EXPERIMENTAL REMOVAL OF SEXUAL SELECTION REVEALS ADAPTATIONS TO POLYANDRY IN BOTH SEXES

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Short title: Adaptations to polyandry in both sexes.
Abstract

Because multiple mating is so common, conflict between the sexes is widespread, with unavoidable consequences for multifarious facets of reproduction. In fact, the evolutionary interests of males and females are only completely unopposed under strict genetic monogamy, where sexual conflict and sexual selection are absent. Investigating the precise consequences of adaptation to the presence of sexual selection and conflict is critical to further understanding of the evolution of polyandry. Here we use an experimental evolution approach applied to the Tribolium castaneum model system to assess adaptations to polyandry, and the presence of sexual selection and conflict, in both sexes. Specifically, we assess male and female reproductive success (RS) in animals from monogamous and polyandrous experimental evolution lines subjected to single vs. multiple male scenarios, as well as longevity and mating behaviors to investigate adaptations to the contrasting selection history. We find that both males and females from the polyandrous regime have greater RS when multiple males are present than monogamous counterparts. Specifically, polyandrous background females produced more offspring, whereas polyandrous males were superior in sperm competition with multiple marker males. In contrast, in the single male scenario, polyandrous and monogamous beetles did not differ in RS. Results from mating trials with multiple males suggest that adaptations to polyandry in both sexes provide benefits when sexual selection is present. In particular, polyandrous females evolved to become choosier while polyandrous males tended to be quicker to achieve matings after mounting. By exposing beetles to different sexual selection intensities, achieved via monogamy vs. polyandry, we reveal adaptations to multiple mating that can have a major impact on reproductive fitness in both sexes.

KEY WORDS: sexual conflict, Tribolium castaneum, Coleoptera, artificial selection, experimental evolution, reproductive success
Introduction

Across the animal kingdom, females commonly mate with more than one male although this is not strictly necessary to cover fertilization needs. Polyandrous mating is especially puzzling given that mating is often associated with considerable costs, such as mechanical damage caused during copulation (Blanckenhorn et al. 2002) or harmful substances transferred with the ejaculate (Chapman et al. 1995; Wigby and Chapman 2005). Although polyandry is so common, the underlying evolutionary causes often remain elusive (Arnqvist and Rowe 2005). Nevertheless, several evolutionary explanations for polyandry have been proposed to date. According to these, polyandry is variously driven by direct material (e.g. Vahed 1998; Hosken and Stockley 2003), indirect genetic benefits (Jennions and Petrie 2000; Simmons 2001; Zeh and Zeh 2001; Neff and Pitcher 2005; but see also Kotiaho and Puurtinen 2007), avoiding costs of not remating (Thornhill and Alcock 1983), or via non-adaptive routes (e.g. Halliday and Arnold 1987). More recently it has been suggested that female polyandry might provide a means to combat the negative consequences of mating with males bearing selfish genetic elements (Price et al. 2008).

Overall, though, despite intense scrutiny, there remains no general consensus regarding the predominant evolutionary mechanisms responsible for counter-intuitively high levels of polyandry found in many animal taxa. There are various obstacles to progress on the way to fully understanding evolutionary causes and consequences of polyandry. At the individual level, there is still a lack of knowledge concerning meaningful natural levels of polyandry and resulting impacts on males and females. In recent studies, this gap has partly been addressed via the use of molecular methods enabling quantification of natural mating rates and sperm utilization (e.g. Bretman and Tregenza 2005; Simmons et al. 2007; Demont et al. 2011; Demont et al. 2012). At a larger scale, we also do not fully understand the reproductive traits involved in male-female coevolution associated with having multiple mates. Specifically, although polyandry enables sexual selection to occur it also provides fertile ground for sexual conflicts over all facets of reproduction to germinate. The interests of the sexes are very frequently in opposition (Parker 1979; Arnqvist and Rowe 2005), thus polyandry can drive adaptations and counter-adaptations to evolve as each sex is selected to gain an edge in this conflict (Chapman et al. 2003; Martin...
Experimental evolution is a particularly powerful tool for the study of male-female co-evolution and responses to sexual conflict, and has been put to good use in a number of species and contexts (reviewed in Arnqvist and Rowe 2005; Edward et al. 2010). Across previous artificial selection experiments, contrasting sexual selection/conflict intensities have been implemented in a variety of ways, such as population size/density (e.g. Martin and Hosken 2003b, 2004b; Gay et al. 2009; Hosken et al. 2009) or through contrasting sex ratios (e.g. Wigby and Chapman 2004; Crudgington et al. 2005). Experimental evolution using regimes with different sex ratios has recently been used in the important model *Tribolium castaneum* to expose evidence for sexual conflict in this system (Michalczyk et al. 2011b). Specifically, it was found that both sexes responded to selection under different sex ratios after only 20 generations. Another particularly clear means of achieving contrasting selection regimes is to apply monogamy vs. polyandry. Under monogamy with random mate allocation, sexual selection and conflict are essentially absent (Rice 2000), so one can assess the presence of sexual selection via an intuitive yes-no dichotomy. This method has been implemented previously in various insect species to illuminate the profound importance of sexual selection and conflict in shaping variation in reproductive traits (e.g. Holland and Rice 1999; Hosken et al. 2001; Hosken and Ward 2001; Martin and Hosken 2003a; Martin et al. 2004).

Here we assess reproductive success (RS) of male and female *T. castaneum* from monogamous and polyandrous experimental evolution lines subjected to single male vs. multiple male scenarios, as well as longevity and mating behaviors to investigate:

a) how selection has shaped male and female traits: e.g. do polyandry animals generally do better, perhaps due to good genes effects of sexual selection, or worse, reflecting costs of sexual conflict?

b) more specifically, whether animals from particular backgrounds are more adapted to that situation: i.e. are monogamous beetles fitter under the single male scenario, and polyandrous beetles better when sexual selection can act in the multiple male scenario?
Methods

EXPERIMENTAL EVOLUTION LINES

*Tribolium castaneum* is an eminent model system for studies of pre- and postcopulatory sexual selection (reviewed in Fedina and Lewis 2008). As the source population for the experimental evolution lines described below, we used the *T. castaneum* wild type strain Georgia 1 (Ga1, initial collection 1980, maintained in culture by the Beeman lab at USDA, Manhattan, Kansas). Experimental evolution lines (initiated in 2005 at the University of East Anglia) and other experimental animals were kept on organic white flour supplemented with 10% brewer’s yeast at 30°C and ca. 65% RH. To achieve contrasting sexual selection intensity, we established the following two regimes: monogamy (= sexual selection/conflict absent) and polyandry (= sexual selection present). Both treatments consisted of three replicate lines each, *i.e.* M A/B/C and P a/b/c. Each monogamy line consisted of twenty pairs (so \(N_e = 40\)) maintained for ca. 7 days. Each polyandry line consisted of 12 females each simultaneously exposed to 5 different males (also \(N_e = 40\)) for the same period.

FEMALE RS IN SINGLE MALE VERSUS MULTIPLE MALE SCENARIOS

To ensure virginity, pupae were collected from the 36th generation and separated by sex. Here, only the females were from the selection lines and these were allowed to mate with tester males (*i.e.* from the unselected source population Ga1) under contrasting single male or multiple male scenarios (single male scenario: \(n=24\) females per line, multiple male scenario: \(n=20\) females per line). In the single male scenario focal females were each maintained with one tester male and in the multiple males scenario females were each housed with 5 tester males. These scenarios were chosen to mirror conditions in the M and P selection regimes, and allow us to investigate how selected females respond to their own vs. opposite conditions. All females were allowed to mate and lay eggs for 8 days in 5 cm diameter Petri dishes containing *ca.* 10 g standard flour-yeast mix, topped with organic rolled oats. Then adults were removed and offspring incubated for additional 29 days before counting reproductive output.

MALE RS IN SINGLE MALE VERSUS MULTIPLE MALE SCENARIOS

Using the males from generation 36, we measured male RS in the single male scenario and paternity share
in the multiple males scenario. Specifically, in the single male scenario, focal males from the selection lines were allowed to reproduce with one tester Ga1-female for 8 days (n=12-17 males per line and scenario). In the multiple males scenario, focal males were given access to one Ga1-female, but were in competition with 4 Rd-males (Rd: Reindeer, easily identifiable phenotypic marker homozygous in a dominant Rd allele, supplied by the Beeman lab), so that sex ratios were F:M=1:5 as in the experiment from the female’s perspective. Here the response measured was the share of paternity accrued by the focal male (= proportion of wild type offspring). Rd has been used successfully with Ga1 in previous experiments (see e.g. Michalczyk et al. 2010; Michalczyk et al. 2011b; Sbilordo et al. 2011). Again offspring were incubated for 29 days after removing adults before counting reproductive output.

**LONGEVITY UNDER STARVATION**

25 pupae per sex and selection line were collected from the 37th generation to determine the longevity of virgin beetles under starvation as a measure of general vigor. All pupae were kept isolated in single wells of 96-well plates without access to flour for emergence and also thereafter. Beetles were checked daily to record time until death after emergence. 15 out of 150 M- and 6 out of 150 P-line pupae did not emerge. This difference is statistically significant (Pearson’s Chi-squared test: $X^2 = 4.15, P = 0.042$) indicating that the M and P selection backgrounds influenced this trait. In our analysis, we only used healthy beetles, i.e. those, which had emerged successfully as adults. Longevity was assessed in virgins, as mating activity is known to dramatically decrease female and male longevity in other species (e.g. Blanckenhorn et al. 2002; Martin and Hosken 2004a). Furthermore, unmated beetles were preferred, as our selection regimes may be expected to alter such traits as female resistance to male-induced harm (see e.g. Martin and Hosken 2003b, 2004b; Michalczyk et al. 2011b). Starvation was deemed necessary, as this stressful treatment should facilitate the detection of subtle longevity effects that may be obscured in overly benign environments (see e.g. Hoffmann and Parsons 1991; Moret and Schmid-Hempel 2000; Martin and Hosken 2003b; Schwarzenbach and Ward 2007). Finally, this assay has also been applied successfully to work focusing on the relationship between inbreeding and polyandry on the same species and strain (Michalczyk et al. 2011a).
MATING BEHAVIORS

Focal beetles for the behavior experiment were collected from generation 53 as pupae and separated by sex. At the same time, test beetles were collected from the Ga1 stock. After emergence, test beetles were marked on the thorax with different colors to be able to discriminate each individual during mating trials. Focal individuals were unmarked. Equal to the RS experiment mating trials were conducted separately for males and females. However, behaviors were only measured in the multiple male scenario to follow up on differences between M and P detected in the RS experiment. In contrast to the experiment on RS tester males in our mating trials were all Ga1 and not Rd, because here it was not necessary to discriminate offspring from different fathers. In detail, female trials were set up with one focal M or P female and three differently marked Ga1 tester males, whereas male trials consisted of one focal M or P male with one tester Ga1 female and two Ga1 competitor males. All beetles were virgins and at least ten days post emergence when the experiments started. On day 1 of the experiment, groups were put together in a Petri dish (Ø 5cm) with filter paper on the bottom and initially without access to food. The first 30 minutes of interactions were observed at room temperature (22-23°C). Specifically, we recorded all behaviors where the focal individual was involved, such as the time of the first mount, all subsequent mounts and all copulation durations. After the first trials a small amount of flour-yeast mix was added to each Petri dish. All groups were observed again for 30 minutes on the 3rd and 6th day, when beetles were potentially more experienced than on the 1st day as virgins. In total, 24 beetles per line and for both male and female perspective were observed.

DATA ANALYSIS

We analyzed the influence of selection history (M vs. P) based on the line means of our response variables using linear models (i.e. lm function) in R version 2.14.1 (R Development Core Team 2011). The residuals of all models were reviewed to fulfill the model assumptions using the Shapiro-Wilk normality test and the Bartlett test of homogeneity of variances. For two response variables, time to the first mount from the female perspective and time of female resistance, the equal variances assumption was violated and a one-way analysis of means (i.e. one-way test in R) was performed instead. Mean paternity shares of
the multiple males scenario were analyzed with a generalized linear model for proportions data using a
quasibinomial distribution to account for overdispersion (i.e. glm function and Chi-square test). In
addition to analyses on line means, we performed analogous analyses on individual-level data using
nested generalized linear mixed models (GLMMs, i.e. lmer function with Poisson distribution for counts,
binomial distribution for proportions and Gamma distribution for survival data; line as random factor
nested in selection history; P-values obtained with pvals.fnc function and 10’000 cycles of MCMC
sampling). There were no qualitative differences between these GLMs and the results based on line means
and also quantitatively the results were similar. However, in most cases the GLMMs did not fulfill the
model assumptions even after transformations of the response variables. Therefore, we only present the
results based on line means.

Results

FEMALE RS

In the single male scenario (focal female & one tester male) the total number of offspring produced by the
female was not influenced by selection history ($F_{1,4} = 0.86, P = 0.407$; Fig. 1a). In contrast, in the multiple
males scenario (focal female & five tester males) females with P selection history had significantly more
offspring than females with M selection history ($F_{1,4} = 31.63, P = 0.005$; Fig. 1b).

MALE RS

In the single male scenario (one tester female & focal male) the total number of offspring produced was
not influenced by the male’s selection history ($F_{1,4} = 0.13, P = 0.733$; Fig. 2a). In contrast, in the multiple
males scenario (one tester female & focal male & four competitor tester males) the paternity share of the
focal male was significantly influenced by selection history ($X^2_{1} = 120.64, P < 0.001$; Fig. 2b), as P-males
achieved more offspring than M-males in competition.

LONGEVITY UNDER STARVATION

Females from M-lines lived on average 24±8 days, which was slightly longer than the males with 23±5
days. Males and females from P-lines had a very similar longevity with females living on average 27±6
days and males 26±6 days. The interaction between sex and selection history on longevity was not significant ($F_{1,8} = 0.10, P = 0.761$). Furthermore, neither sex ($F_{1,8} = 0.22, P = 0.648$) nor selection history ($F_{1,8} = 3.75, P = 0.089$) had a significant effect. When the interaction was removed from the model selection history remained not significant ($F_{1,5} = 4.17, P = 0.072$).

**MATING BEHAVIORS FROM THE FEMALE PERSPECTIVE**

There was no difference in the time elapsed until a M- or P-female was mounted for the first time ($F_{1,2} = 1.67, P = 0.324$; Fig. 3). However, P-females waited longer to copulate for the first time than M-females ($F_{1,4} = 13.24, P = 0.022$; Fig. 3). In total, over all three observation trials, females with M or P selection history were not different in terms of duration of resistance per copulation (M: 11.1±0.2 sec., P: 14.7±4.0 sec., $F_{1,2} = 0.82, P = 0.461$), copulation duration (M: 1.3±0.2 min., P: 1.4±0.2 min., $F_{1,4} = 0.11, P = 0.755$) or the total number of copulations (M: 1.8±0.1, P: 1.4±0.2, $F_{1,4} = 3.72, P = 0.126$).

**MATING BEHAVIORS FROM THE MALE PERSPECTIVE**

On average M- and P-males waited as long until they mounted the tester female for the first time ($F_{1,4} = 0.09, P = 0.781$; Fig. 4). In contrast, P-males started the first copulation earlier than M-males, although the difference was marginally not significant ($F_{1,4} = 6.81, P = 0.059$; Fig. 4). In addition, considering all three observation periods, there were no significant differences between selection histories in the total number of mounts on the female (M: 4.3±0.7, P: 4.8±1.1, $F_{1,4} = 0.12, P = 0.747$), total number of copulations (M: 1.7±0.4, P: 1.9±0.5, $F_{1,4} = 0.12, P = 0.747$) and copulation duration (M: 1.7±0.2 min., P: 1.8±0.1 min., $F_{1,4} = 0.14, P = 0.726$).

**Discussion**

Using contrasting mating scenarios we found adaptation to polyandry in both sexes of our model organism *Tribolium castaneum*. When assessed in single pairs in the absence of sexual selection and conflict, M- and P-line beetles had equal reproductive fitness. However, when multiple males were present, males and females from the P-regime both had greater RS than their M counterparts. P-individuals of both sexes are hence superior in the more natural mating situation including sexual selection and conflict. This mirrors
previous work on *Scathophaga stercoraria* indicating that polyandrous animals require multiple mates in order to profit from their adaptations (Martin et al. 2004). Assessing longevity under starvation in both sexes indicates that results are not due to *P*-individuals being generally fitter than *M*-individuals. Instead, behavioral assays indicate that *P*-females are choosier when in the presence of multiple males. Additionally, *P*-males tended to be more efficient in obtaining copulations swiftly following mounting. Overall, adding to the detection of sexual conflict by Michalczyk et al. (2011b), by enforcing strict monogamy, here, experimental evolution reveals adaptations to pre- and postcopulatory sexual selection in *T. castaneum.*

**FEMALE FITNESS**

Females with *P*-background may have higher reproductive success in the presence of multiple males compared to females with *M*-background, if particular adaptations provide benefits of sexual selection due to polyandry (reviewed *e.g.* in Jennions and Petrie 2000; Zeh and Zeh 2001). Specifically, *P*-females could have higher mating and remating rates thereby increasing not only sperm supply (direct benefit) but also the chance and strength of sperm competition (indirect benefits; Arnqvist and Nilsson 2000). Likewise, *P*-females may be better than *M*-females at making the right mating choice(s) to pick the optimally suited father(s) to gain indirect genetic benefits for their offspring (Tregenza and Wedell 2000; Mays and Hill 2004). It has been shown that in unselected *T. castaneum* females, access to multiple males is neither beneficial nor costly under standard conditions, with no detectable effect on female fitness (Pai and Yan 2003; Grazer and Martin 2012). In contrast, after a rapid temperature increase females in a multiple males scenario achieved higher reproductive success than females in the single male scenario suggesting that environmental change can shift the balance between costs and benefits of multiple mating (Grazer and Martin 2012). In this previous experiment, which used the source population of the present experimental evolution study, the most likely explanation for the observed pattern seemed to be sexual selection for good or compatible genes in the novel environment (reviewed in Mays and Hill 2004; Grazer and Martin 2012). In order to investigate whether sexual selection and more specifically which exact mechanism is responsible for *P*-females profiting more from multiple males it is necessary to have more
information about mating propensities and mating rates. Our behavioral assays indicate that when faced with multiple males, P-females take longer to copulate for the first time. P-females exposed to a multiple male scenario hence appear to be choosier than M-females. Thus, P-females may have evolved to pick the suitable male rather than to mate with multiple males when given a choice (confirmed also by Grazer & Martin, unpublished data).

In contrast, it may not only be benefits of sexual selection explaining our findings for female fitness. As an alternative, it has frequently been shown that P- compared to M-females may evolve to deal better with mating costs due to sexual conflict (Arnqvist and Rowe 2005). In particular, experimental evolution studies have been very useful for revealing hidden costs, and this has successfully been applied to many systems (e.g. Rice 1996; Holland and Rice 1999; Martin and Hosken 2003a; Martin et al. 2004). The preceding experimental evolution study on T. castaneum applying difference sex ratios rather than contrasting mating systems clearly showed that experimental manipulation may be necessary to capture clear evidence of sexual conflict (Michalczyk et al. 2011b). Specifically, females exposed to weak sexual selection intensity (i.e. female-biased sex ratio, contrasted vs. male-biased, high conflict regime) suffered more from exposure to multiple males (Michalczyk et al. 2011b). When exposed to increasing numbers of (control) males, females from female-biased lines showed a steep decline in reproductive fitness (Michalczyk et al. 2011b). This finding seemed to be a response to increased levels of mating vigor and thus harmfulness of males from male-biased selection lines (Michalczyk et al. 2011b). However, in T. castaneum a recent study identified a range of seminal fluid proteins, which are transferred to the female reproductive tract (South et al. 2011). Although it is yet unknown whether any of these proteins have costs for females there might be at least the possibility of finding similar negative effects of postcopulatory sexual conflict as in Drosophila melanogaster (Chapman et al. 1995; Wigby and Chapman 2005). The female behaviors assessed here, however, did not indicate that P-females are under selection pressure to reduce mating frequency, mating duration or increase resistance to male mating attempts.

In sum, improved reproductive success of females under polyandry does not seem to be due to mating more frequently, or a function of superior general vigor, as longevities were similar. Rather differences
seem to relate to increased choosiness and potentially greater resistance to male harm in P-females.

MALE FITNESS

Similar to our findings for females, we find that in the single male scenario (focal male housed with one female) our measure of reproductive success was not influenced by selection history: M- and P-males produced equal numbers of offspring. In contrast, in the multiple males scenario, where focal males were forced to compete with four males for access to a single female, the focal male’s paternity share was significantly influenced by male selection history. P-males had a significantly greater share of paternity than their M counterparts. This finding of superior male competitive ability mirrors findings in previous experiments (e.g. Hosken et al. 2001; Pitnick et al. 2001; Simmons and Garcia-Gonzalez 2008). As for females, these results do not seem to be driven by differences in general vigor across regimes, because longevity of M- and P-males was the same under starvation. Rather they seem to arise from differences in both pre- and/or postcopulatory processes, as in addition to being better sperm competitors, P-males seem be better at achieving matings in the first place. Here we found evidence that, in the face of competition with competitor males, P-males tend to be quicker to copulate successfully than M-males, although P-males were not quicker to mount. This hence indicates greater efficiency at obtaining matings. Further support for this notion comes from the related experimental evolution study using the same source population. There, males from male-biased sex ratios (i.e. exposed to increased sexual selection intensity) obtained greater reproductive success when forced to compete for females with a control male than males from a female-biased background (Michalczyk et al. 2011b). Furthermore, this difference seemed to relate to precopulatory male behavior, as these males were faster to mount females and spent more time mounting and mating (Michalczyk et al. 2011b). Whereas varying sexual selection intensity via sex ratio as in Michalczyk et al. (2011b) allows the study of a range of sexual selection intensities, the use of a monogamy treatment as done here effectively removes sexual selection from the environment beetles evolved under. In the present study, the contrast is hence even clearer, as we can compare situations with vs. without sexual selection in a dichotomous manner (see also Grazer and Martin 2012). Overall, it is conceivable that males from the P-regime (i.e. where sexual selection and conflict were present and...
particularly intense) are not only better in sperm competition, but also better at achieving copulations.

Contrasting with the more subtle results in females (increased choosiness), superior performance of \textbf{P}-males in multiple male situations appears to be a more direct reflection of improved male competitive ability. Interestingly, though, a multiple male scenario is necessary to expose the enhanced pre- and postcopulatory abilities of \textbf{P}-males.

\textbf{CONCLUSIONS}

We find that both males and females from \textbf{P}-lines have greater reproductive success when multiple males are present than their \textbf{M}-counterparts. In contrast, when animals were maintained without choice and competition, \textbf{M}- and \textbf{P}-beetles did not differ in reproductive success. This suggests that in both sexes adaptations to the presence of sexual selection only provide benefits when sexual selection mechanisms are allowed to act. There was, however, no indication that these adaptations bear large costs in either sex, as at least within the confines of the experiment, there was no difference in reproductive output when animals were housed monogamously. Results do not seem to be driven by superior vigor of individuals from the \textbf{P} selection lines as longevity under starvation was equal across regimes. Furthermore, focusing on mating behavior in both sexes when multiple males are present, \textbf{P}-females appear to have evolved to become choosier while \textbf{P}-males tended to achieve copulations more quickly. Overall, our findings underline how differential sexual selection intensity, as implemented here with experimentally enforced monogamy (no sexual selection) \textit{vs.} polyandry (sexual selection present), molds the evolution of reproductive traits and fitness. Future efforts will aim to pinpoint the precise mechanisms responsible for determining reproductive success of both sexes and male competitive ability.

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**LITERATURE CITED**


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**Figure legends**

**Figure 1.** Female reproductive success. Females from monogamous (M) vs. polyandrous (P) selection regimes were exposed to single or multiple tester males to investigate adaptations to the presence of sexual selection/conflict. (a) Single male scenario: focal female & one tester male for 8 days. (b) Multiple males scenario: focal female & five tester males for 8 days.

**Figure 2.** Male reproductive success or paternity shares. Males from monogamous (M) vs. polyandrous (P) selection regimes were assessed in the single male scenario (i.e. no competition over access to female) or the multiple male scenario (i.e. competitor males present) to investigate adaptations to the presence of sexual selection/conflict. (a) Single male scenario: focal male & one tester female for 8 days. (b) Multiple males scenario: focal male & one tester female & four competitor Rd males for 8 days.

**Figure 3.** Female mating behaviors. Focal females from monogamous (M) and polyandrous (P) selection regimes observed in mating trials with three tester males. All beetles were virgins at the first encounter. Here, first mount = female being mounted for the first time.

**Figure 4.** Male mating behaviors. Focal males from monogamous (M) and polyandrous (P) selection regimes observed in mating trials with one tester female and two competitor tester males. All beetles were virgins at the first encounter.