PGE_2 detected in spermatophores and male pronotum width (Pearson correlation: r=0.1776, t=0.8268, d.f.=21, P=0.4176) or mass (Pearson correlation: r=0.0421, t=0.1931, d.f.=21, P=0.8487).

The quantity of PGE_2 in spermatophores collected from females immediately after copulation (15.0±20.6 pg) did not significantly differ from that in spermatophores collected directly from males pre-copula (23.6±36.3 pg; two-sample t-test: t=0.5703, d.f.=12, P=0.5790). Additionally, males did not consistently package the same amount of PGE_2 in sequential spermatophores that they formed (one-way repeated measures ANOVA: F=1.534, d.f.=1, 17, P=0.1917) and spermatophore collection order did not affect the average quantity of PGE_2 contained in subsequent spermatophores (first spermatophore 20.7±18.7 pg, second spermatophore 19.6±12.5 pg; paired t-test: t=−0.2966, d.f.=17, P=0.7704).

Effect of castration on PGE_2 quantity in spermatophores and post-mating oviposition in females

Castrated males (7.5±4.3 pg) had significantly less PGE_2 present in their spermatophores than sham-castrated males (185±110.4 pg; Mann–Whitney test: W=0.00, d.f.=18, P=0.0004). We found a significant effect of mating treatment on the number of eggs laid in the 3 days after mating. Females that mated with sham-castrated males laid significantly more eggs than virgins (z=24.212, d.f.=49, P<0.0001) or females that mated with castrated males (z=14.849, d.f.=44, P<0.0001) (mean±s.e. number of eggs laid: virgin 2.5±1.8, castrated 0.5±0.2, sham-castrated 58.9±12.6).

Analysis of AA in spermathecae

The GC/MS results indicated that spermathecae from virgin females contained only 6±4 ng of AA compared with the 59±15 ng present in the spermathecae of mated females. The level of 5,11,14-20:3 was about 3 times as much as that of AA in both virgin and mated spermathecae (17±14 and 139±65 ng, respectively). This structural analog of AA was also found in large amounts in the spermatophore (see above), but its importance in cricket reproduction is unknown.

Transfer and accumulation of PGE_2 in spermathecae via copulation

Females that mated once tended to have more PGE_2 (15.9±22.5 pg) than virgin females (4.4±5.6 pg), but this difference was only approaching significance (t=−1.823, d.f.=24, P=0.0789; Fig. 2A). Additionally, females that mated four times had significantly more PGE_2 (191.1±137.9 pg) than once-mated females (t=4.254, d.f.=16, P=0.0002).

Post-copula synthesis and presence of PGE_2 in the spermathecae

Spermathecae that were dissected 24 h after copulation (269.9±248.7 pg, N=47) contained significantly more PGE_2 than spermathecae that were dissected only 3 h after copulation (15.9±22.5 pg, N=11; Fig. 2B; t=9.00, d.f.=57, P<0.0001). In contrast, spermathecae dissected 168 h after copulation (13.5±6.5 pg, N=30) had significantly less PGE_2 than females dissected after 24 h (t=14.17, d.f.=76, P<0.0001). As predicted, females that were allowed to mate *ad libitum* throughout the trial period had significantly more PGE_2 in the spermathecae after 168 h (645.0±204.8 pg) than females that were allowed to mate only once (25.4±14.1; Fig. 2C; t=11.411, d.f.=17, P<0.0001).

DISCUSSION

We found that in *G. texensis*, PGE_2 and its precursor AA are present in the spermatophore, passed to females during copulation, and then stored within the spermatheca. Our results also indicate that females synthesize additional PGE_2 within 24 h of mating. This is most likely achieved by females converting the substrate acquired from the accessory fluid into PGE_2, as has been demonstrated in a previous study that used radioactive AA to trace the fate of male-derived AA in *T. commodus* (Stanley-Samuelson et al., 1987). Further, our results are the first to demonstrate that although PGE_2 is depleted through time, its presence can be maintained at high quantities for prolonged periods of time by frequent mating. Our results show that females acquire PGE_2 via mating and support the hypothesis that high mating rates increase the amount and availability of PGE_2 throughout the breeding season.

That fecundity increases with the number of copulations could be due to a greater quantity of accumulated PGE_2. Previous studies have shown that prostaglandin mediates oviposition in crickets (Loher, 1979; Loher et al., 1981), with larger doses increasing the number of eggs a female lays (Destephano and Brady, 1977; Destephano et al., 1982; Tanaka, 2014). We also know that mating more frequently increases the number of eggs females produce and lay (Gershman, 2007, 2010; Loher and Edson, 1973; Murtaugh and Denlinger, 1985) even though a single mating can often provide enough sperm to fertilize all of a female's eggs (Murtaugh and Denlinger, 1985; Simmons, 1988). In our study, we predicted that females that mated at higher rates would have increased quantities of PGE_2 relative to females that mated fewer times, and that only females that mated with males that provided PGE_2 would lay eggs. Our results support this hypothesis and confirm that accessory fluids alone do not induce oviposition; female fecundity increases only after receiving a spermatophore containing preformed

The Journal of Experimental Biology (2015) 218, 2720-2727 doi:10.1242/jeb.121327

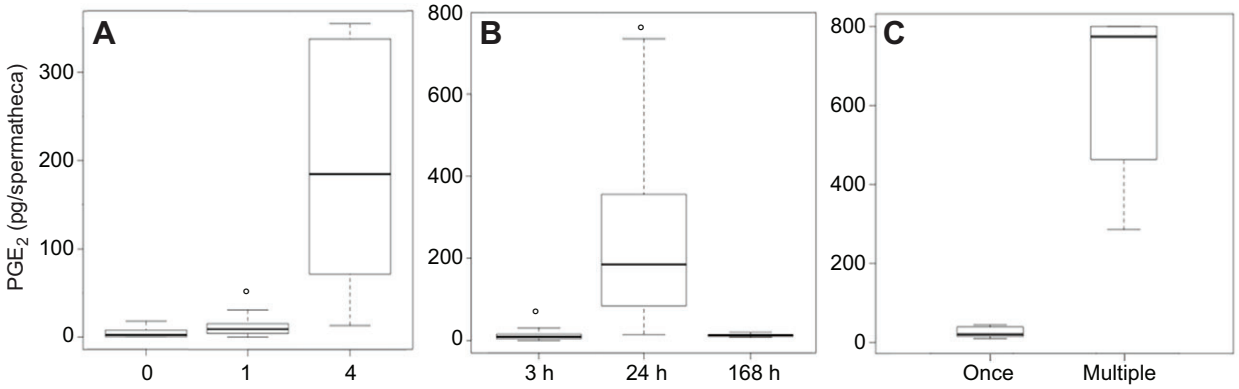

Fig. 2. Average quantity of PGE_2 detected in the spermathecae of females in different experimental mating treatments. Boxplots are shown for spermathecae from females: (A) mated 0, 1 or 4 times then analyzed after 3 h; (B) mated once then analyzed 3, 24 and 168 h post-copula; and (C) mated once at the beginning or multiple times throughout the trial and then analyzed at hour 168 of the trial. Boxes represent the lower (25%) and upper (75%) quartiles, the solid dark horizontal line is the median, and the whiskers indicate 1.5 times the interquartile range. Data beyond the end of the whiskers are outliers and are plotted as open circles.

prostaglandin. However, because both sperm and PGE_2 are derived from the testes, separating the effects of these two factors on female reproductive physiology poses a challenge.

Previous studies on gryllids have used irradiation techniques to kill sperm and prevent spermatogenesis (Murtaugh and Denlinger, 1980, 1987), and alternatively, have directly injected prostaglandin or prostaglandin synthesis inhibitors to manipulate *in vitro* prostaglandin concentrations (Murtaugh and Denlinger, 1980, 1982; Destephano and Brady, 1977). The limited results using these techniques have been mixed and difficult to reproduce. Mating females to irradiated males has led to either normal (Backus, 1986) or decreased (Murtaugh and Denlinger, 1980) rates of oviposition relative to females mated to unmanipulated males. There have been similarly mixed results with the effects of prostaglandin synthesis inhibitors on oviposition (Destephano and Brady, 1977; Murtaugh and Denlinger, 1980), although direct injection of PGE_2 into females consistently results in increased egg laying (Destephano and Brady, 1977; Loher et al., 1981; Stanley-Samuelson et al., 1986a; Tanaka, 2014). Once refined, these techniques will likely be essential in isolating the effects that PGE_2 and sperm have on female reproductive success.

Although previous studies support a major role for ejaculate-derived prostaglandin in mediating oviposition behavior, additional reproductive functions of prostaglandins have been proposed. For example, ovarian follicle development and maturation depend on prostaglandin signaling in *D. melanogaster* (Tootle and Spradling, 2008) and the silkmoth *Bombyx mori* (Machado et al., 2007), and eggshell production is coordinated by prostaglandins in *D. melanogaster* (Tootle et al., 2011). Whether exogenous PGE_2 obtained via mating affects ovarian follicle development and maturation in crickets is unknown, but research has shown that high female mating rates not only increase the rate at which females lay eggs but also induce egg production (Loher, 1979; A.M.W. and C.D.K., unpublished), suggesting that ejaculate-derived PGE_2 has a positive effect on both egg development and oviposition. From the male perspective, prostaglandins play various roles in sperm maintenance in mammals, including sperm motility (Didolkar and Roychowdhury, 1980), viability (Hayashi et al., 1988), protection from phagocytosis (Marey et al., 2013), and enhancing the acrosome reaction necessary for the fusion of sperm and egg (Herrero et al., 1997). To our knowledge, there are no studies examining the importance of prostaglandin on sperm maintenance in insects, so further research will be required to understand these effects.

Finally, theory suggests that male fitness increases with mating rate, while one or a few matings are sufficient for females to gain maximum reproductive success (Bateman, 1948). Empirical research suggests otherwise, as females gain reproductive fitness by mating at high rates despite the costs it has on survival (South and Lewis, 2011). These positive effects of mating multiple times are often the result of ejaculate-derived substances provided by the male, yet whether these substances are a form of nuptial gift or are manipulative substances that evolved out of sexual conflict remains to be seen. Accessory substances in the seminal fluid are sometimes responsible for decreases in female survival (Green and Tregenza, 2009) and increases in female refractory behavior (Gwynne, 1986; Torres-Vila et al., 1997), thereby providing some benefit to the male at a cost to female fitness. Because PGE_2 is required for many essential physiological functions, however, it could provide females with alternative benefits aside from increased fecundity. For example, PGE_2 is a major mediator of the insect immune system (Stanley and Kim, 2014). If females utilize ejaculate-derived PGE_2 to initiate an immune response after mating, then they could gain a significant fitness advantage in fighting off the many pathogens that they face during the breeding season (Knell and Webberley, 2004). In fact, mated female crickets have increased survival after exposure to a bacterial pathogen relative to virgin females (Shoemaker et al., 2006); therefore, it is possible that the functional mechanism for this heightened post-mating immunity is ejaculate-derived PGE_2.

In conclusion, we found that *G. texensis* males provide both AA and PGE_2 in their spermatophores and that these are transferred to the female spermatheca after copulation. Although there is a limited amount of preformed PGE_2 passed immediately after mating, females use the AA and enzyme complex prostaglandin synthetase obtained from males to synthesize more PGE_2 within 24 h of copulation. PGE_2 is found in the spermatheca for extended periods of time; however, that it decreases in quantity as the time since last mating increases suggests it is ephemeral. Most importantly, female *G. texensis* crickets that mate multiple times throughout their lives can maintain continual access to PGE_2. We hypothesize that the continual need for PGE_2 is (at least partially) responsible for the high mating rates observed in crickets and could explain the positive relationship between fecundity and mating rate.

The Journal of Experimental Biology (2015) 218, 2720-2727 doi:10.1242/jeb.121327

MATERIALS AND METHODS

Experimental animals

Crickets (long-winged *G. texensis*) were lab-reared descendants of individuals originally collected in Austin, TX, USA, in the autumn of 2010, 2012 and 2013. The laboratory colony of crickets was reared in large communal bins (73×41×46 cm) until the penultimate instar, after which crickets were kept individually in clear plastic 250 ml containers (10 cm diameter×4.5 cm depth) until sexual maturity. All crickets were housed in an environmentally controlled room (27°C, 12 h:12 h light:dark cycle) and were supplied with cotton-plugged water vials and dry cat food (Special Kitty Premium Cat Food™) *ad libitum*. We checked the crickets daily for eclosion. Once the cuticle of a newly eclosed cricket had hardened (later that same day), we measured its pronotum length (the distance between the anterior and posterior edges of the pronotum) to the nearest 0.001 mm using a stereomicroscope (Leica S6D, Leica Microsystems GmbH, Wetzlar, Germany) equipped with Leica LAS image analysis software (Leica Application Suite V3.8.0, Leica Microsystems). Body mass was recorded to the nearest 0.01 mg on an electronic scale (Denver Instruments TP-64).

AA analysis

AA levels in individual spermatophores and spermathecae were analyzed as methyl esters using the procedure described by Choi et al. (2002). Samples were extracted overnight with 50 µl chloroform/methanol (2/1) containing triheptadecanoin as an internal standard. Methyl esters were made by base methanolysis and analyzed by gas chromatography-mass spectrometry (GC/MS) using a Hewlett-Packard 5890 II mass selective detector coupled with a Hewlett-Packard 5890 GC equipped with a Carbowax (Alltech, Deerfield, IL, USA) capillary column (30 m×0.25 mm). The oven temperature was programmed at 60°C for 1 min, then raised at 10°C min^{-1} to 230°C and held for 10 min.

PGE_2 analysis

To confirm that the prostaglandin present in spermatophores was PGE_2, we used a fluorometric high-pressure liquid chromatography (HPLC) procedure previously described by Jurenka et al. (1999). Briefly, we pulverized 36 spermatophores in 500 µl ethyl acetate, centrifuged them at 10,000 rpm for 2 min, and removed the supernatant. We repeated this three times and then dried the combined extracts under nitrogen. The combined extracts were reconstituted in 4 µl ethyl acetate and derivatized by adding 1 µl of a 9-anthryldiazomethane (Setareh Biotech, Eugene, OR, USA) solution (5 µg $µl^{-1}$ in ethyl acetate) and incubating in the dark at room temperature for 4 h. After 4 h, the sample was diluted with ethyl acetate and injected onto an HPLC silica normal-phase column (Whatman Partisphere Silica, 4.6×125 mm) using a Beckman System Gold solvent delivery system (Fullerton, CA, USA) equipped with a Hewlett-Packard 1046A fluorescence detector (Wilmington, DE, USA) with excitation and emission wavelengths of 250 and 410 nm, respectively. We used a gradient starting at 100% hexane to 20% 2-propanol in 20 min at a flow rate of 1 ml min^{-1} to separate and check for the presence of specific prostaglandins.

We measured the amount of PGE_2 in each sample (spermatophore or spermatheca) using a PGE_2-specific EIA according to the manufacturer's instructions (Amersham Prostaglandin E_2 Biotrak EIA System, GE Healthcare). Samples maintained at −80°C were pulverized using sterile pestles in 120 µl of lysis reagent 1 from the assay kit and then centrifuged at 10,000 rpm for 1 min. Supernatants were analyzed in duplicate in a 96-well assay plate. Serially diluted PGE_2 standards were also run in duplicate starting at 320 pg down to 1.25 pg. We used GraphPad Prism 4 (GraphPad Software 2005) to calculate the concentration of PGE_2 within each well of the assay plate. The minimum level of detection was approximately 1.25 pg PGE_2 per sample. We then calculated the total amount of PGE_2 in each spermatophore or spermatheca based on its initial dilution in lysis reagent 1.

Spermatophore sample collection and analysis

Spermatophores used in some of our analyses were collected from mated females directly after copulation. Copulation consists of the male transferring a spermatophore to the mounted female and securing it in place with an attachment plate inserted above the female's subgenital plate. Then, the male threads the spermatophore through the female's genital opening and into the spermathecal duct. The ejaculate is then transferred and the spermatophore capsule is retained externally (Loher and Dambach, 1989). Using fine forceps, we collected each spermatophore by carefully dislodging the attachment plate from the female immediately post-copula before any of its contents could be transferred. All spermatophores were stored in 1.5 ml centrifuge tubes at −80°C until AA and PGE_2 contents were quantified.

To test our first hypothesis that *G. texensis* package prostaglandin and its precursors into ejaculates, we analyzed individual spermatophores (N=18) using GC/MS to verify the presence and quantity of the prostaglandin precursor AA. Additionally, we collectively analyzed 36 spermatophores using HPLC to verify the presence of PGE_2 within spermatophores, and analyzed individual spermatophores (N=23) using a PGE_2-EIA to quantify the amount of PGE_2 present within a single spermatophore. Because PGE_2 is also required by males to achieve a number of physiological tasks, we recorded the pronotum width and mass for each male to test whether male size affects the quantity of PGE_2 within the spermatophore.

Spermatophores for additional analyses were collected directly from males by pulling down the subgenital plate and gently palpating the abdomen (Kerr et al., 2010). This method was used to standardize the collection time between males. The formation of the spermatophore prior to courtship has been intricately described for *G. bimaculatus* (Hall et al., 2000), yet there has been no research specifically indicating when prostaglandins are incorporated into the spermatophore. To ensure that the collection method did not affect PGE_2 presence and quantity, we collected two spermatophores from 13 mature males, one of which was collected from the female after mating and the other was collected directly from the male using the method described above. We then analyzed the quantity of PGE_2 in each of the spermatophores using a PGE_2-EIA.

To determine whether males consistently package the same quantity of PGE_2 into each of their spermatophores and whether this quantity varies between males, we analyzed two consecutive spermatophores from randomly selected mature males (N=18). Male crickets form and then store a spermatophore for 2–3 days before ejecting it to form a fresh one (Reinhardt and Siva-Jothy, 2005). Because the stability and longevity of PGE_2 within the spermatophore is unknown, we standardized the age of spermatophores by collecting each directly from the male at 13:00 h for three consecutive days. The first spermatophore collected was discarded because of its unknown time of formation, and the quantity of PGE_2 in each of the remaining two spermatophores was analyzed using a PGE_2-EIA.

To test the hypothesis that ejaculate-derived PGE_2 originates in the male gonads, we surgically removed the testes from males (N=13) and compared the quantity of PGE_2 in the spermatophores with that in the spermatophores of sham-castrated males (N=5). We castrated males following the procedure detailed in Larson et al. (2012). Briefly, males 5–6 days post-eclosion were randomly divided into two groups: (1) both testes removed, or (2) sham-castrated to control for the effects that surgery may have on spermatophore PGE_2 content. Prior to surgery, males were cold-anesthetized for 4.5 min in a −20°C freezer. A lateral incision was made through the dorsal intersegmental membrane between the 2nd and 3rd abdominal segments. For castrated males, fine forceps were used to completely remove each testis, whereas sham-castrated males had their body cavity probed in a similar manner but without the removal of any organs. We then used Vetbond™ Tissue Adhesive (3M, St Paul, MN, USA) to seal the incision, and placed each male in a plastic container with a water vial, an egg carton shelter and a piece of cat food. After 2 days recovery, castrated males were paired daily with multiple receptive females and allowed to mate in order to deplete the testes-derived components stored in the seminal vesicles. We regularly examined spermatophores from each male for sperm under a compound light microscope (Leica DM 2500, Leica Microsystems). If sperm were observed, males were provided with virgin females and allowed to continue mating for another 24 h. If no sperm were observed, we considered the males depleted of testes-derived components and ready for sample collection. Sham-castrated males were also allowed to mate with receptive females for 5 days before samples were collected. After mating and sperm depletion, males were again isolated and two spermatophores were collected from each

The Journal of Experimental Biology (2015) 218, 2720-2727 doi:10.1242/jeb.121327

male 24 h apart. Again, only the second spermatophore was analyzed for PGE_2 quantity using a PGE_2-EIA.

To test the hypothesis that females require an intact ejaculate containing testes-derived substances (i.e. PGE_2 and sperm) to stimulate egg laying, we randomly assigned 10 day old, sexually mature female crickets to one of three treatments: (1) unmated (virgins), (2) paired with a castrated male that provided an ejaculate composed of only accessory fluids, or (3) paired with a sham-castrated male that provided an ejaculate composed of both accessory gland and testes-derived substances. Females were transferred to a clear plastic mating arena (10 cm diameter×4.5 cm depth) and placed in a dark room under a 25 W red light. Unmated females (N=25) were paired with a male but were prevented from mating by gently shaking the container. Females assigned to the other treatments were paired with a single sham-castrated (N=24) or castrated male (N=20) and allowed to mate. Once a spermatophore was successfully transferred, the male was removed from the arena and females were monitored to ensure that the spermatophore remained attached for 45 min before we manually detached it. All females were then transferred to individual containers (16.5×10.5×7 cm) and supplied with one water vial, two pieces of dry cat food and a 2 oz plastic dish (3 cm diameter×2 cm depth) of moistened ReptiSand (ZooMed, San Luis Obispo, CA, USA) for oviposition substrate. After 3 days, we dried the sand at room temperature for 24 h and quantified the number of eggs laid by each female.

Spermatheca sample collection and analysis

Spermathecae from virgin and mated females were dissected out of cold-anesthetized individuals through a longitudinal incision in the ventral abdomen. The failure to transfer an ejaculate during copulation frequently occurs in virgins (Loher and Edson, 1973); therefore, we examined the spermatheca of mated females to ensure they contained an ejaculate. This was done visually, as spermathecae from females that have not received an ejaculate are small, flaccid and clear, whereas those from females that have received an ejaculate are large, turgid and opaque. All spermathecae were stored individually in 1.5 ml centrifuge tubes at −80°C. Spermathecae from virgin (N=8) and mated (N=4) females were individually analyzed using GC/MS to measure the quantity of AA present.

To establish that PGE_2 is passed to females during copulation and to test the hypothesis that mating multiple times increases the quantity of PGE_2 found in females, the spermathecae of females mated zero (N=14), one (N=11) or four times (N=6) were analyzed. Females 10–14 days post-eclosion were randomly assigned to a treatment group, and females in the mated treatments were paired with sexually mature virgin males. For females mated four times, a novel male was used for each mating. Spermathecae were dissected and frozen within 3 h of the first copulation, and were later analyzed for PGE_2 quantity using a PGE_2-EIA.

To test the hypotheses that females synthesize additional PGE_2 after copulation and that the PGE_2 stored in the spermatheca is only ephemerally available, we compared the amount of PGE_2 present in the spermathecae of females 3 h (N=11), 24 h (N=47) and 168 h (N=30) post-copula. Females were allowed to copulate with a single virgin male and spermatophores were removed after 45 min of attachment. After spermatophore removal, females were transferred into individual containers and supplied with water vials. After 3 h, 24 h and 168 h, spermathecae from females in the respective groups were dissected and later analyzed for PGE_2 quantity using a PGE_2-EIA.

Finally, to test the hypothesis that repeated mating over time prolongs the presence and maintains the quantity of PGE_2 in the spermatheca, we analyzed the quantity of PGE_2 in the spermathecae of females assigned to one of two treatment groups: (1) mated once and the spermatheca dissected after 168 h (N=9), or (2) reared in the presence of multiple males (i.e. given the opportunity to mate continuously throughout the trial) and the spermatheca dissected after 168 h (N=9). Again, spermatophores were removed from mated females after 45 min of attachment. Females were maintained in individual containers supplied with a water vial and a piece of cat food until the day of dissection. All spermathecae were analyzed for PGE_2 quantity using a PGE_2-EIA.

Statistics

We used Pearson correlations to examine the relationship between male size (i.e. mass and pronotum length) and the amount of PGE_2 present in spermatophores. We used a paired t-test to examine the effect of collection method, either pre- or post-copula, on the amount of PGE_2 detected in each of a male's spermatophores (log-transformed). To examine whether males consistently package the same amount of PGE_2 into each of their spermatophores, we ran a one-way repeated measures ANOVA where male identity was entered as a random factor and spermatophore (first or second) was the main treatment factor. We also used a paired t-test to determine whether the order of spermatophore collection had a significant effect on PGE_2 quantity. We used a non-parametric Mann–Whitney test to determine whether castrated males had significantly different quantities of PGE_2 (log-transformed) in their spermatophores relative to sham-castrated males. Lastly, we investigated the effect of accessory fluids and testes-derived substances on the number of eggs laid by focal females using general linear modeling (GLM) with a Poisson family distribution while statistically controlling for body size.

For females, we used an ANOVA to examine the effect of mating and time since last copulation on the amount of PGE_2 detected in the spermathecae (log-transformed). First, we examined how mating either zero, one or four times affects the quantity of PGE_2 stored in the spermatheca to determine whether there is a positive correlation between PGE_2 and mating rate. Next, we investigated whether additional PGE_2 is synthesized after mating and how long it remains by comparing the amount of PGE_2 in the spermathecae of once-mated females 3, 24 and 168 h post-copula. Lastly, using a one-tailed t-test, we examined whether mating multiple times throughout the 168 h trial period increases the availability of PGE_2 relative to females that mated only once at the beginning of the trial.

We checked that our data met model assumptions by examining the residuals for normality and homogeneity of variances. Summary statistics are reported as means±s.d. All statistical analyses were performed in R version 2.12 (R Development Core Team 2009) with α=0.05.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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