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Developmental Biology of Forensically Important Beetle, *Necrophila* (*Calosilpha*) *brunnicollis* (Coleoptera: Silphidae)

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Abstract

The postmortem interval (PMI) estimation, in cases where the body was discovered in an advanced stage of decomposition, is predominantly based on entomological evidence. However, very few forensically important species are sufficiently known in detail to allow a practical application. One of them is the carrion beetle, *Necrophila* (*Calosilpha*) *brunnicollis* (Kraatz, 1877). Its development from egg to adulthood was studied under a range of ecologically relevant constant temperatures to find parameters of thermal summation models. Developmental sexual dimorphism and the presence of developmental rate isomorphy were investigated. Herein we present the lower developmental thresholds and sum of effective temperatures for all developmental stages of *N. brunnicollis* (egg, first–third larval instar, postfeeding stage, and pupae). We did not find any evidence of developmental sexual dimorphism nor was the presence of developmental rate isomorphy confirmed. Our results present the first thermal summation model of the East Asian carrion beetle that can be used for the PMI estimation.

Key words: Silphinae, forensic entomology, developmental rate, thermal summation model, sexual dimorphism

Estimating the time of death, or postmortem interval (PMI), is a critical part of legal investigations. Such estimate can be used to eliminate suspects or help establish the link between an offender and the victim (Geberth 2006). The PMI can be calculated based on the state of the body itself or by using ecological evidence such as developmental stages of insects (Goff 2010). As the decay progresses, the first method becomes less useful, and the accuracy of the latter becomes superior (Wells and LaMotte 2010). However, the accuracy of PMI estimation using insects largely depends on our knowledge and understanding of necrobiont species biology. The most accurate and precise methods are based on estimating the age of the oldest developmental stages found on the body (Amendt et al. 2011). For correct age calculation, known parameters of thermal summation model (TSM), lower developmental threshold (t_0) and the thermal summation constant (K), are crucial (Ikemoto and Takai 2000). These parameters are species and stage specific, and their establishment is laborious; thus, they are currently available for only a small fraction of all necrobiont insect species (Midgley et al. 2010).

Historically, forensic entomologists preferred flies, especially blowflies (Diptera: Calliphoridae), over carrion beetles for PMI estimation (Midgley et al. 2010, Dekeirsschieter et al. 2013). Only recently, it has become clear that both groups have their advantages and disadvantages depending on specific conditions (Ridgeway et al. 2014). Carrion beetles from the genera *Thanatophilus* Leach, 1815 and *Necrodes* Leach, 1815 were observed to colonize carrion during the first 24 h (Ratcliffe and Luedtke 1969, Midgley and Villet 2009). In addition, many necrobiont beetles are known to have lower developmental rate than most necrophagous flies (e.g., Velásquez and Viloria 2009, Ridgeway et al. 2014, Jakubec 2016, Frątczak-Łagiewska and Matuszewski 2018, Novák et al. 2018, Wang et al. 2018, Jakubec et al. 2019). Hence, beetles can provide more accurate PMI estimates in cases where the body is discovered in later stages of decomposition (Ridgeway et al. 2014). Although many researchers and practitioners are currently well aware of the potential utility of Coleoptera in forensic entomology (Midgley et al. 2010; Charabidze et al. 2014, 2016), the current state of knowledge of their biology and especially the lack of TSMs does not allow their practical use in PMI estimations.

Within the East Asian region, the TSMs covering all developmental stages are known for only four necrobiont beetle species, namely *Aleochara asiatica* Kraatz, 1859 (Coleoptera: Staphylinidae), *Aleochara nigra* Kraatz, 1859, *Creophilus maxillosus maxillosus* (Linnaeus, 1758), and *Sciodrepoides watsoni watsoni* (Spence, 1813) (Coleoptera: Leiodidae) (Lin and Shiao 2013, Jakubec 2016, Frątczak-Łagiewska and Matuszewski 2018, Matuszewski and Fratczak-Łagiewska 2018, Wang et al. 2018). However, none of them represents the ideal bioindicator for PMI. Larvae of *C. maxillosus* are predatory with facultative necrophagy and carrion does not represent their exclusive food source. Hence, they can migrate between various food sources and consequently give inaccurate estimates of PMI. On the other hand, larvae of *S. watsoni* appear to be obligatory necrophagous, yet their preference of later stages of decomposition restricts their application on bodies that have been exposed for less than a month (Růžička 1994, Kočárek 2002). Both *Aleochara* species are parasitoids of blowflies, thus giving PMI estimates with a wider margin of error as TSMs for both species (beetle and fly) must be combined (Lin and Shiao 2013). In this article, we propose a new forensically relevant species that will complement the abovementioned as a new bioindicator for PMI estimation for the region of East Asia.

The East Asia region is considered the biodiversity hotspot for family Silphidae, also known as carrion beetles. Moreover, they include a number of obligatory necrophagous species that colonize carcasses of large vertebrates in the early stages of decomposition. *Necrophila* (*Calosilpha*) *brunnicollis* (Kraatz 1877) appears to have high potential to become widely used as a PMI indicator in this region due to numerous factors. Both adults and larvae of this beetle can be observed soon after death on carcasses of large vertebrates, and the necrophagy of the larvae is considered to be obligatory (Ikeda et al. 2007, 2008; Růžička et al. 2015). The species is quite common, inhabiting mainland China, Bhutan to Far Eastern Russia, Japan, and South and North Korea (Růžička 2015, Růžička et al. 2015). Adults can be found from March to August with peak activity during spring and early summer months (Růžička et al. 2015, Lyu et al. 2016). Nonetheless, despite the considerable potential of *N. brunnicollis* in forensic entomology, the development of its juvenile stages under constant temperatures has never been studied.

The accuracy of the estimate of TSM parameters is highly dependent on the variability occurring in the data. This variability can be higher than expected due to sexual dimorphism in the developmental length (Picard et al. 2013). Therefore, the accuracy and precision of the models could be further improved by examining the difference in developmental lengths between males and females of *N. brunnicollis* that can potentially provide specific TSM parameters for each sex. Testing sexual dimorphism of developmental rate is an important practice that should be more widely adopted, as it can hypothetically have a large impact on PMI estimates, even though not all insect species exhibit one (Frątczak-Łagiewska and Matuszewski 2018).

Last, the effect of temperature on the proportion of time spent in each developmental stage represents another aspect that should be taken into consideration. The state when these proportions do not change with temperature is called development rate isomorphy (DRI; Jarošík et al. 2002). The importance of this relationship lies in the possibility of a highly simplified method for the calculation of TSM parameters (Jarošík et al. 2002). DRI has never been studied in *N. brunnicollis*, nor in any other forensically important species. The possible simplification would allow for faster and more accurate adjustment of TSM parameters to accommodate intraspecific variation displayed by many necrophagous species (Kipyatkov and Lopatina 2010, Amendt et al. 2011, Tarone et al. 2011).

The main aim of this article is to study the development of *N. brunnicollis* in the range of constant temperatures to find parameters of TSM for each of its developmental stages. Furthermore, we would like to test whether the obtained data will show signs of significant developmental sexual dimorphism and if so, provide sex-specific TSM parameters. In addition, the developmental data obtained and specifically the proportion of time spent in individual stages will be tested for compatibility with DRI theory.

Materials and Methods

Colony Establishment and Sampling

We collected adults of *N. brunnicollis* with baited pitfall traps placed in different habitats, from a broad-leaved deciduous forest with *Rhododendron* Linnaeus, 1753 (Ericales: Ericaceae) to a dwarf forest with bamboo shrubs in China, Zhejiang Province, Lin'an County, West Tianmushan Nature Reserve, along the track from the Original Temple of Lion Sect to the Immortal Peak, 1,165–1,470 m a.s.l. (GPS: 30°20′35.16″–30°20′56.76″N 119°25′27.84″–119°25′53.76″E) between the 5 and 6 July 2017. All the beetle specimens collected were transported into the laboratory, identified to species, and sexed using the identification key by Růžička et al. (2015). Adults were then divided into groups of six, with equal number of male and female specimens and held in plastic boxes (185 × 185 × 190 mm) with a 50-mm-thick layer of sterilized gardening soil as substrate. Breeding took place inside four climatic chambers with constant temperature and photoperiod regime (18, 20, 22, and 29°C and 16 h of light followed by 8 h of dark) to simulate the natural environmental conditions during the breeding season of this species. The substrate was regularly moistened by tap water. As a food source, the beetles were provided with fish meat (*Scomber scombrus* Linnaeus, 1758 [Perciformes: Scombridae]) ad libitum.

The breeding boxes were inspected at least once a day, and newly laid eggs were removed and placed in Petri dishes (9 cm diameter) filled up with a thin layer of gardening soil and approximately 5 g of fish meat (*S. scombrus*). The Petri dishes with eggs were placed inside the same climatic chamber as their parents to eliminate heat stress to the developing specimens. Eggs were observed daily, and after their hatching, larvae were transferred into new Petri dishes within the same climatic chamber, filled with gardening soil to two thirds and provided with fish meat (*S. scombrus*).

The layout of larval Petri dishes followed the methodology suggested by Ridgeway et al. (2014), in which the dish is placed on its side and held closed by a rubber band to prevent opening (see Fig. 1). The layout provides enough space for larvae to move around and feed, while it also provides an excellent opportunity to dig or create pupation chambers. The layout improves larval welfare, while the relatively narrow space forces them to pupate next to the dish wall, thus allowing undisturbed observations throughout their development. Moisture was provided by submerging the bottom part of the dish into a 3-cm layer of tap water for a few seconds. The dishes were opened rarely, only to remove unconsumed meat with signs of mold growth and to replace it with a fresh meat. Once the larvae created a pupation chamber, the food source was removed altogether, and the Petri dish remained closed until the development was completed.

Petri dishes with developing specimens were monitored every 24 h. Clutches of eggs from the same breeding pair and day were kept together, and newly hatched larvae were further separated based on the time of hatching or entering the next developmental stage. This allowed for easier observation and collection of their developmental data in the different stages (egg, first- and third-instar larvae, postfeeding stage, and pupa). If multiple larvae hatched from the same brood on the same day, they were purposefully kept together to allow more efficient feeding and to improve their developmental rate and lower mortality. On the other hand, no more than three larvae of the second instar were allowed in one Petri dish to minimize the probability of cannibalism. In the third instar, each larva was moved to its own separate dish, to prevent any disturbance in postfeeding/pupal stages by other individuals.

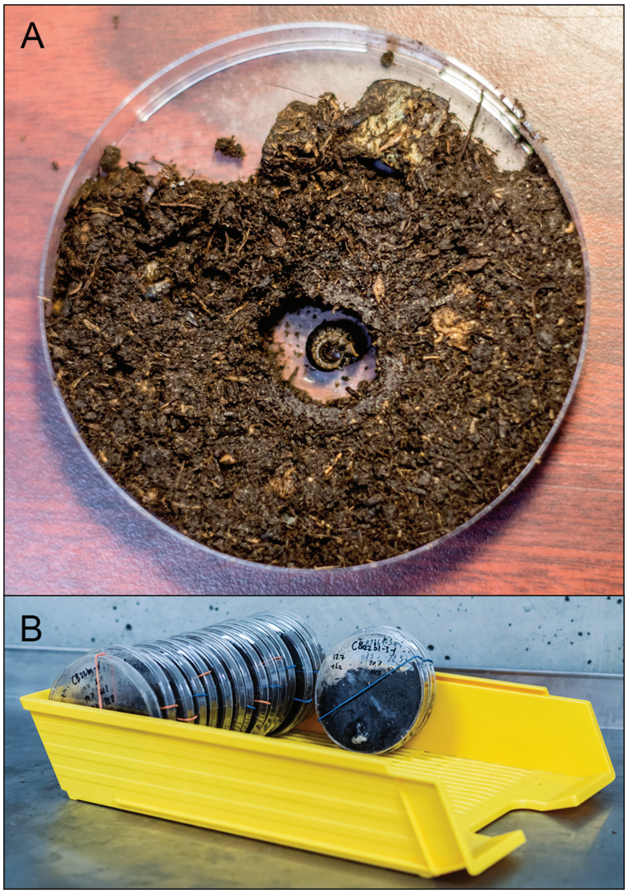

Fig. 1. (A) Layout of larval Petri dish with a pupation chamber with third-instar larvae in postfeeding stage. (B) Documents container tray with larval Petri dishes secured by rubber bands, which was used to allow easier manipulation with large number of dishes.

Statistics and Analysis

To model the relationship between developmental time and accumulated degree days (ADD) for all developmental stages, we applied the linear regression model proposed by Ikemoto and Takai (2000). The main advantage of the Ikemoto and Takai (2000) reparameterization of the relationship between developmental rate and temperature is its robustness and simplicity. Both parameters of TSM (lower developmental threshold [t_0] and thermal summation constant [K]) are defined as a slope and intercept of the linear regression, respectively. Therefore, both t_0 and K can be easily calculated along with their respective SE (Ikemoto and Takai 2000).

To investigate the potential difference in developmental time between males and females, two linear mixed-effect regression models were fitted (null and 'sex' model). The response variable of both models was the development times of six stages (egg, larval instars 1–3, postfeeding stage, and pupae) and their total as a response variable. Null and alternative model also shared two fixed effect explanatory variables (temperature [18, 20, and 22°C] and stage of development). Because the measurements of development times were done repeatedly on the same individual, the identity of that specimen had to be incorporated into the analysis; therefore, we used it as a random effect in both models. The only difference between the alternative and the null models was fitting the latter with sex as a fixed effect explanatory variable. The fit of these two models was compared via Akaike information criteria (AIC) to find out whether the information about the sex of the *N. brunnicollis* does significantly improve it.

The presence of DRI in *N. brunnicollis* was tested by Dirichlet regression using methodology suggested by Boukal et al. (2015). The main advantage of this method is that it does not require the temperatures to be within a range in which the development rate has a positive linear relationship with the temperature. The method consists of fitting three Dirichlet regression models with the same response variable (proportion of time spent in each developmental stage) and with several explanatory variables. The null model (mod0) was fitted without any explanatory variables to simulate the assumption that the proportions of time spent in individual instars are constant and independent of other factors (true presence of DRI in the species). The alternative models were fitted with temperature as explanatory variable (mod1) and the third model with expected quadratic effect of temperature (mod2). Both alternative models suggest that the DRI is not present in the species. Relative quality of each model was evaluated by AIC to allow comparison. The one with the lowest value was considered as the most appropriate description of the underlying relationship.

The analysis was carried out in R program (version 3.5.1; R Core Team 2018). Additionally, we used lme4 package for fitting mixed effect models, and visual outputs were processed via packages ggplot2 and sjPlot (Wickham 2009, Bates et al. 2015, Lüdecke 2018).

Results

Development of *N. brunnicollis*

The development of 481 individuals of *N. brunnicollis* was recorded over four constant temperatures (18, 20, 22, and 29°C). In total, 241 specimens were able to complete their development to adulthood. The difference in development length found between the lowest and highest temperature was 53.05 d; total development duration at 18°C was 71.65 d (SD = 4.96 d, n = 80), whereas at 29°C, it took only 18.60 d (SD = 1.33, n = 8). The development at higher temperature came at the cost in terms of higher mortality, as in 29°C, only 30.8% of all individuals survived to adulthood, compared with 60.9% at 18°C. All adults obtained from the highest temperature had severally deformed elytra and wings. This morphological phenomenon was also recorded in adults from other temperatures (18, 20, and 22°C), but with much lower incidence rate.

The development of the *N. brunnicollis* resembles other species of the subfamily Silphinae. Females lay clutches containing on average 8.4 eggs under the soil surface. The first-instar larvae from the same clutch emerge almost simultaneously, sometimes only minutes apart, followed by immediate search for food and aggregation around its source. The molting between the first–second and second–third instars occurs directly on the food source or very close to it on the surface. Cannibalistic behavior among the larvae has not been directly observed; nonetheless, we found a few dead individuals with feeding marks on their ventral parts. The observed larvae cease eating at the end of the third instar and dig a small, rounded chamber in the substrate, usually at the bottom of the Petri dish where they reside. Shortly after, they become almost immobile and slightly bloated, signaling the progression to prepupal stage, subsequently transitioning to pupa.

The survival rate across all developmental stages (from egg to adulthood) was relatively high (mean = 50.81%; SD = 16.16%). The lowest survival rate (30.77%) was recorded among individuals that were raised at the highest temperature (29°C). Additionally, the transition between the second and third instars was particularly critical (see Table 1).

Thermal Summation Model

The TSMs were established for all six developmental stages (egg, larval instars 1–3, postfeeding stage, and pupae) based on the developmental length at four constant temperatures (18, 20, 22, and 29°C; see Fig. 2). The thermal summation constant (K) and lower developmental threshold (t_0) values were calculated with expected errors (see Table 2). The t_0 varied between 16.74 ± 0.18°C at the first larval instar and 15.36 ± 0.35°C at pupal stage. The lowest amount of ADD that was needed for completion of development was also found at the first larval instar (ADD = 8.35 ± 0.53), whereas the highest value was associated with pupal stage (78.67 ± 6.29). The coefficients of determination (R^2) for all models were above 0.887, indicating very good fit of the models on the data (see Table 2).

Effect of Sex on Development Length

To study the effect of sex on developmental length, we selected a subsample of 193 individuals (100 females and 93 males) that were observed from egg till adulthood in order to determine their sex. The sex ratio was on average 0.93, and the probability of males and females in the sample did not differ significantly from equality (exact binomial test, n = 193, 95% confidence interval [0.41, 0.55], P = 0.666). Moreover, it varied very little with temperature (18°C = 0.88, 20°C = 1.1, and 22°C = 0.84).

The development length was very similar for males and females over all developmental stages and even as a total (see Fig. 3). Comparison of the null model and the model with sex as an explanatory factor by AIC values showed that null model had lower AIC values (null AIC = 8520.9, sex AIC = 8523.0), therefore implying that sex did not improve the fit of the model significantly.

Table 1. Differences in duration of development of *Necrophila brunnicollis* at constant temperatures for specific developmental stages

Temperature (°C)	Developmental stage	Development duration (d)	*N*	Survival rate (%)
18	Egg	6.203 (1.657)	128	100.00
	First-instar larva	4.102 (0.738)	118	92.19
	Second-instar larva	4.842 (1.224)	108	84.38
	Third-instar larva	16.606 (3.956)	94	73.44
	Post-feeding	17.687 (2.547)	90	70.31
	Pupa	22.376 (1.592)	78	60.94
20	Egg	3.672 (0.639)	133	100.00
	First-instar larva	1.844 (0.824)	117	87.97
	Second-instar larva	2.613 (0.557)	95	71.43
	Third-instar larva	7.983 (1.235)	68	51.13
	Post-feeding	9.022 (1.353)	66	49.62
	Pupa	13.587 (1.477)	64	48.12
22	Egg	3.0742 (1.038)	135	100.00
	First-instar larva	2.348 (0.604)	125	92.59
	Second-instar larva	2.301 (0.833)	111	82.22
	Third-instar larva	5.801 (1.736)	100	74.07
	Post-feeding	8.815 (2.254)	96	71.11
	Pupa	16.125 (2.937)	91	67.41
29	Egg	2.068 (0.419)	23	100.00
	First-instar larva	1.073 (0.444)	18	88.46
	Second-instar larva	1.154 (0.3695)	18	69.23
	Third-instar larva	5.281 (1.932)	9	34.62
	Post-feeding	4.758 (1.206)	9	34.62
	Pupa	5.240 (0.916)	8	30.77

Numbers in brackets are SD.

Developmental Rate Isomorphy

We applied Dirichlet regression to individual development data for all six stages (egg, larval instars 1–3, postfeeding stage, and pupae) of *N. brunnicollis* raised in the laboratory at four different temperatures (18, 20, 22, and 29°C). When we compared all three models (mod0, mod1, and mod2), the model with quadratic effect of temperature had the lowest value of AIC (AIC =–5185.04), followed by the model with temperature as explanatory variable (AIC = –5143.326). On the opposite side was the model implying DRI, which was accompanied with the highest value of AIC (AIC = –4837.744), thus suggesting the worst fit to the data. These results imply that the proportion of time spent in each developmental stage is not constant but has a quadratic relationship with temperature.

Discussion

To the best of our knowledge, there are no other reports on the development of *N. brunnicollis* or other members of *Necrophila* Kirby & Spence, 1828, at constant temperatures. Only a few TSMs are available for the members of the family Silphidae in general to allow comparison. The data presented here fit the general pattern reported for other genera of Silphidae (*Oxelytrum* Gistel, 1848 and *Thanatophilus*; Velásquez and Viloria 2009, Ridgeway et al. 2014). Developmental length at 20°C of *N. brunnicollis* was 38.7 d, whereas in *Oxelytrum discicolle* (Brullé, 1836) (Coleoptera: Silphidae), it was substantially shorter, and it took only 29.7 d. The development of *Thanatophilus capensis* (Wiedemann, 1821) and *Thanatophilus micans* (Fabricius, 1794) shows a similar developmental rate at the same temperature, taking about 39.6 and 34.6 d, respectively. However, both had much lower developmental thresholds (*T. capensis*'s t_0 = 8.4°C and *T. micans*'s t_0 = 11.3°C) than *N. brunnicollis* (t_0 = 15.4°C). Reports of development rate at 20°C also exist for two Palearctic species of genus *Thanatophilus: T. rugosus* (Linnaeus, 1758) and *T. sinuatus* (Fabricius, 1775). Both species were able to finish their development in 45.5 and 41.9 d, respectively; therefore, the length of the development of *N. brunnicollis* could be considered very similar to other members of the family Silphidae.

In comparison, the development length of many common blow flies is much shorter. For example, at approximately 20°C, the development of *Calliphora vicina* Robineau-Desvoidy, 1830, *Phormia regina* Meigen, 1826, (Diptera: Calliphoridae), and *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae) can take around 21.45, 20.5, and 24.75 d, respectively (Anderson 2000, Nabity et al. 2006, Roe and Higley 2015). The development among different populations can vary, however not as dramatic as was shown in case of *L. sericata*, where three separate populations developing at 20°C were able to complete their development between 19.1 and 19.8 d on average (Tarone et al. 2011). Thus, in cases when the victim is found after more than 25 d, *N. brunnicollis* can improve the accuracy and precision of the PMI estimates.

To our knowledge, this study represents the first TSM of any member of the family Silphidae from the region of East Asia, although TSMs of several nonsilphid necrophagous beetle species, occurring in the same region, were previously studied, namely *S. watsoni*, *C. maxillosus*, *A. asiatica*, and *A. nigra* (Lin and Shiao 2013, Jakubec 2016, Frątczak-Łagiewska and Matuszewski 2018, Wang et al. 2018).

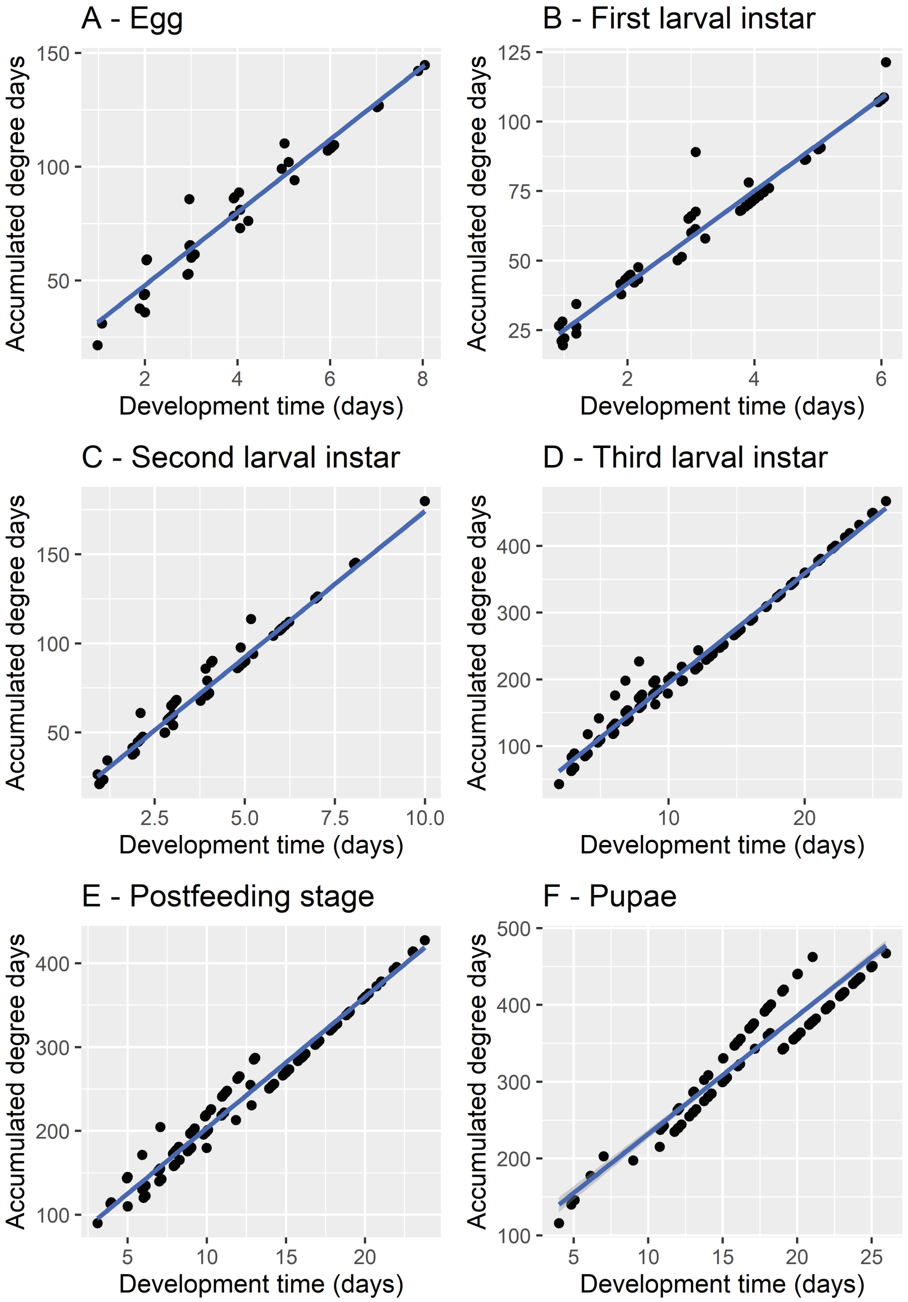

Fig. 2. Thermal summation models for all developmental stages of *Necrophila brunnicollis*: (A) egg, (B) first larval instar, (C) second larval instar, (D) third-larval instar, (E) postfeeding stage, (F) pupae. Points indicate data used in regression analysis.

Table 2. Summary of developmental constants for *Necrophila brunnicollis* for five developmental stages

Developmental stage	Temperature range of model (°C)	R^2	df	*P* value	*K* (°C)	t_0 (°C)
Egg	18–29	0.962	420	>0.001	15.858 (0.700)	16.022 (0.155)
First-instar larva	18–29	0.958	381	>0.001	8.346 (0.527)	16.704 (0.180)
Second-instar larva	18–29	0.974	330	>0.001	10.305 (0.514)	16.401 (0.147)
Third-instar larva	18–29	0.985	269	>0.001	30.406 (1.400)	16.422 (0.122)
Post-feeding	18–29	0.972	259	>0.001	47.004 (2.118)	15.653 (0.166)
Pupa	18–29	0.887	239	>0.001	78.671 (6.294)	15.360 (0.355)

Numbers in brackets are SE.

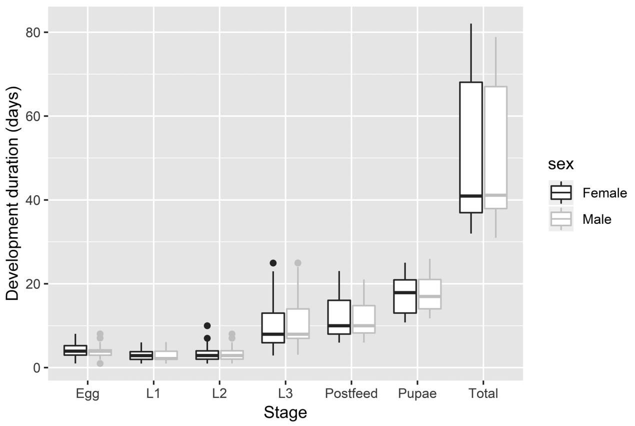

Fig. 3. Boxplots of developmental duration difference between sexes. Horizontal lines within the boxes indicate median values; upper and lower boxes indicate the 75th and 25th percentiles, respectively; whiskers indicate the values within the 1.5 interquartile ranges; small, black, and gray dots are outliers.

The presence of DRI was not confirmed for *N. brunnicollis*; therefore, the proportion of time spent in each developmental stage is not dependent on ambient temperature. This is in line with the recent findings in other species of insects (Boukal et al. 2015, Eliopoulos and Kontodimas 2016). Furthermore, we report quadratic effect of temperature on the proportion of the time spent in each developmental stage. If the relationship is really causal and could be used as a mechanistic description, it would have a significant impact on the way we develop TSMs for insects and how they could be adjusted for local populations. Testing this theory is however beyond the scope of this article. We encourage a follow-up research on the topic as the implications for forensic entomology could be significant.

Necrophagous carrion beetles are common on vertebrate cadavers throughout almost all continents (Africa, America, Asia, Australia, Europe; Sikes 2008). However, they are still overlooked and understudied almost universally, despite general agreement regarding their significance for forensic entomology (Midgley and Villet 2009, Midgley et al. 2010, Ridgeway et al. 2014, Charabidze et al. 2016, Jakubec 2016). From a global perspective, the true state of research on the topic of TSM in carrion beetles could be summarized briefly; there are two TSMs for the African species of genus *Thanatophilus* and one TSM for South American species of genus *Oxelytrum*. In this article, we present the first TSM for necrophagous carrion beetle from Asia, although its distribution covers only a portion of East Asia. There are no TSMs available for the relevant beetle species in Australia, Europe, and North America. In other words, the vast majority of carrion beetles have never been studied, and their larvae are often unknown. However, this represents an opportunity and clear goal for advancement in our knowledge of forensically important beetles and overall enhancements in the field.

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