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Paternity assurance before and after fertilization by male burying beetles (*Nicrophorus quadripunctatus*)

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Running title

Paternity assurance before and after fertilization

Abstract

Parental care requires a large investment of time and energy. This can reduce future parental survival and opportunities for mating. Because males are usually more uncertain of their parentage with respect to the caring of offspring than are females, the reduction in reproductive success is thought to be greater in males. Therefore, males are under selection to ensure paternity of the offspring for which they care. Males can increase paternity before and after fertilization. Before fertilization, males can increase paternity by increasing their competitive ability for fertilization. After fertilization, males can increase paternity by cannibalizing unrelated offspring. Here, we investigated the stage at which male burying beetles, *Nicrophorus quadripunctatus*, increase their paternity by evaluating the number of offspring sired by a nursing male in asynchronously hatched broods in relation to hatching time. We found that nursing males assure a very high level of the paternity of hatching offspring. We also found that the paternity of non-nursing and nursing males remained constant across hatching time within a brood, indicating that it is unlikely that filial cannibalism plays a role in increasing the paternity of offspring. We concluded that ensuring paternity before fertilization is more important in increasing the paternity of offspring.

Keywords: *Nicrophorus*; burying beetle; asynchronous hatching; filial cannibalism

Introduction

Parental care is known across a range of taxa (Zeh & Smith 1985; Clutton-Brock 1991; Beck 1998; Eggert et al. 1998; Tallamy 2000; Reynolds et al. 2002; Cockburn 2006; Summers et al. 2006). Parental care requires a large investment of time and energy, which can reduce future parental survival and opportunities for mating. Because the reproductive success of males is always more limited by mating opportunities than it is for females (Bateman 1948; Wedell et al. 2006), the reduction in reproductive success by lost opportunities for future reproductions is thought to be greater in males. Furthermore, males face a greater risk of caring for unrelated offspring than females (Clutton-Brock 1991; Davies 1992). Therefore, confidence of paternity for males is decreased. In a species whose females have sperm storage organs, some offspring may be fertilized by the sperm stored in a female's reproductive tract from a prior mating experience (Müller and Eggert 1989). Because investing energy and resources in unrelated offspring is costly, males are under selection to ensure the paternity of the offspring for which

they care. Males can increase paternity before and after fertilization. Before fertilization, males can increase paternity by increasing their competitive ability for fertilization (e.g. competition for mating, mate guarding, sperm removal, and sperm competition). After fertilization, males can increase their paternity by cannibalizing some of the unrelated offspring (partial filial cannibalism). In some species, some offspring are cannibalized by their parents (reviewed by FitzGerald 1992; Manica 2002).

Filial cannibalism can be adaptive for parents to minimize parental investment. Particularly, this would applicable to filial cannibalism by females. However, the adaptive causes for filial cannibalism by males may be different from those for females, because males can decrease their parental investment by deserting a female and her offspring. Filial cannibalism by males could be adaptive if males are able to selectively cannibalize unrelated offspring.

Burying beetles, *Nicrophorus* spp., provide elaborate biparental care to their offspring, including provisions of food (Eggert and Müller 1997; Scott 1998). Burying beetles use the carcasses of small vertebrates as food for their larvae. Females copulate with males repeatedly and lay eggs in the soil near the carcass. In *N. quadripunctatus*, each copulatory attempt was completed within 3 min (Takata, unpublished study). Female burying beetles can store transferred sperm within spermatheca. In *Nicrophorus vespilloides*, most females already have fertile sperm stored from a previous mating when they arrive at the carcass, and some of the offspring that hatch arise from eggs fertilized by the stored sperm (Müller and Eggert 1989). The larvae hatch asynchronously over a mean time span of 25 h (range 4–56 h) on a 15-g carcass (in *N. quadripunctatus*; Takata, unpublished study). After hatching, the larvae crawl to the carcass and obtain food by begging for pre-digested carrion from their parents or by self-feeding from the carcass (in *N. vespilloides*; Smiseth and Moore 2002; Smiseth et al. 2003; in *N. quadripunctatus*; Takata, unpublished study). Partial filial cannibalism occurs in *N. quadripunctatus* (Takata, unpublished study). Both males and females cannibalize some of their offspring (in *N. quadripunctatus*; Takata, unpublished study). Previous studies on *N. quadripunctatus* have shown that offspring that hatch later face higher mortality risks when cared for by males (Takata, unpublished study). Burying beetles cannot directly recognize their relatives after the larvae have hatched (in *N. vespilloides*; Müller and Eggert 1990; in *N. quadripunctatus*; Takata, unpublished study). However, if most of the offspring fertilized by stored sperm from a previous mating hatch later, then males could increase their paternity by filial cannibalism. Here, we investigated the stage at which males increase their paternity by

evaluating the number of offspring sired by a nursing male in asynchronously hatched broods in relation to hatching time.

Materials and methods

Sperm storage by females in the field

In October 2012, we collected adult female *Nicrophorus quadripunctatus* Kraatz in the field by using baited pitfall traps to determine the proportion of female beetles that have fertile sperm in store. Burying beetles cannot escape from the traps. Female beetles caught in the traps without conspecific males were chosen for use in this experiment. We collected 16 such females. Each female was individually placed in a plastic cup (height 8 cm, diameter 15 cm) containing 2 cm of moist peat and 15 ± 0.5 g of carcass (a piece of fresh chicken meat). All females were given this carcass in the same state of freshness. After 93 h, which is the approximate time when females typically finish laying eggs (Takata, unpublished study), the female and the carcass were removed from the cup. The eggs were left in the plastic cup to hatch. To determine whether the female had fertile sperm, the eggs were checked for hatching larvae at 12-h intervals. We defined reproductively active females and non-reproductive females by following the definition criteria described by Müller and Eggert (1989). A female that had laid eggs within 4 days was defined as a reproductively active female, and a female that had not was defined as a non-reproductive female. A female was considered to have fertile sperm in store if one or more larvae hatched from her eggs.

Behavioural observation and maintenance of beetles

We collected adult *N. quadripunctatus* in baited pitfall traps in Chiba, Japan, and reared the first-generation offspring in the laboratory. The beetles were maintained individually in small transparent plastic cups (height 4 cm, diameter 6 cm) at 20 ± 1 °C under a 14:10 h light:dark cycle. They were fed small pieces of fresh chicken meat twice a week. All males and females used in this experiment were sexually mature and between 21 and 35 days of age.

We used six observation groups in this study. In the first and second groups, we observed the copulation frequency of non-nursing males that were allowed to copulate with females without a carcass but did not provide parental care. We observed the copulation frequency at 1 h (first group) or 24 h (second group) following initial placement of the males with the females. Pairs of randomly selected, non-sibling, virgin male (non-nursing males) and female beetles were

each placed in a plastic cup (height, 8 cm; diameter, 15 cm) containing 2 cm of moist peat. The first group was composed of 20 pairs of males and females, and the second group was composed of 30 pairs. The pairs were allowed to copulate at 20 ± 1 °C in a dark incubator. The number of copulatory attempts (i.e. male mounting a female) was counted during a 30-min period at 1 h (first group) or 24 h (second group) following initial placement in the incubator.

In the third to sixth groups, we observed the copulation frequency of nursing males in different breeding periods. Females of *N. quadripunctatus* typically start laying eggs around 48 h after introduction and finish laying eggs around 84 h after introduction (Takata, unpublished study). We set up observation periods during pre-oviposition (1 h and 24 h after introduction), oviposition (72 h after introduction), and post-oviposition (120 h after introduction). We first prepared previously mated females by following the same experimental manipulation for the first and second observation groups. The males and females were allowed to copulate for 24 h. The females were used in the following experiment immediately after this manipulation. Pairs of randomly selected, non-sibling, virgin males (nursing males) and the previously mated female beetles were each placed in a plastic cup (height 8 cm, diameter 15 cm) containing 2 cm of moist peat and 15 ± 0.5 g of carcass (a piece of fresh chicken meat). All pairs were given this carcass in the same state of freshness. The third group was composed of 20 pairs of males and females, and the fourth to sixth groups were composed of 42 pairs each. The pairs were allowed to copulate at 20 ± 1 °C in a dark incubator. The number of copulatory attempts was counted during a 30-min period at 1 h (third group) or 24 h (fourth group) or 72 h (fifth group) or 120 h (sixth group) following initial placement in the incubator. All observations were conducted under red light. Following our observations, we confirmed that all females actually did not lay eggs at 1 h and 24 h after introduction, laid eggs at 72 h after introduction, and finished laying eggs before 120 h.

Parentage analysis by using amplified fragment length polymorphism

Eleven pairs of randomly selected, non-sibling, virgin male (non-nursing male) and virgin female beetles were each placed in a plastic cup (height 8 cm, diameter 15 cm) containing 1 cm of moist peat. They were allowed to copulate for 24 h at 20 ± 1 °C in a dark incubator. At 24 h following placement in the incubator, the females were placed in a new plastic cup containing 2 cm of moist peat and 15 ± 0.5 g of carcass (a piece of fresh chicken meat) with a non-sibling, virgin male (nursing male). All pairs were given this carcass in the same state of freshness. The male, female, and carcass were removed from the cup at 93 h, and the eggs were left in the plastic cup

to hatch. The hind legs of male and female beetles were surgically removed and immediately stored in 99.5 % ethanol. We collected newly hatching offspring at 4-h intervals until all the offspring had hatched and stored them in 99.5 % ethanol. We obtained 248 larvae [20.7 ± 8.5 larvae (mean ± SD) from 11 clutches] and analyzed their paternity by using amplified fragment length polymorphism (AFLP). The hatching rate was 81.0 ± 3.7 % (mean ± SD).

DNA extraction

The extraction of DNA from the legs of adult beetles or the whole body of larvae was performed using a DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Approximately 3 mm of the legs of the adult beetles or the whole body of larvae were transferred to a sterile 1.5-ml microcentrifuge tube containing 180 µl of ATL buffer (Qiagen) and 20 µl of proteinase K (Qiagen) and incubated at 56 °C in a water bath to disperse the sample overnight until the tissue was completely lysed. The mixture was mixed by vortexing for 15 s. A total of 200 µl of AL buffer (Qiagen) was added to the sample and mixed thoroughly by vortexing. The mixture was then added to 200 µl of ethanol (99.5 %, Wako Pure Chemical Industries, Osaka, Japan) and mixed by vortexing to yield a homogenous solution. The homogenous solution was pipetted into the DNeasy® mini column in a 2-ml collection tube and centrifuged at 8,000 rpm for 1 min. The DNA bound to the column was washed in two centrifugation steps by using 500 µl of AW1 buffer and AW2 buffer, to improve the purity of the eluted DNA. The purified DNA was then eluted from the column in 200 µl of AE buffer and stored at 4 °C until further use.

AFLP procedure

The AFLP technique was performed by following the AFLP Core Reagent Kit protocol (Invitrogen, Carlsbad, CA, USA), according to the method of Vos et al. (1995), as follows: 1 µl of the total cellular DNA sample was double-digested with 0.4 µl of *Eco*RI/*Mse*I (Invitrogen), 1 µl of 5× reaction buffer, and 2.6 µl of distilled water. Adapters specific to *Eco*RI and *Mse*I digested DNA were ligated to the restriction fragments. After incubation at 37 °C for 24 h, 4.8 µl of the adapter ligation mixture and 0.2 µl of T4 DNA ligase (Invitrogen) were added and ligated for 2 h at 20 °C.

Pre-amplification

178 The resulting products were diluted tenfold, and 10 µl of reaction mixtures containing 1 µl
179 of DNA solution were used for PCR reactions in 1 µl of 10× PCR buffer, 1 µl of 10 mM dNTP
180 mix (200 µM each), 0.25 µl of 10 µM *Eco*RI (plus A) and *Mse*I (plus C) primers, 0.05 µl of
181 TaKaRa Ex Taq (1.25 U; Takara Bio, Shiga, Japan), and 4.95 µl of distilled water. After an
182 initial denaturation at 95 °C for 5 min, PCR was performed using 30 successive cycles of 94 °C
183 for 30 s, annealing at 56 °C for 60 s, and 72 °C for 60 s. Chain elongation at 72 °C was extended
184 to 5 min after the final cycle. The PCR was performed using a PCR thermal cycler (BioRad,
185 Richmond, CA, USA). The sequences of the primers *Eco*RI-A and *Mse*I-C were 5′-
186 GACTGCGTACCAATTCA-3′ and 5′-GATGAGTCCTGAGTAAC-3′, respectively. The pre-
187 selective amplification products were electrophoresed, and amplification was confirmed to
188 minimise genotyping errors.
189
190 Selective amplification
191 The pre-selective amplification products were diluted tenfold, and 10 µl of reaction mixtures
192 containing 1 µl of DNA solution were used in selective PCR amplification reactions in 1 µl of
193 10× PCR buffer, 1 µl of 10 mM dNTP mix (200 µM each), 0.05 µl of 10 µM *Eco*RI (plus AGG
194 or AAG) and 0.25 µl of 10 µM *Mse*I (plus CTA) primers, 0.05 µl of TaKaRa Ex Taq (1.25 U),
195 and 5.15 µl of distilled water. After an initial denaturation at 95 °C for 5 min, PCR was
196 performed using 30 successive cycles of 94 °C for 30 s. The annealing temperature in the first
197 cycle was 66 °C, which was subsequently reduced in each cycle by 1 °C for the next 12 cycles
198 and was continued at 57 °C for 60 s and 72 °C for 60 s. Chain elongation at 72 °C was extended
199 to 5 min after the final cycle. PCR was performed using a PCR thermal cycler (BioRad). The
200 sequences of the primers *Eco*RI-AGG, *Eco*RI-AAG, and *Mse*I-CTA were 5′-
201 GACTGCGTACCAATTCAGG-3′, 5′-GACTGCGTACCAATTCAAG-3′, and 5′-
202 GATGAGTCCTGAGTAACTA-3′, respectively.
203
204 Fragment analysis
205 AFLPs were detected using fragment analysis with the ABI PRISM3500 system. The S500 ROX
206 (PE Applied Biosystems, Foster City, CA, USA) fragment size standards were included in each
207 sample. Amplified fragments with fluorescent signals were identified using GeneScan 3.2.1 (PE
208 Applied Biosystems). All steps throughout the AFLP protocol were conducted to minimize
209 genotyping errors.

Assessment of reproducibility

To assess the reproducibility of our AFLP data (Crawford et al. 2012), 13 samples (5.2 % of the total sample size) were replicated from the stage of restriction enzyme digestion by using the same DNA extract. The genotyping error rate in this study was 3.2 % (total number of mismatched genotypes, 2; number of replicated genotypes, 63). The genotyping error rate was calculated, according to the method described by Pompanon et al. (2005), as the ratio of the total number of mismatched genotypes (band presence vs. band absence) to the number of replicated genotypes.

Parentage analysis

To detect paternity, all peaks were scored for presence/absence in each individual by using the GeneScan analysis software in the 40- to 700-bp range. The presence of 2 diagnostic peaks appears to be sufficient for detecting parentage, as reported in previous studies (Questiau et al. 1999; García-González et al. 2003, 2005; Simmons et al. 2004; Suzuki et al. 2006). In the current study, for small fragments, we considered all peaks with a height above 150 fluorescent units. All fragments present in the offspring, the two potential fathers, and the mothers were scored. For paternity assignment, fragments present in larvae but absent in mothers were assumed to be derived from the father. When 1 male and a larva had 2 or more common diagnostic peaks that were absent in the other male and the mother, the former male was assigned as the father of the larva. The total number of loci obtained was 944 loci. The number of polymorphic loci retained for parentage analysis was 112 loci. We determined the paternity of 79 % of the offspring, but we could not determine the paternity of the remaining offspring because they did not show any diagnostic peaks. We excluded these offspring from the following analysis.

Statistical analysis

First, one-way ANOVA was used to investigate temporal change in the frequency of copulation. The number of copulatory attempts observed in each mating period was treated as a response variable and mating periods were treated as an explanatory variable. Pairwise Wilcoxon rank sum tests were then used to examine the differences in copulation frequency between observation groups. A conservative Bonferroni adjustment for multiple testing (Zar 1984) was used for the analysis.

Second, to test the paternity bias towards non-nursing and nursing males, we compared the number of offspring sired by non-nursing males with the number of offspring sired by nursing males by using Wilcoxon rank test.

Finally, to demonstrate whether the hatching of offspring of non-nursing males was skewed towards the earlier or later hatching period, we examined the effect of hatching time on parentage distribution by using a generalized linear mixed model (GLMM) with the lme4 package (Bates and Maechler 2010). Paternity of each offspring of non-nursing or nursing males was treated as a response variable assuming a binomial distribution. Hatching time (i.e. the point in time when each larva hatched) was treated as an explanatory variable and family identity was treated as a random factor. All analyses were performed using R 2.12.1 GUI 1.35 (http://cran.r-project.org). The lme4 package was used for GLMM.

Results

Sperm storage in the field

Eleven of the 16 wild-caught females were reproductively active. Nine of the 11 reproductively active females (82 %) had fertile sperm in store. Therefore, most of the reproductively active females had stored fertile sperm in their reproductive tracts when they arrived at the carcass.

Copulatory attempts

Mating period had a significant effect on the frequency of copulation (ANOVA: $F_{5,190} = 12.932$, $p < 0.001$). Non-nursing males copulated with females 0.6 ± 0.2 times (mean ± SD) per 30 min at 1 h after introduction (Fig. 1; Table 1). However, the number of copulatory attempts significantly decreased at 24 h after introduction (i.e. no copulatory attempts were observed).

Nursing males copulated with females 0.7 ± 0.1 times (mean ± SD) per 30 min at 1 h after introduction, and 0.7 ± 0.1 (mean ± SD) times at 24 h following introduction (Fig. 1; Table 1). However, the number of copulatory attempts significantly decreased at 72 h (0.2 ± 0.1 times, mean ± SD) and 120 h (0.2 ± 0.1 times, mean ± SD) after introduction (Fig. 1, Table 1).

Parentage analysis

Paternity assignment to non-nursing and nursing males was biased towards the nursing male (Wilcoxon rank test, $z = -4.318$ $p < 0.001$); 0.4 ± 1.2 larvae (mean ± SD) were sired by non-nursing males and 15.8 ± 5.7 larvae (mean ± SD) were sired by nursing males. On average, 97 %

of the offspring (190/195 larvae) were sired by the nursing males. In 7/11 clutches, nursing males had 100 % paternity.

Asynchronous hatching and parentage distribution

The proportion of offspring sired by nursing males was not significantly affected by hatching time (GLMM: estimate = 0.015, $z = 0.011$, $p = 0.99$; Fig. 2); in other words, paternity of 2 groups of males remained constant across hatching time within a brood.

Discussion

Our study showed that majority of the offspring were sired by nursing males. We also found that the paternity of 2 groups of males remained constant across hatching time within a brood. Our study demonstrates at which stage males increase their paternity in *N. quadripunctatus*.

Our data shows that nursing males achieved a high level of paternity. On average, 97 % of the hatching larvae were sired by nursing males. Similar results have been reported in other species of the same genus (*N. vespilloides*: Müller and Eggert 1989; *N. orbicollis*: Trumbo and Fiore 1991; *N. tomentosus*: Scott and Williams 1993). Caring for unrelated offspring is costly for nursing males, and competition for fertilizations arises when females mate with more than one male during a single reproductive cycle (Parker 1970). In this study, most of the reproductively active, wild-caught females had fertile sperm in store when they arrived at the carcass. Previous studies on *N. vespilloides* have shown that all reproductively active males use two alternative mate-finding tactics: (1) search for carcasses that serve as oviposition sites or (2) attract mates via pheromone emission without a carcass (Pukwski 1933; Müller and Eggert 1987; Eggert and Müller 1989). Females readily mate with pheromone-emitting males (Müller and Eggert 1987; Eggert and Müller 1989). Males of *N. quadripunctatus* also use two alternative mate-finding tactics throughout the active seasons (Takata, unpublished study). Therefore, although we collected adult beetles in only one season, October, it is likely that the high level of sperm storage in the field remains constant throughout the breeding season. Therefore, nursing males need to ensure paternity by improving their competitive ability for fertilization against previously mated males. In this study, nursing males assured a very high level of paternity. In 7 out of 11 clutches, nursing males achieved 100 % paternity. Our experimental design made conditions more advantageous for non-nursing males to sire offspring than would have been the case in wild conditions, when taking into account the number of copulations and the freshness of sperm. Non-

306 nursing males were allowed to copulate with females over a 24-h period, and, as our
307 observational data show, they indeed copulated with females frequently. Furthermore, the
308 copulated females started breeding immediately after the copulatory attempt with non-nursing
309 males. However, nursing males achieved a high level of paternity, suggesting that males can
310 ensure a high level of paternity regardless of female mating history if they remain on the carcass
311 with the female.
312 Nursing males copulate with females most frequently during the pre-oviposition period. In
313 contrast, their copulation frequency significantly decreased after the oviposition period in which
314 the eggs were fertilized, and, therefore, males cannot increase offspring paternity by copulation.
315 Müller and Eggert (1989) found that high levels of paternity in nursing males coincided with the
316 increased matings of nursing males. These findings suggest that paternity assurance in
317 *Nicrophorus* is caused by repeated mating. Future studies are required to determine the proximate
318 cause of paternity assurance.
319 The proportion of offspring sired by nursing males was not significantly affected by
320 hatching time (i.e. the point in time when each larva hatched). These data indicate that paternity
321 of 2 groups of males remained constant across hatching time within a brood. A previous study
322 revealed that offspring that hatch later face higher mortality risks when cared for by male parents
323 (Takata, unpublished study). Therefore, if filial cannibalism by male parents plays a role in
324 increasing the paternity of offspring, the hatching of offspring sired by nursing males would
325 skew toward an earlier hatching period. However, our data do not support this prediction.
326 Nursing males assured very high levels of paternity (i.e. 100 % paternity in 7/11 clutches).
327 Furthermore, paternity of 2 groups of males remained constant across hatching time within a
328 brood. These results suggest that male parents of *N. quadripunctatus* do not increase their
329 paternity share through filial cannibalism.
330 In conclusion, we found that nursing males assure a very high level of paternity of
331 hatching offspring. We also found that the paternity of 2 groups of males remained constant
332 across hatching time within a brood, indicating that it is unlikely that filial cannibalism plays a
333 role in increasing the paternity of the offspring. Our study shows that, in *N. quadripunctatus*,
334 ensuring paternity before fertilization is more important in increasing the paternity of offspring
335 than filial cannibalism after fertilization.
336

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Ethics declarations

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Consent to participate

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Consent for publication

Not applicable.

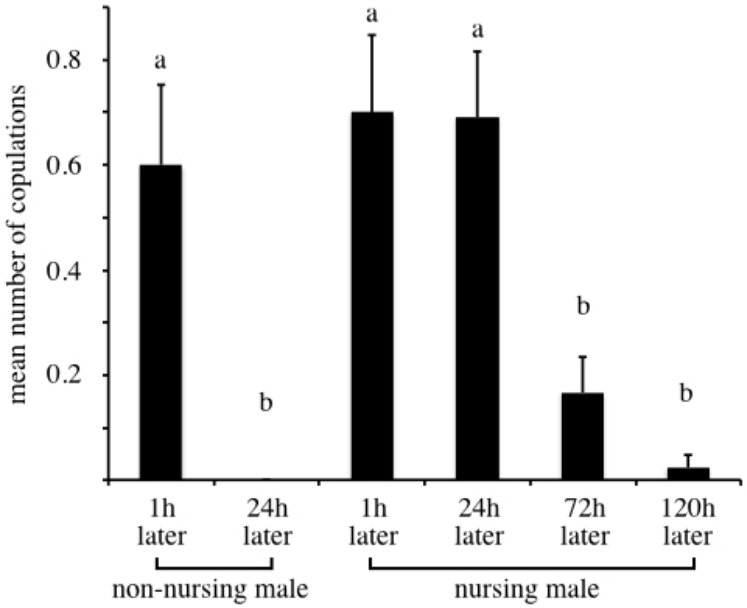
Competing interests

433 The authors declare no competing interests.

434

435 **Figures**

436

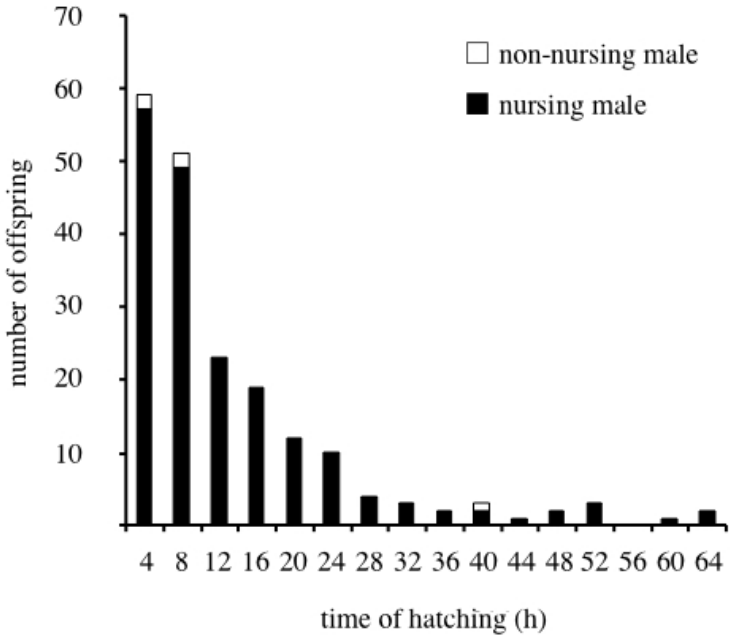


437

438 **Figure 1** Mean (±SD) number of copulations by *Nicrophorus quadripunctatus* observed in different
439 mating periods. The mating periods are expressed as hours after introduction of males and females.
440 Different subscripted letters are significantly different from each other, following Bonferroni
441 correction for multiple comparisons ($p < 0.05$).

442

443



444

445 **Figure 2** Asynchronous hatching and parentage distribution. The bars denote the number of hatching
446 larvae at 4-h intervals. The number of hatching larvae sired by non-nursing and nursing males is
447 denoted by white and black bars, respectively.

448

449 **Table 1.** Pairwise Wilcoxon rank sum tests for each of the 15 pair-wise comparisons between each
450 mating period of non-nursing and nursing male units

	Non-nursing male 1 h later	Non-nursing male 24 h later	Nursing male 1 h later	Nursing male 24 h later	Nursing male 72 h later
Non-nursing male 24 h later	**<0.001**				
Nursing male 1 h later	1.000	**<0.001**			
Nursing male 24 h later	1.000	**0.001**	1.000		
Nursing male 72 h later	**0.045**	0.492	**0.004**	**0.006**	
Nursing male 120 h later	**<0.001**	1.000	**<0.001**	**<0.001**	0.748

451

452 Bonferroni-adjusted *p*-values are shown. Values < 0.05 in **bold** are statistically significant.