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The proximate cause of asynchronous hatching in the burying beetle *Nicrophorus quadripunctatus*

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Running title

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Abstract

In some species, a brood hatches or is born asynchronously over an extended period of time. This asynchronous hatching establishes competitive asymmetries between offspring, and younger offspring usually exhibit a higher mortality risk and/or lower growth rate. A large body of research suggests selection favours asynchronous hatching, though a consensus is yet to be reached on the nature of its adaptive benefits. One of the possible causes of this is that previous studies have focused mostly on avian species, who must incubate their eggs. Thus, studying non-avian species may provide an opportunity to improve our understanding of the wider importance of asynchronous hatching, for example in invertebrates, which do not incubate their eggs. In the burying beetle, *Nicrophorus quadripunctatus,* asynchronous hatching has been shown to maximize parental fitness. However, there is currently no information on the proximate cause of asynchronous hatching in this species. Here we investigated the influence of timing of egg-laying and the duration of embryonic development on the timing of hatching. We found that the timing of egg-laying by the parent was a major determinant of the timing of hatching, although the duration of embryonic development was also important. Our results suggest that the principal proximate cause of asynchronous hatching is parental asynchronous egg-laying.

Keywords: *Nicrophorus*; burying beetle; asynchronous hatching; proximate cause; parent-offspring conflict

Introduction

Asynchronous hatching is a phenomenon where offspring from a single reproductive attempt hatch or are born asynchronously over an extended period of time in a species (Stoleson and Beissinger 1995). Although asynchronous hatching is reported from wide range of taxa, for example altricial birds (Magrath 1990; Stoleson and Beissinger 1995; Stenning 1996), amphibians (Ryan and Plague 2004), reptiles (While et al. 2007), and insects (Nalepa 1988; Smiseth et al. 2006; Takata et al. 2013), previous studies have focused almost exclusively on avian species. In avian species, asynchronous hatching causes an age hierarchy in the offspring and usually results in establishment of competitive asymmetries within the brood. Consequently, older siblings obtain a larger amount of parental provisioning and grow faster and/or have a higher survival rate (Mock and Forbes 1995; Forbes et al. 1997; Mock and Parker 1997; Forbes and Glassey 2000; Hall et al. 2010). Many hypotheses have been proposed to explain why selection might favour asynchronous hatching, despite producing offspring of a lower quality (Magrath 1990; Stoleson and Beissinger 1995; Stenning 1996). One set of hypotheses suggest that asynchronous hatching provides a mechanism for increasing fitness (Lack 1954; Hussell 1972; Stinson 1979; Hahn 1981; Slagsvold and Lifjeld 1989), while another set of hypotheses suggest that asynchronous hatching is a by-product of physiological constraints on the timing of egg-laying and occurs due to selection for the early onset of incubation (Clark and Wilson 1981; Arnold et al. 1987; Beissinger and Waltman 1991). Despite intensive research, a consensus regarding the adaptive benefits of asynchronous hatching is lacking. The close relationship between the onset of incubation and asynchronous hatching in altricial birds is a major obstacle in understanding the reason for asynchronous hatching. Because of this physiological constraint, separating these two groups of hypotheses is difficult in altricial birds (Stenning 1996). Studying non-avian species may provide an opportunity to improve our understanding of the wider importance of asynchronous hatching, because the timing of hatching is uncoupled from parental incubation, so hypotheses for the evolution of hatching asynchrony that involve incubation are not relevant.

The burying beetle *Nicrophorus quadripunctatus* is one non-avian species in which the ultimate causes of asynchronous hatching are studied. The burying beetle, like other species of the same genus, uses the carcasses of small vertebrates as a food resource for their larvae. Eggs are laid in the soil near the carcass and the larvae hatch asynchronously (Takata et al. 2013). After hatching, the larvae crawl onto the carcass and obtain food by begging for pre-digested carrion from their parents or by directly feeding from the carcass themselves (in *N. vespilloides*; Smiseth & Moore, 2002; Smiseth et al., 2003; in *N. quadripunctatus*; Takata, unpublished data). The larvae compete for parental food provisioning and early hatching larvae typically obtain more food and grow to a larger size than those that hatch later (Takata et al. 2013; Takata et al. 2014). Resource allocation among offspring is affected by the age hierarchy of offspring caused by asynchronous hatching and a natural age hierarchy maximizes the total weight of the brood (Takata et al. 2014). However, the proximate cause of asynchronous hatching is unknown in this species. In our first experiment, we investigated the influence of the timing of egg-laying and the duration of embryonic development on the timing of hatching, to demonstrate the proximate cause of asynchronous hatching in *N. quadripunctatus*. Additionally, factors affecting egg survival were investigated, as they may also affect the age hierarchy of offspring. In our second experiment, we investigated the temperature gradient in the soil and whether the distance eggs were laid from the carcass changes with the timing of egg-laying, to demonstrate whether female parents use the temperature gradient to control the timing of hatching.

Materials and methods

Origin and maintenance of the beetles

The beetles used in these experiments were the fourth generation offspring of over 200 wild-caught adult *Nicrophorus quadripunctatus* Kraatz collected by baited pitfall traps in June 2013 in Tokyo, Japan. 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 emerged as adults, they were fed freshly killed mealworms (*Zophobas atratus*) three times a week. All males and females used in this experiment were sexually mature and between 14 and 21 days of age after adult eclosion.

General experimental procedure

Experiment 1

Firstly, we measured the thoracic width of female parents as a proxy for body size using an electronic vernier caliper (Mitutoyo Corp., Kanagawa, Japan) with an accuracy of 0.1 mm. Then, 22 pairs of non-sibling, virgin male and female beetles were randomly selected, and each pair was placed in a plastic cup (height 8 cm, diameter 15 cm) with moist peat. They were provided with 4.0 ± 0.5 g of a whole body mouse carcass supplied by Cyber Cricket, Shiga, Japan. The beetles in the plastic cups were kept in a dark incubator at 20 ± 1 °C. To record the timing of egg-laying, we checked for eggs at 1-hourly intervals, starting 10 hours after pairing. The male beetles were removed from the plastic cup 10 hours after pairing, to exclude potential effects of male presence on egg-laying. When eggs were found, the lengths of their major and minor axis were measured using light microscopy (cellSens Standard Olympus software version 1.6, Olympus Optical Co., Tokyo, Japan) to an accuracy of 0.01 mm. Eggs were individually transferred to a new plastic cup with moist filter paper and left to hatch. This procedure was continued until females finished laying eggs. To record the timing of hatching, we checked for hatching at one-hourly intervals until all the eggs had hatched or died. Eggs that turned brown or black were recorded as dead eggs.

Experiment 2

Seventeen pairs of non-sibling, virgin male and female beetles were placed in a plastic cup with moist peat and 4.0 ± 0.5 g of a whole body mouse carcass using the same experimental procedure as Experiment 1. To measure the temperature gradient of the peat and how it changed over time, we measured the temperature using a digital thermometer (CT-220, CUSTOM, Tokyo, Japan) in 2-cm increments from the edge of the carcass at 24-hourly intervals, starting at 24 hours after pairing and terminating 96 hours after pairing. We measured the temperature at a distance of 1 mm from the bottom of the plastic cup where most of the eggs were laid. To investigate whether the distance eggs were laid from the carcass changes with the timing of egg-laying, we checked for eggs at 24-hourly intervals, and measured the position of the eggs (distance from the edge of the carcass). The position of carcass was stationary in the post egg-laying period in all of our samples.

Statistical analysis

We calculated egg volume using the formula for the volume of an ellipsoid: $V\ (mm^3) = 4/3 \times \pi \times a/2 \times (b/2)^2$, where π is circumference ratio, and a and b are the lengths of the major and minor axes of the egg respectively.

We used a generalized linear mixed model (GLMM) to investigate the effects of the timing of egg-laying on the timing of hatching. The timing of hatching, measured as the duration of time between the first egg being laid and the hatching of each egg, was treated as a response variable assuming a Gaussian distribution. The timing of egg-laying was treated as an explanatory variable and clutch identity as a random factor. The coefficient of determination (R^2) between the timing of hatching and egg-laying was calculated.

We used a generalized linear model (GLMM) to investigate how egg-laying spread, defined as the length of time between the initiation and the termination of egg laying, related to egg-hatching spread, defined as the length of time between the initiation and the termination of egg hatching. The laying spread was treated as a response variable assuming a Gaussian distribution. The hatching spread was treated as an explanatory variable. The coefficient of determination (R^2) between the laying and hatching spread was calculated.

A GLMM was used to investigate the influence of egg volume and timing of egg-laying on the duration of embryonic development. We defined timing of egg-laying as the duration from first egg-laying to the laying of each egg, and the duration of embryonic development as the duration from laying to hatching of each egg. The duration of embryonic development was treated as a response variable assuming a Gaussian distribution. Egg volume and timing of egg-laying were treated as explanatory variables and clutch identity as a random factor.

A GLMM was used to investigate the influence of egg volume and timing of egg-laying on egg survival. Egg survival was treated as a response variable assuming a binomial distribution. Egg volume and timing of egg-laying were treated as explanatory variables and clutch identity as a random factor.

A GLMM was used to investigate the influence of female body size on egg volume. Egg volume was treated as a response variable assuming a Gaussian distribution. Female body size was treated as an explanatory variable and clutch identity as a random factor.

A GLMM was used to investigate the temperature gradient in the soil and how it changed over time. The measured temperature was treated as a response variable assuming a Gaussian distribution. The distance from the edge of the carcass and elapsed time from pairing were treated as explanatory variables and clutch identity as a random factor.

A GLMM was used to investigate whether the distance eggs were laid from the carcass changes with the timing of egg-laying. The distance eggs were laid from the carcass was treated as a response variable assuming a Gaussian distribution. The timing of egg-laying was treated as a categorical explanatory variable and clutch identity as a random factor.

Interaction terms were excluded from these analyses, because none of them had significant effects. P values were calculated using the likelihood ratio test. GLMMs were conducted with the lme4 package (Bates & Maechler, 2010) using R 2.12.1 GUI 1.35 (http://cran.r-project.org).

Results

Experiment 1

The thoracic width of female parents used in this study was 4.10 ± 0.10 mm (mean ± SE, $N = 22$). Egg laying started 20.8 ± 1.6 h (mean ± SE, $N = 22$ broods) and finished 79.3 ± 3.3 h after pairing. The average egg-laying spread was 58.5 ± 3.6 h (mean ± SE, $N = 22$ broods). Female parents laid 22.8 ± 1.6 eggs (mean ± SE, $N = 22$ clutches) during this period. Mean egg volume was 1.33 ± 0.01 mm^3 ($N = 455$ eggs).

The timing of egg-laying (GLMM: estimate = 1.071, $\chi^2 = 1049.0$, *d.f.* = 1, 316, $P < 0.001$, Fig. 1) had significant effects on the timing of hatching. The estimated slope of regression line is > 1, meaning that eggs that are laid later take longer to hatch. The coefficient of determination between the timing of egg-hatching and egg-laying was 0.965. As predicted from this result, the egg-laying spread had a significant and strong positive effect on the egg-hatching spread (GLM: estimate = 1.105, $\chi^2 = -7406.9$, *d.f.* = 1, 21, $P < 0.001$, Fig. 2). The coefficient of determination between the egg-laying and egg-hatching spread was 0.792.

The mean duration of embryonic development was 73.0 ± 0.3 h (mean ± SE, $N = 317$). Our analyses showed that the timing of egg-laying (GLMM: $P < 0.001$, Table 1; Fig. 3) had a significant positive effect on the duration of embryonic development. Egg volume did not have a significant effect on the duration of embryonic development (GLMM: $P = 0.099$, Table 1).

In this study, 317 out of 392 eggs succeeded in hatching. We investigated the influence of egg volume and timing of egg-laying on egg survival. Egg volume had a significant positive effect on egg survival (GLMM: $P < 0.001$, Table 2; Fig. 4). Timing of egg-laying (GLMM: $P = 0.859$; Table 2) did not have a significant effect on egg survival.

We also investigated the influence of female body size on egg volume. Female body size had a significant positive effect on egg volume (GLMM: estimate = 0.210, $\chi^2 = 17.078$, *d.f.* = 1, 451, $P < 0.001$, Fig. 5).

Experiment 2

Temperature did not change with increasing distance from the carcass (GLMM; $P = 0.542$, Table 3), though it fluctuated slightly during the observed period (GLMM; $P < 0.001$, Table 3), but the differences were marginal (24 h: 19.76 ± 0.12 °C, 48 h: 19.82 ± 0.13 °C, 72 h: 19.75 ± 0.17 °C, 96 h: 19.71 ± 0.16 °C, mean ± SE). The distance eggs were laid from the carcass did not change with the timing of egg-laying (GLMM: estimate = -0.003, $\chi^2 = 1.172$, *d.f.* = 1, 311, $P = 0.279$).

Discussion

In this study, we investigated the influence of the timing of egg-laying and the duration of embryonic development on the timing of hatching in *N. quadripunctatus*, to demonstrate proximate causes of asynchronous hatching. We found that eggs hatched in the order in which they were laid, and asynchronous egg-laying is the proximate mechanism underlying asynchronous hatching. The duration of embryonic development was longer in eggs that were laid later. Egg survival also modifies the age hierarchy of offspring, but egg survival was not affected by the timing of egg-laying. A temperature gradient was not detected in the soil. Furthermore, we found no evidence that female parents use temperature gradient to control the timing of hatching because the distance eggs were laid from the carcass did not change with the timing of egg-laying. These results suggest that the age hierarchy of offspring is controlled mostly, if not completely, by the parent.

Although both the timing of egg-laying and the duration of embryonic development affected the timing of hatching, our results suggest that the timing of hatching is mostly determined by the timing of parental egg-laying (Fig. 1, 2). A similar result is reported from the congeneric species of the burying beetle, *N. vespilloides* (Smiseth et al. 2006). These results suggest that the principal proximate cause of asynchronous hatching in burying beetles is fundamentally different from that in altricial birds, where timing of hatching is determined by the onset of parental incubation (Stoleson and Beissinger 1995). A recent study showed that the age hierarchy of offspring caused by asynchronous hatching affects the relative fitness of *N. quadripunctatus* parents and offspring, and the natural age hierarchy is optimal for the parents (Takata et al. 2014). These results suggest that the parents receive fitness benefits by controlling the timing of egg-laying, although further studies are needed to investigate whether parents incur fitness costs through synchronous egg-laying.

The duration of embryonic development was longer in eggs that were laid later. The duration of embryonic development was also affected by the timing of egg-laying in *N. vespilloides*, though in contrast, eggs that were laid later developed slightly more quickly (Smiseth et al. 2006). These findings suggest eggs are qualitatively different depending on the timing of egg-laying. In some species, the duration of embryonic development is affected by egg volume (Gillooly and Dodson 2000a; Gillooly and Dodson 2000b; Schenk and Söndgerath 2005). However, there is no evidence for this in burying beetles (Smiseth *et al.* 2006 and this study).

Larger females laid larger eggs, and offspring that hatched from larger eggs had higher survival rates. The findings of the current study correspond with previous studies of other organisms (e.g. Berrigan 1991; Bernardo 1996; Fox and Czesak 2000; Gilbert and Manica 2010). In burying beetles, egg size positively correlated with larval body size at hatching, but did not affect subsequent larval growth when the larvae are cared for by parents of similar body size (Monteith et al. 2012; Steiger 2013). This suggests the major benefit of producing large eggs is an increased chance of egg survival. Positive correlations between egg size and survival rates are also reported from other species such as turtles and birds (Gutzke and Pachard 1985; Packard et al. 1989; Potti and Merino 1996).

In conclusion, the timing of parental egg-laying was a major determinant of asynchronous hatching in *N. quadripunctatus*. The timing of egg-laying may play a similar role in resource allocation among offspring in other species. As asynchronous hatching is known in various taxa, further studies are encouraged to investigate both the ultimate and proximate causes of asynchronous hatching in other phylogenetically distinct species.

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Ethics declarations

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

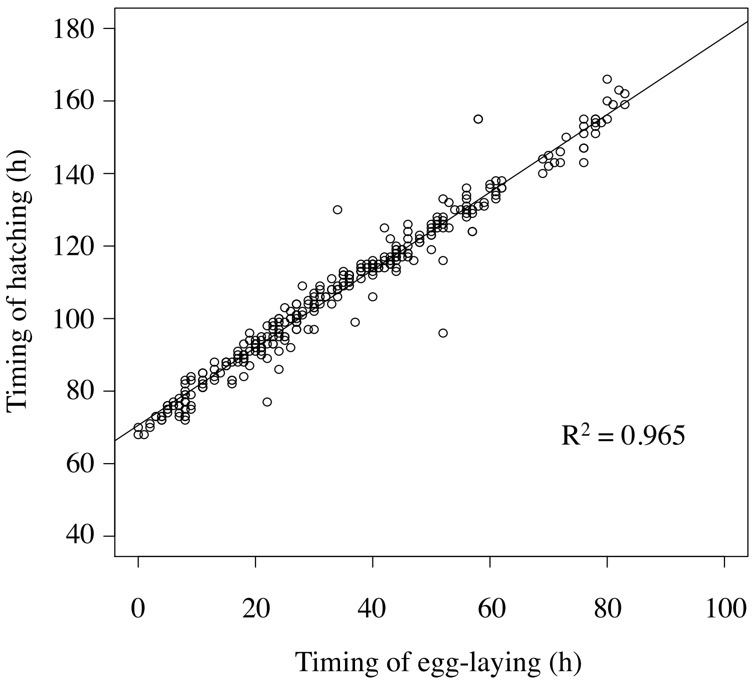

Figure 1. Influence of the timing of egg-laying on the timing of hatching in *N. quadripunctatus*. The timing of egg-laying is measured as the duration of time from the first egg being laid to each subsequent egg being laid. The timing of hatching is measured as the duration of time from the first egg being laid to the hatching of each egg. The coefficient of determination (R^2) is shown in the graph. The regression line estimated from GLMM is shown in the graph (y = -70.588 + 1.071x).

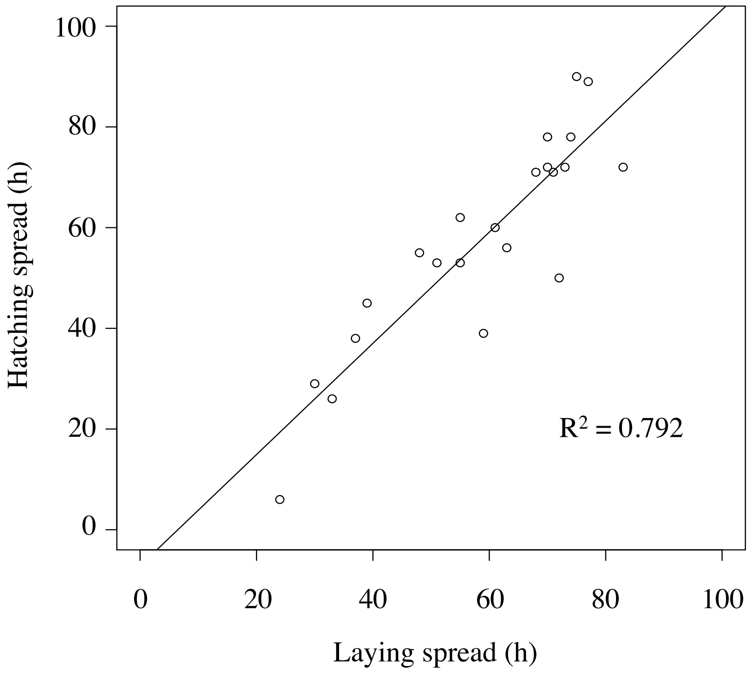

Figure 2. The influence of laying spread (i.e. the time over which the eggs of a given clutch were laid) on hatching spread (i.e. the time over which the eggs of a given clutch hatched) in *N. quadripunctatus*. The coefficient of determination (R^2) is shown in the graph. The regression line estimated from GLMM is shown in the graph ($y = -7.164 + 1.105x$).

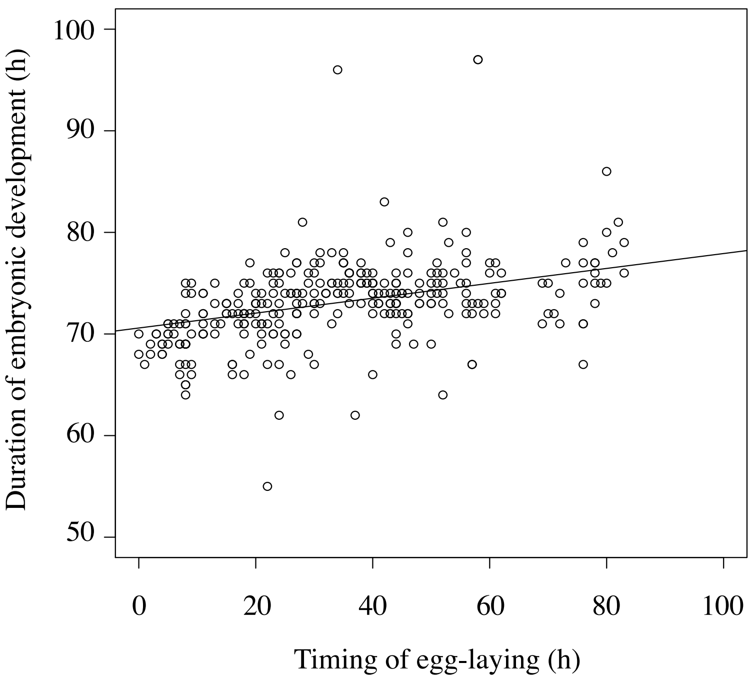

Figure 3. The influence of timing of egg-laying on the duration of embryonic development in *N. quadripunctatus*. The timing of egg-laying is measured as the duration of time from the first egg being laid to each subsequent egg being laid. The regression line estimated from GLMM is shown in the graph ($y = 70.589 + 0.733x$).

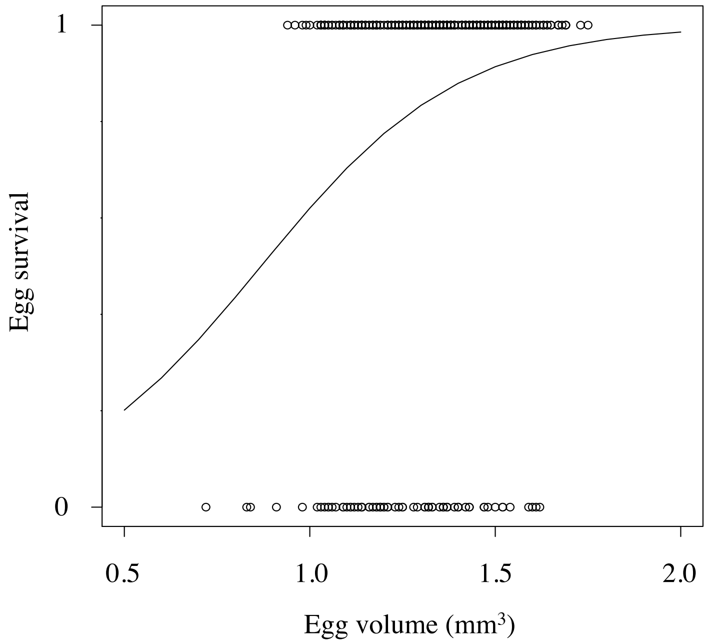

Figure 4. The influence of egg volume on egg survival in *N. quadripunctatus*. Each plot shows the survival data of each egg, success is measured as hatching (1) or death (0). The logistic regression curve is shown in the graph.

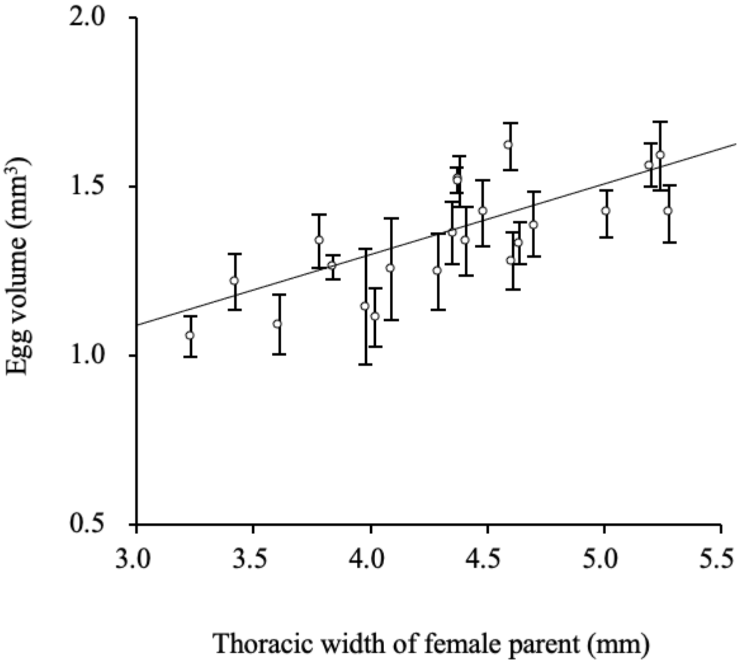

Figure 5. The influence of body size of the female parent on egg volume in *N. quadripunctatus*. Data are presented as mean ± SD. The thoracic width of the female parents was used as an indicator of body size. The regression line estimated from GLMM is shown in the graph ($y = 0.468 + 0.210x$).

Table 1. GLMM results for the effect of timing of egg-laying and egg volume on the duration of embryonic development.

Factor	Estimate	χ^2	*df*	*p*
Timing of egg-laying	0.073	36.303	1, 315	**< 0.001**
Egg volume	3.365	2.715	1, 315	0.099

P values in **bold** indicate statistically significant results.

Table 2. GLMM results for the effect of timing of egg-laying and egg volume on egg survival.

Factor	Estimate	χ^2	*df*	*p*
Timing of egg-laying	3.738	14.341	1, 390	**< 0.001**
Egg volume	-0.001	0.032	1, 390	0.859

P values in **bold** indicate statistically significant results.

Table 3. GLMM results for the effect of the distance from the edge of the carcass and elapsed time on the temperature in the soil.

Factor	Estimate	χ^2	*df*	*p*
Distance from carcass	0.003	0.372	1, 202	0.542
Timing of measurement	-	19.078	1, 202	**< 0.001**

P values in **bold** indicate statistically significant results.