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**Polyandry and fitness in female horned flour beetles (Gnatocerus cornutus).**

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ABSTRACT

Although polyandry is common, it is often unclear why females mate with multiple males, because although polyandry may provide females with direct or indirect fitness benefits, it can also be costly. Our understanding of polyandry is also restricted by the relative paucity of studies that disentangle the fitness effects of mating more than once with a single male and mating with multiple males. Here we investigated potential benefits and costs of polyandry in the horned beetle (Gnatocerus cornutus) while controlling for number of matings. We found that female lifespan was independent of mating frequency, indicating that mating itself is not very costly. However, females that mated more than once laid more eggs and had greater lifetime reproductive success than singly mated females. Because the magnitude of these effects was similar in monandrous and polyandrous females, this improved fertility was due to multiple mating itself, rather than mating with multiple males. However, polyandrous females produced more attractive sons, but they tended to have smaller mandibles and so may fare less well in male-male competition. These results indicate that polyandry is relatively cost free, at least in the laboratory, and has direct and indirect benefits to female fitness. However, because the attractive sons produced by polyandrous females may fight less well, the indirect benefits of polyandry will depend on the intensity of male-male competition and how free females are to exert mate choice. Where competition between males is intense, polyandry benefits via son attractiveness may be reduced and perhaps even carry costs to female fitness.

KEYWORDS

Coleoptera, Female choice, Fisherian process, Good genes, Insecta, Polyandry, Monandry, Sexual selection
INTRODUCTION

Polyandry, where females mate with multiple males, is common in many species of birds, mammals and insects (Arnqvist & Nilsson, 2000; Hosken & Stockley, 2003; Jennions & Petrie, 2000; Taylor, Price, & Wedell, 2014). However, we do not fully understand why females mate with more than one male, especially because mating can be costly. Mating requires time and energy (Huchard et al., 2012) and can increase the risk of parasitism (Kemp, 2011), predation (Rowe, 1994), disease (Poiani & Wilks, 2000) and injury (Arnqvist, Nilsson, & Katvala, 2005). Polyandry can also elevate the intensity of sexual conflict (Holman & Kokko, 2013; but see Pizzari & Wedell, 2013) and select against male parental care (Kokko & Jennions, 2008; Queller, 1997). However, given that polyandrous mating is so common both across and within taxa (Taylor et al., 2014), its benefits must sometimes outweigh these considerable costs.

Polyandry may allow females to minimize the costs of male harassment if resisting courting males is more expensive than accepting these extra males as mates (Harano, 2015; Panova et al., 2010; Rivera & Andrés, 2002; Thornhill & Alcock 1983). Alternatively, polyandry could offer direct, material benefits transferred at or after mating that improve female lifespan or fecundity (reviewed in Arnqvist & Nilsson, 2000; Hosken & Stockley, 2003).

For example, in decorated crickets males transfer a nutrient rich nuptial gift to females at mating and females that mate multiple times and receive many such gifts, live longer than females who mate less frequently (Burpee & Sakaluk, 1993). Polyandry could also provide indirect heritable, genetic benefits that improve the survival or reproductive success of offspring (Arnqvist & Nilsson, 2000; Hosken & Stockley, 2003; Jennions & Petrie, 2000; Simmons, 2001; Taylor et al., 2014; Zeh & Zeh, 2001). This is because polyandry creates the opportunity for post-copulatory sexual selection, potentially improving the fertilization success
of the sperm of genetically compatible (Zeh & Zeh, 2001), diverse (Taylor et al., 2014),
unrelated (Hosken & Blanckenhorn, 1999; Tregenza & Wedell, 2002), viable or attractive
(Slatyer, Mautz, Backwell, & Jennions, 2012) males. While there is evidence for indirect
benefits of polyandry (Simmons, 2005; Slatyer et al., 2012), their role in the evolution of
polyandry remains controversial (Reding, 2014; Uller & Olsson, 2008).
To understand why females mate polyandrously the costs and benefits (both direct
and indirect) of polyandry on female fitness need to be estimated (Arnqvist & Nilsson, 2000;
Jennions & Petrie, 2000). The problem is that very few studies have successfully decoupled
the female fitness effects of mating more than once (e.g. multiple times with a single male)
and mating with multiple males (Slatyer et al., 2012). Studies that have made this distinction
have been conducted in a very few species and have often only measured a small number of
fitness related traits, over a short period of time (Slatyer et al., 2012). Collecting these data is
vital to improving our understanding of polyandry (Slatyer et al., 2012; Taylor et al., 2014) as
well as its evolutionary and ecological consequences (Holman & Kokko, 2013).
Here, we investigated polyandry and its effects on female fitness in the polyandrous
beetle *Gnatocerus cornutus* (Okada & Miyatake, 2009; Yamane, Okada, Nakayama, & Miyatake,
2010). We use a long-running laboratory population that has been kept in large numbers
(approx. Ne > 1000), and has been able to exert free mate choice and express other behaviours
in conditions that closely mimic their natural habitat. This regime has ensured the
persistence of ample genetic diversity, as shown by rapid phenotypic responses to selection on
mandible size (Harano, Okada, Nakayama, Miyatake, & Hosken, 2010). Additionally, traits
including mass, development time and mandible size in these beetles overlap or are very close
to trait estimates from independent laboratories (Demuth, Naidu, & Mydlarz, 2012; Holloway
et al., 1987). This indicates that the beetles used in this study are a good representative of
natural populations of *G. cornutus*. In this species females exert mate choice and gain genetic benefits via the attractiveness of their sons (Okada, Katsuki, Sharma, House, & Hosken, 2014). However, female choice is limited by male-male competition as unpreferred males, those with larger mandibles, gain more mates through their superior competitive abilities (Okada et al., 2014). We compared the fitness of singly mated females with those that mated multiply with a single male (monandry) or with multiple males (polyandry) using direct (e.g. lifespan, fecundity) and indirect (e.g. offspring lifespan, fertility and attractiveness) fitness measures. We then discuss the costs and benefits of polyandry in the female *G. cornutus*.

**MATERIALS AND METHODS**

*Stock Culture Maintenance*

*G. cornutus* larvae do not pupate at high densities and so following Okada and Miyatake (2010) final instar larvae were individually housed in wells of a 24-well tissue culture plate with 1g of food (Cellstar; Greiner Bio-One, Frickenhausen, Germany). After adult emergence, *G. cornutus* males can take up-to seven days to attain sexual maturity (Katsuki, Harano, Miyatake, Okada, & Hosken, 2012; Katsuki, Okada, & Okada, 2012) and so we allowed individuals from both sexes to mature for 14 days before using them in experiments. All experiments within this study follow this maintenance protocol unless stated otherwise.

*Mating Regimes*

Experimental animals were removed from the stock population at their final instar stage and then randomly allocated to one of three experimental treatments: monandry (multiple mated to one male), polyandry (multiply mated with multiple males) or single mating (one copulation with one male) (n = 50 / treatment). Two weeks after adult emergence,
experimental females were weighed to the nearest 0.01 mg on an electronic balance (Mettler-Toledo AG, Laboratory and Weighing Technologies, Greifensee, Switzerland) and then individually aspirated into plastic containers (17 mm diameter, 20 mm high), which contained a disc of filter paper for traction (17 mm diameter). After thirty minutes, a virgin stock-population male was added to each dish and the pair were observed until they finished mating. After copulating, pairs were separated and females placed in a well of the tissue culture plate with 1g of food. Mating began on day 14 post eclosion, such that singly mated females were mated once on day 14, while monandrous and polyandrous females were mated once every day between days 14 and 17 post-eclosion to either a single male (monandry) or to each of four different males (polyandry).

Assaying Direct Effects on Female Fitness

Fifty females from each treatment were divided into two groups (n = 25/group). One of these groups was used to measure lifetime reproductive success (LRS - the number of adult offspring that hatch from eggs), while the other group was used to assay fecundity (the number of eggs laid). Lifespan was examined in females from both of these groups.

Females used to measure LRS were transferred after mating into an egg-laying vial (70 mm diameter, 25 mm high) containing excess food (20 g) for two months. LRS of each female was used as a proxy for female fitness (Katsuki, Harano, et al., 2012; Katsuki, Okada, et al., 2012; Tsuda & Yoshida, 1985) and measured as the total number of adult offspring that emerged from these vials. Females from all treatment groups (singly mated, monandry or polyandry) were transferred into egg-laying vials at the same age i.e. 18 days post-eclosion. This ensured that females had the same amount of time in which to lay eggs and therefore, all fertility measures are directly comparable across treatment groups. After two months,
7 females were moved into new survival assay vials (40 mm high, 15 mm diameter) containing
an excess of the culture medium (4 g).

Females in the fecundity assay group were transferred after mating into an egg laying
vial containing excess food (20 g) for two months. Their eggs were separated from this food by
sieving and were counted twice a week, because eggs take 4.6 days to hatch (Tsuda & Yoshida,
1984). After two months, females were also moved into survival assay vials. Their survival
after mating in both groups monitored weekly until death.

Lifespan and fecundity (e.g. egg counts) measure the direct effects of polyandry on
female fitness, while LRS may indicate indirect effects of polyandry if differences between
females are due to variation in embryo survival or direct effects if differences are due to how
many ova are fertilized. Our mating regime allows us to distinguish between these
possibilities: direct benefits of multiple mating are likely to improve fertility in polyandrous and
monandrous females to a similar extent relative to singly mated females, while indirect genetic
benefits will improve fertility in polyandrous females relative to other treatment regimes. It
is important to highlight that our mating regime means that monandrous multiple-mating
females (mated four times to a single male), could potentially suffer from the effects of male
sperm depletion more than polyandrous females, mated to four different, virgin males.
However, past work has shown that sperm counts do not differ between a male's first and
second mating, 24 hours later. This reduces the potential for sperm depletion effects on
fertility in our monandrous females but does not preclude it completely.

Assaying Indirect Effects on Female Fitness

From the females that were used to assay fecundity, twenty eggs were randomly
selected per female (N=20 / treatment) and each of these newly laid eggs were reared in a
glass vial (15 mm diameter, 40 mm high) with an excess of the culture medium (4 g). We recorded development time from egg to adult by monitoring these larvae daily. On the day of adult emergence, offspring were weighed to the nearest 0.01 mg. Longevity and LRS were measured in daughters (mean = 8/dam), all of which were mated just once, but were otherwise treated in the same way as their mothers, while additional traits were assayed in sons. To assay son attractiveness and courtship behaviors, virgin females were randomly chosen from the stock population and individually aspirated into in plastic dishes (17 mm diameter, 20 mm high) lined with filter paper. After 30 minutes, one experimental male was added to each dish. The pair were continuously observed until the end of mating, when sons were removed to avoid re-mating. Following Okada et al. (2014), copulation latency (the time from male introduction to commencement of copulation) was measured as an indicator of son attractiveness. This is a widely used measure of male attractiveness (Okada, Blount, Sharma, Snook, & Hosken, 2011; Shackleton, Jennions, & Hunt, 2005; Taylor, Wedell, & Hosken, 2007). We also measured courtship rate as a measure of courtship quality (e.g. Simmons & Holley, 2011). Copulation rate is measured as the number of bouts of tapping of the female’s body a male performs per unit time. We measure this by counting how many times a male performs this courtship behavior from the point at which they are introduced to a female until the point at which copulation begins. Both copulation latency and courtship rate are highly repeatable measures (Okada et al., 2014). After mating trials, mandible length (±0.01 mm) of each male was measured using a dissecting microscope linked to a PC (VM-60, Olympus, Tokyo, Japan). Longevity was then assayed in these sons, as described for their mothers.

Statistical Analysis

The LRS, fecundity and longevity of mothers were analyzed using analysis of variance
ANOVA with mating treatment (monandry, polyandry or singly mated) and female body size as fixed effects. Sex-specific mean values of each trait measured in sons and daughters (development time, body size, longevity, and LRS) and for traits only assayed in sons (copulation latency, courtship rate, mandible size) were calculated for each mother. Each trait mean was then analyzed using an ANOVA with mating treatment as a fixed effect. Student’s t tests were used to compare means, correcting the significance level for multiple comparisons by the sequential Bonferroni method (Rice, 1989). All analyses were performed using JMP 7.0 for windows (SAS Institute 2007).

**Ethical Note**

The stock population of *G. cornutus* used in this study originated from adults collected in Miyazaki City, Japan (31° 54’N, 131° 25’ E) in June 1957. This population has been maintained on whole-meal flour enriched with yeast (Okada & Miyatake, 2010; Okada, Yamane, & Miyatake, 2010) at 25°C and 60% relative humidity under a 14 : 10 h light / dark cycle. These laboratory conditions closely resemble natural conditions of this stored product pest. All individuals in the experiment were handled with care and handling time was kept to an absolute minimum. Note that the use of these beetles conforms to the University of Exeter’s Animal Ethics Policy.

**RESULTS**

**Direct Effects on Female Fitness**

We found significant effects of female mating treatment and female body size on female fecundity (treatment, $F_{2,71} = 16.91, P < 0.001$; mass, $F_{1,71} = 21.59, P < 0.001$) and LRS (treatment, $F_{2,71} = 15.523, P < 0.0001$; body size, $F_{1,71} = 22.651, P < 0.0001$). Multiple
comparisons showed that the number of eggs laid and LRS was significantly higher in monandrous and polyandrous females than in singly mated animals (Table 1). This comparable LRS in both monandrous and polyandrous females suggests that this elevated LRS is due to direct effects of mating. Crucially, this result also suggests that monandrous females (mated four times to one male) did not experience reduced fertility relative to polyandrous females (mated four times, to four virgin males), as a result of sperm depletion in their mate.

In the LRS and fecundity groups, female longevity was not affected by mating treatment or body size (Table 1; LRS, treatment, F2, 71 = 0.951, P = 0.3912; body size, F1, 71 = 3.187, P = 0.0785; fecundity, F2, 71 = 0.361, P = 0.6982; body size, F1, 71 = 0.012, P = 0.9131). Moreover, within each treatment, there was no significant difference in longevity between both the LRS and fecundity assay groups (single mating, t = 1.007, P = 0.3191; monandry, t = -0.301, P = 0.7649; polyandry, t = -0.174, P = 0.8628).

**Indirect Effects on Female Fitness**

Sons of polyandrous females developed faster than sons from any other treatment groups and were more attractive (i.e. had shorter copulation latency) and had a greater courtship rate than other sons (Tables 2 & 3). However, sons of polyandrous females also had significantly shorter mandibles compared to sons produced from other mating treatments (Tables 2 & 3). Female mating treatment had no significant effect on sons’ body size or longevity. Daughters of polyandrous females developed more quickly than other female offspring but daughters did not differ in any other trait assayed (Tables 2 & 3).
DISCUSSION

We demonstrate a clear, direct fitness advantage to multiple mating in *G. cornutus*.

Irrespective of whether females mate with one or four males, mating multiply improved female fecundity and LRS. In insects in general it appears that mating just once does not always maximize female fertility (Arnqvist & Nilsson, 2000), and that it is often multiple mating, rather than polyandry, that has direct positive effects on female fecundity (South & Lewis, 2011). These increases in female fecundity may be driven by accessory substances transferred with a male’s ejaculate (Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011) or resources, such as amino acids or water, that trigger or elevate egg laying (Vahed, 1998).

Alternatively, improved female fecundity may simply reflect that a single mating does not provide females with enough sperm to fertilize all of their eggs (Kraus, Neumann, Praagh, & Moritz, 2004; Slatyer et al., 2012). Consistent with this latter explanation, *G. cornutus* copulation is brief and relatively few sperm are transferred (Okada et al., 2010; Yamane et al., 2010). Irrespective of the underlying mechanism, multiple mating is often associated with reduced female lifespan (Arnqvist & Nilsson, 2000; Slatyer et al., 2012). This can either be due to the survival costs of mating itself (e.g. injury) (Blanckenhorn et al., 2002) or the direct costs of elevated egg production following multiple mating (Chapman, Takahisa, Smith, & Partridge, 1998). In *G. cornutus*, neither multiple mating nor the subsequent increases in fecundity reduced female survival: females that mated multiply, laid more eggs than once mated females, without experiencing significant reductions in lifespan. Overall, this suggests that the costs of both mating and of laying eggs are low in this species (at least in the laboratory) and that mating offers females direct fitness benefits.

In addition to these direct benefits of mating multiply, polyandry had clear indirect benefits for female fitness. Firstly, polyandry was associated with rapid development to
adulthood, something seen in other taxa (Hosken, Garner, Tregenza, Wedell, & Ward, 2003).

The fitness consequences of rapid growth are unknown in flour beetles (Okada et al., 2014), but we did not detect any costs to fast development later in life (e.g. reduced lifespan or LRS) and rapidly developing genotypes are probably generally advantageous in grain pests (Jayas, White, & Muir, 1994). Thus rapid development may improve offspring fitness in natural populations, although this needs to be tested. Less ambiguously, polyandry clearly improved the attractiveness of sons: males whose mothers mated polyandrously invested more intensely in courtship behaviors and were more attractive to females. Investment in courtship is under strong pre-copulatory sexual selection via female choice in this species (Okada et al., 2014) and so it appears that polyandrous mating in G. cornutus can indirectly elevate fitness. It is unclear if this is driven by cryptic female choice for attractive males or because these males produce highly competitive sperm (Jennions & Petrie, 2000) but there is some evidence for the latter idea. Attractive males produced by polyandrous mothers tended to have smaller mandibles. Mandible size is positively genetically associated with competitive ability in males (Okada & Miyatake, 2009; Yamane et al., 2010) and mating success (Harano et al., 2010) and so is a major determinant of male fitness. However, males with large mandibles transfer fewer sperm per ejaculate and have smaller testes (Yamane et al., 2010). This may mean males with large mandibles are disadvantaged in sperm competition – if sperm competition proceeds via the raffle principle whereby the more sperm a male transfers, the greater the probability of fertilization success (Parker, 1990).

These results highlight the complex interplay between male-male competition, female choice and sexual conflict over mating in this species. Males with large mandibles gain high reproductive success by out-competing other males and by coercing females into mating but transfer few sperm. Males that invest heavily in courtship are highly attractive to
females, despite having smaller mandibles, and transfer more sperm, which may improve their paternity during sperm competition. Clearly there are different routes to reproductive success in *G. cornutus*. Crucially, the benefits of either of these male strategies (attractive versus competitive phenotypes) probably depend on population density. This is because the probability of males gaining reproductive success by outcompeting their rivals (e.g. via combat) are reduced when rivals are rare (Kokko & Rankin, 2006). This means that at high densities, the benefits of having large mandibles are likely to increase because male reproductive success increasingly depends on the outcome of male-male competition. At low population densities, males that are very attractive may gain reproductive success by readily attracting females without having to frequently fight off competitors. This means that as population density increases, the indirect fitness benefits of polyandry (i.e. producing attractive but less competitive sons) may decrease and it is easy to imagine that this increased offspring attractiveness could even carry fitness costs in male biased, high density populations.

Environmental factors often influence female remating decisions (Bleu, Bessa-Gomes, & Laloi, 2012; Pai & Yan, 2002) and social situation (e.g. sex ratio), mate encounter rates and demography can all affect the costs and benefits of polyandry (Bleu et al., 2012; Holman & Kokko, 2013; Kokko & Mappes, 2013). Clearly, to understand the evolution of polyandry, the costs and benefits of multiple mating need to be considered in an ecological context. Indeed, while recent meta-analyses that have shown a weak, positive effect of polyandry on offspring viability (Slatyer et al., 2012), indirect genetic benefits to polyandrous mating are controversial (Reding, 2014; Uller & Olsson, 2008). Perhaps this controversy persists because the magnitude of the indirect benefits of polyandry depend enormously on population ecology and demography, factors that are often overlooked in laboratory research.
REFERENCES


Evolutionary Biology, http://doi.org/10.1111/jeb.12563


Table 1. Means ± SE of fitness measures for mothers.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Singly mated</th>
<th>Mating Treatment</th>
<th>Polyandry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lifetime reproductive success (LRS)</td>
<td>158.276 ± 5.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>189.36 ± 5.94&lt;sup&gt;b&lt;/sup&gt;</td>
<td>191.84 ± 4.32&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Fecundity</td>
<td>142.20 ±5.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>170.40±4.30&lt;sup&gt;d&lt;/sup&gt;</td>
<td>174.76±6.21&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>Longevity – fecundity group (weeks)</td>
<td>33.60 ± 1.00</td>
<td>31.56 ± 1.59</td>
<td>30.64 ± 2.03</td>
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<tr>
<td>Longevity – LRS group (weeks)</td>
<td>32.08 ± 1.13</td>
<td>32.08 ± 0.69</td>
<td>30.64 ± 2.03</td>
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</table>

Different letters indicate a significant difference at P < 0.05 by Student’s t test with the sequential Bonferroni method (Rice, 1989).
Table 2. Means ± SE of offspring traits of each treatment.

<table>
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<tr>
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<td>Singly mated</td>
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<td><strong>Male offspring</strong></td>
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<tr>
<td>Development time (days)</td>
<td>46.07 ± 0.33⁹</td>
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<td>Body mass (mg)</td>
<td>2.72 ± 0.006</td>
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<td>Longevity (weeks)</td>
<td>29.78 ± 0.51</td>
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<td>Copulation latency (seconds)</td>
<td>1181.38 ± 38.01³</td>
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<td>Courtship rate (per second)</td>
<td>0.06 ± 0.003³</td>
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<td>Mandible length (mm)</td>
<td>0.40 ± 0.007²</td>
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<td><strong>Female offspring</strong></td>
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<td>Development time (days)</td>
<td>44.49 ± 0.40³</td>
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<td>Body mass (mg)</td>
<td>2.70 ± 0.007</td>
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<td>Longevity (weeks)</td>
<td>34.49 ± 0.46</td>
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<tr>
<td>LRS</td>
<td>124.23 ± 2.79</td>
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Different letters indicate a significant difference at P < 0.05 by Student’s t test with the sequential Bonferroni method (Rice, 1989). Average values for offspring of 20 mothers/treatment.
Table 3. Analysis of variance in traits measured in sons and daughters

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<tr>
<td>Development Time</td>
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