

The adaptive significance of asynchronous hatching and filial cannibalism
in the burying beetle, *Nicrophorus quadripunctatus*

(ヨツボシモンシテムシにおける非同調孵化と子殺しの適応的意義)

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General introduction

Paternal care including food provisioning has evolved in many animal lineages, such as in mammals and birds (reviewed in Clutton-Brock 1991; Royle *et al.* 2012), as well as in some amphibians (Weygoldt 1980), insects (Field 2005), crustaceans (Diesel 1989), and leeches (Kutchera and Wirtz 1987). Parental care increases the fitness of offspring by investing some parental expenditure (time, energy or other resources) called parental investment. The optimal levels of parental investment can be found at the point at which they experience greatest benefit for least cost. In general, the optimal level of parental investment for offspring is greater than that of its parent. The disparity between the optimal levels of parental investment for parent and offspring generates evolutionary conflict. This evolutionary conflict is called parent-offspring conflict (Trivers 1974). Parent-offspring conflict causes co-evolution of reciprocally acting traits in parents and offspring. In principle, parent-offspring conflict selects offspring to develop mechanisms to increase total amount of parental investment to current reproduction and skew parental investment towards the offspring, whereas parent-offspring conflict selects parents to develop mechanisms to withhold parental investment. Evolutionary biologists and behavioural ecologists have studied that which party control the amount of parental investment as a result of this co-evolution. Recent studies have demonstrated the out come of co-evolution between parents and offspring. Parents control the amount of parental investment in mice *Mus musculus* (Hager and Johnstone 2003; Curley *et al.* 2004), earwigs *Forficula auricularia* (Mas *et al.* 2009), burying beetles *Nicrophorus vespilloides* (Lock *et al.* 2004; 2007), great tits *Parus major* (Kölliker *et al.* 2000) and canaries

Serinus canaria (Hinde *et al.* 2010), whereas offspring control the amount of parental investment in macaques *Macaca mulatta* (Kölliker *et al.* 2005) and burrower bugs *Sehirus cincta* (Agrawal *et al.* 2001). However, there is currently no information on what kind of ecological and physiological conditions affect the outcome of co-evolution between parents and offspring.

In this study, the author focused on hatching pattern of offspring and filial cannibalism by parents, both of which potentially influence total amount of parental investment to current reproduction and allocation of resources to individual offspring. Asynchronous hatching refers to the time span across which a clutch hatches, from the hatching of the first egg to the hatching of the last egg (Stenning 1996). Asynchronous hatching is known over a range of taxa, e.g. in the avians (Stenning 1996), in the White's skink *Egernia whitii* (While *et al.* 2007; While and Wapstra 2008), in the woodroach *Cryptocercus punctulatus* (Nalepa 1988) and in the burying beetle *Nicrophorus vespilloides* (Müller 1987; Müller and Eggert 1990; Smiseth *et al.* 2006). In asynchronous hatching species, early and late hatching offspring obtain different amount of parental investment (Mock and Forbes 1995; Forbes *et al.* 1997; Mock and Parker 1997; Forbes and Glassey 2000; Hall *et al.* 2009). Thus, the age composition of offspring established by asynchronous hatching may influence total amount of parental investment to current reproduction. Filial cannibalism is the act of eating one's own offspring (Manica 2002), and known in a wide range of taxa (Polis 1981). Parents can save the amount of parental care to current reproduction by cannibalizing some of their offspring. Thus, filial cannibalism can also influence the amount of parental investment to current reproduction. Here, the author investigated the role of asynchronous hatching and filial cannibalism

in the regulation of the amount of parental investment, to understand the adaptive significance of asynchronous hatching and filial cannibalism. In the present study, the author investigated the burying beetle *N. quadripunctatus*, a species in which the parent can eliminate less-adaptive offspring (e.g. slower-growing offspring) by filial cannibalism and adjust the age structure of offspring to adaptive pattern.

In Chapter 1, the author investigated how the point in time at which each group of larvae hatched affects filial cannibalism by the female parent. The main aim of the present study was to determine the age composition of offspring that survived and to determine the effect of larval growth on filial cannibalism. In Chapter 2, the author investigated the influence of hatching patterns on offspring growth and survival, to demonstrate how hatching patterns affects the allocation of parental investment. In Chapter 3, the author investigated the adaptive significance of filial cannibalism by male parent. Filial cannibalism by males could be adaptive if males are able to selectively cannibalize unrelated offspring. 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, the author investigated the stage at which male burying beetles, *N. quadripunctatus*, increase their paternity by evaluating the number of offspring sired by a nursing male in asynchronously hatched broods in relation to hatching time.

Chapter 1

Asynchronous hatching and brood reduction by filial cannibalism in the burying beetle, *Nicrophorus quadripunctatus*

Abstract

Despite decades of intensive research, there is much debate about the adaptive significance of asynchronous hatching. A major obstacle in understanding the significance of this process is the difficulty in separating the hypotheses that explain asynchronous hatching as an adaptive trait from those that explain it as a by-product of physiological constraints on hatching or egg laying patterns. The author investigated the burying beetle *N. quadripunctatus*, a species in which the parent can eliminate less-adaptive offspring (e.g. slower-growing offspring) by filial cannibalism and adjust the age structure of offspring to adaptive pattern. The main aim of the present study was to determine the age composition of offspring that survived and to determine the effect of larval growth on filial cannibalism. The author investigated how the point in time at which each group of larvae hatched affects filial cannibalism by the female parent. The author found that *N. quadripunctatus* exhibited asynchronous hatching, and reared larvae of different ages. Furthermore, the larvae hatching at latter intervals had lower survival and growth rates; therefore, filial cannibalism plays a role in eliminating later-arriving, slower-growing, and hence less-adaptive offspring.

Introduction

Asynchronous hatching refers to the time span across which a clutch hatches, from the hatching of the first egg to the hatching of the last egg (Stenning 1996). This process usually establishes competitive asymmetries within the brood, with the younger siblings facing higher risk of mortality from starvation and showing slower growth, because they typically obtain less food. Many hypotheses have been proposed to explain how selection might favour asynchronous hatching in spite of the higher mortality risk of the youngest siblings (Magrath 1990; Stoleson and Beissinger 1995; Stenning 1996). There are 2 main groups of hypotheses. One group of hypotheses explains that asynchronous hatching provides a mechanism to increase fitness [e.g. peak-load–reduction hypothesis (Hussell 1972), brood-reduction hypothesis (Lack 1954), sibling-rivalry hypothesis (Hahn 1981), insurance hypothesis (Stinson 1979) and sex-ratio–manipulation hypothesis (Slagsvold and Lifjeld 1989)]. The other group of hypotheses explains that asynchronous hatching is a by-product of physiological constraints on egg laying patterns and selection for the early onset of incubation [nest-failure hypothesis (Clark and Wilson 1981), limited-breeding–opportunity hypothesis (Beissinger and Waltman 1991) and egg-viability hypothesis (Arnold *et al.* 1987)]. Despite decades of intensive research on asynchronous hatching in birds, none of the hypotheses has gained overall support, and the reason why asynchronous hatching has evolved in altricial birds is still unclear (Magrath 1990; Stoleson and Beissinger 1995; Stenning 1996). A major reason for the failure to verify the reason is the close relationship between the onset of incubation and asynchronous hatching in birds. Because of this physiological constraint, separating the hypotheses

explaining asynchronous hatching as an adaptive trait and those explaining it as a by-product of selection for the early onset of incubation is difficult in altricial birds (Stenning 1996). However, asynchronous hatching is not restricted to altricial birds, but is known over a range of taxa, e.g. in the White's skink *Egernia whitii* (While *et al.* 2007; While and Wapstra 2008), in the woodroach *Cryptocercus punctulatus* (Nalepa 1988) and in the burying beetle *Nicrophorus vespilloides* (Müller 1987; Müller and Eggert 1990; Smiseth *et al.* 2006). Using a non-avian species allowed us to test each hypothesis separately from the onset of incubation.

The burying beetles, *Nicrophorus* spp., provides a particularly valuable system because, as in altricial birds, both parents provide elaborate care to their offspring, including food provisioning (Eggert *et al.* 1998). *N. quadripunctatus*, alike other species of the same genus, uses the carcass of small vertebrates (e.g. bird chick and small mouse) as a food resource for their larvae. Females adapt the number of eggs laid to the available carcass size. The eggs are laid in the soil near the carcass asynchronously. This extended period of egg laying is the proximate cause of asynchronous hatching in this species. In *N. vespilloides*, the larvae hatch asynchronously over a mean period of 27 h (range 8–56 h) on a 10-g carcass (Müller and Eggert 1990) and a mean period of 30 h (range 8–56 h) on a 25-g carcass (Smiseth *et al.* 2006). After hatching, the larvae crawl to the carcass and obtain some food by begging for pre-digested carrion from their parents and some by self-feeding on the carcass. *Nicrophorus* spp. can directly regulate the number of offspring by filial cannibalism (Bartlett 1987; Trumbo 1990c); therefore, the parent can eliminate less-adaptive offspring (e.g. slower-growing offspring) from an asynchronously hatching brood and adjust the age structure of

offspring to adaptive pattern. To address whether asynchronous hatching in *Nicrophorus* is an adaptive trait or a by-product of physiological constraints on egg laying patterns, it is important to demonstrate how the point in time at which each larva hatches affects filial cannibalism by the parent; however, there is currently no information on these effects. The author predicts that if asynchronous hatching is a by-product of physiological constraints on egg laying patterns and incur a fitness cost to parents, parents tend to kill later hatching offspring and synchronize the age structure of offspring. Conversely, if asynchronous hatching in *Nicrophorus* has been evolved as an adaptive trait, the author predicts that parents maintain the age structure of offspring.

The purposes of the present study were to (1) determine the age composition of larvae that survive until they grow to the stage at which no parental care is required, (2) determine the effect of larval growth on filial cannibalism and (3) determine the effect of clutch size on filial cannibalism in *N. quadripunctatus*. To my knowledge, this is the first report that documents the consequence of direct parental regulation on asynchronous broods.

Materials and methods

Collection and maintenance of the beetles

The author collected 60 adult *N. quadripunctatus* Kraatz in baited pitfall traps in Chiba prefecture, Japan, and reared 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 chicken meat twice a week. All males and females used in this experiment were sexually mature and ranged between 21 and 35 days of age.

General experimental procedure

Twenty-three pairs (10 pairs for behavioural observation, 13 pairs for measuring survival rate and growth) of randomly selected, non-sibling, virgin male and female beetles were each placed in a plastic cup (height 8 cm, diameter 15 cm) filled with 2 cm of moist peat and were provided with 15 ± 0.5 g of chicken meat [15 g of meat is an appropriate amount for rearing larvae (Suzuki and Nagano 2007)]. The plastic cups with beetles were placed in a dark incubator at 20 ± 1 °C. After 93 h, the female and the meat were transferred to a new plastic cup filled with 2 cm of moist peat. The male beetles were removed from the old plastic cup at this stage because parental care by male parents has no effect on larval growth or survival under laboratory conditions (Takata, unpublished data). The eggs were left to hatch.

Parent and offspring behaviour were recorded under infrared light to investigate cause of larval death. First of all, the author checked for hatching at 8-h intervals and transferred newly hatching

offspring to the cup containing their mother. Secondly, parent and offspring behaviour were recorded using a video camera (HOGA, HCIR-41F690) under infrared light until the age at which the larvae dispersed from the carcass. The number of hatching and surviving larvae were noted at 8-h intervals. If some larvae disappeared from the brood, the author checked the video and investigated the cause of the larvae's death. A larva that was bitten by the parent was defined as one that died because of filial cannibalism. A larva that appeared shrunken and that died near the carcass was defined as one that died because of hunger or infection. Additionally, the author weighed larval body mass at 8-h intervals until dispersal, to obtain general information on larval growth. The age of dispersal is defined as the day at which the larvae left the crypt surrounding the carcass. Dispersal from the carcass is synchronous and occurs normally when the earliest hatched larvae are 144 h old.

To investigate the hatching pattern and measure the growth and survival rate of different-aged larvae, the author noted the number of hatching and surviving larvae at 4-h intervals and measured their body mass. First of all, the author checked for hatching larvae at 4-h intervals and transferred newly hatching offspring to the cup containing their mother. Then, the hatching time of each larva from the onset of hatching was used as the time of hatching in the subsequent analysis. Secondly, to measure the growth of each group of larvae that hatched at the same time interval, living larvae that were on the carcass were individually weighed at 6-h intervals until the age at which they dispersed from the carcass. Because of their rapid growth, different-aged larval body mass was determinably different from each other; therefore, the hatching time of each larva in each measurement of body

mass could be identified by the methods. The author already had confirmed in the pilot study that no larvae caught up to the body weight of earlier hatching larvae, therefore the time of hatching of each larva and growth of each group of larvae could be identified by the methods. But, the body mass of individual larva could not be identified, because the larvae which hatched at the same time interval exhibited similar growth, mean body mass of each group of larvae were calculated and used to calculate the growth rate of each group of larvae in the subsequent analysis. The number of each group of larvae in each brood that had survived to the age at which the larvae dispersed from the carcass was used in the subsequent analysis for survival.

To determine whether female parents cannibalize offspring to regulate the number of offspring to the amount of carcass provided as food, the author investigated the effect of offspring number on filial cannibalism. The author checked for hatching larvae at 4-h intervals and transferred newly hatching offspring to the cup containing their mother. The author already had confirmed in the pilot study that *N. quadripunctatus* lays 21 ± 8 (mean \pm SD) eggs on the 15 g carcass. In the present study, two experimental groups with different brood size were set up according to the number of eggs. Brood with normal number of offspring was generated by placing 15 larvae simultaneously, and brood with large number of offspring was generated by placing 30 larvae simultaneously. The author used the number of each group of larvae in each brood that had survived to the age at which the larvae dispersed from the carcass in the subsequent analyses for survival. The author already had confirmed in the pilot study that my experimental manipulation did not affect larval survival.

Statistical analysis

First of all, the effects of the point in time at which each group of larvae hatched and clutch size on offspring survival were analysed using a generalized linear mixed model (GLMM) with the lme4 package (Bates and Maechler 2010). Survival rate was treated as a response variable assuming a binomial distribution, hatching time and clutch size as an explanatory variable and brood identity as a random factor.

Secondly, to test the effect of clutch size on hatching pattern, two different indices for asynchronous hatching patterns, hatching spread and hatching skew, were used in the following analysis. Hatching spread is the time between hatching of the first and that of the last larva from each brood (Smiseth *et al.* 2006). Hatching skew is an index of the degree to which hatching was skewed towards the part of hatching period. Hatching skew index, $V(t)$, was calculated as follows:

$$V(t) = \sum(T_i - T_m)/T_m \times P_i$$

where T_i refers to a particular time interval of the hatching period ranging between 0 and n , T_0 refers to the first time interval of the hatching period, T_n refers to the last time interval of the hatching period, T_m refers to the midst time interval of hatching period ($T_m = (T_n - T_0)/2$), and P_i refers to the proportion of the larvae that hatched a particular time interval of the hatching period. A hatching skew index approaching a value of -1 indicate that hatching was skewed toward the earlier hatching period, and it approaching a value of 1 indicate that hatching was skewed toward the later hatching period. More detailed information is presented on Smiseth *et al.* (2008). The effects of clutch size on hatching spread and hatching skew were analysed using a generalized linear model

(GLM), hatching spread and hatching skew was treated as a response variables assuming a binomial distribution, clutch size as an explanatory variable.

Thirdly, correlation between the hatching spread and hatching skew was analyzed using a GLM. Hatching skew was treated as response variables assuming a Gaussian distribution, hatching spread as an explanatory variable.

Fourthly, the effects of the point in time at which each group of larvae hatched on the growth rates of each group of larvae were analysed using a GLMM. Growth rate was treated as a response variable assuming a Gaussian distribution, hatching time as an explanatory variable and brood identity as a random factor. Methods for calculation of the growth rates were described below. In the present study, the body mass of each larva which hatched at the same time interval could not be identified. So, mean body mass of each group of larvae were calculated and used to calculate the growth rate of each group of larvae. The body mass of *N. quadripunctatus* larvae increased exponentially within the first few days. The first 24 h of larval body masses which were weighed 6 h intervals were transformed into logarithmic values to calculate growth rate; therefore there were 4 data points for each group of larvae. Then, slopes of the regression line of log (body mass) on time were calculated as growth rates. The slope of the regression line for each group of larvae was calculated as follows:

$$\sum(X_i - X_m)(\log Y_i - \log Y_m)/(X_i - X_m)^2 \times 100$$

where X_i is the time after hatching, Y_i is mean body mass (mg) for each group of larvae at time X_i , and X_m and Y_m are the mean values of X and Y . All correlation coefficient values were >0.97 .

Fifthly, difference in the offspring survival between treatments were analysed using GLMM, offspring survival was treated as a response variables assuming a binomial distribution, treatment group for brood size as an explanatory variable and brood identity as a random factor.

Finally, difference in the number of surviving offspring between treatments were analysed using GLM, number of surviving offspring was treated as a response variables assuming a gaussian distribution, treatment group for brood size as an explanatory variable. All analyses were performed using R 2.12.1 GUI 1.35 (<http://cran.r-project.org>). Data are expressed as mean \pm SD.

Results

The cause of larval death and general information on larval body mass

In the present study, 156 larvae hatched from 10 clutches were observed. Sixteen out of 156 larvae had died before dispersing from the carcass. Video analysis revealed that 14 larvae (1.4 ± 1.7 per clutch) were killed by their female parent and 1 larva died as a result of hunger or infection.

The cause of death of the remaining dead larva could not be determined.

The mean larval body mass at hatching was 1.9 ± 0.3 mg and larvae grew up to 13.1 ± 3.0 mg by age 24 h, 38.5 ± 14.0 mg by age 48 h, 103.8 ± 38.2 mg by age 72 h and 172.7 ± 53.5 mg by age 96 h. The mean larval weight at the dispersal stage (at age 120 h) was 193.1 ± 57.9 mg.

Number of hatching larvae and larvae that survived

N. quadripunctatus exhibits asynchronous hatching (Fig. 1-1, sample size: 13 clutches). The number of hatching larvae was skewed towards earlier hatching periods. Hatching spread ranged from 4 to 56 h (25 ± 12 h). The mean number of hatching larvae was 22 ± 8 . Hatching skew ranged from -0.52 to 0.06 (-0.15 ± 0.27). Clutch size did not have a significant effect on hatching spread (GLM: estimate = 0.368, $t = 0.942$, $p = 0.365$) and hatching skew (GLM: estimate = -0.006, $t = -0.372$, $p = 0.719$). There was no significant correlation between hatching spread and hatching skew (GLM: estimate = -0.004, $t = -0.900$, $p = 0.378$).

Larvae of different ages survived until dispersal (Fig. 1-1). The hatching spread across larvae that survived (17 ± 9 h) was lower than the hatching spread across the entire brood. Larvae hatching at

latter intervals had lower survival (Fig. 1-2, GLMM: estimate = -0.098, $z = -4.380$, $p < 0.001$,

sample size: 219 larvae from 13 clutches). The mean number of larvae that survived was 11 ± 4 .

Clutch size did not have a significant effect on offspring survival (GLMM: estimate = -0.036, $z = -0.858$, $p = 0.391$).

Effect of hatching time on growth rate

The point in time at which each group of larvae hatched had a significant negative effect on the growth rate (Fig. 1-3, GLMM: estimate = -0.001, $F_{1, 40} = 4.435$, $p = 0.042$).

Effect of brood size on filial cannibalism

Offspring in the brood with 15 offspring had a significantly higher survival than offspring in the brood with 30 offspring (Fig. 1-4, GLMM: estimate = -0.200, $p = 0.849$). There was no statistically significant difference in the number of surviving offspring between treatments (Fig. 1-5, GLM: estimate = 0.335, $p < 0.001$).

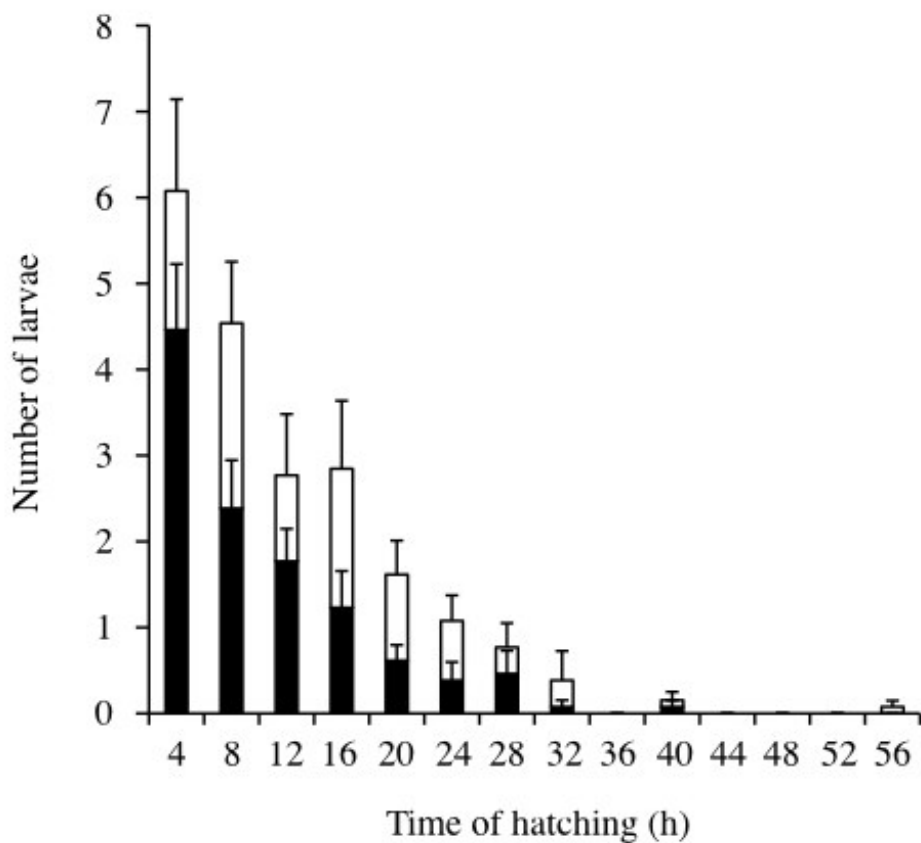


Fig. 1-1. Number of hatching larvae and larvae that survived during 4-h intervals. The time of hatching denotes the time elapsed from the onset of hatching. The black bars denote the mean number of larvae that survived; the white bars denote the mean number of dead larvae. Data are presented as mean \pm SE.

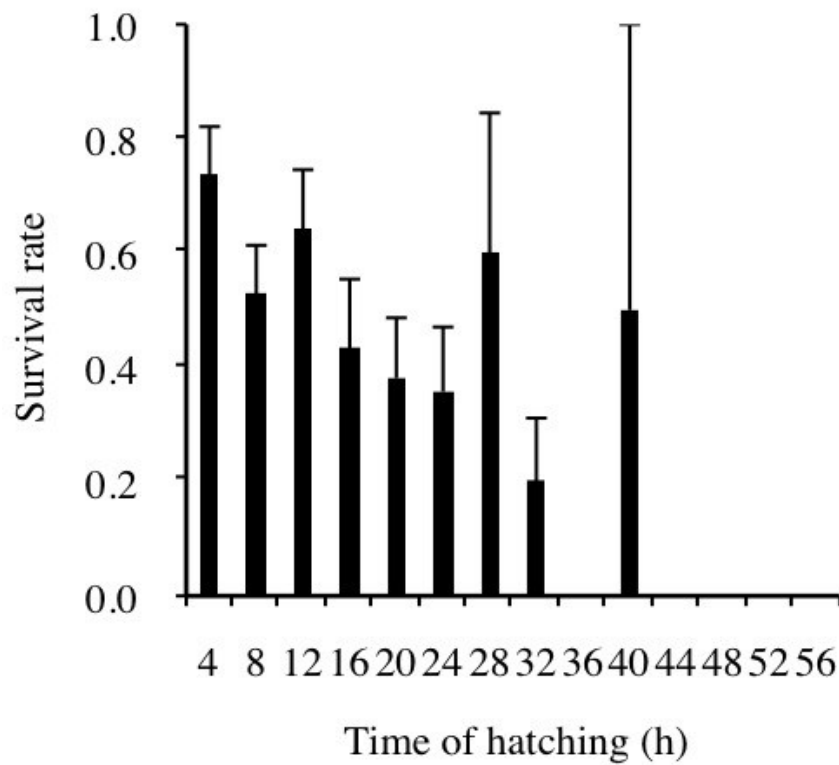


Fig. 1-2. The effect of the point in time at which each group of offspring hatched on offspring survival rate. The time of hatching denotes the time elapsed from the onset of hatching. Each bar denotes the mean survival rate of hatching larvae at 4-h intervals. Data are presented as mean \pm SE.

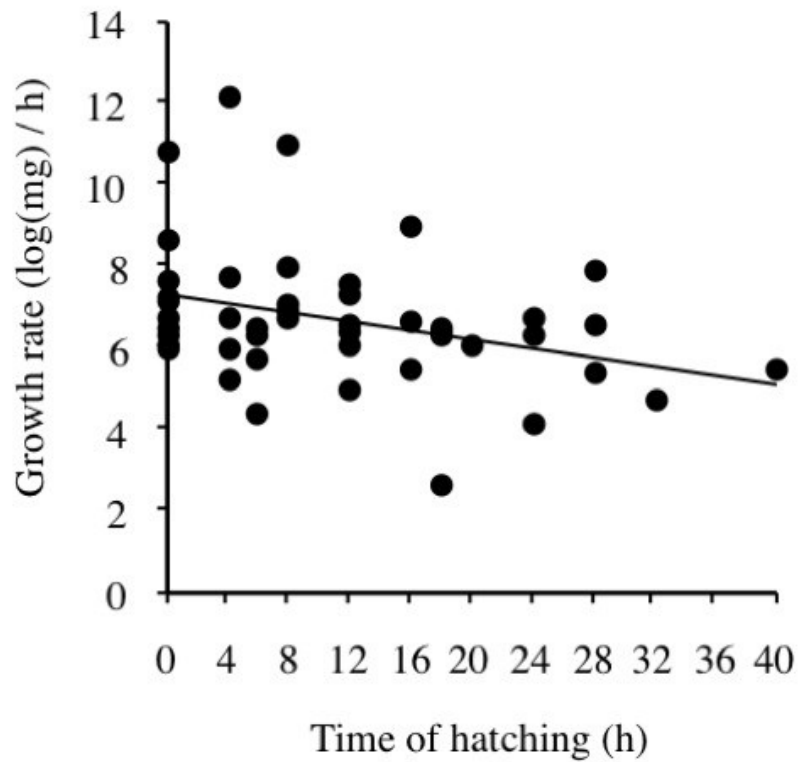


Fig. 1-3. The effect of the point in time at which each group of offspring hatched on offspring growth rate. The time of hatching denotes the time elapsed from the onset of hatching. Each plot denotes the mean growth rate of larvae in each brood at 4-h intervals. The growth rate was calculated as the slope of the regression line of log (body mass) on time for each group of offspring.

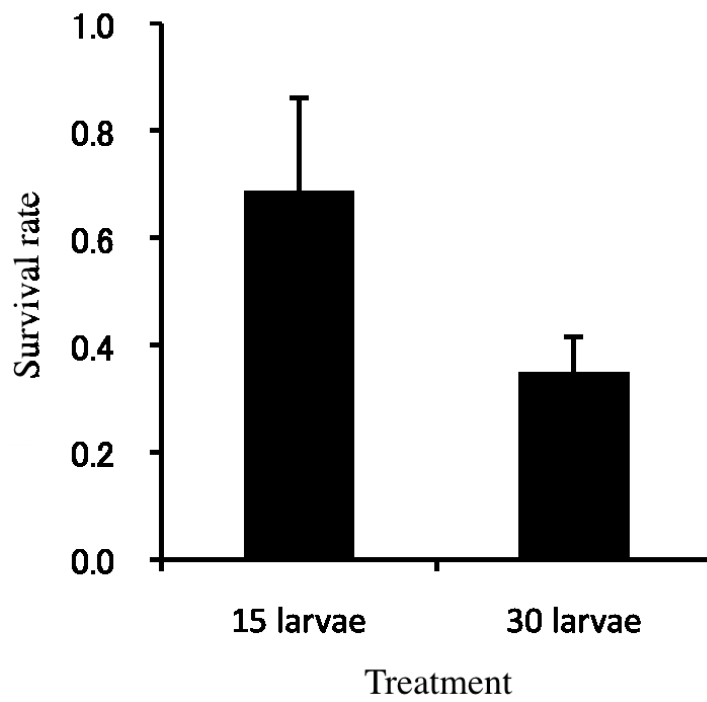


Fig. 1-4. The survival rate of larvae in the brood with 15 and 30 offspring. Data are presented as mean \pm SD.

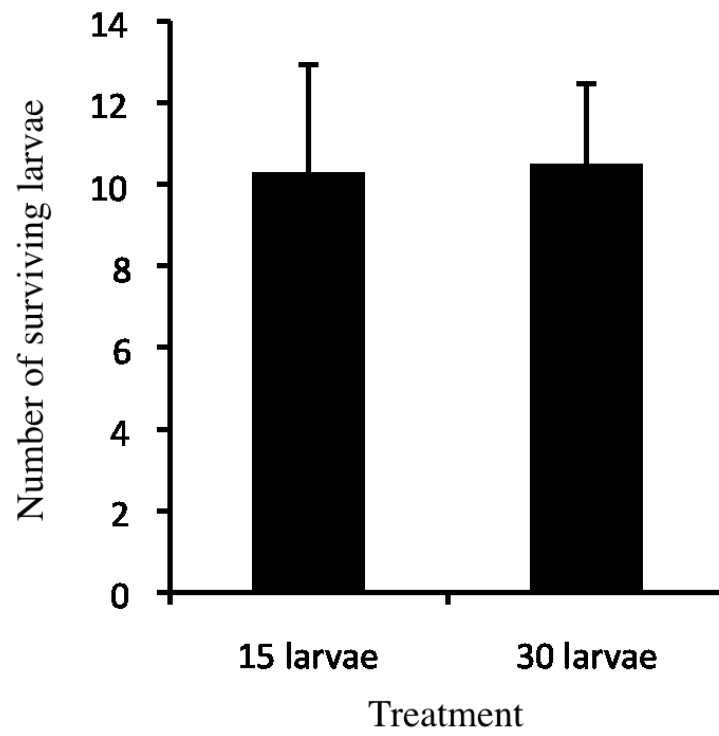


Fig. 1-5. Number of surviving larvae in the brood with 15 and 30 offspring. Data are presented as mean \pm SD.

Discussion

The author found that *N. quadripunctatus* exhibited asynchronous hatching, and the number of hatching larvae was skewed towards earlier hatching periods. These results demonstrated that the female parent decreases hatching spread by filial cannibalism, but still rears larvae of different ages. The point in time at which each group of larvae hatched had a significant negative effect on the growth rate. Additionally, the later hatching offspring faced higher risk of mortality from filial cannibalism by the female parent; therefore, filial cannibalism plays a role in eliminating later-arriving, slower-growing, and hence less-adaptive offspring. To my knowledge, this is the first demonstration of how the point in time at which each group of larvae hatched influences larval growth and filial cannibalism by the female parent in an asynchronous hatching brood.

The point in time at which each group of larvae hatched had a significant negative effect on the growth rate, suggesting that larvae hatching at latter intervals had lower growth rate. In the present study, mean body mass of each group of larvae were calculated and used to calculate the growth rate of each group of larvae. Since the number of larvae that hatched was not the same in each interval, the mean body mass used to calculate the growth rate is based on inevitably unbalanced group size. The results could be biased due to the unbalanced group size. However, the results are corresponding to the results reported in the recent study on other species of burying beetle *N. vespilloides* (Smiseth *et al.* 2007). Smiseth *et al.* (2007) used an experimentally established brood of *N. vespilloides* and found that later hatching larvae grew less than earlier hatching larvae when the female parent provided care for them. These findings suggest that there is age-based

asymmetric sibling competition. Interestingly, asynchronous hatching also forms competitive asymmetries among siblings in many altricial birds (Magrath 1990; Stoleson and Beissinger 1995; Mock and Paker 1997). The brood-reduction hypothesis explains that asynchronous hatching provides a mechanism by which asymmetric sibling competition can reduce broods when resources are limited (Lack 1954). It predicts that competitively disadvantaged offspring have a higher mortality risk resulting from sibling competition when resources are limited. Coincidentally, my results show that later hatching offspring faced higher mortality risk; however, the burying beetle directly reduces its brood by filial cannibalism (Bartlett 1987; Trumbo 1990c). It is therefore unlikely that the mechanism for brood reduction promotes the evolution of asynchronous hatching in the burying beetle.

Larvae hatching at latter intervals had lower survival. Video analysis revealed that the major cause of larval death in this species was from filial cannibalism by the female parent; therefore, the negative effect of hatching time on survival rate suggests that later hatching offspring face a higher risk of mortality from filial cannibalism. Furthermore, larvae hatching at latter intervals had lower survival. These findings suggest that the female parent is more likely to kill the offspring that exhibit a slower growth rate; therefore, these results revealed that filial cannibalism by the female parent plays a role in eliminating later-arriving, slower-growing, and hence less-adaptive offspring. Additionally, the present study also demonstrated that offspring in the brood with 15 offspring had a significantly higher survival than offspring in the brood with 30 offspring, and there was no statistically significant difference in the number of surviving offspring between treatments. Similar

results were reported from Bartlett (1987) and Trumbo (1990c). Thus, these findings suggesting that filial cannibalism also had the role to regulate the number of offspring to the amount of carcass provided as food.

In the present study, clutch size did not have a significant effect on offspring survival, hatching spread and hatching skew. Moreover, there was no significant correlation between hatching spread and hatching skew. These findings suggest that hatching spread and hatching skew are independent variables. Thus, further studies to investigate the adaptive consequence of asynchronous hatching need to consider not only hatching spread but also hatching skew. Similar results was reported from Smiseth *et al.* (2008). They investigated the effect of clutch size on hatching spread and hatching skew and the correlation between hatching spread and hatching skew on five different carcass sizes in *N. vespilloides*. They found that there was no correlation between hatching spread and hatching skew, but these two indices were significantly influenced by clutch size. In contrast to Smiseth *et al.* (2008), clutch size did not have significant effect on hatching spread and hatching skew in the present study. This difference may be due to a difference in methodology. Smiseth *et al.* (2008) have tested the effect on five different carcass sizes (5 – 25 g), while my study tested the effect of clutch size on hatching spread and hatching skew only on 15 g carcass. Therefore, the smaller variations in clutch size may reduce statistical power in my study. Further studies are needed to investigate the potential linkage between hatching spread and hatching skew.

My study demonstrated that the female parent rears larvae of different ages. Smiseth *et al.* (2008) and Smiseth and Morgan (2009) established three types of broods with different hatching pattern,

synchronous, moderately asynchronous and highly asynchronous, with a hatching span of 0, 24 and 48 h. Smiseth and Morgan (2009) found that offspring survival is lower in highly asynchronous broods than in synchronous or asynchronous broods and Smiseth *et al.* (2008) found that offspring survival is higher in moderately asynchronous broods than in either synchronous or highly asynchronous broods. These findings suggest that there is an optimal length of hatching spread. In the present study, the hatching spread across larvae that survived was lower than the hatching spread across the entire brood. Furthermore, larvae hatching at latter intervals had lower survival. My results suggest that the female parent decreases hatching spread by filial cannibalism. These findings imply that the optimal length of hatching span in *N. quadripunctatus* is lower than the observed length of hatching span and female parent regulate the length of hatching span to optimal length by filial cannibalism. To understand the adaptive significance of asynchronous hatching in *Nicrophorus*, further studies are needed to investigate the effect of hatching pattern on offspring survival and growth. Additionally, in the present study, the author focused on the effect of filial cannibalism by the female parent on age structure of offspring, and male beetles were removed from experimental system. Although parental care by male parents has no effect on larval growth or survival under laboratory conditions (Takata, unpublished data), the presence of male may affect pattern of filial cannibalism by female parent. Further studies are needed to investigate the potential effects of male presence on the pattern of filial cannibalism by female.

Chapter 2

Asynchronous hatching maximizes offspring growth and survival by optimizing the allocation of parental investment among offspring in the burying beetle, *Nicrophorus quadripunctatus*

Abstract

In many asynchronous hatching species, hatching of offspring is skewed towards the earlier part of hatching period and majority of offspring hatch early on. Because of early and late hatching offspring obtain different amount of parental investment, it is likely that the age composition of offspring affects total amount of parental investment to current reproduction. Thus, asynchronous hatching patterns may contribute to optimize the amount of parental investment. To address the adaptive significance of asynchronous hatching, it is important to demonstrate how hatching patterns affect the allocation of parental investment. Here, the author investigated the influence of hatching patterns on offspring growth and survival. The author found that asynchronous hatching pattern, in which hatching of offspring is skewed towards the earlier part of hatching period, maximizes offspring growth and survival. Additionally, hatching patterns had a significant effect on growth of individual offspring. Thus, the present study demonstrates that asynchronous hatching pattern maximizes offspring growth and survival by affecting the allocation of parental investment.

Introduction

Asynchronous hatching refers to the time span across which a clutch hatches, from the hatching of the first egg to the hatching of the last egg (Stenning 1996). Because offspring are fed and gain weight from the time they hatch, a size difference is established within the brood. Consequently, asynchronous hatching usually establishes competitive asymmetries within the brood, with the younger offspring facing higher risk of mortality from starvation and showing slower growth, because they typically obtain less parental investment (Mock and Forbes 1995; Forbes *et al.* 1997; Mock and Parker 1997; Forbes and Glassey 2000; Hall *et al.* 2009). It is a central yet controversial issue in evolutionary and behavioral ecology that explaining how selection might favour asynchronous hatching pattern in spite of the higher mortality risk of the youngest siblings (Magrath 1990; Stoleson and Beissinger 1995; Stenning 1996).

In many asynchronous hatching species, hatching of offspring is skewed towards the earlier part of hatching period and majority of offspring hatch early on (Magrath 1990; Stoleson and Beissinger 1995). Because of early and late hatching offspring obtain different amount of parental investment (Mock and Forbes 1995; Forbes *et al.* 1997; Mock and Parker 1997; Forbes and Glassey 2000; Hall *et al.* 2009), it is likely that the age composition of offspring affects total amount of parental investment to current reproduction. Thus, asynchronous hatching patterns may contribute to optimize the amount of parental investment.

Similar asynchronous hatching pattern, in which hatching of offspring is skewed towards the earlier part of hatching period, is known in burying beetles (Smiseth *et al.* 2006; Takata *et al.* 2013).

Nicrophorus quadripunctatus, alike other species of the same genus, uses the carcass of small vertebrates (e.g. bird chick and small mouse) as a food resource for their larvae. Females lay eggs in the soil near the carcass. The larvae hatch asynchronously over a mean time span of 25 h (range 4–56 h) on a 15-g carcass (Takata *et al.* in press). 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 data). The larvae compete for parental food provisioning and earlier hatching offspring typically obtain more food and grow better (Takata *et al.* in press). To address the adaptive significance of asynchronous hatching, it is important to demonstrate how hatching patterns affect the allocation of parental investment; however, there is currently no information on these effects. Here, the author investigated the influence of hatching patterns on offspring growth and survival.

Materials and methods

Collection and maintenance of the beetles

In September 2012, 200 adult *Nicrophorus quadripunctatus* Kraatz were collected in baited pitfall traps in Tokyo prefecture, Japan, and reared 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. After they emerge to adult, they were fed red roaches (*Rutilus arcasii*) three times a week. All males and females used in this experiment were sexually mature and ranged between 21 and 35 days of age.

General experimental procedure

Pairs of randomly selected, non-sibling, virgin male and female beetles were each placed in a plastic cup (height 8 cm, diameter 15 cm) filled with 2 cm of moist peat, and were provided with 15 ± 0.5 g of chicken meat (15 g of meat is an appropriate amount for rearing larvae (Suzuki and Nagano 2007)). All females were given this carcass in the same state of freshness. The plastic cup with beetles were placed in a dark incubator at 20 ± 1 °C. After 93 h, the female and the meat were transferred to a new plastic cup filled with 2 cm of moist peat. The male beetles were removed from the old plastic cup at this stage because parental care by male parents has no effect on larval growth or survival under laboratory conditions (Takata, unpublished data). The eggs were left to hatch. Hatching of offspring was checked at 12-h intervals and used the newly hatched larvae to generate experimental broods.

In the present study, to investigate the influence of hatching pattern on larval growth and survival, 5 experimental treatments with different hatching pattern were set up (1 treatment for synchronous hatching treatment and 4 treatments for asynchronous hatching treatments). In all experimental groups, 15 larvae were provided to female parent. Treatment for synchronous hatching [treatment (A)] were generated by placing 15 larvae simultaneously. Treatments for asynchronous hatching [treatment (B) – (E)] were generated by placing early hatching larvae followed by another middle hatching larvae 12 h later and late hatching larvae 24 h later. In each treatment group, different number of early, middle and late hatching larvae were added to generate different hatching pattern (see Table 2-1 for details of experimental design).

To discriminate early, middle and late hatching larvae, each group of larvae were randomly marked by cutting the outer part of either the right or the left hind or middle leg when the larvae were 12 h of age. All larvae were marked by cutting the leg including treatment (A). The author already had confirmed in the pilot study that this treatment does not affect on larval growth and survival.

All treatments for asynchronous broods were well within the natural variation for hatching span (25 ± 12 h, mean \pm SD; Takata *et al.* in press) and brood size (number of hatching larvae was 22 ± 8 , mean \pm SD; number of surviving larvae was 11 ± 4 , mean \pm SD; Takata *et al.* in press) observed when *N. quadripunctatus* reared on 15 g of carcass in the laboratory. Hatching in natural broods on 15 g of carcass occurs continuously and is biased towards the early part of the hatching period

(hatching skew = -0.15 ± 0.27 , mean \pm SD; Takata *et al.* in press). The treatment (C) well mimics the natural hatching pattern.

To measure the growth of each group of larvae that hatched during the same time interval, living larvae that were on the carcass were weighed individually at 12-h intervals until the age at which they dispersed from the carcass. Dispersal from the carcass is synchronous and occurs normally when the earliest hatched larvae are 144 h old (Takata, unpublished data). Larval body weight normally reaches to the peak when the larvae are 120 h old (Takata *et al.* in press), and it well predicts their adult body size (Takata, unpublished data). Thus, the body weight is an accurate indication of the fitness return to parent. So, the body weight was used as an index of larval quality in the following analyses. The number of each group of larvae in each brood that had survived to the age at which the larvae dispersed from the carcass was used in the subsequent analyses for survival. The author already had confirmed in the pilot study that the experimental manipulation did not affect larval survival.

Statistical analysis

First of all, the differences in growth of individual offspring between treatments were analyzed using a generalized linear mixed model (GLMM) with the lme4 package (Bates and Maechler 2010). Offspring body weight at 120 h old including early, middle and late hatching larvae was treated as a response variable assuming a gaussian distribution, experimental treatments as an explanatory variable and brood identity as a random factor.

Secondly, the differences in survival rate between treatments were analyzed using a GLMM. Offspring survival (1 = survived or 0 = dead) including early, middle and late hatching larvae was treated as a response variable assuming a binomial distribution, experimental treatments as an explanatory variable and brood identity as a random factor.

Thirdly, the differences in total offspring weight between treatments were analyzed using a GLMM. Total value of offspring body weight at 120 h old was treated as a response variable assuming a gaussian distribution, experimental treatments as an explanatory variable and brood identity as a random factor. These analyses were performed multiple times to assess difference between treatments, Bonferroni adjusted significance level was adopted for each comparison.

Fourthly, the effects of hatching skew on offspring growth were analysed using a GLMM. Offspring body mass at 120 h old was treated as a response variable assuming a gaussian distribution, hatching skew as an explanatory variable and brood identity as a random factor. The effects of hatching skew on offspring growth were analyzed separately for early, middle or late hatching larvae.

Fifthly, the effects of hatching skew on offspring survival were analyzed using a GLMM. Offspring survival (1 = survived or 0 = dead) was treated as a response variable assuming a binomial distribution, hatching skew as an explanatory variable and brood identity as a random factor. The effects of hatching skew on offspring survival were analysed separately for early, middle or late hatching larvae.

Sixthly, the effect of hatching order on offspring growth were analyzed using a GLMM.

Offspring body mass at 120 h old was treated as a response variable assuming a gaussian distribution, the hatching order (early hatching larvae = 1, middle hatching larvae = 2, late hatching larvae = 3) as an explanatory variable and brood identity as a random factor.

Finally, the effect of hatching order on offspring survival were analyzed using a GLMM.

Offspring survival (1 = survived or 0 = dead) was treated as a response variable assuming a binomial distribution, the hatching order (early hatching larvae = 1, middle hatching larvae = 2, late hatching larvae = 3) as an explanatory variable and brood identity as a random factor. All analyses were performed using R 2.12.1 GUI 1.35 (<http://cran.r-project.org>).

Results

Differences in offspring growth and survival between treatments

Total offspring body weight was largest in asynchronous hatching treatment group (C) and (E) (Fig. 2-1). Offspring in the treatment group (A), (C) and (E) grew significantly better than offspring in the treatment group (B) and (D) (Fig. 2-2; Table 2-2). Offspring in the asynchronous hatching treatment group (B) – (E) had significantly higher survival than offspring in the synchronous treatment group (A) (Fig. 2-3; Table 2-3).

The effect of hatching pattern on offspring growth

Hatching skew had a significant effect on growth of individual offspring in early (Fig. 2-4, GLMM: estimate = 66.345, $F_{1, 168} = 20.385$, $p < 0.001$), middle (Fig. 2-4, GLMM: estimate = 82.785, $F_{1, 120} = 30.467$, $p < 0.001$) and late hatching offspring (Fig. 2-4, GLMM: estimate = 46.904, $F_{1, 60} = 6.453$, $p = 0.014$).

The effect of hatching pattern on offspring survival

Hatching skew did not have a significant effect on offspring survival in early hatching offspring (Fig. 2-5, GLMM: estimate = -0.9251, $F_{1, 180} = 0.644$, $p = 0.424$). But, hatching skew had a significant effect on offspring survival in middle hatching offspring (Fig. 2-5, GLMM: estimate = 3.872, $F_{1, 148} = 14.531$, $p < 0.001$) and marginally significant effect in late hatching offspring (Fig. 2-5, GLMM: estimate = 1.372, $F_{1, 116} = 3.224$, $p = 0.075$).

Hatching order and offspring growth and survival

Hatching order had a statistically significant effect on growth of individual offspring and survival (Table 2-4). Earlier hatching offspring had a significantly higher survival and grew better than later hatching offspring in all treatment groups.

Table 2-1. Experimental treatments used to investigate the influence of hatching pattern on larval growth and survival.

Treatment	Early	Middle	Late	Hatching skew
A	15	0	0	–
B	9	5	1	-0.53
C	7	5	3	-0.27
D	5	5	5	0.00
E	3	5	7	0.27

The number of early, middle and late hatching larvae added in each treatment group and hatching skew of each hatching pattern are shown. (A) is synchronous hatching treatment group, (B) – (E) are asynchronous hatching treatment groups. In treatment group (B), hatching is highly biased towards the early part of the hatching period. In treatment group (C), hatching is biased towards the early part of the hatching period with a normal level. In treatment group (D), hatching is not biased towards any part of the hatching period. In treatment group (E), hatching is biased towards the late part of the hatching period.

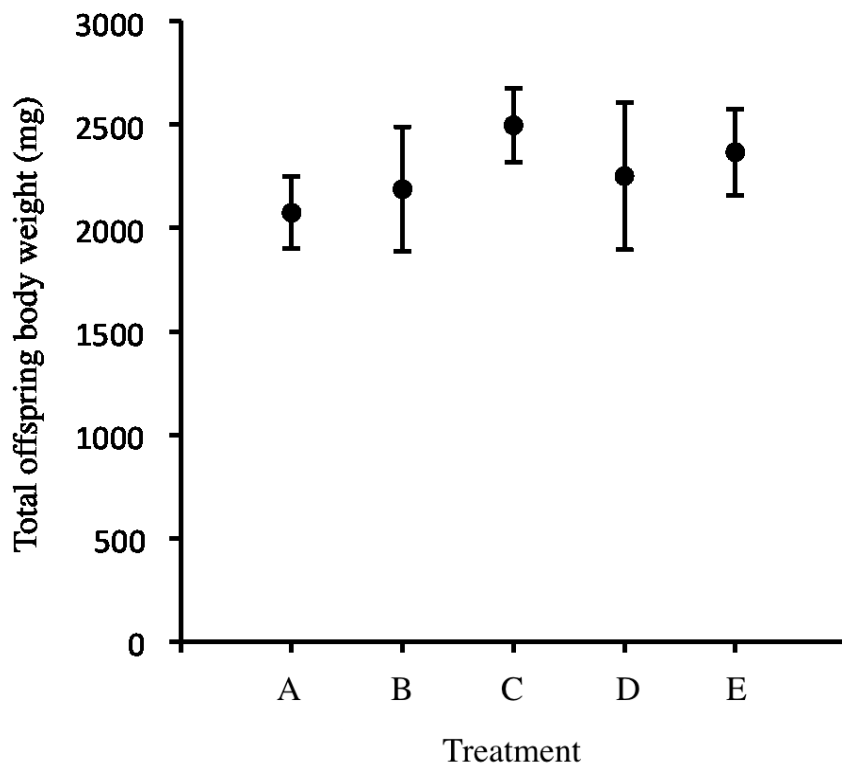


Fig. 2-1. Hatching patterns and total offspring body weight. Data are presented as mean \pm SE. See Table 2-1 for details of treatment groups.

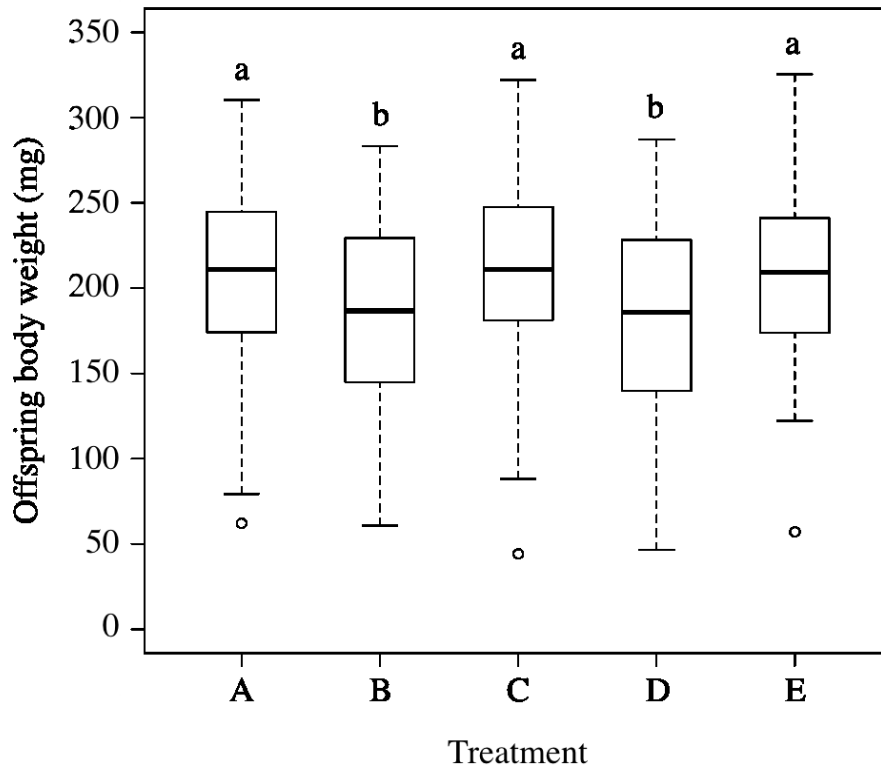


Fig. 2-2. Hatching patterns and offspring growth. Box plot showing the offspring body weight at 120 h old in each treatment group. Different subscripted letters are significantly different from each other, following Bonferroni correction for multiple comparisons ($p < 0.005$). See Table 2-1 for details of treatment groups.

Table 2-2. GLMM testing the difference in offspring body weight for each of the 10 pair-wise comparisons between each treatment group units.

	Treatment (A)	Treatment (B)	Treatment (C)	Treatment (D)
Treatment (B)	0.0018			
Treatment (C)	0.9437	0.0012		
Treatment (D)	0.0045	0.8585	0.0031	
Treatment (E)	0.9317	0.0011	0.9881	0.0030

Bonferroni significance level is < 0.005 . Values in **bold** are statistically significant. See Table 2-1 for details of treatment groups.

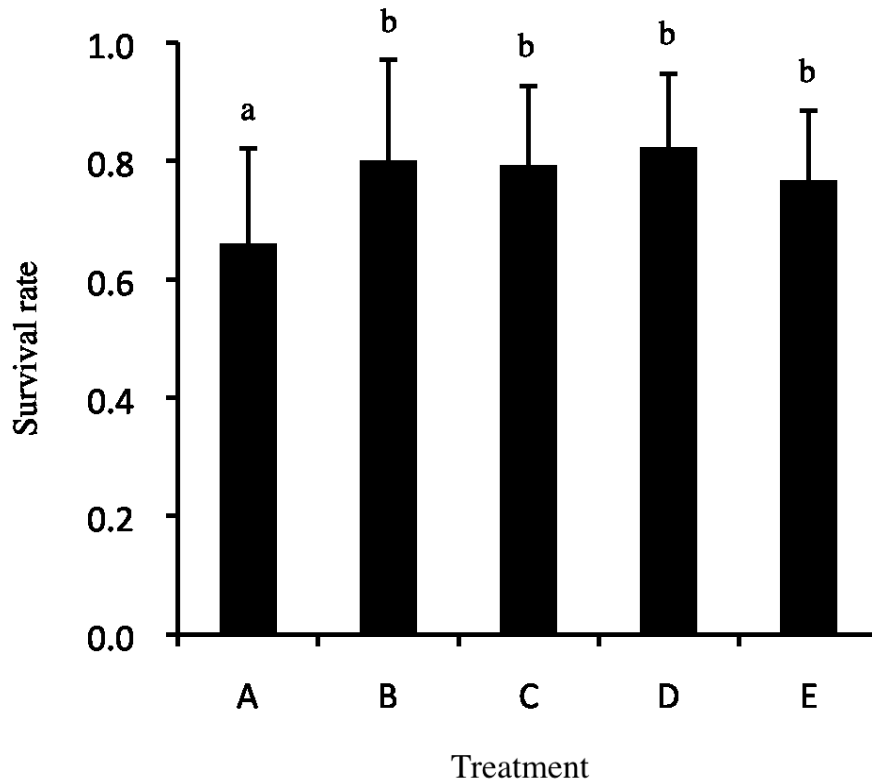


Fig. 2-3. Hatching patterns and offspring survival rate. Data are presented as mean \pm SE. Different subscripted letters are significantly different from each other, following Bonferroni correction for multiple comparisons ($p < 0.005$). See Table 2-1 for details of treatment groups.

Table 2-3. GLMM testing the difference in offspring survival for each of the 10 pair-wise comparisons between each treatment units.

	Treatment (A)	Treatment (B)	Treatment (C)	Treatment (D)
Treatment (B)	0.0118			
Treatment (C)	0.0185	0.8734		
Treatment (D)	0.0072	0.6866	0.5825	
Treatment (E)	0.0596	0.5328	0.6423	0.3299

Bonferroni significance level is < 0.005 . Values in **bold** are statistically significant. See Table 2-1 for details of treatment groups.

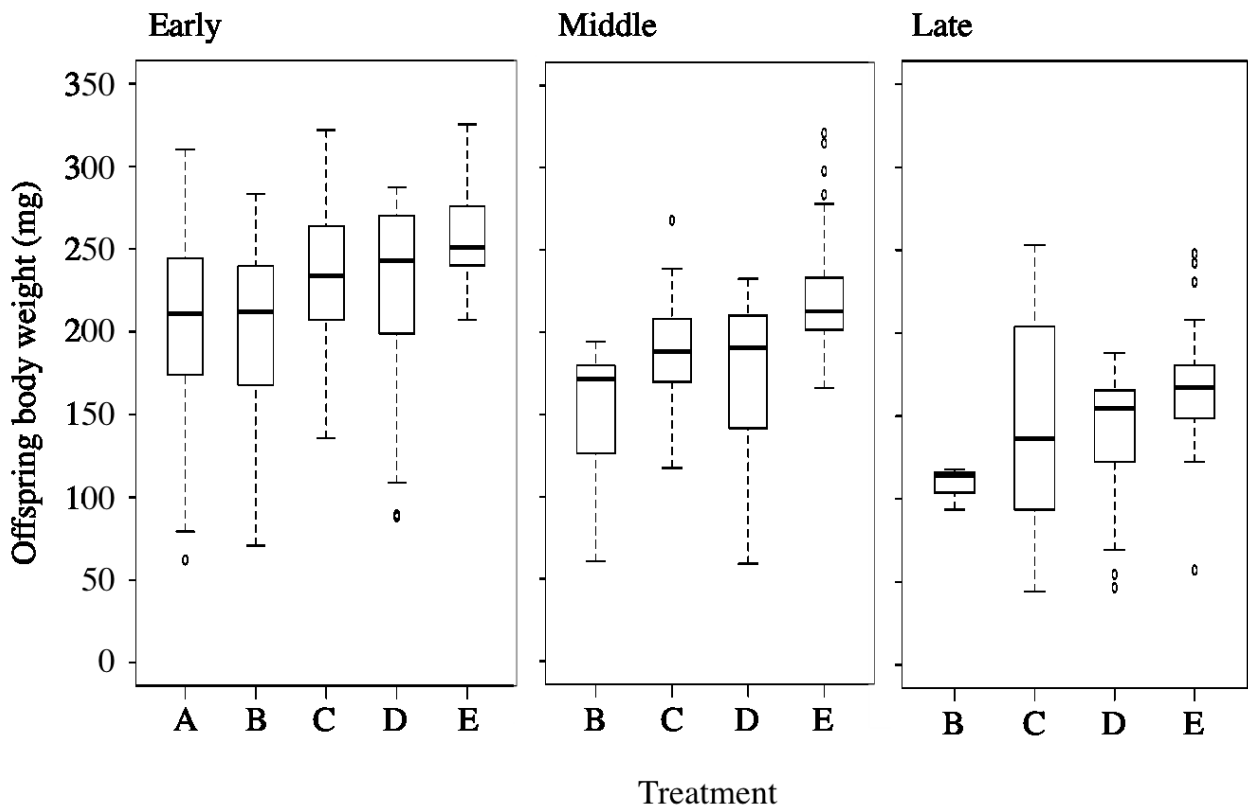


Fig. 2-4. The effect of hatching skew on offspring growth. Box plot showing the offspring body weight at 120 h old in each treatment group. See Table 2-1 for details of treatment groups.

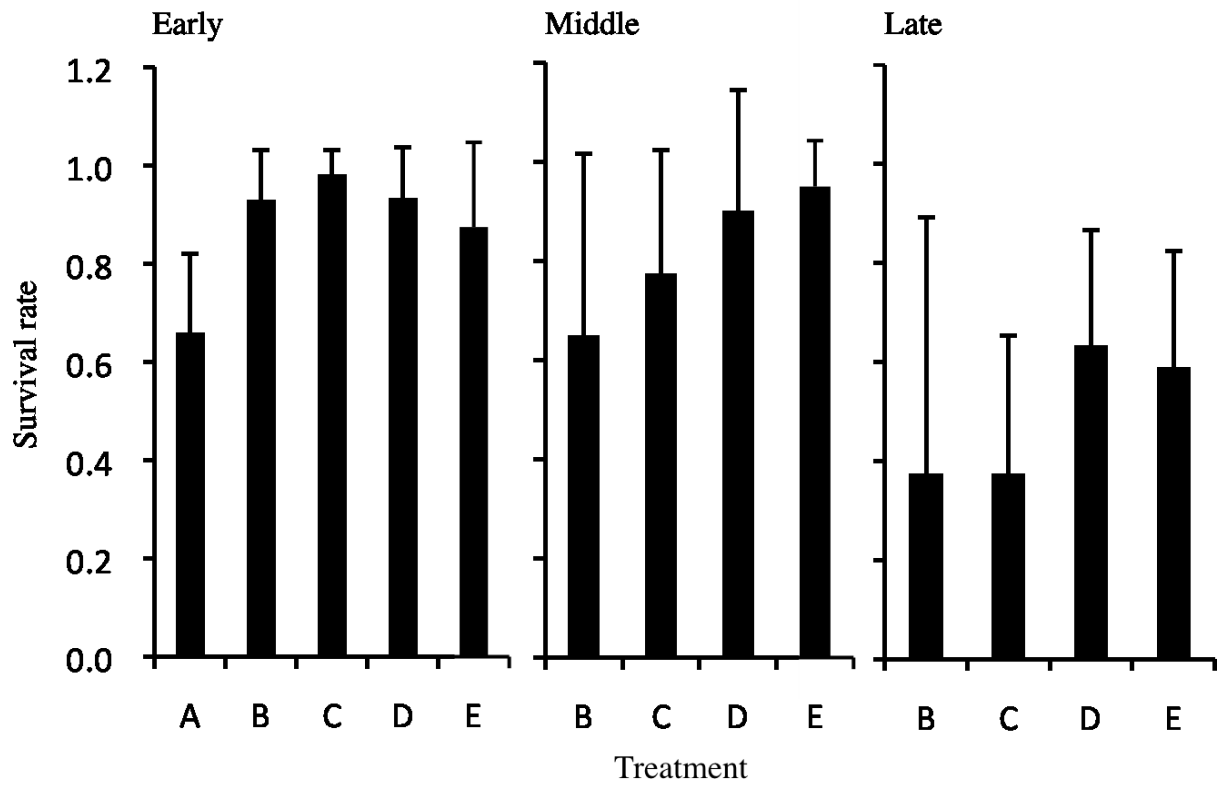


Fig. 2-5. The effect of hatching skew on offspring survival rate. Data are presented as mean \pm SE.

See Table 2-1 for details of treatment groups.

Table 2-4. GLMM testing for the effect of hatching order on offspring growth and offspring survival in each treatment group.

Factor	Treatment group	Estimate	df	<i>p</i>
Body weight	B	-62.610	1, 93	< 0.001
	C	-48.320	1, 93	< 0.001
	D	-51.930	1, 72	< 0.001
	E	-52.710	1, 88	< 0.001
Survival	B	-2.279	1, 118	< 0.001
	C	-2.387	1, 118	< 0.001
	D	-1.239	1, 88	0.002
	E	-1.282	1, 118	< 0.001

Values in **bold** are statistically significant. See Table 2-1 for details of treatment groups.

Discussion

Asynchronous hatching and hatching patterns affected offspring growth and survival. The author found that total offspring body weight is largest in asynchronous hatching treatment group (C) and (E). Offspring in the treatment group (A), (C) and (E) grew significantly better than offspring in the treatment group (B) and (D). Offspring in the asynchronous hatching treatment group (B) – (E) had significantly higher survival than offspring in the synchronous treatment group (A). Furthermore, hatching skew had a significant effect on growth of individual offspring. The more hatching of offspring was skewed towards the earlier part of hatching period, the more growth of individual offspring was reduced. The effect is similar in early, middle and late hatching offspring. Additionally, hatching order had significant effect on growth of individual offspring, and earlier hatching offspring grew better than later hatching offspring in all treatments. The present study demonstrates that asynchronous hatching pattern, in which hatching of offspring is skewed towards the earlier part of hatching period, maximizes offspring growth and survival, and how hatching patterns affects the allocation of parental investment.

Total offspring body weight is largest in asynchronous hatching treatment group (C) and (E). Offspring in the treatment group (C) and (E) grew significantly better than offspring in the treatment group (B) and (D), and had significantly higher survival than offspring in the synchronous treatment group (A). Hatching in natural broods is biased towards the early part of the hatching period (Takata *et al.* in press), and the treatment group (C) well mimics the natural hatching pattern in *N. quadripunctatus*. Thus, the present study demonstrates that asynchronous

hatching pattern, in which hatching of offspring is skewed towards the earlier part of hatching period, maximizes offspring growth and survival. Total offspring body weight is similar in treatment group (C) and (E). Interestingly, offspring in the treatment group (D) grew significantly less than offspring in the treatment group (C) and (E), suggesting that there is a fitness valley between the natural hatching pattern (C) and experimentally created hatching pattern (E). This may be the reason why hatching pattern like (E) is not observed today. Alternatively, in the field condition, in which adult beetles are facing intense competition for carcass and take over of carcass by conspecific competitors occurs (Wilson and Fudge 1984; Bartlett 1988; Scott 1990; Trumbo 1990 a, b; Scott and Gladstein 1993), the fitness return from hatching pattern like treatment group (C), in which majority of offspring hatch early on, may be higher than treatment group (E), in which majority of offspring hatch in the later part of hatching period. This is because the probability of take over of carcass by conspecific competitors decreases as the carcass is consumed and the larvae are large (Scott and Gladstein 1993).

Which hypothesis can explain the result that total offspring body weight is largest in asynchronous hatching treatment group (C) and (E)? Peak load reduction hypothesis (Hussel 1972) and sibling–rivalry hypothesis (Hahn 1981) explains that asynchronous hatching provides a mechanism to increase offspring growth. Peak load reduction hypothesis explains that asynchronous hatching increases offspring growth by reducing the parent’s workload during the peak in demand from offspring. This hypothesis predicts that growth of individual offspring will be largest in treatment group (D), in which the number of hatching offspring is evenly distributed and

not skewed any part of hatching period. However, in contrast to the prediction, growth of individual offspring is largest in the treatment group (E). Therefore, there is no evidence that asynchronous hatching increases offspring growth by reducing the parent's workload during the peak in demand from offspring, and thus the results obtained here provide no support for peak load reduction hypothesis. Sibling-rivalry hypothesis explains that asynchronous hatching increases offspring growth by reducing sibling rivalry over parental investment and minimizing the energetic cost for wasteful competition, because asynchronous hatching establishes a size hierarchy within the brood and smaller offspring tend to avoid competition against larger offspring. This hypothesis predicts that growth of individual offspring will be smallest in synchronous hatching treatment group (A). However, in contrast to the prediction, growth of individual offspring in treatment group (A) is similar or even better than some of asynchronous hatching treatment groups. Therefore, there is no evidence that asynchronous hatching increases offspring growth by reducing energy waste through sibling aggression by producing a size hierarchy within the brood, and thus the results obtained here provide no support for sibling-rivalry hypothesis.

Alternative to these hypotheses, the results obtained here can explain by focusing on sibling competition and allocation of parental investment among offspring. In the present study, earlier hatched offspring had a significantly higher survival and grew better than later hatching offspring in all treatment groups. This result suggests that there is age-based asymmetric sibling competition, and earlier hatching offspring have higher competitive ability. Similar results are reported from previous studies in burying beetles (Smiseth *et al.* 2007; Takata *et al.* in press) and also from avians

(Mock and Forbes 1995; Forbes *et al.* 1997; Mock and Parker 1997; Forbes and Glassey 2000; Hall *et al.* 2009). Thus, the more hatching of offspring is skewed towards the earlier part of hatching period, the more number of offspring with the higher competitive ability will increase within the brood and result in severe competition for parental investment. The results obtained here are corresponding to this prediction. In the present study, the more hatching of offspring is skewed towards the earlier part of hatching period, the more growth of individual offspring was reduced.

Total offspring body weight changes intricately as a function of allocation of parental investment among different age offspring. This is because growth of individual offspring increases with decrease of number of early hatching offspring, whereas number of early hatching larger offspring within the brood decrease and late hatching smaller offspring increase. In the present study, total offspring body weight is largest in treatment group (C), suggesting that the allocation of parental investment among offspring is thought to be the best in treatment group (C). Interestingly, hatching pattern like treatment group (C), in which hatching of offspring is skewed towards the earlier part of hatching period, is similar in avian asynchronous hatching (Magrath 1990; Stoleson and Beissinger 1995). Previous studies have assessed the adaptive significance of asynchronous hatching by changing the time span from the hatching of the first egg to the hatching of the last egg. Further studies need to consider not only the time span across which a clutch hatches, but also the effect of hatching skew on the allocation of parental investment among offspring.

Chapter 3

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

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, the author investigated the stage at which male burying beetles, *N. quadripunctatus*, increase their paternity by evaluating the number of offspring sired by a nursing male in asynchronously hatched broods in relation to hatching time. The author found that nursing males assure a very high level of the paternity of hatching offspring. 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. The author concluded that ensuring paternity before fertilization is more important in increasing the paternity of offspring.

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 be 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 *et al.* 2013). 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 *et al.* in press). 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 data). Partial filial cannibalism occurs in *N. quadripunctatus* (Takata *et al.* in press). Both males and females cannibalize some of their offspring (in *N. quadripunctatus*; Takata, unpublished data). Previous studies on *N. quadripunctatus* have shown that offspring that hatch later face higher mortality risks when cared for by males (Takata, unpublished data). Burying beetles cannot directly recognize their relatives after the larvae have hatched (in *N. vespilloides*; Müller and Eggert 1990; in *N. quadripunctatus*;

Takata *et al.* in press). 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, the author 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, adult female *Nicrophorus quadripunctatus* Kraatz were collected 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. Sixteen such females were collected. 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 data), 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. Reproductively active females and non-reproductive females were defined 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

Adult *N. quadripunctatus* were collected in baited pitfall traps in Chiba prefecture, 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.

In the present study, 6 observation groups were set up. In the first and second groups, the copulation frequency of non-nursing males were observed. The non-nursing males were allowed to copulate with females without a carcass, but did not provide parental care. The copulation frequency was observed 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, the copulation frequency of nursing males was observed 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 data). Observation periods were set up during pre-oviposition (1 h and 24 h after introduction), oviposition (72 h after introduction), and post-oviposition (120 h after introduction). Previously mated females were first prepared 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. The author already had 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. Newly hatching offspring were collected at 4-h intervals until all the offspring had hatched and stored them in 99.5 % ethanol. In the present study, 248 larvae [20.7 ± 8.5 larvae (mean \pm SD) from 11 clutches] were obtained and analyzed their paternity by using amplified fragment length polymorphism (AFLP). The hatching rate was 81.0 ± 3.7 % (mean \pm 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 μ l of ATL buffer (Qiagen) and 20 μ l 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 μ l of AL buffer (Qiagen) was added to the sample and mixed thoroughly by vortexing. The mixture was then added to 200 μ l 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 μ l 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 μ l 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 μ l of the total cellular DNA sample was double-digested with 0.4 μ l of *EcoRI/MseI* (Invitrogen), 1 μ l of 5 \times reaction buffer, and 2.6 μ l of distilled water. Adapters specific to *EcoRI* and *MseI* digested DNA were ligated to the restriction fragments. After incubation at 37 °C for 24 h, 4.8 μ l of the adapter ligation mixture and 0.2 μ l of T4 DNA ligase (Invitrogen) were added and ligated for 2 h at 20 °C.

Pre-amplification

The resulting products were diluted tenfold, and 10 μ l of reaction mixtures containing 1 μ l of DNA solution were used for PCR reactions in 1 μ l of 10 \times PCR buffer, 1 μ l of 10 mM dNTP mix (200 μ M each), 0.25 μ l of 10 μ M *EcoRI* (plus A) and *MseI* (plus C) primers, 0.05 μ l of TaKaRa Ex Taq (1.25 U; Takara Bio, Shiga, Japan), and 4.95 μ l of distilled water. After an initial denaturation at 95 °C for 5 min, PCR was performed using 30 successive cycles of 94 °C for 30 s, annealing at 56 °C for 60 s, and 72 °C for 60 s. Chain elongation at 72 °C was extended to 5 min after the final cycle. The PCR was performed using a PCR thermal cycler (BioRad, Richmond, CA, USA). The

sequences of the primers *EcoRI*-A and *MseI*-C were 5'-GACTGCGTACCAATTCA-3' and 5'-GATGAGTCCTGAGTAAC-3', respectively. The pre-selective amplification products were electrophoresed, and amplification was confirmed to minimise genotyping errors.

Selective amplification

The pre-selective amplification products were diluted tenfold, and 10 µl of reaction mixtures containing 1 µl of DNA solution were used in selective PCR amplification reactions in 1 µl of 10× PCR buffer, 1 µl of 10 mM dNTP mix (200 µM each), 0.05 µl of 10 µM *EcoRI* (plus AGG or AAG) and 0.25 µl of 10 µM *MseI* (plus CTA) primers, 0.05 µl of TaKaRa Ex Taq (1.25 U), and 5.15 µl of distilled water. After an initial denaturation at 95 °C for 5 min, PCR was performed using 30 successive cycles of 94 °C for 30 s. The annealing temperature in the first cycle was 66 °C, which was subsequently reduced in each cycle by 1 °C for the next 12 cycles and was continued at 57 °C for 60 s and 72 °C for 60 s. Chain elongation at 72 °C was extended to 5 min after the final cycle. PCR was performed using a PCR thermal cycler (BioRad). The sequences of the primers *EcoRI*-AGG, *EcoRI*-AAG, and *MseI*-CTA were 5'-GACTGCGTACCAATTCAGG-3', 5'-GACTGCGTACCAATTCAAG-3', and 5'-GATGAGTCCTGAGTAACTA-3', respectively.

Fragment analysis

AFLPs were detected using fragment analysis with the ABI PRISM3500 system. The S500 ROX (PE Applied Biosystems, Foster City, CA, USA) fragment size standards were included in each

sample. Amplified fragments with fluorescent signals were identified using GeneScan 3.2.1 (PE Applied Biosystems). All steps throughout the AFLP protocol were conducted to minimize genotyping errors.

Assessment of reproducibility

To assess the reproducibility of the 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 the present 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, all peaks with a height above 150 fluorescent units were considered. 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. The paternity of 79 % of the offspring were determined, but the paternity of the remaining offspring could not be determined because they did not show any diagnostic peaks. These offspring were excluded from the following analysis.

Statistical analysis

First of all, 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.

Secondly, to test the paternity bias towards non-nursing and nursing males, the number of offspring sired by non-nursing males with the number of offspring sired by nursing males were compared 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, the effect of hatching time on parentage distribution

was examined 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 \pm SD) per 30 min at 1 h after introduction (Fig. 3-1; Table 3-1). 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 \pm SD) per 30 min at 1 h after introduction, and 0.7 ± 0.1 (mean \pm SD) times at 24 h following introduction (Fig. 3-1; Table 3-1). However, the number of copulatory attempts significantly decreased at 72 h (0.2 ± 0.1 times, mean \pm SD) and 120 h (0.2 ± 0.1 times, mean \pm SD) after introduction (Fig. 3-1, Table 3-1).

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 \pm SD) were sired by non-nursing males and 15.8 ± 5.7 larvae (mean \pm 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. 3-2); in other words, paternity of 2 groups of males remained constant across hatching time within a brood.

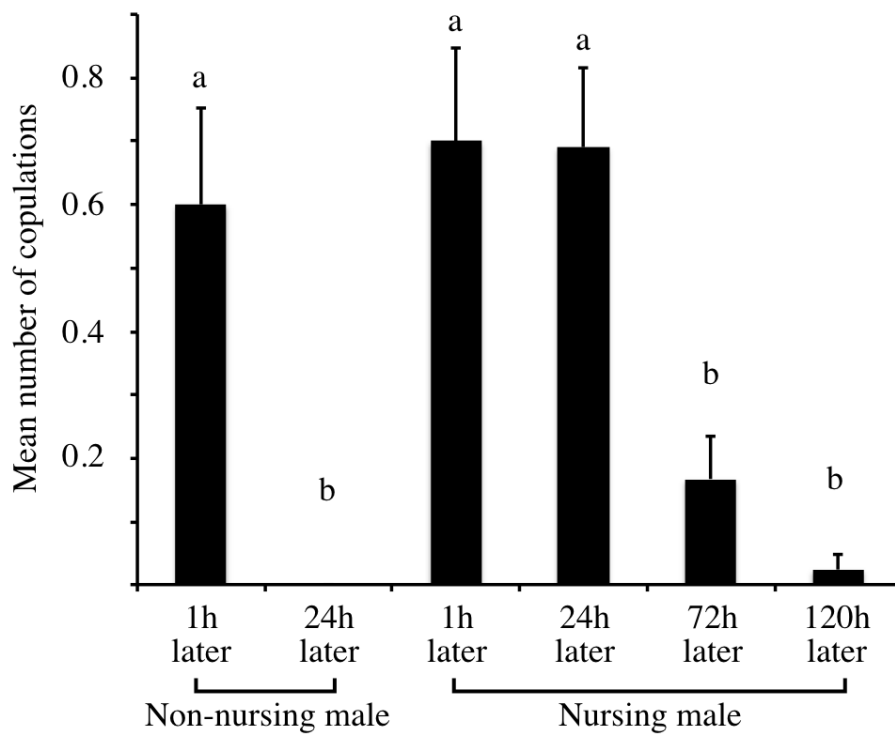


Fig. 3-1. Mean (\pm SD) number of copulations by *Nicrophorus quadripunctatus* observed in different mating periods. The mating periods are expressed as hours after introduction of males and females. Different subscripted letters are significantly different from each other, following Bonferroni correction for multiple comparisons ($p < 0.05$).

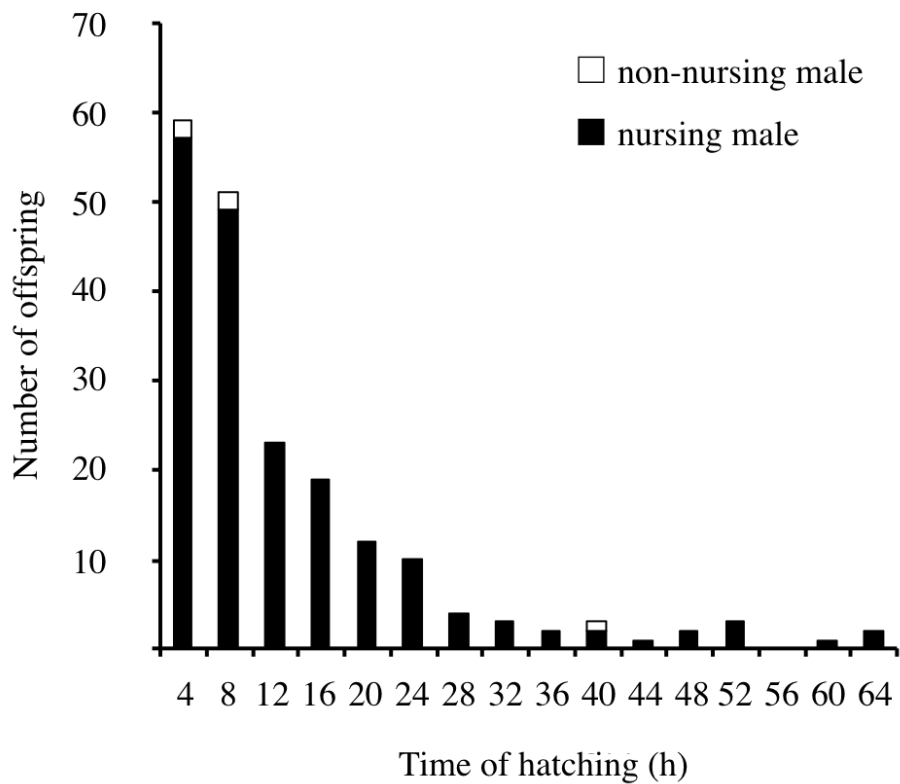


Fig. 3-2. Asynchronous hatching and number of hatching larvae sired by non-nursing and nursing males. The time of hatching denotes the time elapsed from the onset of hatching. The bars denote the number of hatching larvae at 4-h intervals. The number of hatching larvae sired by non-nursing and nursing males is denoted by white and black bars, respectively.

Table 3-1. Pairwise Wilcoxon rank sum tests for each of the 15 pair-wise comparisons between each 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

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

Discussion

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

The data obtained here 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 the present 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 data). Therefore, although the adult beetles were collected 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 the present

study, nursing males assured a very high level of paternity. In 7 out of 11 clutches, nursing males achieved 100 % paternity. The experimental design used in the present study 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-nursing males were allowed to copulate with females over a 24-h period, and, as the observational data show, they indeed copulated with females frequently. Furthermore, the copulated females started breeding immediately after the copulatory attempt with non-nursing males. However, nursing males achieved a high level of paternity, suggesting that males can ensure a high level of paternity regardless of female mating history if they remain on the carcass with the female.

Nursing males copulate with females most frequently during the pre-oviposition period. In contrast, their copulation frequency significantly decreased after the oviposition period in which the eggs were fertilized, and, therefore, males cannot increase offspring paternity by copulation. Müller and Eggert (1989) found that high levels of paternity in nursing males coincided with the increased matings of nursing males. These findings suggest that paternity assurance in *Nicrophorus* is caused by repeated mating. Further studies are required to determine the proximate cause of paternity assurance.

The proportion of offspring sired by nursing males was not significantly affected by hatching time (i.e. the point in time when each larva hatched). These data indicate that paternity of 2 groups of males remained constant across hatching time within a brood. A previous study revealed that offspring that hatch later face higher mortality risks when cared for by male parents (Takata,

unpublished data). Therefore, if filial cannibalism by male parents plays a role in increasing the paternity of offspring, the hatching of offspring sired by nursing males would skew toward an earlier hatching period. However, the data obtained here do not support this prediction. Nursing males assured very high levels of paternity (i.e. 100 % paternity in 7/11 clutches). Furthermore, paternity of 2 groups of males remained constant across hatching time within a brood. These results suggest that male parents of *N. quadripunctatus* do not increase their paternity share through filial cannibalism.

In conclusion, the author found that nursing males assure a very high level of paternity of hatching offspring. Furthermore, the paternity of 2 groups of 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 the offspring. The present study shows that, in *N. quadripunctatus*, ensuring paternity before fertilization is more important in increasing the paternity of offspring than filial cannibalism after fertilization.

General discussion

The present study has demonstrated that (1) filial cannibalism by female parents has the role to regulate the number of offspring to the amount of carcass provided as food, (2) asynchronous hatching pattern maximizes offspring growth and survival by affecting the allocation of parental investment, (3) nursing males assure a very high level of the paternity of hatching offspring and filial cannibalism does not play a role in increasing the paternity of offspring.

The findings obtained from the present study provide two important insights into the regulation of the amount of parental investment and the outcome of parent-offspring conflict. First of all, the present study demonstrated the adaptive significance of asynchronous hatching, which has long been controversial issue in evolutionary and behavioural ecology. The present study sheds light into the adaptive significance of asynchronous hatching from new angle, sibling competition and allocation of parental investment among offspring. The experimental methods used in the present study are applicable to phylogenetically distinct species, thus, by studying wide range of animal lineages, further studies can assess the generality of the role of asynchronous hatching on the allocation of parental investment.

Another important insight the author gained is that parents can regulate the amount of parental investment by two different ways. Parental care increases the fitness of offspring by investing some parental investment. In general, the optimal level of parental investment for offspring is greater than that of its parent. The disparity between the optimal levels of parental investment for parent and offspring generates parent-offspring conflict (Trivers 1974). In principle, parent-offspring

conflict selects offspring to develop mechanisms to increase total amount of parental investment to current reproduction and skew parental investment towards the offspring, whereas parent-offspring conflict selects parents to develop mechanisms to withhold parental investment. Recent studies have demonstrated the out come of co-evolution between parents and offspring. Parents control the amount of parental investment in mice *Mus musculus* (Hager and Johnstone 2003; Curley *et al.* 2004), earwigs *Forficula auricularia* (Mas *et al.* 2009), burying beetles *Nicrophorus vespilloides* (Lock *et al.* 2004; 2007), great tits *Parus major* (Kölliker *et al.* 2000) and canaries *Serinus canaria* (Hinde *et al.* 2010), whereas offspring control the amount of parental investment in macaques *Macaca mulatta* (Kölliker *et al.* 2005) and burrower bugs *Sehirus cincta* (Agrawal *et al.* 2001). However, there is currently no information on what kind of ecological and physiological conditions affect the out come of co-evolution between parents and offspring.

The present study presents one ecological factor which might affect the out come of the co-evolution. The present study demonstrated that parents can regulate the amount of parental investment to current reproduction by two different ways, (1) by regulating the number of offspring by filial cannibalism, (2) by affecting the allocation of parental investment by asynchronous hatching. These mechanisms set up the level of offspring competition and allocation of parental investment among them, and offspring cannot affect these mechanisms. In contrast to parents, offspring can increase the amount of parental investment only by soliciting more investment. Thus, even if offspring can increase the amount of parental investment by soliciting more investment,

parents can control the total amount and allocation of parental investment. The existence of these superior regulation mechanisms may contribute to parental control of the amount of investment.

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Summary

Paternal care including food provisioning has evolved in many animal lineages. Parental care increases the fitness of offspring by investing some parental expenditure (time, energy or other resources) called parental investment. The optimal levels of parental investment can be found at the point at which they experience greatest benefit for least cost. In general, the optimal level of parental investment for offspring is greater than that of its parent. The disparity between the optimal levels of parental investment for parent and offspring generates evolutionary conflict, which is called parent-offspring conflict. In principle, parent-offspring conflict selects offspring to develop mechanisms to increase total amount of parental investment to current reproduction and skew parental investment towards the offspring, whereas parent-offspring conflict selects parents to develop mechanisms to withhold parental investment. Recent studies have demonstrated the outcome of co-evolution between parents and offspring. In some species, parents control the amount of parental investment, whereas in other species, offspring control the amount of parental investment. However, there is currently no information on what kind of ecological and physiological conditions affect the outcome of co-evolution between parents and offspring.

In the present study, the author focused on hatching pattern of offspring and filial cannibalism by parents, both of which potentially influence total amount of parental investment to current reproduction and allocation of resources to individual offspring. Asynchronous hatching refers to the time span across which a clutch hatches, from the hatching of the first egg to the hatching of the last egg. In asynchronous hatching species, early and late hatching offspring obtain different

amount of parental investment. Thus, the age composition of offspring established by asynchronous hatching may influence total amount of parental investment to current reproduction. Filial cannibalism is the act of eating one's own offspring. Parents can save the amount of parental care to current reproduction by cannibalizing some of their offspring. Thus, filial cannibalism can also influence the amount of parental investment to current reproduction. Here, the author investigated the role of asynchronous hatching and filial cannibalism in the regulation of the amount of parental investment, to understand the adaptive significance of asynchronous hatching and filial cannibalism. In the present study, the author investigated the burying beetle *N. quadripunctatus*, a species in which the parent can eliminate less-adaptive offspring (e.g. slower-growing offspring) by filial cannibalism and adjust the age structure of offspring to adaptive pattern.

First of all, the author investigated how the point in time at which each group of larvae hatched affects filial cannibalism by the female parent. The main aim of the present study was to determine the age composition of offspring that survived and to determine the effect of larval growth on filial cannibalism. The author found that *N. quadripunctatus* exhibited asynchronous hatching, and reared larvae of different ages. Furthermore, the larvae hatching at latter intervals had lower survival and growth rates; therefore, filial cannibalism plays a role in eliminating later-arriving, slower-growing, and hence less-adaptive offspring.

Secondly, the author investigated the influence of hatching patterns on offspring growth and survival, to demonstrate how hatching patterns affects the allocation of parental investment. The author found that asynchronous hatching pattern, in which hatching of offspring is skewed towards

the earlier part of hatching period, maximizes offspring growth and survival. Additionally, hatching patterns had a significant effect on growth of individual offspring. Thus, the present study demonstrates that asynchronous hatching pattern maximizes offspring growth and survival by affecting the allocation of parental investment.

Thirdly, the author investigated the adaptive significance of filial cannibalism by male parent. Filial cannibalism by males could be adaptive if males are able to selectively cannibalize unrelated offspring. Here, the author investigated the stage at which male burying beetles, *N. quadripunctatus*, increase their paternity by evaluating the number of offspring sired by a nursing male in asynchronously hatched broods in relation to hatching time. The author found that nursing males assure a very high level of the paternity of hatching offspring. 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. Thus, ensuring paternity before fertilization is more important in increasing the paternity of offspring.

The findings obtained from the present study provide important insights into the mechanisms to regulate the amount of parental investment and the outcome of parent-offspring conflict. The present study demonstrated that parents can regulate the amount of parental investment to current reproduction by two different ways, (1) by regulating the number of offspring by filial cannibalism, (2) by affecting the allocation of parental investment by asynchronous hatching. These mechanisms set up the level of offspring competition and allocation of parental investment among them, and offspring cannot affect these mechanisms. In contrast to parents, offspring can increase the amount

of parental investment only by soliciting more investment. Thus, even if offspring can increase the amount of parental investment by soliciting more investment, parents can control the total amount and allocation of parental investment. The existence of these superior regulation mechanisms may contribute to parental control of the amount of parental investment.

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