UNIWERSYTET IM. ADAMA MICKIEWICZA W POZNANIU

WYDZIAŁ BIOLOGII

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Development models of necrophilous beetle *Creophilus maxillosus* (L.) (Staphylinidae) – comparison of efficiency in estimation of immature insect age

Modele rozwoju chrząszcza nekrofilnego Creophilus maxillosus (L.) (Staphylinidae) – porównanie ich skuteczności w szacowaniu wieku form preimaginalnych

Rozprawa doktorska

Rozprawa doktorska została przygotowana w Zakładzie Taksonomii i Ekologii Zwierząt
Wydział Biologii, Uniwersytet im. Adama Mickiewicza w Poznaniu
Poznań, 2020

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SPIS TREŚCI

STRESZCZENIE

Rozwój gatunków nekrofilnych chrząszczy o wykazanej użyteczności w entomologii sądowej jest słabo poznany. Brak modeli rozwoju dla tych gatunków często uniemożliwia wykorzystanie w praktyce entomologicznych metod szacowania czasu zgonu. Niewiele wiadomo także na temat czynników, które wpływają na jakość danych rozwojowych, a w konsekwencji na jakość modeli rozwoju. *Creophilus maxillosus* (Linnaeus, 1758) jest drapieżnym chrząszczem z rodziny kusakowatych (Staphylinidae) obecnym niemal zawsze na zwłokach dużych kręgowców w środowiskach naturalnych. Pojawia się na nich później niż muchówki z rodziny plujkowatych (Calliphoridae), które są owadami najczęściej wykorzystywanymi na potrzeby wymiaru sprawiedliwości. Późniejsza kolonizacja zwłok przez *C. maxillosus*, znacznie wydłuża okres po śmierci, w którym można wnioskować o czasie zgonu. W ramach pracy doktorskiej zrealizowane zostały następujące cele: 1) opracowanie, porównanie i częściowa walidacja temperaturowych modeli rozwoju (liniowych, nieliniowych i graficznych) *C. maxillosus*, 2) określenie wpływu rodzaju pokarmu na śmiertelność, wymiary ciała i czas rozwoju chrząszczy tego gatunku, 3) sprawdzenie, czy istnieje związek pomiędzy czasem całego rozwoju preimaginalnego, a płcią i rozmiarem imagines *C. maxillosus*, oraz czy cechy te mogą pomóc w dokładniejszym oszacowaniu wieku chrząszczy w momencie zakończenia rozwoju, 4) sprawdzenie, czy wielokrotne, przyżyciowe pomiary chrząszczy wpływają na ich rozwój.

Przeprowadzone zostały dwa eksperymenty. W ramach pierwszego, dokonywano pomiarów długości i masy ciała larw oraz masy poczwarek w trakcie rozwoju w 10 stałych temperaturach (10-32,5 °C; co 2,5 °C). Dla każdego osobnika określano czas potrzebny do osiągnięcia każdego z 5 punktów orientacyjnych w rozwoju (wylęganie larw, pierwsze linienie, drugie linienie, przepoczwarczenie, pojawienie się imago). Eksperyment drugi przeprowadzony został w stałej temperaturze 24 °C. Wszystkie chrząszcze były badane pod kątem czasu potrzebnego do osiągnięcia punktów orientacyjnych rozwoju. Ponadto mierzono i ważono larwy po przejściu do trzeciego stadium larwalnego i ważono poczwarki w momencie ich pojawienia się. Larwy *C. maxillosus* były karmione w tym eksperymencie jednym typem pokarmu (larwy trzeciego stadium lub puparia *Calliphora* sp. Robineau-Desvoidy lub *Lucilia* sp. Robineau-Desvoidy (Diptera: Calliphoridae), mieszanka larw pierwszego i drugiego stadium chrząszcza *Necrodes littoralis* (Linnaeus, 1758) (Silphidae)).

Badania wykazały, że żaden chrząszcz nie osiągnął stadium imago w 10, 12, i 32,5 °C. Najniższa śmiertelność występowała w 25 °C. Postać dorosła pojawiła się w hodowlach prowadzonych w siedmiu na dziesięć temperatur (15–30 °C). Całkowity czas rozwoju wahał się od około 122 dni w temperaturze 15°C do około 22 dni w temperaturze 30°C. Dolne progi rozwojowe wyprowadzone z modelu liniowego wahały się od 8,1 ± 0,4 °C dla drugiego linienia do 12,0 ± 0,4 °C dla przepoczwarczenia. Stałe cieplne (*K* (SE)) dla kolejnych punktów orientacyjnych rozwoju wyniosły: 49,2 (4,0), 81,7 (6,0), 122,9 (7,8), 274,8 (14,5), 405,2 (14,6). Podczas walidacji modeli sumowania cieplnego, największe błędy szacowania wieku stwierdzono dla chrząszczy hodowanych w temperaturach 10 i 12,5 °C (od 21 do 43% dla wszystkich punktów orientacyjnych rozwoju). Larwy *C. maxillosus* karmione stadiami larwalnymi *N. littoralis* wykazywały bardzo wysoką śmiertelność i żaden osobnik nie

osiągnął stadium imago. Najniższa śmiertelność była obserwowana, gdy larwy karmione były larwami muchówek, niezależnie od ich rodzaju. Całkowity czas rozwoju był najkrótszy, gdy larwy *C. maxillosus* karmione były poczwarkami *Lucilia* sp.. Wyniki wykazały, że samce rozwijają się istotnie dłużej niż samice. Mimo tych różnic, walidacja wykazała tylko małe i statystycznie nieistotne różnice w dokładności szacowania wieku owadów z wykorzystaniem modeli ogólnych i modeli specyficznych dla płci. Stwierdzono, że liczba jednostek cieplnych (*K*), które owad musi skumulować żeby zakończyć rozwój, oszacowana w oparciu o rozmiar ciała osobnika dorosłego, jest istotnie bliższa rzeczywistemu *K* niż *K* uzyskane w oparciu o ogólny model rozwoju. Wykorzystanie rozmiaru owada dorosłego jako zmiennej predykcyjnej i osobnych modeli regresji dla samców i samic istotnie poprawiło dokładność oszacowania wieku w przypadku tego gatunku. Mierzone osobniki rozwijały się dłużej niż niemierzone. Opracowano modele dla osobników mierzonych oraz dla osobników niemierzonych, a następnie przeprowadzono ich walidację wykorzystując osobniki niemierzone. Dokładność oszacowania wieku była większa w przypadku użycia modelu opracowanego dla larw niemierzonych, a różnice w dokładności były bliskie istotnym statystycznie.

Podsumowując, w ramach pracy doktorskiej zbadałam rozwój drapieżnego chrząszcza nekrofilnego *C. maxillosus*, opracowałam ogólne modele rozwoju dla tego gatunku, a także modele uwzględniające czynniki mogące wpływać na ich jakość i porównałam ich skuteczność w szacowaniu wieku form preimaginalnych. Najważniejszym efektem moich badań są temperaturowe modele rozwoju gatunku *C. maxillosus*. Są to jedne z pierwszych modeli opracowanych dla europejskich gatunków chrząszczy nekrofilnych, a ich wykorzystanie na potrzeby entomologii sądowej jest możliwe w skali co najmniej środkowoeuropejskiej. Podkreślić należy, że modele rozwoju specyficzne dla płci owada są jednymi z pierwszych, a modele regresji dla zależności między rozmiarem imagines, a stałą *K*, pierwszymi w skali ogólnoświatowej. Ostatnim efektem są zaproponowane przeze mnie sposoby modyfikacji protokołu laboratoryjnego badań rozwojowych, które zostały opracowane w związku z wykazaniem szkodliwego wpływu wielokrotnych pomiarów na rozwój *C. maxillosus.*

ABSTRACT

The development of necrophilous beetles with demonstrated utility in forensic entomology is poorly understood. The lack of development models for these species often prevents the use of entomological methods of estimating the time of death in forensic cases. Also, little is known about the factors that influence the quality of development data and, consequently, the quality of development models. *Creophilus maxillosus* (Linnaeus, 1758) is a predatory beetle species that regularly visits large vertebrate cadavers in natural environments. It appears on them later than Calliphoridae flies, which are most often used in forensic entomology. Subsequent colonization of the corpse by *C. maxillosus* significantly extends the period after death in which, among others, the time of death can be estimated. As part of my doctoral dissertation, the following objectives were achieved: 1) development, comparison and partial validation of temperature models of development (linear, non-linear and graphical) of *C. maxillosus*, 2) determination of the effect of the food type on mortality, body size and development time of this species, 3) testing whether there is a relationship between the entire immature development time and the sex and size of *C. maxillosus* imagines, and whether these features can help to more accurately estimate the age of beetles at the end of development, 4) testing whether multiple, *in vivo* measurements of beetles affect their development.

Two experiments were conducted. In the first experiment the length and weight of the larvae and the weight of pupae were measured during development at 10 constant temperatures (10-32.5 °C; every 2.5 °C). For each beetle the time needed to reach each of the 5 development landmarks (hatching, first ecdysis, second ecdysis, pupation, eclosion) was determined. The second experiment was carried out at a constant temperature of 24 °C. All beetles were examined to determine the time needed to reach developmental landmarks. In addition, the larvae were measured and weighed after passing to the third instar, and pupae were weighed when they appeared. In this experiment *C. maxillosus* larvae were fed with one food type (third instar larvae or puparia of *Calliphora* sp. Robineau-Desvoidy or *Lucilia* sp. Robineau-Desvoidy (Diptera: Calliphoridae), or a mix of first- and second-instar larvae of the beetle *Necrodes littoralis* (Linnaeus, 1758) (Silphidae)).

Studies have shown that no beetle reached the adult stage at 10, 12, and 32.5 °C. The lowest mortality was at 25 °C. Beetles reached the adult stage in cultures at seven out of ten temperatures (15–30 °C). Total development time ranged between 122 days at 15 °C and 22 days at 30 °C. Lower developmental thresholds derived from the linear model ranged from 8.1 ± 0.4 °C for the second ecdysis to 12.0 ± 0.4 °C for pupation. The thermal constants (*K* (SE)) for the consecutive development landmarks were: 49.2 (4.0), 81.7 (6.0), 122.9 (7.8), 274.8 (14.5), 405.2 (14.6). In the validation of thermal summation models, the highest errors were recorded for beetles reared at temperatures of 10 and 12.5 °C (for all developmental landmarks errors were between 21 and 43%). Larvae fed with larvae of *N. littoralis* showed a very high mortality and no beetles reached the adult stage. The lowest mortality was observed when the *C. maxillosus* larvae were fed with blow fly larvae, regardless of their genus. Total development time was the shortest when *C. maxillosus* larvae were fed with *Lucilia* sp. puparia. Results demonstrate that males developed significantly longer than

females. Despite these differences, the validation study showed just minimal and statistically insignificant differences in the accuracy of age estimates using sex-specific and general thermal summation models. It was found that the number of thermal units (*K*) that insect must accumulate to complete its development, estimated based on the adult insect size was significantly closer to the true *K* as compared to *K* from the general thermal summation model. Using the beetle length at emergence as a predictor variable and male or female specific models regressing *K* against beetle size significantly improved the accuracy of age estimation for this species. Measured beetles developed longer than non-measured beetles. Models for measured and non-measured insects were developed and then validated using non-measured insects. The accuracy of age estimates was higher while using the model for the non-measured beetles and the differences in the accuracy were close to statistically significant.

In conclusion, as part of my doctoral dissertation, I investigated the development of the predatory necrophilous beetle *C. maxillosus*, I developed general development models for this species, as well as models that take into account factors that may affect their quality, and I also compared their accuracy in estimating the age of immature insects. The most important result of my research are temperature models of *C. maxillosus* development. They are among the first models developed for the European necrophilous beetles, and it is possible to use them in forensic entomology at least in Central Europe. It should be emphasized that the sex-specific models are among the first such models on a global scale and the regression models for the relationship between the size of adult beetles and the development constant *K* are the first on a global scale. An additional achievement of the experiment are my proposals to modify the laboratory protocols for development studies in forensic entomology, which were developed as an effect of the demonstration that multiple measurements of immature *C. maxillosus* have detrimental effects on its development.

WPROWADZENIE

Wiedza na temat biologii owadów ma ogromne znaczenie poznawcze, ale może być wykorzystana także w praktyce. Doskonałym tego przykładem jest entomologia sądowa, zajmująca się metodami ustalania prawnie istotnych faktów na podstawie śladów entomologicznych (Greenberg 1991, Amendt 2004, Gennard 2007). Jest to nauka interdyscyplinarna, która wykształciła się na styku entomologii, kryminalistyki oraz medycyny sądowej. Z reguły dzielona jest na entomologię produktów magazynowych (ang. *stored products entomology*), entomologię miejską (ang. *urban entomology*) oraz entomologię medyczno-kryminalną (ang. *medicocriminal entomology*) (Hall 2010). W praktyce, entomolodzy sądowi zajmują się przede wszystkim ustalaniem czasu śmierci, a ściślej okresu pośmiertnego, który oznaczany jest skrótem PMI (łac. *post mortem intervallum*) i definiowany jako okres od śmierci do zabezpieczenia śladów entomologicznych (Matuszewski 2017). Do śladów entomologicznych zaliczamy żywe i martwe owady, ich części (np. puste puparia, wylinki), a także ślady ich aktywności (np. uszkodzenia zwłok) (Frątczak-Łagiewska 2016, Matuszewski 2017).

Oszacowania czasu zgonu metodami entomologicznymi dokonuje się poprzez analizę składu gatunkowego owadów obecnych na zwłokach (metoda sukcesyjna) lub poprzez określenie wieku najstarszych stadiów preimaginalnych owadów zabezpieczonych na zwłokach (metoda rozwojowa) (Matuszewski 2010). Podstawę szacowania czasu zgonu metodą sukcesyjną stanowią zmiany struktury entomofauny w trakcie rozkładu. Wykorzystanie tej metody jest uwarunkowane istnieniem odpowiedniego modelu sukcesji, który opracowywany jest w oparciu o wyniki eksperymentalnych badań terenowych. Biegły sądowy wybierając model, z którego będzie korzystał, musi wziąć pod uwagę czynniki determinujące czas pojawu owadów na zwłokach i długość ich obecności (pora roku, masa zwłok, środowisko wyeksponowania zwłok itd.). Tylko model adekwatny, czyli uzyskany w warunkach podobnych do warunków rozkładu zwłok, w przypadku których biegły opiniuje, pozwala na dokładne oszacowanie czasu zgonu w podejściu sukcesyjnym.

Metoda rozwojowa polega na oszacowaniu wieku najstarszych stadiów preimaginalnych owadów znalezionych na zwłokach, który wyznacza minimalny czas zgonu (minPMI) (Higley i Haskell 2010). W tym celu wykorzystuje się modele rozwoju (głównie modele kumulacji cieplnej) opracowane dla danego gatunku owada. Szacowanie wieku przy ich użyciu polega na określeniu wartości wskaźnika wieku (np. stadium rozwoju), liczby jednostek cieplnych (ilość ciepła w czasie) niezbędnych dla osiągnięcia tej wartości, i w końcu na retrospektywnym obliczeniu momentu rozpoczęcia rozwoju badanego osobnika (Villet i Amendt 2011) Modele rozwoju owadów uzyskiwane są w efekcie prowadzenia hodowli w kontrolowanych warunkach laboratoryjnych, które pozwalają na częste pomiary wskaźników wieku owadów (Villet i Amendt 2011). Głównym powodem, dla którego nie zawsze można skorzystać z tej metody jest brak modeli rozwoju dla ujawnianych na zwłokach gatunków owadów. Obecnie ograniczenie to dotyczy głównie chrząszczy.

Na potrzeby entomologii sądowej rozwój owadów modelowany jest na wiele sposobów. Jedną z najstarszych metod jest prosty model regresji liniowej zwany modelem kumulacji cieplnej (Richards i in. 2008, Richards i Villet 2009). Zapewnia on bezpośrednie

oszacowanie stałej cieplnej *K*, czyli liczby jednostek cieplnych powyżej dolnego progu rozwojowego potrzebnych do osiągnięcia danego punktu w rozwoju. Innym liniowym podejściem, które zyskało ogromną popularność w ostatnich latach, jest metoda zaproponowana prze Ikemoto i Takai (2000). Pozwala ona na dokładniejsze obliczenie stałej cieplnej *K* i jej przedziałów ufności. W tej metodzie czas wymagany do osiągnięcia danego punktu w rozwoju (D) jest modelowany przy użyciu analizy regresji względem iloczynu tego czasu i temperatury (DT). Nachylenie linii regresji to dolny próg rozwojowy (D_0), a miejsce przecięcia z osią Y to stała cieplna *K*. W związku z faktem, że są to parametry oszacowanego modelu regresji, wykonując taką analizę w prosty sposób uzyskujemy przedziały ufności zarówno dla progu rozwojowego jak i dla stałej cieplnej *K*. Modele nieliniowe (np. Analytis 1981, Lactin i in. 1995, Briére i in. 1999, Shi 2011), które wykorzystywane są często w badaniach nad rozwojem owadów, w entomologii sądowej stosowane są znacznie rzadziej. Chociaż opisują one zależność rozwoju od temperatury bardziej dokładnie niż modele liniowe, są mniej użyteczne w praktyce, ponieważ nie pozwalają na oszacowanie stałej cieplnej *K* dla punktów rozwojowych. Wyniki eksperymentów rozwojowych przedstawiane są także na diagramach graficznych – izomegalenicznych i izomorfenicznych. Na diagramach izomegalenicznych pokazane są krzywe, które reprezentują długość ciała larwy. Znając temperaturę, w jakiej rozwijał się dany osobnik, można z nich odczytać ile czasu upłynęło od rozpoczęcia rozwoju do momentu osiągnięcia określonej wielkości ciała. Natomiast na diagramach izomorfenicznych przedstawione są krzywe reprezentujące kluczowe momenty w trakcie rozwoju, tj. wylęganie larw, pierwsze linienie, drugie linienie, przepoczwarczenie, pojawienie się imago. Obszary między krzywymi to odpowiadające im stadia rozwoju. Korzystając z takiego diagramu również można oszacować ile czasu upłynęło od początku rozwoju do osiągnięcia danego momentu w rozwoju (Grassberger i Reiter 2004). Wiele modeli rozwoju użytecznych dla celów sądowych opracowano dla muchówek nekrofagicznych. Niewiele jest natomiast takich modeli dla nekrofilnych chrząszczy (tabela 1). W momencie rozpoczęcia przeze mnie eksperymentów w ramach rozprawy doktorskiej (2014 rok), dla gatunków europejskich nie było żadnego takiego modelu.

Tabela 1. Gatunki chrząszczy nekrofilnych, dla których dostępne są dane rozwojowe.

Rodzina i gatunek	**Występowanie**	**Dane dla populacji**	**Temperatury, w których badano rozwój (°C)**	**Źródło**
Cleridae				
Necrobia rufipes (De Geer, 1775)	Kosmopolityczny	USA (Madison)	27	Hasan i Philips 2010
		Chiny	19, 22, 25, 28, 31, 34, 36, 39	Hu i in. 2020
Dermestidae				
Dermestes frischii Kugelann, 1792	Kosmopolityczny	Hiszpania	15, 20, 25, 30, 35	Martín-Vega i in. 2017
Dermestes maculatus DeGeer, 1774	Kosmopolityczny	Hiszpania	15, 20, 25, 30, 35	Martín-Vega i in. 2017
		USA (Hawaje)	15, 20, 25, 30, 35	Richardson i Goff 2001

Rodzina i gatunek	**Występowanie**	**Dane dla populacji**	**Temperatury, w których badano rozwój (°C)**	**Źródło**
		Ghana (Akra)	30	Zakka i in. 2013
		Argentyna	15, 20, 22, 24, 27, 30	Zanetti i in. 2015
Dermestes undulatus Brahm, 1790	Azja Centralna, Europa, Ameryka Północna	Hiszpania	15, 20, 25, 30, 35	Martín-Vega i in. 2017
Dermestes haemorrhoidalis Küster, 1852	Kosmopolityczny	Anglia	15, 20, 25, 30, 32,5	Coombs 1997
Dermestes peruvianus Laporte de Castelnau, 1840	Region holarktyczny, kraina neotropikalna	Anglia	15, 20, 25, 30, 32,5	Coombs 1997
Histeridae				
Euspilotus azureus (Sahlberg, 1823)	Argentyna, Brazylia, Wenezuela	Brazylia	10, 15, 20, 25, 30, 35	Caneparo i in. 2017
Leiodidae				
Sciodrepoides watsoni (Spence, 1815)	Region holarktyczny	Czechy	12, 15, 18, 21, 28	Jakubec 2016
Nitidulidae				
Omosita colon (Linnaeus, 1758)	Region holarktyczny	Chiny	16, 19, 22, 25, 28, 31, 34	Wang i in. 2020
Silphidae				
Oxelytrum discicolle (Brullé, 1836)	Kraina neotropikalna	Wenezuela	15, 20, 28	Velásquez i Viloria 2008
Thanatophilus micans (Fabricius 1794)	Afryka	Afryka Południowa	15, 17, 18, 19, 20, 25, 28,4, 35	Midgley i Villet 2008
Thanatophilus mutilatus (Castelneau, 1840)	Afryka	Afryka Południowa	14, 15, 17,5, 19, 20, 22,5, 25, 27.5, 30	Ridgeway i in. 2013
Necrophila (*Calosilpha*) *brunnicollis* (Kraatz, 1877)	Azja Środkowa i Wschodnia	Chiny	18, 20, 22, 29	Jakubec i in. 2020
Necrodes littoralis (Linnaeus, 1758)	Kraina palearktyczna	Polska	14, 15, 16, 17, 18, 19, 20, 22, 26, 30	Gruszka i Matuszewski 2020
Staphylinidae				
C. maxillosus	Kraina palearktyczna	Chiny	17,5, 20, 22,5, 25, 27,5, 30, 32,5	Wang i in. 2016
		Południowe Stany Zjednoczone	16, 24, 32	Watson-Horzelski 2012
Aleochara nigra Kraatz, 1859	Tajwan	Tajwan	17,5, 20, 22,5, 25, 27,5, 30	Lin i Shiao 2013
Aleochara asiatica Kraatz, 1859	Tajwan	Tajwan	17,5, 20, 22,5, 25, 27,5, 30	Lin i Shiao 2013

Wiele czynników wpływa na rozwój owadów i w konsekwencji na jakość danych rozwojowych. Są to m.in. metody pomiaru larw (Bugelli i in. 2017), częstość pomiarów (Richards i Villet 2008), warunki hodowli (np. zagęszczenie larw) (Charabidze i in. 2011, Johnson i Wallman 2014), metoda zabijania i konserwowania owadów (Midgley i Villet 2009, Richards i in. 2013), czy jakość pokarmu (Thomas i in. 2016, Bernhardt i in. 2017). Dobre dane referencyjne powinny odzwierciedlać rozwój owadów na zwłokach w warunkach naturalnych. Niestety uzyskanie takich danych w warunkach laboratoryjnych nie jest możliwe. Z tego powodu dane wykorzystywane przez entomologów sądowych mają pewne wady wynikające ze sposobu w jaki zostały pozyskane. Aktualnie, duży nacisk kładziony jest na identyfikację czynników, które potencjalnie mogą wpływać na jakość danych rozwojowych i określenia wielkości ich wpływu.

Creophilus maxillosus (Staphylinidae) to drapieżny chrząszcz, który odżywia się głównie larwami muchówek nekrofagicznych (Greene 1996, Watson-Horzelski 2012, Wang i in. 2017). Jest to gatunek regularnie kolonizujący zwłoki dużych kręgowców, w tym ludzkie (Dekeirsschieter i in. 2013, Charabidze i in. 2016, Wang i in. 2017) w środowiskach naturalnych (pozamiejskich) (Tabor i in. 2005, Mądra i in. 2014, Charabidze i in. 2016, Matuszewski i in. 2016, Salimi i in. 2018, Jarmusz i in. 2020). Jest to jeden z liczniejszych gatunków chrząszczy na zwłokach, a dzięki dużym rozmiarom ciała łatwo go zauważyć i odłowić. Zaczyna być on obecny na zwłokach znacznie później niż większość muchówek, które są owadami pojawiającymi się na zwłokach jako pierwsze, zatem wykorzystanie go znacznie wydłuża okres, w którym można wnioskować o czasie zgonu stosując metodę rozwojową (Wang i in. 2017). Dodatkowo okres przed pojawieniem się *C. maxillosus* na zwłokach (PAI - *pre appearance interval*) można łatwo oszacować przy użyciu metod temperaturowych (Matuszewski i Szafałowicz 2013). Tak więc, jest to gatunek bardzo przydatny do celów szacowania czasu zgonu w oparciu o połączenie metody rozwojowej oraz temperaturowej metody szacowania PAI. W literaturze można znaleźć pewne dane na temat rozwoju *C. maxillosus* (Voris 1939, Krammer 1954, Watson-Horzelski 2012, Wang i in. 2017). Niektóre z nich uzyskane zostały w efekcie badań w niekontrolowanych warunkach hodowli (Voris 1939, Krammer 1954). Watson-Horzelski (2012) badała rozwój populacji *C. maxillosus* z południowych obszarów Stanów Zjednoczonych Ameryki Północnej w trzech temperaturach. Z kolei Wang i in. (2017) opracowali modele rozwojowe dla populacji *C. maxillosus* pochodzącej z Chin. Jednak w związku z tym, że różne populacje mogą odmiennie reagować nawet na podobne warunki środowiskowe, szacując wiek owadów należy korzystać z danych rozwojowych uzyskanych na podstawie badań lokalnych populacji (Gallagher i in. 2010, Grzywacz 2019).

Pierwszym i głównym celem moich badań było opracowanie, porównanie i częściowe zwalidowanie temperaturowych modeli rozwoju (liniowych, nieliniowych i graficznych) dla *C. maxillosus*. Drugim celem było określenie wpływu rodzaju pokarmu na śmiertelność, rozmiar i czas rozwoju chrząszczy. Trzecim celem było sprawdzenie, czy istnieje związek pomiędzy czasem całego rozwoju preimaginalnego, a płcią i rozmiarem imagines *C. maxillosus*. Czwartym celem było sprawdzenie, czy wielokrotne, przyżyciowe pomiary wpływają na rozwój *C. maxillosus*.

Do prawidłowego obliczenia wieku owada wymagana jest znajomość parametrów modelu kumulacji cieplnej (dolnego progu rozwojowego (D_0) i stałej cieplnej *K*) (Ikemoto i Takai 2000). Parametry te są specyficzne dla gatunku i stadium. Ich ustalenie jest pracochłonne, więc są one obecnie dostępne dla niewielu gatunków owadów nekrofilnych. Dla środkowoeuropejskiej populacji *C. maxillosus* takie dane nie zostały dotychczas opracowane. Stałe cieplne mogą być szacowane na podstawie różnych danych rozwojowych. W związku z tym mogą reprezentować prawdziwą stałą cieplną dla danej populacji z różną dokładnością. Niektóre badania walidacyjne w entomologii sądowej pokazały niską jakość danych rozwojowych uzyskiwanych w warunkach laboratoryjnych (Harnden i Tomberlin 2016). W związku z tym niezwykle ważna jest walidacja uzyskiwanych wyników. Modele rozwojowe (liniowe, nieliniowe i graficzne), ich porównanie oraz wyniki walidacji modeli liniowych dla populacji środkowoeuropejskiej *C. maxillosus* opublikowano w artykule: Frątczak-Łagiewska K., Grzywacz A., Matuszewski S. 2020. Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae). International Journal of Legal Medicine, 134: 1531-1545.

Jakość pokarmu wpływa na rozwój i przeżywalność owadów (Bong i in. 2014), co było wielokrotnie wykazywane dla muchówek nekrofagicznych (Kaneshrajah i Turner 2004, Clark i in. 2006, Harnden i Tomberlin 2016, Bernhardt i in. 2017). Owady drapieżne były dotychczas w badaniach tego typu pomijane. Jakość ofiary ma bezpośredni wpływ na wzrost i rozwój owadów drapieżnych (Thompson 1999, Mirhosseini i in. 2015). Zakłada się, że nekrofilne owady drapieżne (Staphylinidae, Histeridae) polują głównie na larwy i puparia plujek. Jednak ich preferencje pokarmowe nie były dotychczas zbadane w eksperymentach laboratoryjnych. Możliwe, że typ pokarmu wpływa na ich rozwój. W konsekwencji dokładność oszacowania ich wieku może być obniżona poprzez wykorzystanie danych rozwojowych uzyskanych w badaniach owadów karmionych pokarmem nieoptymalnym dla ich rozwoju. Wyniki eksperymentów nad wpływem różnych diet na śmiertelność, rozmiar i czas rozwoju *C. maxillosus* opublikowano w artykule: Frątczak-Łagiewska K., Grzywacz A., Matuszewski S. 2020. Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae). International Journal of Legal Medicine, 134: 1531-1545.

Płeć dorosłych owadów nekrofilnych jest zazwyczaj łatwa do identyfikacji. W przypadku osobników preimaginalnych zebranych ze zwłok płeć może więc być oznaczona po wyhodowaniu w warunkach laboratoryjnych osobników dorosłych. Istnieją także metody molekularne oznaczania płci, które mogą być wykorzystane do określania płci martwych osobników młodocianych (Smith i Wells 2016). U owadów powszechnie występują różnice wielkości pomiędzy samcami i samicami (*SSD – sexual size dimorphism*) (Teder i Tammaru 2004, Blackenhorn i in. 2007, Jarošik i Honek 2007). Mogą one wynikać z różnic w wielkości płci już na etapie rozwoju embrionalnego, z różnic w tempie rozwoju larwalnego, długości tego rozwoju, a także dowolnej kombinacji tych trzech czynników (Teder i Tammaru 2004). Dotychczas różnice w długości rozwoju pomiędzy płciami stwierdzano u wielu gatunków owadów, w tym również u gatunków wykorzystywanych w entomologii sądowej (Zuha i Omar 2014, Picard i in. 2014). Założono zatem, że różnice takie wystąpią również u *C. maxillosus*, u którego występują różnice płciowe w rozmiarze

ciała. W tym zakresie testowano ogólną hipotezę, że stworzenie odrębnych modeli rozwoju dla samców i samic wpłynie na poprawę ich jakości, a dokładność szacowania wieku owadów przy pomocy takich modeli będzie większa niż przy użyciu modeli ogólnych. Postawiono następujące hipotezy szczegółowe:

1) samce *C. maxillosus* rozwijają się dłużej;
2) różnice w tempie rozwoju pomiędzy płciami kumulują się przez cały okres preimaginalny, największe są jednak w stadium poczwarki;
3) stosowanie osobnych modeli rozwoju dla samców i samic poprawia dokładność szacowania wieku owadów.

Wyniki tej części badań opublikowano w artykule: Frątczak-Łagiewska K., Matuszewski S. 2018. Sex-specific developmental models for *Creophilus maxillosus* (L.) (Coleoptera: Staphylinidae): searching for larger accuracy of insect age estimates. International Journal of Legal Medicine, 132 (3): 887-895.

U owadów przydatnych do celów sądowych stwierdzano znaczną zmienność wewnątrzgatunkową rozwoju (Anderson 2000, Tarone i Foran 2008, Aubernon i in. 2015, Kotzé i in. 2015). W obrębie gatunku zmienność czasu rozwoju jest jednym z czynników o największym wpływie na dokładność szacowania wieku owadów (Tarone i Foran 2006, Richards i Villet 2009). Część zmienności może wynikać z różnic między lokalnymi populacjami (Gallagher i in. 2010), przedwczesnego rozwoju jaj, w wyniku którego niektóre larwy wylęgają się wcześniej (zjawisko powszechne u muchówek z rodziny Sarcophagidae) (Wells i King 2001), a także z różnic w jakości i ilości pobieranego pokarmu (Tarone i Forane 2006, Kaneshrajah i Turner 2004, Clark i in. 2006, Bernhardt i in. 2017). U owadów powszechna jest korelacja między czasem rozwoju owadów, a ostatecznym rozmiarem owadów dorosłych. Wśród owadów roślinożernych i drapieżnych zaobserwowano korelację ujemną, natomiast pozytywna korelacja występuje powszechnie u parazytoidów (Teder i in 2014). Uzasadnione jest zatem założenie, że rozmiar owada w momencie zakończenia rozwoju może być wykorzystany do poprawienia dokładności oszacowania wieku owada. W badaniach testowano więc koncepcję, że rozmiar owada (długość i waga) po zakończeniu rozwoju preimaginalnego może zostać wykorzystany do oszacowania jego wieku fizjologicznego, a uzyskane w ten sposób wyniki będą dokładniejsze niż te uzyskiwane w drodze szacowania ogólnymi modelami kumulacji cieplnej. Sformułowano następujące hipotezy szczegółowe:

1) liczba jednostek cieplnych, które owad musi skumulować żeby zakończyć rozwój (tzw. stała cieplna *K*) jest skorelowana z jego rozmiarem w momencie zakończenia rozwoju, i w związku z tym

2) wykorzystanie rozmiaru imago w celu oszacowania *K* może znacząco poprawić dokładność szacowania wieku owadów w entomologii sądowej oraz

3) związek między rozmiarem owada, a liczbą jednostek cieplnych (*K*) potrzebnych do zakończenia rozwoju będzie bardziej ścisły w przypadku osobnych modeli dla samców i samic niż modeli ogólnych.

Wyniki tej części badań opublikowano w artykule: Matuszewski S., Frątczak-Łagiewska K. 2018. Size at emergence improves accuracy of age estimates in forensically-useful beetle *Creophilus maxillosus* L. (Staphylinidae). Scientific Reports, 8: 2390.

Podczas tworzenia modeli rozwojowych dla nekrofilnych chrząszczy stosowano dotychczas dwa podejścia: pomiar owadów po zabiciu ich w alkoholu (Richardson i Goff 2001) lub pomiary przyżyciowe (Ridgeway i in. 2013). Pomiary *in vivo* są użyteczne w przypadku ciągłego monitorowania rozwoju owadów. Takie podejście umożliwia uzyskanie dokładnych danych na temat rozwoju każdego osobnika. Ponadto, eksperymenty laboratoryjne mogą być prowadzone wtedy z wykorzystaniem mniejszej liczby owadów. Podczas badań rozwojowych owady są mierzone wiele razy w trakcie rozwoju larwalnego, co może wpływać na ich rozwój i rozmiar osobników dorosłych. Chociaż wielokrotnie już w trakcie badań nad rozwojem różnych gatunków owadów nekrofilnych dokonywano pomiarów wskaźników wieku *in vivo* (Midgley i Villet 2009, Ridgeway i in. 2013, Wang i in. 2017), wpływ takich pomiarów na rozwój nie został jeszcze zbadany. W związku z tym przeprowadzono eksperyment, w którym część osobników była wielokrotnie mierzona (pomiar długości oraz masy), natomiast część monitorowana była wyłącznie pod kątem przejścia do kolejnego stadium rozwojowego. Postawiono następujące hipotezy:

1) wielokrotne pomiary *in vivo* wydłużają całkowity czas rozwoju i zwiększają śmiertelność;

2) pomiary w stadium larwy wpływają na wielkość dorosłego chrząszcza;

3) protokół wielokrotnych pomiarów *in vivo* obniża jakość danych rozwojowych. Wyniki tej części badań zostały przedstawione w pracy: Frątczak-Łagiewska K., Matuszewski S. 2019. The quality of developmental reference data in forensic entomology: Detrimental effects of multiple, *in vivo* measurements in *Creophilus maxillosus* L. (Staphylinidae). Forensic Science International, 298: 316-322.

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LISTA PUBLIKACJI

1. **Frątczak-Łagiewska K.**, Grzywacz A., Matuszewski S. 2020. Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae). International Journal of Legal Medicine, 134: 1531-1545.

2. **Frątczak-Łagiewska K.**, Matuszewski S. 2018. Sex-specific developmental models for *Creophilus maxillosus* (L.) (Coleoptera: Staphylinidae): searching for larger accuracy of insect age estimates. International Journal of Legal Medicine, 132 (3): 887-895.

3. Matuszewski S., **Frątczak-Łagiewska K.** 2018. Size at emergence improves accuracy of age estimates in forensically-useful beetle *Creophilus maxillosus* L. (Staphylinidae). Scientific Reports, 8: 2390.

4. **Frątczak-Łagiewska K.**, Matuszewski S. 2019. The quality of developmental reference data in forensic entomology: Detrimental effects of multiple, *in vivo* measurements in *Creophilus maxillosus* L. (Staphylinidae). Forensic Science International, 298: 316-322.

International Journal of Legal Medicine (2020) 134:1531–1545
https://doi.org/10.1007/s00414-020-02275-3

ORIGINAL ARTICLE

Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae)

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Received: 21 October 2019 / Accepted: 12 March 2020 / Published online: 8 April 2020

Abstract

The hairy rove beetle, *Creophilus maxillosus* (Linnaeus) (Staphylinidae), is recognized for its use in forensic entomology. However, insufficient developmental data exist for the Central European population of this species. Accordingly, we studied the development of *C. maxillosus* at ten constant temperatures (10–32.5 °C). Based on these results, linear and nonlinear developmental models were created and validated. We also studied the effect of different homogenous diets (third-instar larvae or puparia of *Calliphora* sp. Robineau-Desvoidy or *Lucilia* sp. Robineau-Desvoidy (Diptera: Calliphoridae) or mix of first- and second-instar larvae of *Necrodes littoralis* (Linnaeus) (Coleoptera: Silphidae)) on the development and mortality of *C. maxillosus.* Average total development times ranged between 122.21 days at 15 °C and 22.18 days at 30 °C. Beetles reached the adult stage in seven out of ten temperatures (15–30 °C). No beetles reached the adult stage when fed with larvae of *N. littoralis*; their development times at first and second larval stage were also significantly longer than in other food conditions. When *C. maxillosus* larvae were fed with blowfly larvae, the highest mortality was observed at the pupal stage, as compared when they were fed with blowfly puparia—at the first larval stage. While validating thermal summation models, the highest age estimation errors were found for beetles bred at 10 and 12.5 °C (between 21 and 43% for all developmental events). Age estimation errors were on average higher for pupation and eclosion than hatching and first and second ecdyses. While validating the models with specimens fed with different diets, the highest errors were recorded for beetles fed with *N. littoralis* larvae (22% for the first ecdysis and 33% for the second ecdysis) and *Lucilia* sp. puparia (32% for pupation and 22% for eclosion). Implications for *C. maxillosus* use in forensic entomology are discussed.

Key Points

- Development of the Central European population of *C. maxillosus* was studied at ten constant temperatures and using different homogenous diets.
- Thermal summation models were validated with insects reared at different temperatures and fed with different diets.
- Total development times ranged between 122 days at 15 °C and 22 days at 30 °C. Beetles reached the adult stage in seven temperatures (15–30 °C).
- The highest age estimation errors were found for beetles bred at 10 and 12.5 °C (21–43%) and for beetles fed with *Necrodes littoralis* larvae (22–33%).
- The lowest mortality was observed for beetles fed with *Calliphora* sp. and *Lucilia* sp. larvae. Estimation errors were generally low for beetles fed with blowfly larvae or *Calliphora* sp. puparia.

Keywords Forensic entomology · Developmental models · *Creophilus maxillosus* · Staphylinidae · Minimum postmortem interval · Validation study

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00414-020-02275-3) contains supplementary material, which is available to authorized users.

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Introduction

Development time of insects depends mostly on temperature [1, 2]. Poikilotherms operate within species-specific temperature ranges associated with their local temperatures. Development beyond these limits may be harmful or even lethal for them [3, 4]. Predicted thermal tolerance for development of insects, the range in temperature between the minimum and the maximum rate of development, is about 20 °C [5]. Thermal development of insects is a powerful tool in forensic entomology. Insect development models may be used to estimate the minimum time that elapsed from death until the body discovery (called min PMI) [2]. This involves estimating the age of the oldest immature insects found on a cadaver based on age indicators such as development stage or larval length [2]. The possibility to use the method depends on the availability of developmental data for the species collected on a crime scene. Observed values of insect age indicators are compared with the reference developmental data. Such data are collected during laboratory experiments where the development of insects is studied under controlled conditions [6]. Results of such experiments are presented using graphical development representations (i.e., isomegalen and isomorphen diagrams) or mathematical development models (i.e., linear or nonlinear development equations) [7].

Developmental data used in forensic entomology are collected using different insect rearing protocols, sampling frequency, sample sizes, killing or preservation techniques etc. As a consequence, the data used in casework may differ in quality with likely detrimental effects on the accuracy or precision of min PMI estimates [8]. The ideal reference data should accurately reflect the development of insects on cadavers under natural conditions. However, developing such perfect reference data in the laboratory is simply impossible. Therefore, all data used by forensic entomologists in casework have limitations resulting from their laboratory origin. Currently, one of the most pressing needs of forensic entomology is to identify factors affecting the quality of reference developmental data and to determine the magnitude of their impact.

Insect development was analyzed and modeled in different ways in forensic entomology [9]. In most cases, linear development models were used, i.e., simple regression model relating developmental rate with rearing temperature [2] or the thermal summation model sensu Ikemoto and Takai [10] (e.g., [11–16]). Although frequently used in insect developmental studies, nonlinear models were developed less frequently in forensic entomology–oriented experiments (but see [9, 17–19]). Although they describe the relationship more accurately than linear models, they are less practically useful as they do not provide thermal constant K for the developmental landmarks [17]. The constant K for a landmark may be divided into smaller parts, enabling retrospective calculation of the time needed to reach the landmark in changing temperature conditions. Moreover, it may be used to estimate the age of the most problematic pieces of insect evidence, i.e., postfeeding larvae or pupae, through subtracting thermal units which insects collected on a crime scene have accumulated in the laboratory from the constant K [20]. The thermal constants may be estimated using different methods and from different data. Accordingly, they may represent the true thermal constants for an insect population with different accuracy. For this reason, it is necessary to validate thermal constants or any other reference developmental data used by forensic entomologists [21]. Some validation studies in forensic entomology revealed low accuracy of the laboratory-produced data when applied to insects reared in naturally occurring temperatures [21]. Although there are different ways to validate reference developmental data [21–25], the minimum standard should be a validation using insect laboratory sample different than the one used for the derivation of the data.

Another group of factors affecting the quality of developmental data is related to laboratory protocols, e.g., methods of larval measurement, conditions of insect colony maintenance, or food quality and quantity [26]. Food quality influences the survival and development of insects in nature [27]. Its effect on the development was demonstrated in several species of necrophagous flies [21, 28–32]. No previous work tested such effects in forensically useful insect predators. Prey quality has a direct impact on growth and development of predatory insects [33, 34]. It is not clear what exactly predatory beetle species (e.g., staphylinids or histerids) eat on carrion. Based on the literature [35, 36], they should prey mostly on larvae and puparia of blowflies. However, their food preferences have not been investigated. Moreover, it is possible that food type affects their development. Consequently, in casework, the accuracy of insect age estimation may be reduced by using developmental data derived from insects fed with non-optimal food type.

Creophilus maxillosus (Linnaeus) (Coleoptera: Staphylinidae) is a common predator of fly larvae and puparia associated with carrion [7, 37]. It abundantly visits and breeds in large vertebrate cadavers and is recognized as highly forensically useful [38–41]. Early works analyzed the development of *C. maxillosus* in uncontrolled rearing conditions [42, 43]. Watson-Horzelski [44] investigated the development of *C. maxillosus* at 3 temperatures for the population from Southern United States. Wang et al. [45] provided more robust development models of the species for the population from China. However, distinct populations may respond differently to similar environmental conditions. Thus, to get accurate insect age estimates, calculations should use developmental data for the local insect population [17, 46]. Recently more attention has been paid to the Central European population of *C. maxillosus* [7, 8, 47]. Differences in development time

between males and females of *C. maxillosus* were reported, however with no significant influence on the accuracy of age estimates using sex-specific as compared with general thermal summation models [7]. *Creophilus maxillosus* size at emergence was demonstrated to be useful for physiological age prediction and accordingly for the improvement of the age estimate accuracy in forensic entomology [47]. Moreover, it was found that the multiple measurement protocol affects the accuracy of insect age estimates using the resultant reference developmental data [8]. However, none of these studies derived developmental models for all developmental landmarks of Central European *C. maxillosus*.

Consequently, the aim of this study was to create full set of developmental models for the central European population of *C. maxillosus* and to validate the models with insects reared at different temperature conditions and fed with different diets. Additionally, we investigated how different homogenous diets affect development and mortality of *C. maxillosus*.

Materials and methods

Insect colony establishment and maintenance

Insects were collected from rabbit carcasses placed in a xerothermic grassland (Biedrusko military range, western Poland: 52 31′ N, 16 55′ E). Carcasses were exposed in spring and summer of 2015 and 2016 every few days to have permanent access to adult beetles. Beetles were collected during 5–7 inspections each year, 10–15 specimens at a time. A colony consisted of 25–30 individuals with a more or less equal proportion of males and females. New beetles sampled in the field and first generations bred in the laboratory were used to permanently renew the colony. Adult beetles were kept in plastic containers (30 × 20 × 20 cm) with 6–7 cm layer of moist soil and access to water. They were fed once a day with a mix of blowfly third-instar larvae and puparia. Containers were kept at room temperature (20–22 °C) and humidity (50–60%) and cleaned once a week to avoid the appearance of mites and mold.

Methods common for temperature and food type experiments

Rearing

Females of *C. maxillosus* lay singular eggs in small clumps of soil which makes them difficult to be found. Eggs in the same age were obtained by placing adult insects from a single colony into 3-l container filled halfway with soil for 4 h. Containers were kept in the dark at a room temperature (20–22 °C). Afterwards, adult beetles were pulled out and containers were placed in insect incubators (ST 1/1 BASIC or +, POL-EKO, Poland) set for the specific temperature. After 70% of the average egg stage duration, containers were inspected for the presence of first-instar larvae at intervals equal to 10% of the average egg stage duration. Freshly hatched first-instar larvae are creamy-white and very active, so it is easy to find them while searching the soil. Only freshly hatched larvae were sampled and transferred to separate cups.

First- and second-instar larvae were kept in 80-ml containers filled with 1.5 cm of soil. Third-instar larvae, immediately after the second ecdysis, were transferred to 120-ml containers with 5–6 cm of soil and were kept there until adults emerged. Containers were placed in insect incubators (ST 1/1 BASIC or +, POL-EKO, Poland). Humidity in incubators was maintained at 60–70% and a photoperiod (h) was set on 12:12 (L:D).

Inspections and measurements

All individuals were inspected for developmental landmarks: hatching, first and second ecdysis, pupation, adult emergence. After 60% of the average stage duration, insects were checked every 10% of stage duration. Containers were taken out of the incubator, and beetles were inspected for developmental stage. In each stage, 4–5 inspections were made. After noticing the landmark, the midpoint between current and previous inspection was used as the actual time of the landmark occurrence. Transitions between larval stages were determined based on the creamy-white color of a larva (appearing shortly after ecdysis) and the width of the mesonotum.

A geometrical micrometer was used to measure in vivo larval length. The larva was placed in a 1.5-ml Eppendorf tube, and after it had become immobile and fully erected, its length (from clypeus to the last abdominal segment) was measured with a micrometer. The analytical balance AS 82/220.R2 (Radwag, Poland) was used to weigh larvae and pupae in a 1.5-ml Eppendorf tube.

Experiment 1: Effect of temperature on development

Development was studied at ten constant temperatures: 10–32.5 °C, in 2.5 °C intervals. Two or three temperatures were studied at the same time. Larvae were fed once a day with third-instar larvae of blowflies punctured to make feeding easier for the first- and second-instar larvae of *C. maxillosus*.

Forty larvae per temperature were used. Insects were randomly allocated to temperatures. All individuals were inspected for developmental landmarks. Twenty individuals were also repeatedly measured and weighted. Containers were placed on two shelves inside the incubator. Container positions were rearranged every few inspections.

Inspection intervals were calculated based on the results of pilot tests at 5 temperatures (12.5, 17.5, 22.5, 27.5, 32.5 °C; 10 insects per temperature). Intervals established at a specific

temperature were also used for the lower adjoining temperature (e.g., intervals at 12.5 °C were also used for 10 °C).

Differences in mortality between specimens bred at different temperatures were evaluated using the chi-squared test. Percentage mortality was defined as [(number of dead specimens × 100%)/number of sampled larvae]. Differences in time of development and differences in length or weight between specimens bred at different temperatures were evaluated using one-way analysis of variance. All analyses were conducted using Statistica 13.1 (StatSoft).

Models for particular developmental events were developed using data for randomly selected non-measured beetles (usually 10 per temperature), chosen for each model from the entire insect pool. We derived the linear Ikemoto and Takai model [10] (Eq. 1) and nonlinear models: Analytis [48] (Eq. 2), Brière-2 [49] (Eq. 3), Lactin-2 [50] (Eq. 4), and SSI (Sherpe-Schoolfield-Ikemoto) [51] (Eq. 5).

$$DT = k + T_{\min}D \tag{1}$$

where D is the duration of development (in hours or days), T is the rearing temperature, $T_{\min}$ is the lower developmental threshold, and k is thermal summation constant. It is recommended to calculate these parameters by means of the reduced major axis (RMA) instead of the ordinary least squares (OLS) regression [10, 17]. Model was calculated with the *lmodel2* package [52] in *R*, where the RMA is called a standard major axis (SMA) regression.

$$\frac{1}{D} = a \times (T - T_{\min})^{n} \times (T_{\max} - T)^{m} \tag{2}$$

$$\frac{1}{D} = a \times (T - T_{\min}) \times (T_{\max} - T)^{\frac{1}{d}} \tag{3}$$

$$\frac{1}{D} = e^{(p \times T)} - e^{\left(p \times T_{\max} - \left(\frac{T_{\max} - T}{\Delta T}\right)\right)} + \lambda \tag{4}$$

$$\frac{1}{D} = \frac{\rho_{\varphi}\left(\frac{T}{T_{\varphi}}\right) \times e^{\left[\frac{\Delta H_A}{R} \times \left(\left(\frac{1}{T_{\varphi}}\right) - \left(\frac{1}{T}\right)\right)\right]}}{1 + e^{\left[\frac{\Delta H_L}{R} \times \left(\left(\frac{1}{T_L}\right) - \left(\frac{1}{T}\right)\right)\right]} + e^{\left[\frac{\Delta H_H}{R} \times \left(\left(\frac{1}{T_H}\right) - \left(\frac{1}{T}\right)\right)\right]}} \tag{5}$$

where $1/D$ is development rate, T is the rearing temperature, $T_{\min}$ is the lower developmental threshold, and $T_{\max}$ is the upper developmental threshold [53]. In the Analytis model a, n, and m are constants [48, 54]. In the Brière-2 model, a and d are empirical constants [49, 55]. In the Lactin-2 model, p is a constant defining rate of optimum temperature, ΔT is the temperature range across which physiological breakdown becomes the overriding influence, and λ allows the curve to intercept the x-axis allowing the estimation of lower temperature threshold ($T_{\min}$) [50, 54]. In the SSI model, ρ_{φ} is the development rate at the intrinsic optimum temperature T_{φ}, ΔH_A is the change in enthalpy of activation of the reaction that is catalyzed by the enzyme, ΔH_L is the change in enthalpy associated with low temperature inactivation of the enzyme, and ΔH_H is the change in enthalpy associated with high temperature inactivation of the enzyme, R is the gas constant (1.987 cal/deg/mol), T_L is the temperature at which the enzyme is 1/2 active and 1/2 low temperature inactive, and T_H is the temperature at which the enzyme is 1/2 active and 1/2 high temperature inactive (both in Kelvin degrees) [51, 53].

Fitting of the nonlinear Analytis, Brière-2, and Lactin-2 models was done using the Levenberg-Marquardt algorithm with the *minpack.lm* package [56] and the SSI model using the *SSI* package [51], both in *R*.

Experiment 2: Effect of food type on development

For the experiment, we chose five different types of food, i.e., third-instar larvae of *Calliphora* sp. Robineau-Desvoidy and *Lucilia* sp. Robineau-Desvoidy (Diptera: Calliphoridae), puparia of *Calliphora* sp. and *Lucilia* sp., mix of first- and second-instar larvae of *Necrodes littoralis* (Linnaeus) (Coleoptera: Silphidae), as they are present on carrion at the same time as larval stages of *C. maxillosus* [57]. In these food type conditions, we reared 25, 20, 20, 25, and 20 larvae of *C. maxillosus*, respectively. Third-instar larvae of blowflies were purchased from a fishing shop. Genus determinations were made using the identification key by Szpila [58]. In order to get puparia, larvae were bred in the laboratory. First- and second-instar larvae of *N. littoralis* were sampled from our laboratory colony.

Development was studied under constant temperature conditions of 24 °C. Larvae were fed once a day ad libitum. All beetles were inspected for developmental landmarks. Additionally, third-instar larvae were measured and weighed at the beginning of the third larval stage and pupae were weighed at the beginning of the pupal stage.

Differences in mortality between specimens fed with different types of food were evaluated using the chi-squared test. Percentage mortality was defined as [(number of dead specimens × 100%)/number of sampled larvae]. Differences in time of development and differences in length or weight between specimens fed with different types of food were evaluated using one-way analysis of variance. All analyses were conducted using the Statistica 13.1.

Validation of development models

Models for particular developmental events were validated using different number of specimens originating from different temperature ranges, i.e., for hatching—241 specimens bred at 10–32.5 °C, for first ecdysis—77 specimens bred at 12.5–32.5 °C, for second ecdysis—75 specimens bred at 12.5–32.5 °C, for pupation—46 specimens bred at 17.5–30 °C, and for eclosion—29 beetles bred at 20–27.5 °C. Due to large mortality at extreme temperatures, some of them were poorly represented or not represented in the validation sample.

Models were also validated using beetles fed with different food types from our experiment 2, with 104, 102, 100, 82, and 72 beetles respectively for hatching, first ecdysis, second ecdysis, pupation, and eclosion.

The validation included a comparison of the thermal units needed to reach a particular developmental landmark with the thermal constant from the model. For this purpose, we calculated absolute differences between actual and model thermal units and divided them by actual thermal units.

Results

Development of *C. maxillosus* across temperatures

Mortality significantly varied across the temperatures ($\chi^2 = 420.0579$, $P < 0.001$; Fig. 1). No beetle reached the adult stage at 10, 12.5, and 32.5 °C. The lowest mortality was at 25 °C (Fig. 1). The highest mortality was recorded for third-instar larvae and pupae (Fig. 1). Mortality of third-instar larvae was the highest at low and high extreme temperatures, whereas pupae revealed an increase in mortality with increase in temperature (Fig. 1).

Beetles reached the adult stage in seven out of ten temperatures (15–30 °C) (Table 1, Figs. 1 and 2). Total development time ranged between 122.21 days at 15 °C and 22.18 days at 30 °C (Table 1). At 10 and 12.5 °C, most larvae died in the postfeeding phase (Table 1, Figs. 1 and 2). At 32.5 °C, most larvae pupated but then pupae died (Table 1, Figs. 1 and 2). Fresh pupae reared at 30 and 32.5 °C often failed to shed the third-instar exuvia.

Larval size, pupal weight, and adult size differed significantly between temperatures (larval length $F_{9.164} = 13.267$, $P < 0.001$, larval weight $F_{9.164} = 26.944$, $P < 0.001$, pupal weight $F_{7.95} = 22.188$, $P < 0.001$, adult length $F_{6.81} = 13.243$, $P < 0.001$, adult weight $F_{6.81} = 11.777$, $P < 0.001$; Table 2). The largest size of pupae and adult beetles was recorded at 17.5 °C, and then, beetle size decreased with temperature (Table 2). Larval size changed similarly; however, the largest larvae were reared at 15 °C (Table 2).

Differences in the development of *C. maxillosus* fed with different types of food

Mortality significantly varied between types of food ($\chi^2 = 131.4858$, $P < 0.001$; Fig. 3). No beetles reached the adult stage when fed with larvae of *N. littoralis* (Fig. 3). The lowest mortality was observed for beetles fed with *Calliphora* sp. and *Lucilia* sp. larvae (Fig. 3). In the case of beetles fed with blowfly puparia, the highest mortality was observed for first-instar larvae (Fig. 3). In the case of beetles fed with blowfly larvae, the highest mortality was observed at the pupal stage (Fig. 3).

First- and second-instar larvae of *C. maxillosus* fed with *N. littoralis* larvae developed significantly longer than beetles fed with other types of food (first instar $F_{4.89} = 87.38$, $P < 0.001$; second instar $F_{4.86} = 228.79$, $P < 0.001$; Fig. 4). Total development time was the shortest when *C. maxillosus* were fed with *Lucilia* sp. puparia ($F_{3.68} = 13.166$, $P < 0.001$; Fig. 4).

Length and weight of third-instar larvae were the lowest when they were fed with *Necrodes littoralis* larvae (length $F_{4.85} = 59.456$, $P < 0.001$, weight $F_{4.85} = 44.607$, $P < 0.001$;

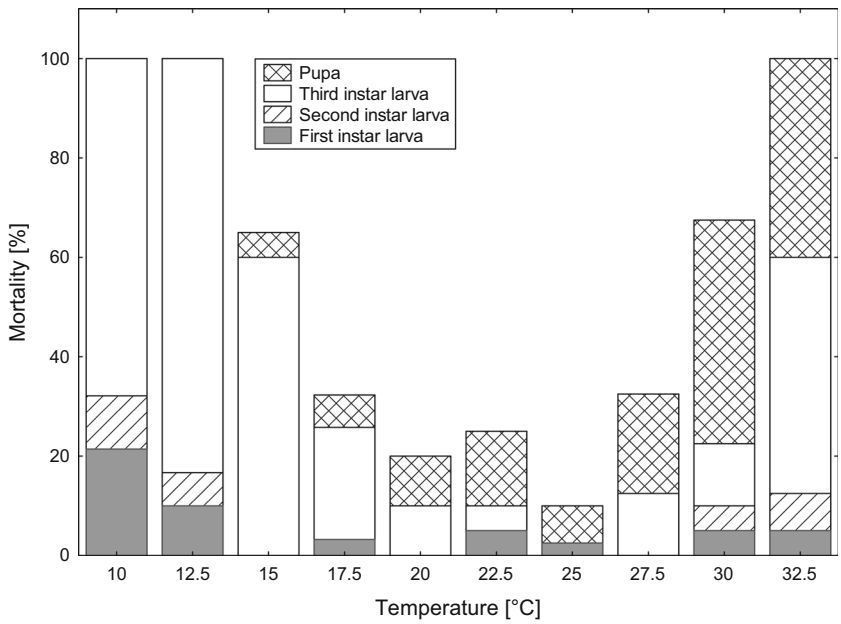


Fig. 1 Mortality of *C. maxillosus* developmental stages at different rearing temperatures

Table 1 Time of development (mean (SE; *N*)) of *C. maxillosus* at 10 constant temperatures. Mean for the egg stage duration was calculated on the basis of measured and non-measured individuals. For other stages, mean was calculated using only non-measured individuals

Temperature (°C)	Egg	1st-instar larva	2nd-instar larva	3rd-instar larva	Pupa	Total development
10	22.9 (0.24; 19)	12.9 (0.54; 9)	14.06 (0.27; 8)	-	-	-
12.5	16.58 (0.18; 25)	8.92 (0.14; 13)	9.89 (0.22; 14)	-	-	-
15	8.41 (0.04; 40)	4.99 (0.1; 20)	5.09 (0.12; 20)	67.74 (10.81; 5)	24.97 (1.83; 5)	122.21 (6.63; 4)
17.5	5.9 (0.08; 29)	4.05 (0.14; 14)	4.34 (0.12; 14)	42.74 (4.51; 11)	19.11 (0.43; 10)	76.88 (4.99; 10)
20	4.29 (0.02; 40)	2.65 (0.04; 20)	3.12 (0.07; 19)	18.4 (0.5; 19)	15.51 (0.21; 17)	43.91 (0.57; 17)
22.5	3.33 (0.03; 40)	2.28 (0.07; 18)	2.46 (0.08; 18)	17.74 (0.62; 16)	12.25 (0.19; 13)	37.63 (0.58; 13)
25	2.82 (0.02; 37)	1.86 (0.04; 20)	2.32 (0.04; 20)	13.8 (0.38; 20)	9.57 (0.17; 18)	29.91 (0.74; 18)
27.5	2.58 (0.02; 40)	1.62 (0.04; 19)	2.08 (0.05; 19)	13.66 (0.49; 17)	8.07 (0.25; 13)	27.64 (0.48; 13)
30	2.18 (0.02; 36)	1.38 (0.02; 17)	1.66 (0.08; 17)	10.56 (0.47; 16)	7.48 (0.16; 8)	22.18 (0.42; 7)
32.5	2.1 (0.02; 35)	1.33 (0.03; 17)	1.78 (0.04; 17)	9.02 (0.58; 6)	-	-

Table 3). Pupal and adult length and weight were the highest when larvae were fed with *Calliphora* sp. puparia; the differences between beetles fed with different blowfly-related food types were however insignificant (pupal weight $F_{3.69} = 1.0929$, $P = 0.35805$; adult length $F_{3.63} = 0.30579$, $P = 0.82110$; adult weight $F_{3.63} = 1.2683$, $P = 0.29293$; Table 3).

Development models

All temperature points were included while calculating linear model parameters for developmental events (Fig. 5). Lower developmental thresholds calculated from linear model ranged from 8.085 ± 0.365 °C for the second ecdysis to 11.98 ±

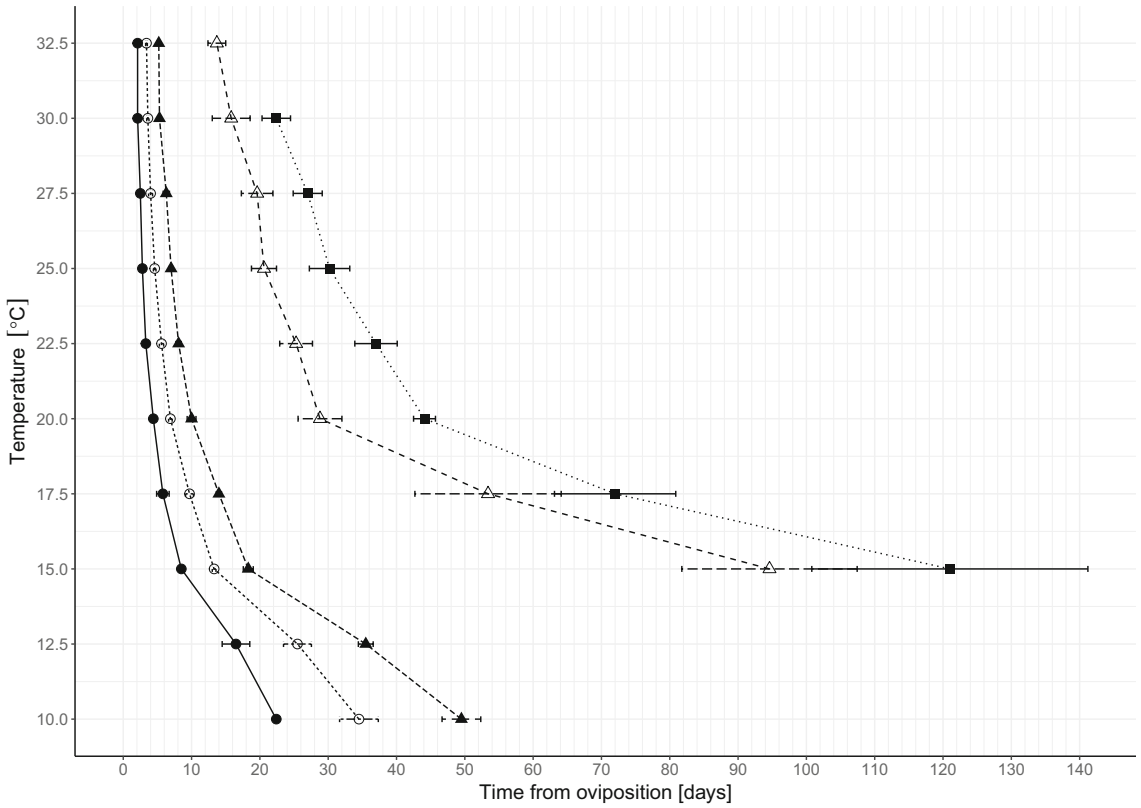


Fig. 2 Isomorphen diagram for *C. maxillosus* based on median times to reach particular developmental events at each of the rearing temperature conditions. Horizontal bars represent interquartile ranges. Areas between lines represent developmental stages; symbols represent developmental events. Black circle—hatching. White circle—first ecdysis. Black triangle—second ecdysis. White triangle—pupation. Black square—eclosion

Table 2 Length and weight (mean (SE; *N*)) of *C. maxillosus* bred at different temperatures. Means were calculated based on the length and weight measurements conducted at the beginning of particular stage

Temperature (°C)	3rd-instar length	3rd-instar weight	Pupal weight	Adult length at emergence	Adult weight at emergence
10	16.85 (0.21; 10)	45.91 (1.97; 10)	–	–	–
12.5	18.29 (0.32; 12)	56.67 (2.79; 12)	–	–	–
15	20.53 (0.36; 20)	74.83 (2.82; 20)	177.9 (3.69; 12)	19.67 (0.58; 9)	147.69 (4.29; 9)
17.5	20.28 (0.29; 16)	69.72 (2.68; 16)	185.01 (6.63; 11)	21.1 (0.39; 10)	158.83 (6.01; 10)
20	19.88 (0.26; 20)	65.14 (1.48; 20)	160.86 (4.47; 16)	20.9 (0.34; 15)	140.73 (4.14; 15)
22.5	19.78 (0.29; 20)	62.88 (2.65; 20)	148.16 (4.83; 19)	19.65 (0.35; 17)	127.72 (3.95; 17)
25	19.39 (0.12; 19)	60.06 (1.19; 19)	146.7 (3.06; 19)	18.11 (0.26; 18)	124.65 (2.6; 18)
27.5	18.6 (0.29; 20)	51.07 (1.83; 20)	130.45 (5.25; 15)	17.61 (0.45; 14)	114.57 (8.04; 14)
30	19.13 (0.44; 19)	48.87 (1.88; 19)	110.16 (5.23; 10)	17.5 (0.59; 5)	94.56 (2.48; 5)
32.5	17.5 (0.25; 18)	38.18 (2.05; 18)	-	-	-

0.351 °C for the pupation (Table 4). Although lower development threshold calculated from linear model was slightly below 12 °C, beetles failed to reach the adult stage already at 12.5 °C. Estimated thermal summation constants required to reach certain developmental events are presented in Table 4.

In general, upper developmental thresholds were higher and lower developmental thresholds were lower when estimated with the Analytis, Brière-2, and Lactin-2 nonlinear models (Online Resource 1 and 2). Moreover, for pupation and eclosion, parameters of nonlinear models showed unrealistically high values of T_{max}, reaching up to 185.8 °C (Online Resource 1). The SSI model provided more reliable parameter values, and the estimated intrinsic optimum temperature, T_{φ}, ranged from 18.71 to 20.96 °C. Lower developmental thresholds T_{min} calculated from linear model and T_L calculated from the SSI model were highly congruent (Tables 4 and 5). Values of T_H calculated using the SSI model ranged from 32.35 °C for hatching to 35.03 °C for pupation (Table 5, Fig. 6).

Validation

The highest errors were recorded for beetles bred at low temperatures, i.e., for all developmental event errors were between 21 and 43% (Fig. 7). Starting from 15 °C, mean error rates were usually below 10% (Fig. 7). Mean error rates were higher for pupation and eclosion than those for hatching and first and second ecdyses ($F_{4.25} = 16.421$, $P = 0.00000$; Fig. 7).

The highest errors were recorded for beetles fed with *N. littoralis* larvae (22% for the first ecdysis and 33% for the second ecdysis) (Fig. 8). High error rates were also recorded for beetles fed with *Lucilia* sp. puparia (32% for pupation and 22% for eclosion) (Fig. 8).

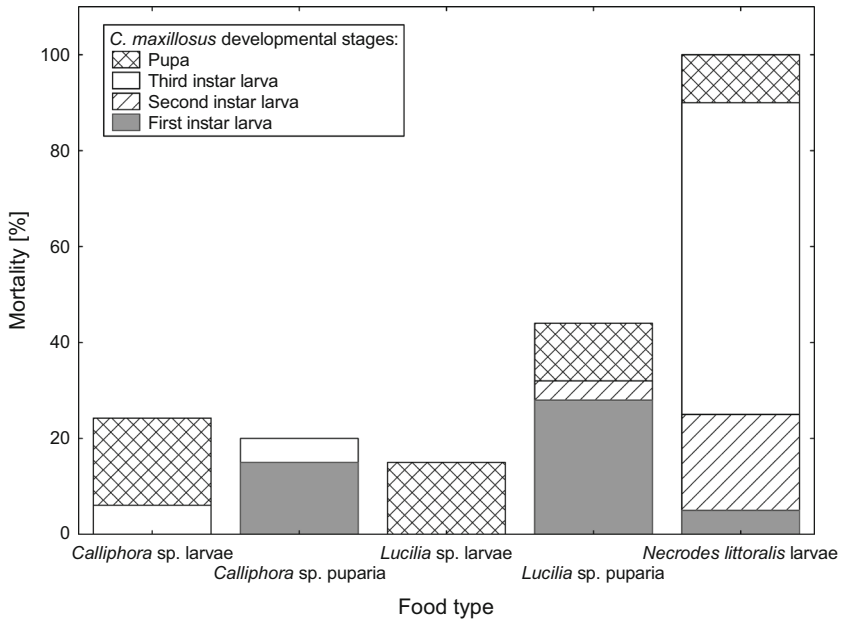


Fig. 3 Mortality of *C. maxillosus* developmental stages reared at 24 °C and fed with different food types

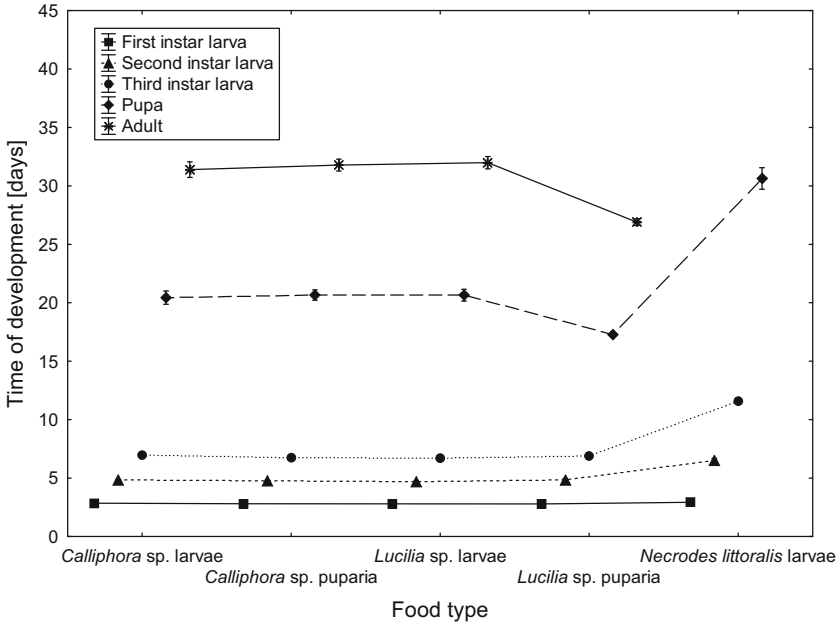


Fig. 4 Duration of *C. maxillosus* developmental stages reared at 24 °C and fed with different food types. Vertical bars represent standard errors; symbols represent mean developmental times of different instars

Discussion

We found that immature stages of *C. maxillosus* reached the adult stage in seven out of ten rearing temperatures (15–30 °C). The thermal requirements for *C. maxillosus* development have previously been investigated for populations from China [45] and Southern United States [44]. Wang et al. [45] investigated the development at the temperature range 17.5–32.5 °C and *C. maxillosus* reached the adult stage at all temperatures. Development times from oviposition to adult emergence for *C. maxillosus* in the present study were similar to these reported by Wang et al. [45]. The differences were found only at 17.5 °C and 20 °C. Total development time at 16 °C from Watson-Horzelski's study [44] was half shorter (65.47 days) than in the present study (122.21 days at 15 °C). It is possible that these large differences are a result of the shift in the linear portion of the relation between temperature and rate of development. Perhaps in the case of the *C. maxillosus* population from the USA, 16 °C lies within the liner portion of the relationship, whereas in the case of the European population of *C. maxillosus*, 15 °C lies beyond this portion. Consequently, at the same low temperatures, Central European beetles should develop longer than beetles from the USA. In the current study, no specimen reached the adult stage when bred at 32.5 °C. At the same temperature, Wang et al. [45] and Watson-Horzelski [44] obtained adult specimens. It is probably due to the fact that these populations have wider ranges of acceptable temperatures. The differences in development of *C. maxillosus* between the studies could be also due to a number of other factors, e.g., differences in beetle diets or differences in rearing conditions. It is difficult to compare such research because of the large differences of the methods used. However, such comparisons reveal that standardization of the insect rearing protocols seems to be one of the most pressing needs in forensic entomology.

Size of pupae and emerging adult beetles was inversely related to temperature. Starting from 17.5 °C, pupae and adult beetles became smaller. Similarly, larval size decreased with temperature starting from 15 °C. These findings are consistent with the temperature-size rule ("hotter is smaller") and represent a form of phenotypic plasticity commonly occurring in ectotherms [19].

As expected, the food type influenced the development of *C. maxillosus*. First- and second-instar larvae of the beetles fed

Table 3 Length and weight (mean (SE; *N*)) of *C. maxillosus* reared at 24 °C fed with different types of food. Means were calculated based on the length and weight measurements conducted at the beginning of particular stage

Food type	3rd-instar length	3rd-instar weight	Pupal weight	Adult length at emergence	Adult weight at emergence
Calliphora sp. larvae	20.08 (0.2; 24)	65.75 (2.38; 24)	174.72 (4.28; 23)	20.38 (0.28; 21)	149.52 (3.61; 21)
Calliphora sp. puparia	20.68 (0.26; 17)	71.17 (3.9; 17)	184.86 (9.12; 16)	20.66 (0.37; 16)	160.1 (8.84; 16)
Lucilia sp. larvae	20.6 (0.21; 20)	69.63 (1.96; 20)	176.24 (4.08; 20)	20.35 (0.28; 17)	149.01 (3.84; 17)
Lucilia sp. puparia	19.29 (0.23; 14)	59.71 (1.76; 14)	169.67 (4.25; 14)	20.65 (0.25; 13)	145.11 (4.42; 13)
Necrodes littoralis larvae	16.07 (0.18; 15)	28.19 (0.97; 15)	58.6 (15.75; 2)	-	-

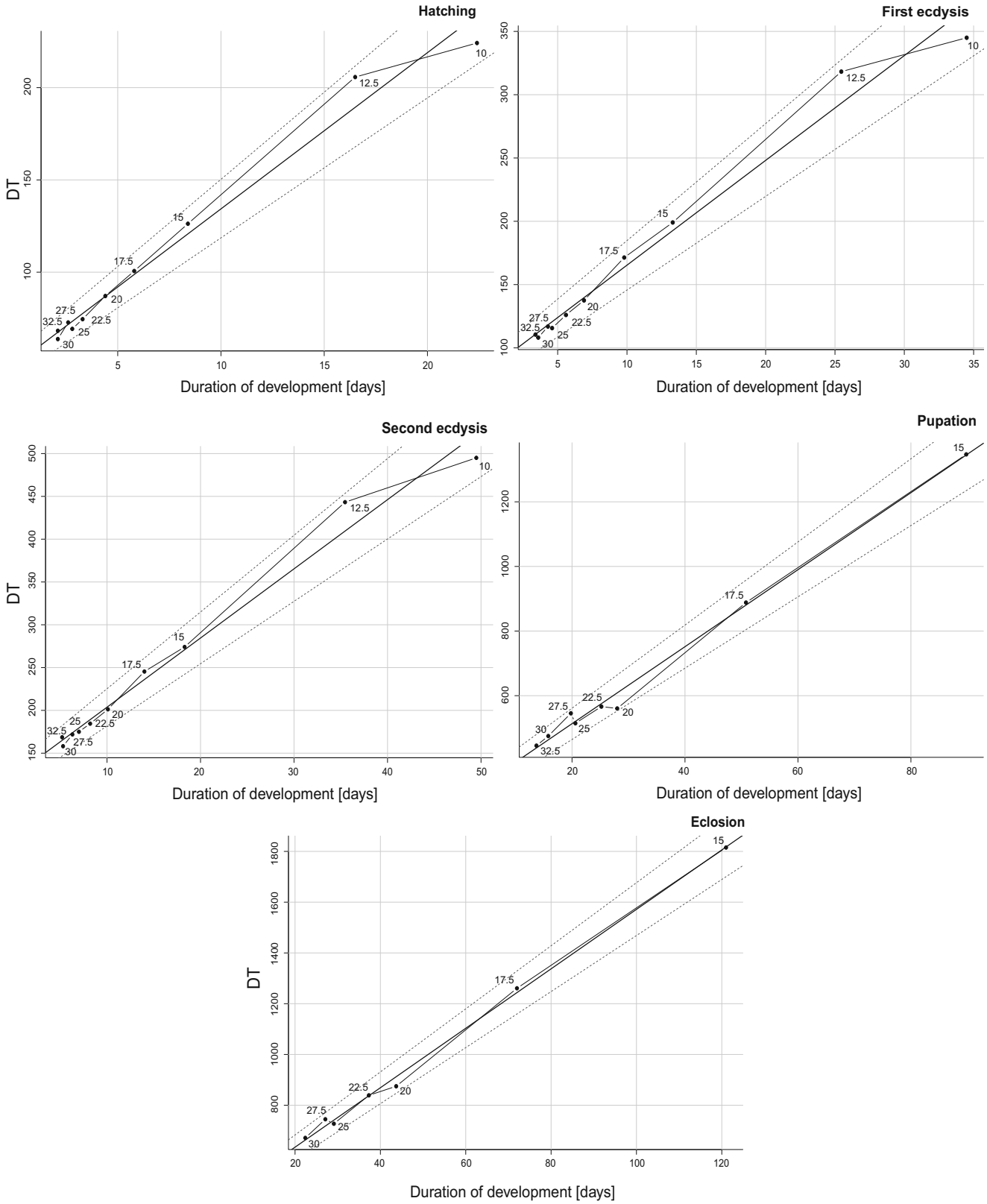


Fig. 5 Reduced major axis (RMA) regression lines sensu Ikemoto and Takai [10] with 95% confidence intervals were used to determine thermal constants for five developmental events; DT is the time in days to reach the adult stage multiplied by the constant rearing temperature

Table 4 Thermal summation models for five developmental events of *C. maxillosus* calculated using Ikemoto and Takai [10] method

	Model	Temp. range	*N*	Thermal summation constant *K* (SE)	Developmental threshold D_0 (SE)	r^2
Hatching	DT = 49.225 + 8.506 × D	10–32.5	10	49.225 (4.009)	8.506 (0.415)	0.981
First ecdysis	DT = 81.688 + 8.314 × D	10–32.5	10	81.688 (6.033)	8.314 (0.402)	0.981
Second ecdysis	DT = 122.883 + 8.085 × D	10–32.5	10	122.883 (7.80)	8.085 (0.365)	0.984
Pupation	DT = 274.825 + 11.984 × D	15–32.5	8	274.825 (14.49)	11.984 (0.351)	0.995
Eclosion	DT = 405.156 + 11.660 × D	15–30	7	405.156 (14.63)	11.660 (0.243)	0.998

Table 5 Estimated parameters and goodness of fit (AICc) of the SSI model for five developmental events of *C. maxillosus*

Parameter	Hatching	First ecdysis	Second ecdysis	Pupation	Eclosion
ρ_φ	0.213748	0.1312873	0.08648179	0.032643	0.022
ΔH_A	19260.18	18671.95	18005.09	18217.24	19132.11
ΔH_L	− 52357.1	− 51441.47	− 53625.17	− 77343.7	− 73724.8
ΔH_H	41899.41	40602.36	40045.81	44281.39	52283.35
T_φ	19.0278	19.0387	18.7123	20.9551	20.5736
T_L	8.506	8.3141	8.0852	11.9839	11.6601
T_H	32.3455	32.7719	32.9047	35.0283	32.906
AICc	− 9.85	− 19.78	− 27.9	− 34.9725	− 35.48
r^2	0.9946	0.99687	0.9936287	0.965429	0.981282

ρ_φ—mean development rate at the intrinsic optimum temperature (1/day)

ΔH_A—enthalpy of activation of the reaction that is catalyzed by the enzyme (cal/mol)

ΔH_L—change in enthalpy associated with low temperature inactivation of the enzyme (cal/mol)

ΔH_H—change in enthalpy associated with high temperature inactivation of the enzyme (cal/mol)

T_φ—intrinsic optimum temperature at which no enzyme inactivation is hypothesized (°C)

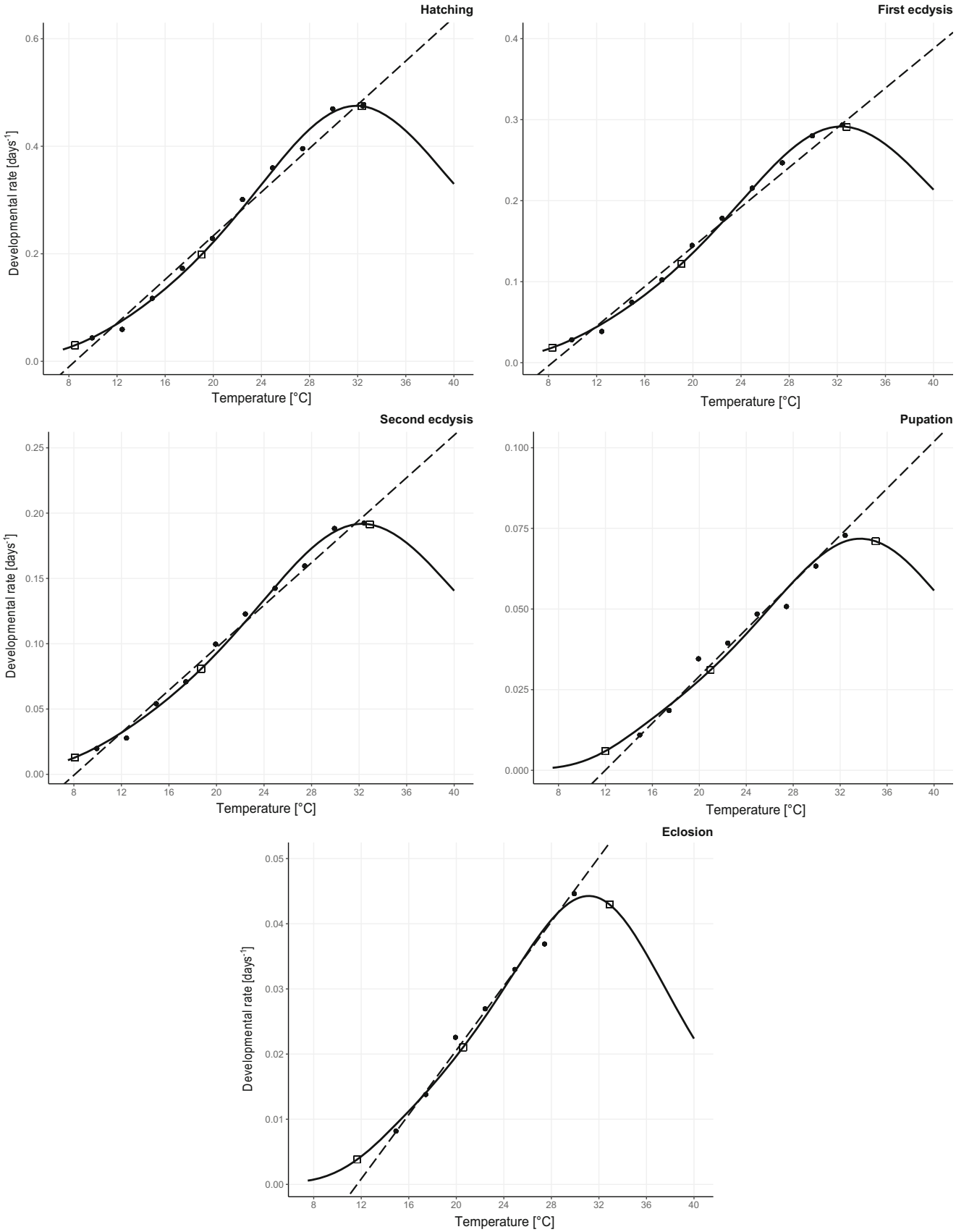
T_L—temperature at which the enzyme is 1/2 active and 1/2 low temperature inactive (°C)

T_H—temperature at which the enzyme is 1/2 active and 1/2 high temperature inactive (°C)

with *N. littoralis* larvae developed significantly longer than larvae fed with other diets. Additionally, no specimen reached the adult stage when fed with *Necrodes littoralis* larvae. These results indicate that in natural and typical conditions, *C. maxillosus* larvae do not prey on larval *Necrodes littoralis*. When larvae were fed with blowfly larvae, the highest mortality was observed at the beetle pupal stage (Fig. 3). However, for *C. maxillosus* larvae fed with blowfly puparia, the highest mortality was observed at the first larval stage of *C. maxillosus* (Fig. 3). Probably, only some of the first-instar larvae were able to puncture the blowfly puparium, and for this reason, beetle mortality at this stage was so high. These findings indicate that *C. maxillosus* may change food preferences during its development. First-instar larvae may feed on blowfly larvae only, while second- and third-instar larvae may switch to a more diverse diet, with larger contribution of blowfly puparia. These patterns are actually consistent with successional patterns of blowflies and *C. maxillosus* as recorded on pig carcasses [57].

Current linear models are useful for minimum PMI estimation in Central Europe. The thermal constant *K* for the total development time was lower than that in Wang et al.'s study [45] (405.16 ± 14.63 and 492.06 ± 23.61 ° days, respectively). Lower developmental threshold (T_{min}) was however higher than the one presented by Wang et al. [45] (11.6 ± 0.24 °C and 9.6 ± 0.58 °C, respectively). Due to the lower T_{min} for *C. maxillosus* from China, it should logically take longer to reach the adult stage. These differences may therefore result from differences in thermal requirements of the Chinese and Central European populations of the beetle. However, lower developmental threshold for the Chinese population of *C. maxillosus* might have been underestimated due to the poor representation of the low temperatures in this study (i.e., 17.5–32.5 °C).

Fig. 6 SSI models for five developmental events of *C. maxillosus*. Black ▶ circles represent observed developmental rates at particular rearing temperatures; open squares denote the predicted development rates at temperature at which the enzyme is 1/2 active and 1/2 low temperature inactive (°C) (T_L), intrinsic optimum temperature at which no enzyme inactivation is hypothesized (°C) (T_φ), and temperature at which the enzyme is 1/2 active and 1/2 high temperature inactive (°C) (T_H)


Hatching
Developmental rate [days-1]
Temperature [°C]
First ecdysis
Developmental rate [days-1]
Temperature [°C]
Second ecdysis
Developmental rate [days-1]
Temperature [°C]
Pupation
Developmental rate [days-1]
Temperature [°C]
Eclosion
Developmental rate [days-1]
Temperature [°C]

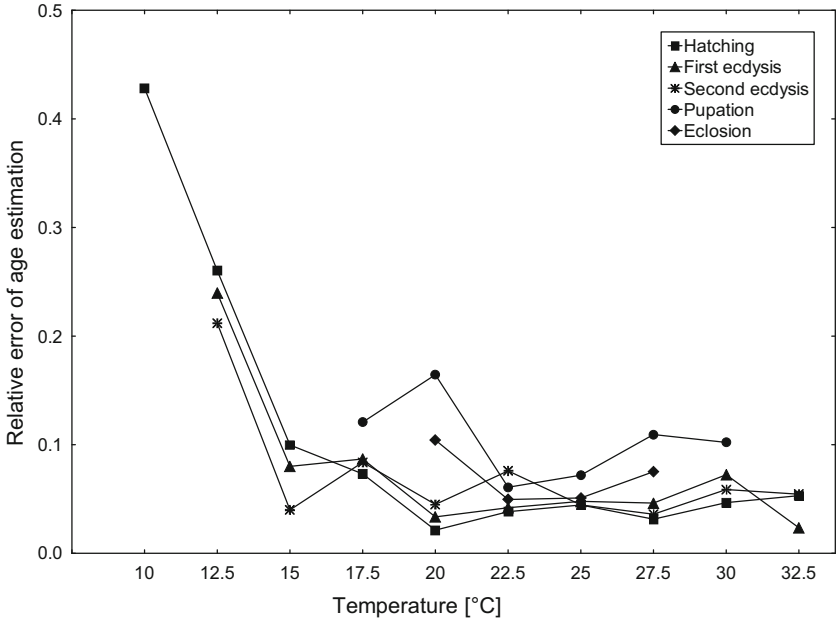


Fig. 7 Relative error of age estimation for *C. maxillosus* validation specimens (reared at ten constant temperatures) using thermal summation data from our Table 4

In our study, T_{min} and T_{max} for pupation and eclosion obtained from nonlinear models (Analytis, Brière-2, and Lactin-2) were not satisfactory, since some of them represented biologically unrealistic values (see Online Resource 1), e.g., very low values for T_{min}, some even below 0 °C (see Online Resource 1). Our fit of nonlinear models was biased in the region close to upper development rate limits. On the other hand, in the similar study on *Fannia canicularis* (Linnaeus) (Diptera: Fanniidae) [17], nonlinear models were biased in the area of lower development rate limits. Because of the high mortality of *C. maxillosus* in the highest examined temperatures, data used for nonlinear modeling represented a rather straight line and, in consequence, led to a failure in nonlinear model fitting. Estimating lower and upper thermal thresholds with the use of empirical nonlinear models, e.g., the Analytis, Brière-2, or Lactin-2, may not provide reliable results [59]. Application of nonlinear models to determine thermal tolerance of forensically useful insects, i.e., the temperature range between the minimum and the maximum rate of development is therefore not a useful approach. Simpler linear models provide more reliable estimations of T_{min} and additionally allow estimating K, a constant of primary usefulness for forensic practice. On the other hand, the theoretical SSI model may provide forensically useful information on the development of examined insect species [59].

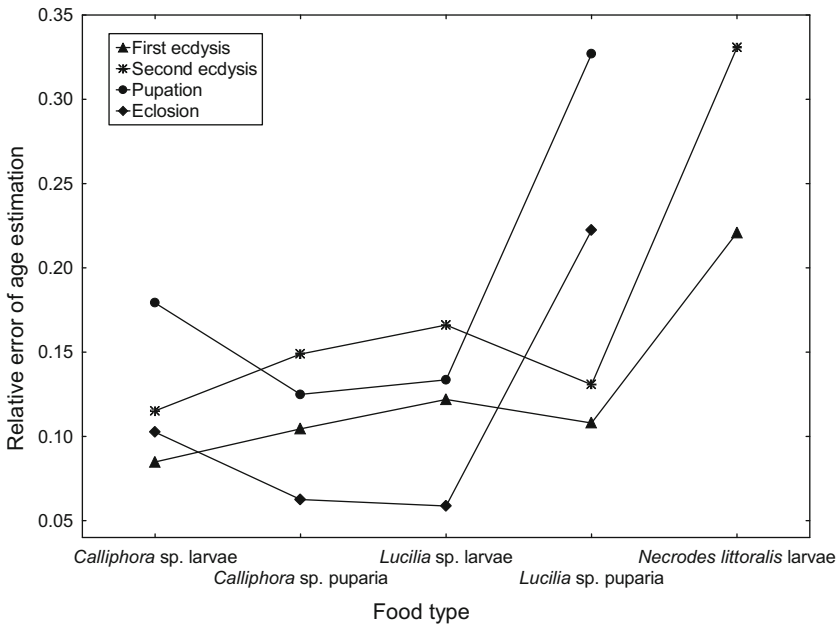


Fig. 8 Relative error of age estimation for *C. maxillosus* validation specimens reared at 24 °C and fed with different food types. Thermal summation data from Table 4 were used for the estimation

Environmental conditions used in reference developmental studies are rarely the same as those experienced during casework. Thus, accuracy of lab-generated data should be determined preferably in a robust validation study. Validation part of this study revealed that accuracy in age estimation was lower using models for final development landmarks, i.e., pupation or eclosion than models for earlier events. Interestingly, age estimation errors were the largest while using model for pupation (Fig. 7). This may be due to the large variation in duration of the third larval stage resulting from surprisingly high variation in the postfeeding larval phase (Table 1).

Because the food type influenced the development of *C. maxillosus* similarly, it must have affected the accuracy of age estimation for specimens reared on different diets. Developmental models were created based on specimens fed with *Calliphora* sp. larvae. When models were validated using specimens fed with the same type of food and in the same laboratory trials, error rates were below 10% for all developmental events (see data for 22.5 and 25 °C in Fig. 7). However, when we used specimens fed with different diets and reared under 24 °C in different trials, error rates were generally larger (usually between 10 and 15%), and for some diets, they were very much larger, e.g., 32% for pupation and 22% for eclosion for larvae fed with *Lucilia* sp. puparia (Fig. 8). As for the fly-related diets, total development times were the shortest (Table 3), mortality was the highest (Fig. 3), beetle size was the smallest (Table 4), and estimation errors were the largest (Fig. 8) for *C. maxillosus* fed with *Lucilia* puparia. These findings indicate that in natural and typical conditions, larvae of *C. maxillosus* do not prey solely on *Lucilia* puparia, probably due to the low quality of this diet. Some studies suggest that food quality affects thermal constants in insects [60–62]. Additionally, Jarošík et al. [60] showed that aphidophagous ladybirds develop significantly faster and start developing at significantly lower temperature on a good-quality diet. Consequently, the food quality may affect lower development threshold, indicating that the threshold may change depending on a diet. Similar results were reported for moths [61, 62] and aphids [63]. It is therefore possible that similar effects may occur in case of *C. maxillosus* and other forensically useful insects. In natural conditions, carrion insects usually have access to optimal diet. Therefore, insect evidences encountered in casework are usually specimens fed with optimal food type. However, the problem may arise when we estimate their age with developmental data obtained using non-optimal diet. This source of estimation error may be particularly important in case of predatory insects, as was demonstrated in this article for predatory *C. maxillosus* beetle. Moreover, these findings indicate that collecting reference developmental data using optimal diet is crucial and that forensic entomologists should pay more attention to the quality of food used in developmental studies and in rearing insects during casework.

Funding information The study was funded by the Ministry of Science and Higher Education (grant no. DI2013011043).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The current study involved laboratory experiments using insect species *Creophilus maxillosus* (Coleoptera: Staphylinidae). The species is not under protection. No permissions or approval from Ethic Commission were needed.

Informed consent Not applicable.

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SUPPLEMENTARY TABLES FOR

Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae)

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Content

Table 1. Estimated parameters and goodness of fit (*AICc*) of the nonlinear developmental models for five developmental events of *C. maxillosus*.

Model	Parameter	Hatching	First ecdysis	Second ecdysis	Pupation	Eclosion
Analytis	a	0.0006601	0.00001843	0.00003636	$2.665*10^{-24}$	$2.75*10^{-33}$
	n	0.5332	0.4214	0.4868	0.1004	0.1003
	m	3.721	1.458	1.493	0.1709	0.1155
	T_{min}	2.7	2.46	3.63	-2.027	-16.73
	T_{max}	42.27	42.89	44.71	48.43	77.99
	T_{fast}	33.17	33.83	34.60763	29.75	33.98
	AICc	-85.19	-104.52	-102.91	-65.05	-82.08
Brière-2	a	0.003927	0.000210755	0.00008685	$4.994*10^{-18}$	$1.185*10^{-7}$
	d	3.638	3.30341	2.227	0.1643	0.5709
	T_{min}	6.298	5.8819	5.663	10.78	10.64
	T_{max}	36.16	37.36	40.62	185.8	69.73
	T_{fast}	32.23	32.89	33.75	50.45	40.04
	AICc	-87.03	-105.57	-104.85	-86.05	-87.6
Lactin-2	P	0.1206	0.11875	0.10366	0.01043	0.008049
	ΔT	8.1806	8.35294	9.56126	52.63348	12.35552
	λ	-0.1166	-0.06909	-0.06475	-0.71485	-0.03712
	T_{max}	40.15	40.65775	42.34	129.31	46.84
	T_{fast}	31.85	32.21	32.67	35	34.37
	AICc	-85.08	-101.79	-103.62	-87.06	-86.81

T_{min} is the lower developmental threshold, T_{max} is the upper developmental threshold, T_{fast} is the temperature of the fastest development the and a, n, m, d, p, ΔT and λ are estimated model parameters.

SUPPLEMENTARY FIGURES FOR

Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae)

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Content

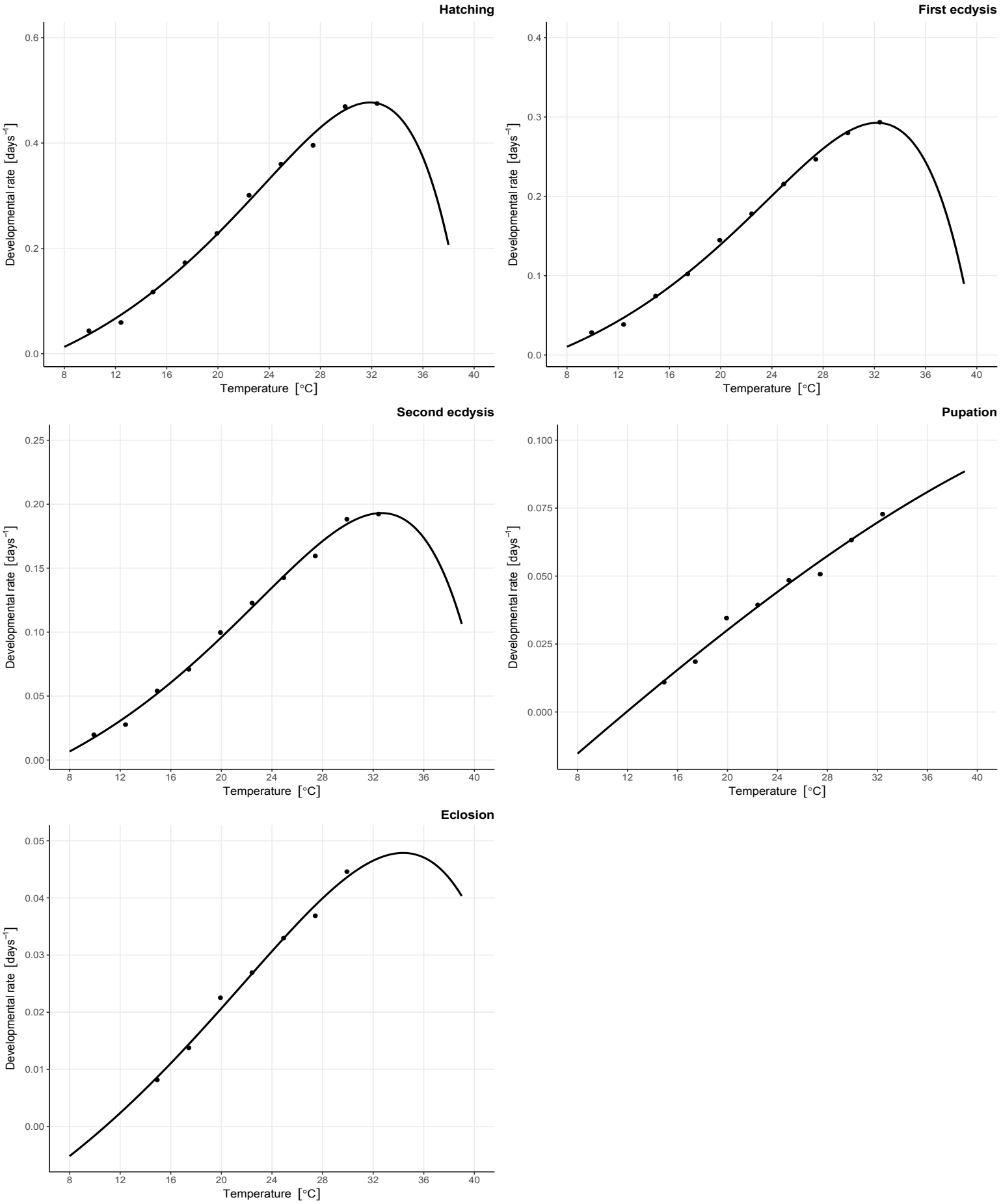

Fig. 1. Lactin-2 models fit to observed developmental rates of five development events of *C. maxillosus.* Black circles indicate observed data.

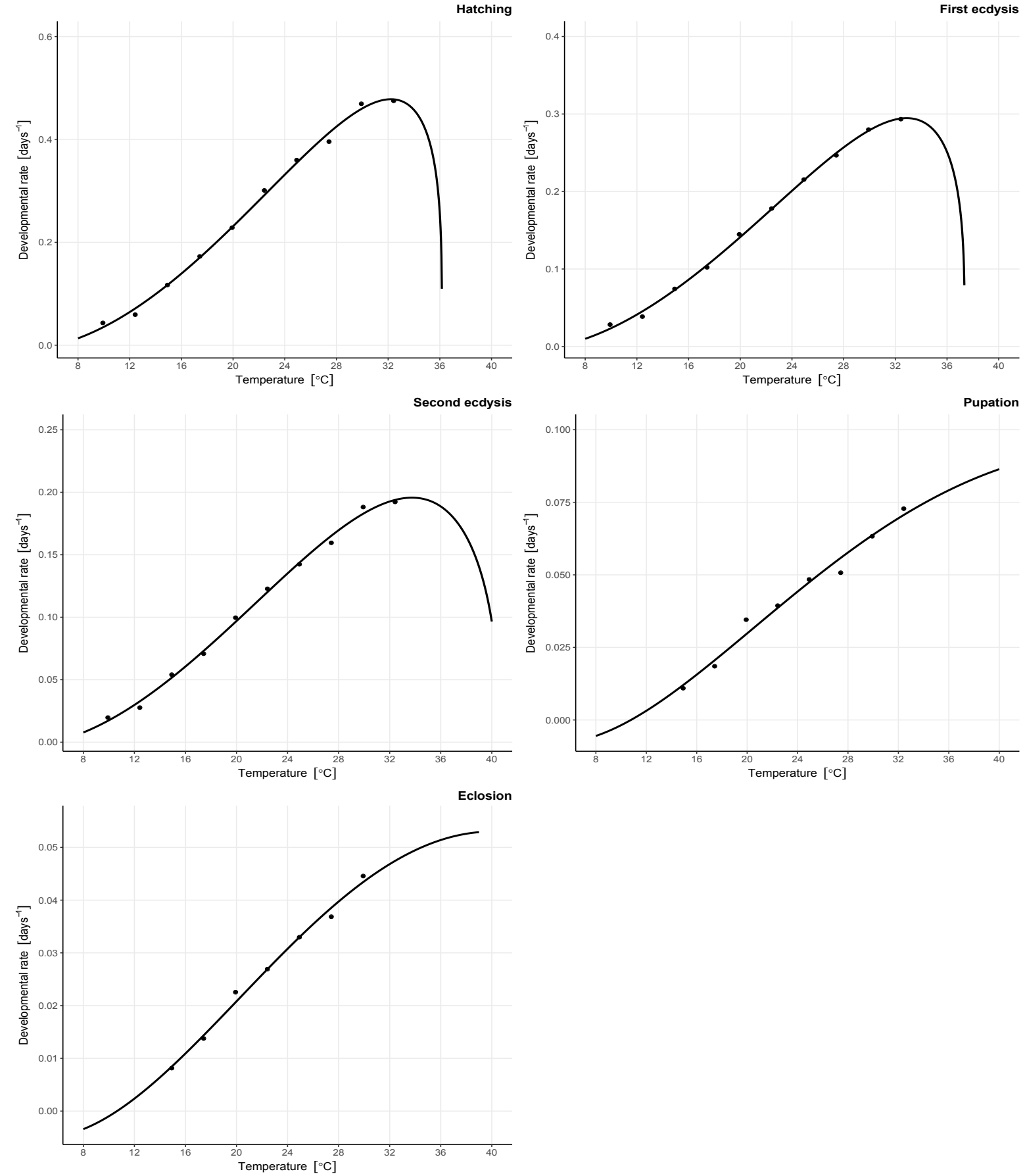

Fig. 2. Brière-2 models fit to observed developmental rates of five development events of *C. maxillosus*. Black circles indicate observed data.

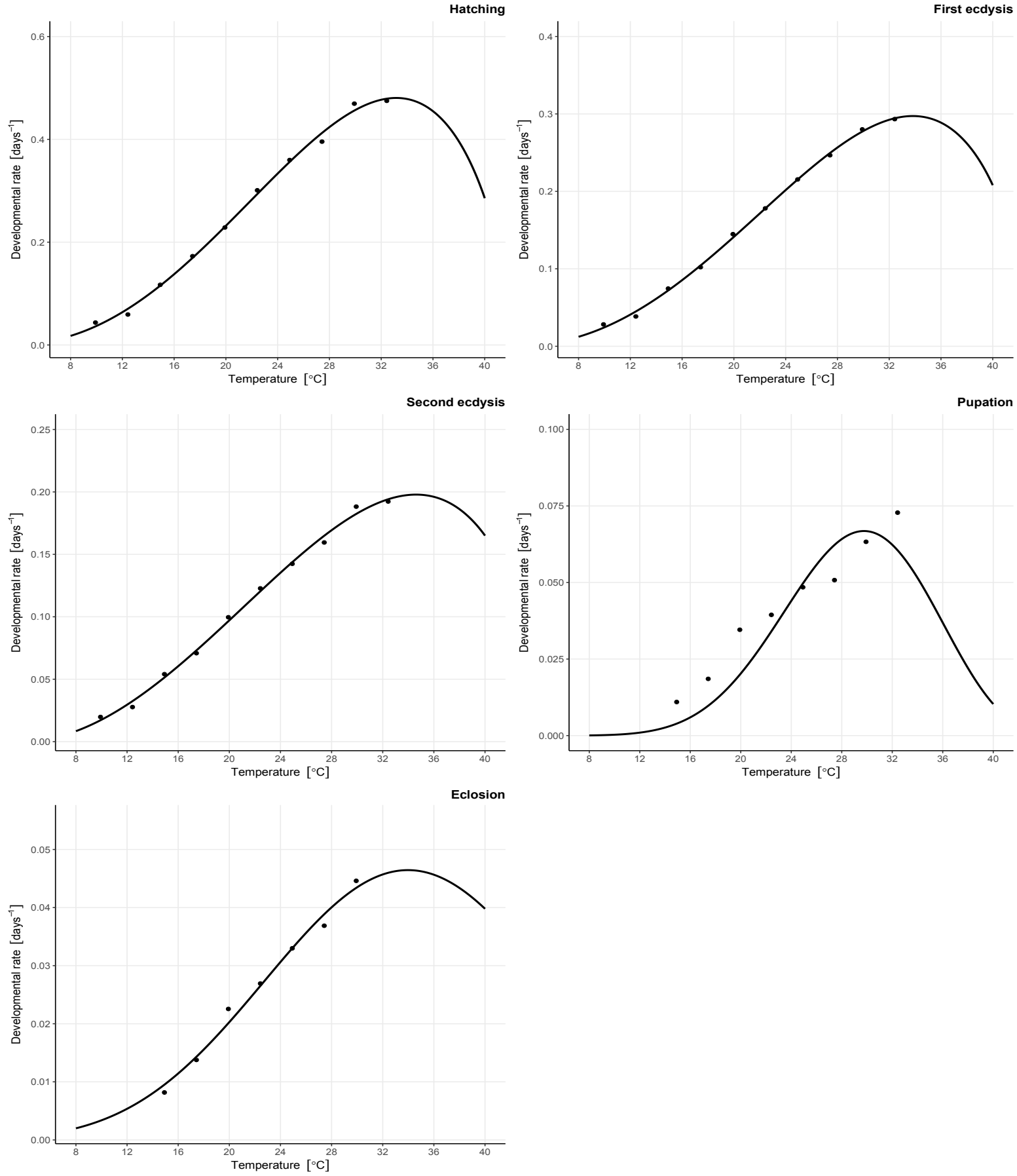

Fig. 3. Analytis models fit to observed developmental rates of five development events of *C. maxillosus.* Black circles indicate observed data.

Int J Legal Med (2018) 132:887–895
https://doi.org/10.1007/s00414-017-1713-4

ORIGINAL ARTICLE

Sex-specific developmental models for *Creophilus maxillosus* (L.) (Coleoptera: Staphylinidae): searching for larger accuracy of insect age estimates

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Received: 10 July 2017 / Accepted: 11 October 2017 / Published online: 24 October 2017

Abstract Differences in size between males and females, called the sexual size dimorphism, are common in insects. These differences may be followed by differences in the duration of development. Accordingly, it is believed that insect sex may be used to increase the accuracy of insect age estimates in forensic entomology. Here, the sex-specific differences in the development of *Creophilus maxillosus* were studied at seven constant temperatures. We have also created separate developmental models for males and females of *C. maxillosus* and tested them in a validation study to answer a question whether sex-specific developmental models improve the accuracy of insect age estimates. Results demonstrate that males of *C. maxillosus* developed significantly longer than females. The sex-specific and general models for the total immature development had the same optimal temperature range and similar developmental threshold but different thermal constant *K*, which was the largest in the case of the male-specific model and the smallest in the case of the female-specific model. Despite these differences, validation study revealed just minimal and statistically insignificant differences in the accuracy of age estimates using sex-specific and general thermal summation models. This finding indicates that in spite of statistically significant differences in the duration of immature development between females and males of *C. maxillosus*, there is no increase in the accuracy of insect age estimates while using the sex-specific thermal summation models compared to the general model. Accordingly, this study does not support the use of sex-specific developmental data for the estimation of insect age in forensic entomology.

Keywords Forensic entomology · Postmortem interval · Development · Age estimates · Insect sex · Estimation accuracy

Introduction

One of the entomological methods for postmortem interval (PMI) estimation is the developmental method. It involves estimating the age of the oldest immature stages of insects found on a cadaver [1–3]. For this purpose, indicators of insect age, such as larval length or weight, are measured. Observed values of indicators are then compared with developmental data included in a species-specific developmental model [4, 5]. The whole procedure requires access to case-specific temperature data [6, 7]. Developmental models are created in laboratory experiments where insects are usually kept in constant temperatures and measured frequently [8]. Their results are presented using graphical (e.g., isomegalen and isomorphen diagram) or mathematical models [3, 4]. In practice, age of insects is usually estimated using linear models (i.e., thermal summation models) [3, 9]; however, recently forensic entomologists have become more interested in non-linear models [10, 11].

The variety of factors affects accuracy of the development-based PMI estimates. Many of them influence directly the accuracy with which insect age is estimated, and the quality of the developmental model is one of the highest importance

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00414-017-1713-4) contains supplementary material, which is available to authorized users.

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[12, 13]. Developmental models should be based on experiments carried out in at least six temperatures, age indicators should be measured at intervals representing no more than 10% of the stage duration, and the median should be the statistic used to characterize duration of development [12]. Apart from these factors, accuracy of insect age estimation depends on the appropriateness of the model used (i.e., the degree of match between the conditions under which the model was developed and the conditions under which insects from this particular cadaver have been developing) as well as the quality of temperature data used [6, 7]. Nonetheless, even a very accurate estimate of insect age indicates just the minimum PMI, which may substantially differ from the actual PMI. Therefore, apart from the factors indicated above, the accuracy of PMI estimation using the developmental method may depend on factors affecting length of the period preceding appearance of insects on a cadaver (i.e., the pre-appearance interval, PAI) [14]. In the case of insects colonizing cadavers shortly after death (e.g., blowflies), their age is usually very close to the actual PMI. However, in the case of insects colonizing carcasses later in decomposition, it is usually necessary to estimate PAI in some way, for example, with the use of the temperature methods [14]. Additionally, it should be evaluated how accurately the entomofauna of a cadaver is represented by the insect sample (in particular, whether the oldest insects were sampled) and what factors may have affected colonization of a body by insects in this particular case. In cases of negligence, a body may be colonized before death [15], whereas in a burial scenario [16], after wrapping in material [17], in an indoor scenario [18, 19], in a car [19], or in a suitcase [20], colonization by insects may be delayed. The same effect may result from the bad weather [21], or a nighttime cadaver exposure [22, 23].

In forensic entomology, efforts are now being made to improve the accuracy of insect age estimation through, among others, improving the quality of developmental models. From this point of view, insect sex has recently focused attention of researchers [11, 24]. Usually, sex may easily be identified in the adult stage. Accordingly, sex of immature insects collected from the cadaver may be determined after breeding them to the adult stage in the laboratory. There are also molecular methods of sex determination [25]. There are widespread differences in size between males and females of insects, called the sexual size dimorphism [26–29]. They may result from differences between sexes in size at hatching, rate of development, length of development, or any combination of these factors [30, 31]. Accordingly, sex-specific differences in length of development have been found in many insect species [27, 28], including species used in forensic entomology [11, 24]. It can therefore be assumed that such differences may occur in many other species of forensically important insects. Sexual size dimorphism is present in many species of necrophilous beetles, e.g., *Necrodes littoralis* (Linnaeus, 1758), *Creophilus maxillosus* [32], *Dermestes maculatus* (DeGeer, 1774) [33, 34], and flies, e.g., *Chrysomya megacephala* (Fabricius, 1794) [35] or *Megaselia scalaris* Loew, 1866 [11]. We believe that these differences in size are followed by differences in the duration of development. It is however unclear whether these differences may increase the accuracy of age estimates while using sex-specific models of development. Although some researchers suggested that assessment of sex-specific growth may reduce noise in minimum PMI estimates [24], no previous study validated sex-specific developmental data or created sex-specific developmental models for PMI estimation.

Table 1 Inspection/measurement intervals (hours) in each temperature

Developmental stage	Temperature (°C)						
	15	17.5	20	22.5	25	27.5	30
Egg	12	12	7	7	5	5	4
1st instar larva	9	9	6	6	4	4	3
2nd instar larva	11	11	8	8	6	6	5
3rd instar larva	40	40	30	30	24	24	18
Pupa	30	30	24	24	16	16	12

C. maxillosus is a predatory beetle that feeds mainly on larvae of necrophagous flies [36–38]. It regularly visits large vertebrate cadavers in natural (non-urban) environments [39–42]. Moreover, due to its large size, it may be easily sampled during cadaver inspection. It colonizes cadavers much later than most flies, so its use may substantially prolong the period when PMI is estimated using the developmental method [37, 43]. Additionally, PAI of *C. maxillosus* may easily be estimated using temperature methods [44]. It is therefore a perfect species for the PMI estimation based on the

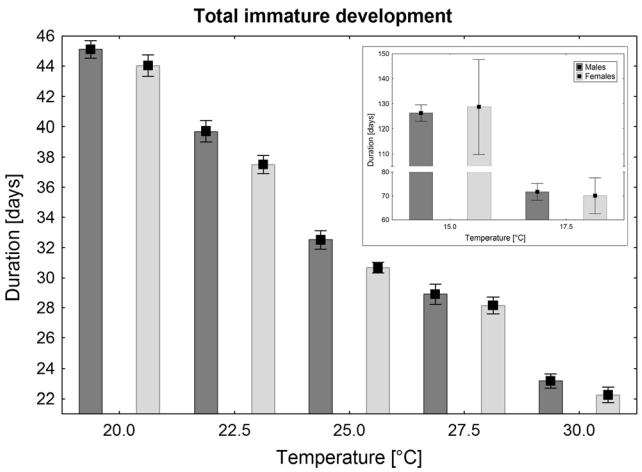

Fig. 1 Differences between males and females of *C. maxillosus* in duration of total immature development at seven constant temperatures; black box—mean, whiskers—standard error of the mean

combination of the developmental method and the PAI method. Although *C. maxillosus* was regularly sampled from human cadavers, it was rarely used to estimate PMI (mostly with succession-based approach) [39, 45]. Its infrequent use resulted from lack of developmental data for this species and colonization of cadavers exclusively in the natural habitats.

The aim of this study was to test whether there are differences in development time between sexes of *C. maxillosus*

Table 2 Differences in the duration of development between males and females of *C. maxillosus* at different temperatures and for different stages

Temperature (°C)	Developmental stage	Mean duration (days) (SD; *N*)		*t*	*P*
		Females	Males		
15	Egg	8.36 (0.13; 3)	8.53 (0.35; 11)	− 0.81	0.43
	1st instar larva	4.95 (0.24; 3)	5.23 (1.01; 11)	− 0.45	0.66
	2nd instar larva	5.00 (0.46; 3)	5.38 (0.40; 11)	− 1.40	0.19
	3rd instar larva	85.23 (29.46; 2)	81.06 (8.46; 10)	0.44	0.67
	Pupa	23.54 (3.15; 3)	26.23 (2.55; 11)	− 1.55	0.15
	Total	128.75 (26.81; 2)	126.24 (10.58; 10)	0.25	0.81
17.5	Egg	5.94 (0.51; 6)	5.86 (0.41; 15)	0.35	0.73
	1st instar larva	4.26 (0.70; 6)	4.21 (0.60; 15)	0.14	0.89
	2nd instar larva	4.13 (0.31; 6)	4.25 (0.28; 15)	− 0.90	0.38
	3rd instar larva	37.44 (17.09; 6)	38.17 (13.87; 15)	− 0.10	0.92
	Pupa	18.33 (0.68; 6)	19.21 (1.27; 15)	− 1.58	0.13
	Total	70.10 (18.23; 6)	71.71 (13.67; 15)	− 0.22	0.83
20	Egg	4.31 (0.13; 16)	4.30 (0.16; 16)	0.30	0.76
	1st instar larva	2.74 (0.22; 16)	2.79 (0.27; 16)	− 0.51	0.61
	2nd instar larva	3.12 (0.21; 16)	3.06 (0.26; 16)	0.74	0.47
	3rd instar larva	18.29 (2.86; 16)	19.15 (1.75; 16)	− 1.02	0.31
	Pupa	15.56 (0.63; 16)	15.81 (0.97; 16)	− 0.88	0.39
	Total	44.04 (2.88; 16)	45.11 (2.34; 16)	− 1.16	0.25
22.5	Egg	3.34 (0.11; 17)	3.37 (0.21; 13)	− 0.41	0.69
	1st instar larva	2.26 (0.22; 17)	2.37 (0.35; 13)	− 1.05	0.30
	2nd instar larva	2.55 (0.19; 17)	2.47 (0.29; 13)	0.86	0.40
	3rd instar larva	17.26 (2.43; 17)	19.06 (2.68; 13)	− 1.92	0.06
	Pupa	12.10 (0.80; 17)	12.43 (0.48; 13)	− 1.34	0.19
	Total	37.51 (2.52; 17)	39.70 (2.50; 13)	− 2.37	0.02
25	Egg	2.80 (0.09; 22)	2.86 (0.10; 14)	− 1.76	0.09
	1st instar larva	1.90 (0.15; 22)	2.00 (0.37; 14)	− 1.09	0.28
	2nd instar larva	2.24 (0.16; 22)	2.40 (0.14; 14)	− 3.09	0.004
	3rd instar larva	14.12 (1.50; 22)	14.98 (1.86; 14)	− 1.52	0.14
	Pupa	9.60 (0.63; 22)	10.26 (0.51; 14)	− 3.32	0.002
	Total	30.67 (1.62; 22)	32.51 (2.28; 14)	− 2.84	0.008
27.5	Egg	2.57 (0.14; 15)	2.60 (0.13; 12)	− 0.74	0.47
	1st instar larva	1.70 (0.30; 15)	1.72 (0.24; 12)	− 0.16	0.88
	2nd instar larva	2.21 (0.31; 15)	2.21 (0.26; 12)	< 0.01	0.99
	3rd instar larva	13.56 (1.91; 15)	13.79 (1.75; 12)	− 0.33	0.75
	Pupa	8.58 (0.78; 15)	8.12 (1.13; 12)	− 1.26	0.22
	Total	28.15 (2.13; 15)	28.9 (2.34; 12)	− 0.87	0.39
30	Egg	2.20 (0.15; 10)	2.21 (0.07; 3)	− 0.05	0.96
	1st instar larva	1.41 (0.14; 10)	1.52 (0; 3)	− 1.37	0.20
	2nd instar larva	1.69 (0.14; 10)	1.83 (0; 3)	− 1.74	0.11
	3rd instar larva	9.63 (1.65; 10)	9.98 (0.43; 3)	− 0.35	0.73
	Pupa	7.32 (0.42;10)	7.62 (0.87; 3)	− 0.86	0.41
	Total	22.25 (1.61;10)	23.17 (0.81; 3)	− 0.92	0.37

and at what stage of development they appear. Moreover, we created separate developmental models for males and females, and made validation studies to test whether such models improve the accuracy of age estimation. The following predictions were formulated: (1) Males of *C. maxillosus* develop longer than females. (2) Sex-specific differences in development time accumulate across all stages; however, they are the largest at the third larval and pupal stages. (3) The use of sex-specific developmental models substantially improves the accuracy of insect age estimates and consequently minimum PMI.

Materials and methods

Maintaining *C. maxillosus* colony in the laboratory

A laboratory colony was established twice, in 2015 and 2016. In each year, about 50 adult beetles were collected manually from rabbit carcasses placed in a xerothermic grassland in the Biedrusko military range (Western Poland, Europe; 52 31′ N, 16 55′ E) during spring and summer. All the time, the colony consisted of 25–30 individuals (more or less equal ratio of males and females). New beetles sampled from the field carcasses and individuals bred in the laboratory were added to the colony. Insects were kept in plastic containers (30.4 × 20 × 20.1 cm) and were fed once a day with blowfly pupae or third instar larvae. Moist soil (6–7 cm) was used, and containers were cleaned every 6–8 days to avoid appearance of mites and mold. Insects were kept at room temperature and humidity (20–22 °C, 50–60%).

Laboratory protocol

Development was studied at seven constant temperatures: 15, 17.5, 20, 22.5, 25, 27.5, and 30 °C. In order to get eggs, all adult insects from the colony were put into a 3-l container filled halfway with soil (temperature 20–22 °C). After 4 h, adult beetles were pulled out and containers were placed in insect incubators (ST 1/1 BASIC or +, POL-EKO, Poland) with the predefined temperature. After 70% of the average egg stage duration, containers were inspected for the presence of first instar larvae, at intervals equal to 10% of the average egg stage duration. We used such methods, as *C. maxillosus* lay singular eggs in small clumps of soil which makes them very difficult to be found. Freshly hatched first instar larvae are very active and creamy-white in color, so it is not possible to omit them while searching the soil. Only freshly hatched larvae were sampled and transferred to separate cups, each larva to a single cup. Forty larvae per temperature were used. Immature beetles came from separate ovipositions (laid by different females originating from the highly variable colony). Two or three temperatures were studied at the same time, and insects were randomly allocated to temperatures. First and second instar larvae were kept in 80-ml containers with 1.5 cm of soil, third instar larvae and pupae in 120-ml containers with 5 cm of soil. Larvae were fed once a day with third instar larvae of blowflies killed and punctured to make feeding easier for the first and second instar *C. maxillosus*. Humidity in insect incubators was maintained at 60–70%, and a photoperiod (h) was set on 12:12 (L/D).

Five developmental landmarks were defined: hatching, first ecdysis, second ecdysis, pupation, and adult emergence. All individuals were inspected for developmental landmarks; half of them (chosen at random) were also repeatedly measured and weighed. After a landmark had been recorded, the

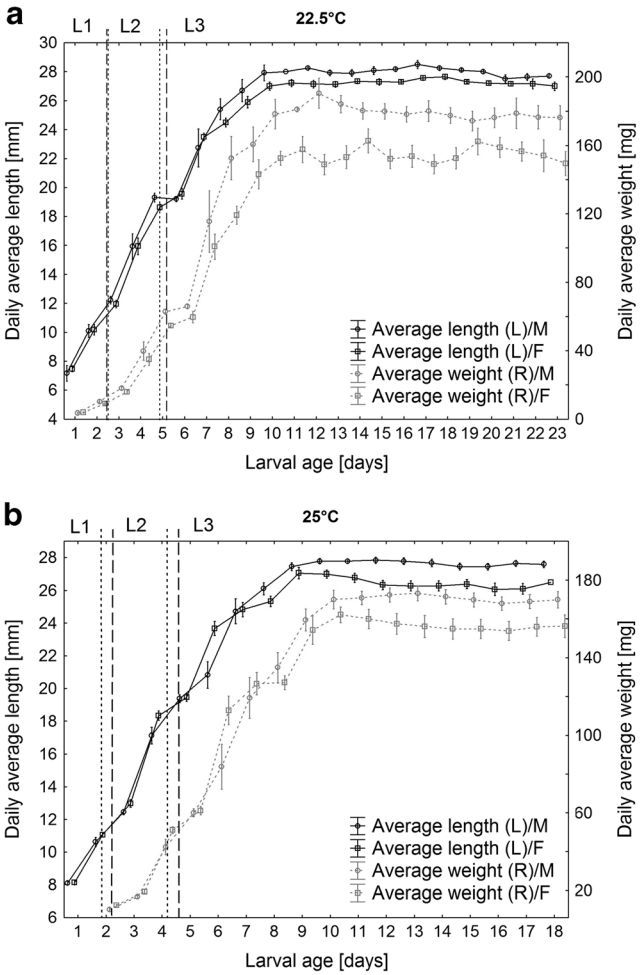

Fig. 2 Growth curves for males and females of larval *C. maxillosus* at constant temperatures of 22.5 °C (**a**) and 25 °C (**b**); symbols—mean, whiskers—standard error of the mean; L1—first larval stage, L2—second larval stage, L3—third larval stage; dotted lines indicate the average moment of transition to the next stage for females (- - - -) and males (— —); M-males, F-females, L-left *y* axis, R-right *y* axis. Daily average length and weight are average values calculated across all measurements of the given larva in a given day

midpoint between the current and the previous inspection was used as the actual time of the landmark occurrence. Transitions between larval stages were determined based on the color of a larva (creamy-white shortly after ecdysis) and the width of the mesonotum [46]. Sex of beetles was determined after emergence on the basis of the shape of the eighth abdominal sternite.

Inspections and measurements of larvae and pupae

Inspections and measurements were carried out at intervals representing 10% of the life stage duration [12] (Table 1). A geometrical micrometer was used to measure in vivo larval length [47]. A larva was placed in a 1.5-ml Eppendorf tube, and after it had become immobile and fully erected, its length (from clypeus to the last abdominal segment) was measured with a micrometer. An analytical balance AS 82/220.R2 (Radwag, Poland) was used to weigh larvae and pupae while being kept in a 1.5-ml Eppendorf tube.

Statistical analyses

Differences between males and females in the duration of developmental stages and total immature development as well as in the adult insect length and weight at emergence were evaluated using the *t* test for independent samples. Thermal summation models for the total immature development were calculated separately for males and females, as well as for the pooled sample (i.e., the general model), using the equation proposed by Ikemoto and Takai [9]. Eight insects per temperature and sex were randomly selected to be used for the modeling purposes; the rest of the specimens were used to test performance of the models in the age estimation task. Due to the large mortality at extreme temperatures, 15 and 30 °C were poorly represented in the validation sample. The validation included comparison of the thermal units needed to reach the adult stage, as calculated for each specimen at relevant developmental threshold, with the thermal constant from the model. The accuracy with which the model represented the actual thermal units needed for the emergence of the adult stage was compared across models using the *t* test for correlated samples. All analyses were performed using Statistica 12 (StatSoft, Inc., 2014) at 5% level of significance.

Results

Differences in development between sexes of *C. maxillosus*

As a rule, males of *C. maxillosus* developed longer than females (Fig. 1, Table 2, electronic supplementary material). Differences in the duration of development between females and males were largest at 22.5 and 25 °C and in the third larval and pupal stages (Fig. 1, Table 2, electronic supplementary material). The differences were statistically insignificant in most single-stage comparisons, partly due to their small size and partly due to the small size of samples used in the comparisons (Table 2). Because the differences have accumulated over the entire premature development, at eclosion they were quite large and in the case of 22.5 and 25 °C statistically significant, for example, at 25 °C males of *C. maxillosus* emerged on average almost 2 days later than females (Table 2). Males were distinctly larger (longer and heavier)

Table 3 Differences between males and females of *C. maxillosus* in length and weight at emergence

Insect size	Temperature (°C)	Mean (SD; *N*)		*t*	*P*
		Females	Males		
Length (mm)	15	18.2 (2.1; 3)	20.7 (1.6; 11)	− 2.32	0.04
	17.5	19.7 (1.0; 6)	21.3 (1.3; 15)	− 2.76	0.01
	20	21.1 (1.2; 16)	22.3 (1.4; 16)	− 2.53	0.02
	22.5	19.7 (1.5; 17)	21.1 (1.6; 13)	− 2.43	0.02
	25	17.7 (1.0; 22)	18.6 (0.8; 14)	− 2.81	0.008
	27.5	17.0 (1.3; 15)	18.5 (1.7; 12)	− 2.65	0.01
	30	17.8 (1.4; 10)	17.8 (0.8; 3)	0.02	0.98
Weight (mg)	15	142.5 (23.6; 3)	160.6 (25.1; 11)	− 1.12	0.29
	17.5	136.6 (13.9; 6)	158.0 (16.6; 15)	− 2.77	0.01
	20	146.5 (17.3; 16)	166.4 (28.4; 16)	− 2.40	0.02
	22.5	127.8 (14.7; 17)	149.7 (20.0; 13)	− 3.46	0.002
	25	120.1 (10.4; 22)	130.2 (8.5; 14)	− 3.05	0.004
	27.5	107.1 (16.4; 15)	117.7 (23.4; 12)	− 1.38	0.18
	30	97.3 (10.1; 10)	98.9 (10.9; 3)	− 0.23	0.82

than females from the beginning of the third larval stage until eclosion (Fig. 2). After eclosion, adult males were about 1.5 mm longer and about 20 mg heavier than adult females; however, at higher temperatures, starting from 25 °C, these differences were smaller (Table 3).

Sex-specific developmental models of *C. maxillosus*

All temperature points were included while calculating model parameters (Fig. 3). The models have the same optimal temperature range, similar developmental threshold, and different thermal constant *K*, which was the largest in the case of the male-specific model and the smallest in the case of the female-specific model (Table 4). Despite these differences, validation study revealed just minimal and statistically insignificant differences in the accuracy of age estimates using sex-specific and general models (*t* test for correlated samples; $t = -0.25$, $P = 0.80$, Fig. 4).

Discussion

Differences in development between sexes of *C. maxillosus*

Differences in duration of development between sexes were in line with our expectations. Final size of an insect should be proportional to the duration of growth [48]. As *C. maxillosus* males are larger than females, we have correctly assumed that they will develop longer. Sexual differences in the duration of development have already been studied in necrophagous fly species *Lucilia sericata* (Meigen, 1826) [24] and *M. scalaris* [11]. In both species, females are larger and thus develop longer than males. This pattern is much more common among various insect species [28, 31], including forensically important ones, e.g. *D. maculatus* [33, 34] or *C. megacephala* [35]. Large females gain more than males in terms of fitness [49] as they can lay more eggs of good quality [50]. In the case of males, large body size may be crucial while competing for limited resources, i.e., food or mating candidates. Larger males are common among predacious species (e.g., Staphylinidae) probably because of the high level of intrasexual competition for limited resources [32]. All these studies suggest that sexual differences in development time may be prevalent in forensically important insects, as many of them are characterized by the sexual size dimorphism, e.g., *N. littoralis* [32].

The developmental differences between sexes were not large, but at eclosion they were already substantial due to their accumulation during development. The differences were the largest for the third larval and pupal stages, as these life stages are the longest in the case of *C. maxillosus* [37, 38]. In the case of *M. scalaris*, differences in development between males and females were the largest in the pupal stage [11]. For many fly species, the pupal stage is the longest life stage, frequently representing about 50% of the total immature development [51, 52]. For many beetle species, the longest life stage is

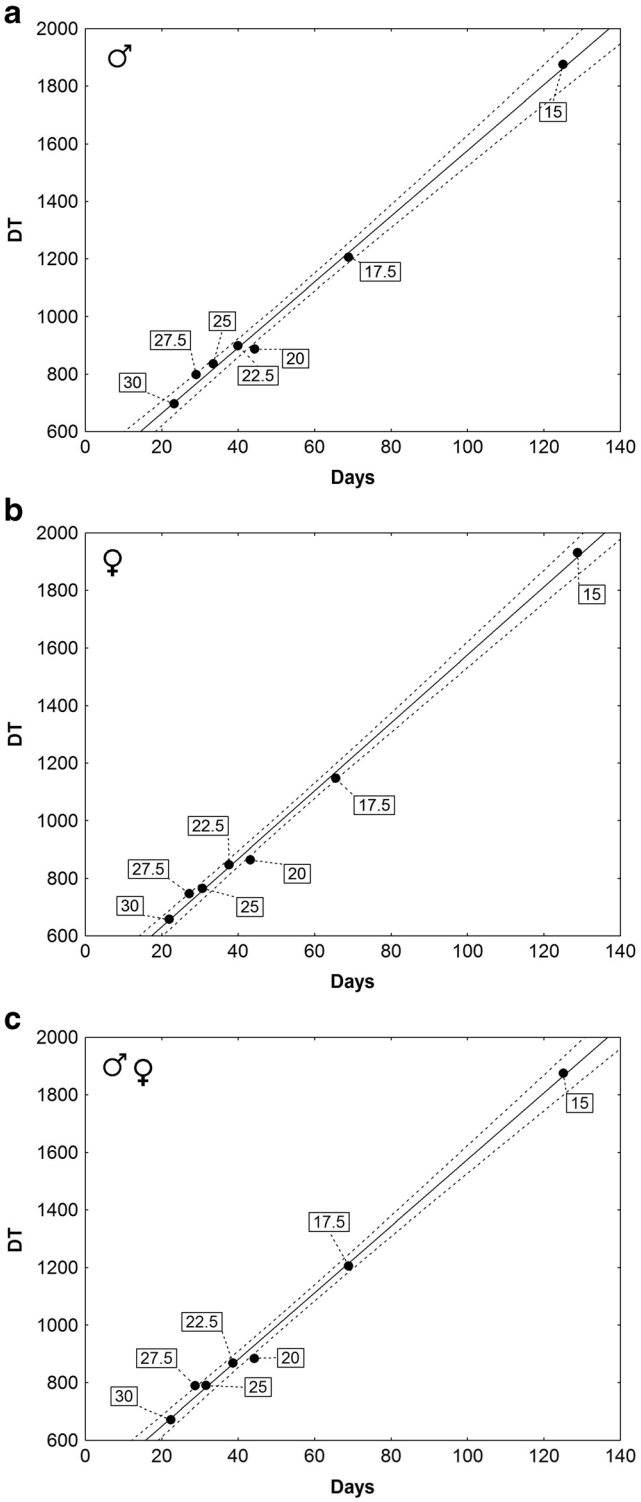

Fig. 3 Thermal summation models for the total immature development of *C. maxillosus* males (**a**), females (**b**), and pooled sample (**c**); DT is the time in days to reach the adult stage multiplied by the constant rearing temperature

Table 4 Thermal summation models for the total immature development of *C. maxillosus*

Model	Temperature range (°C)	Thermal summation constant—K (SE) (days °C)	Developmental threshold—D_0 (SE) (°C)	r^2	N	P
Sex-specific (males)	15–30	434.25 (22.06)	11.43 (0.36)	0.995	7	< 0.001
Sex-specific (females)	15–30	395.27 (17.83)	11.81 (0.29)	0.997	7	< 0.001
General (males and females)	15–30	417.33 (19.52)	11.58 (0.32)	0.996	7	< 0.001

the third larval stage [53, 54]. Consequently, it is likely that sexual differences in duration of development will be the largest in the third larval stage in the case of beetles and in the pupal stage in the case of flies.

Sex-specific developmental models of *C. maxillosus*

We expected that the use of sex-specific developmental models will substantially improve the accuracy of insect age estimates. Despite significant differences in the duration of development between males and females, the improvement has not been achieved. The differences in duration of development are probably too small to have consequences for the accuracy of age estimates using sex-specific developmental models. This finding draws attention to a very important issue in the case of any new technique in forensic science, that is, its validation. Current results demonstrate that statistically significant effect is not always equivalent to practically relevant effect, which has recently been highlighted by Wells and LaMotte [55].

Sexual differences in the duration of particular life stages were small. Consequently, it is not worth creating sex-specific developmental models for particular stages, possibly just for the entire development. In this study, the largest differences between males and females occurred at 22.5 and 25 °C. In both temperatures, females completed development in about 95% of the time required by males. Similar differences in the duration of development between sexes were reported for *M. scalaris* [11] and *L. sericata* [24], except that females developed longer than males. In the case of *M. scalaris*, males completed development in 92.5% of the time required by females [11], and in the case of *L. sericata*, males completed development in 94.5% of that time [24]. Although these authors either did not create sex-specific developmental models or did not validate them, the differences reported are similar to the current differences. Therefore, it is probable that in the case of *L. sericata* and *M. scalaris* sexual differences in developmental time are, similarly, too small to improve the accuracy of insect age estimates.

Because our results do not support the use of sex-specific developmental data in forensic entomology, it would be useful to test the effect the sex-specific developmental models may have on the accuracy of age estimates in the case of insect species with larger size differences between males and females. Moreover, other techniques to improve the accuracy of insect age estimates in forensic entomology are necessary. Because insect size is highly intra-sexually variable, the better solution may be to use the size of an insect instead of its sex.

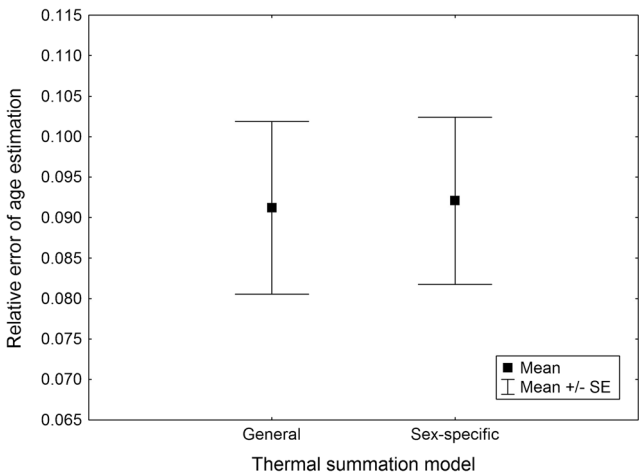

Fig. 4 Relative error in age estimation of *C. maxillosus* at emergence using sex-specific (for males and females) and general models (for pooled sample)

Self-critique

Fewer individuals at extreme temperatures In low and high temperatures, the development of fewer individuals has been analyzed due to the high mortality. Moreover, females and males survived differently in different temperatures, with more females surviving in high temperatures and more males in low temperatures. Although we had enough data from all temperatures to create sex-specific models, extreme temperatures were underrepresented in the validation part of the study.

Study of development at constant temperatures Although insects develop in natural environment at fluctuating temperatures, the study was made at constant temperatures to enable comparison with results of other studies (development of forensically useful insects is usually studied under constant

temperature) and make it possible to create accurate thermal summation models avoiding rate summation effect [3].

Acknowledgements We would like to thank A. Mądra-Bielewicz (Poznań, Poland) and M. Nawrocka (Poznań, Poland) for the assistance in field and laboratory work. Thanks are also extended to O. Łagiewski (Pobiedziska, Poland) for the Visual Basic scripts used while analyzing raw data.

Funding information The study was funded by the Ministry of Science and Higher Education (grant no. DI2013011043).

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SUPPLEMENTARY FIGURES FOR

"Sex-specific developmental models for *Creophilus maxillosus* (L.) (Coleoptera: Staphylinidae): searching for larger accuracy of insect age estimates"

International Journal of Legal Medicine

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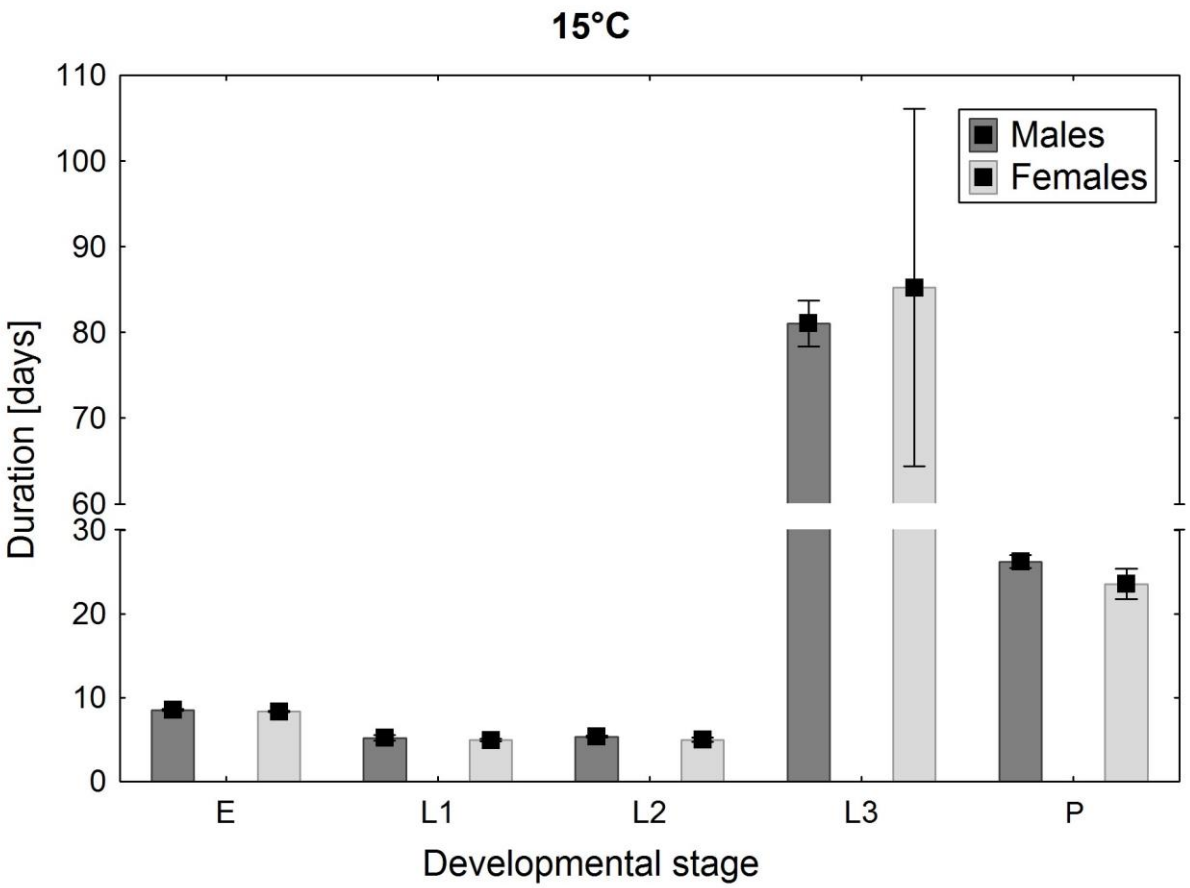
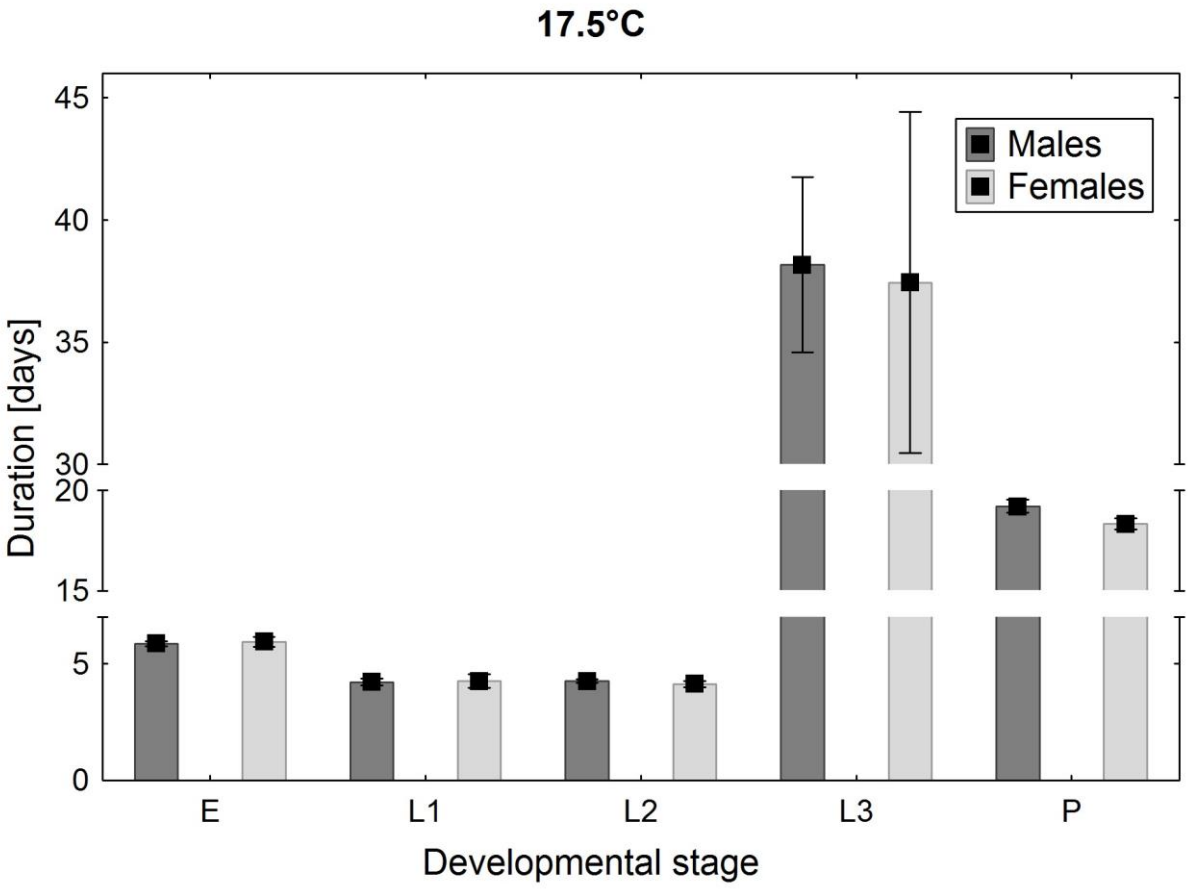
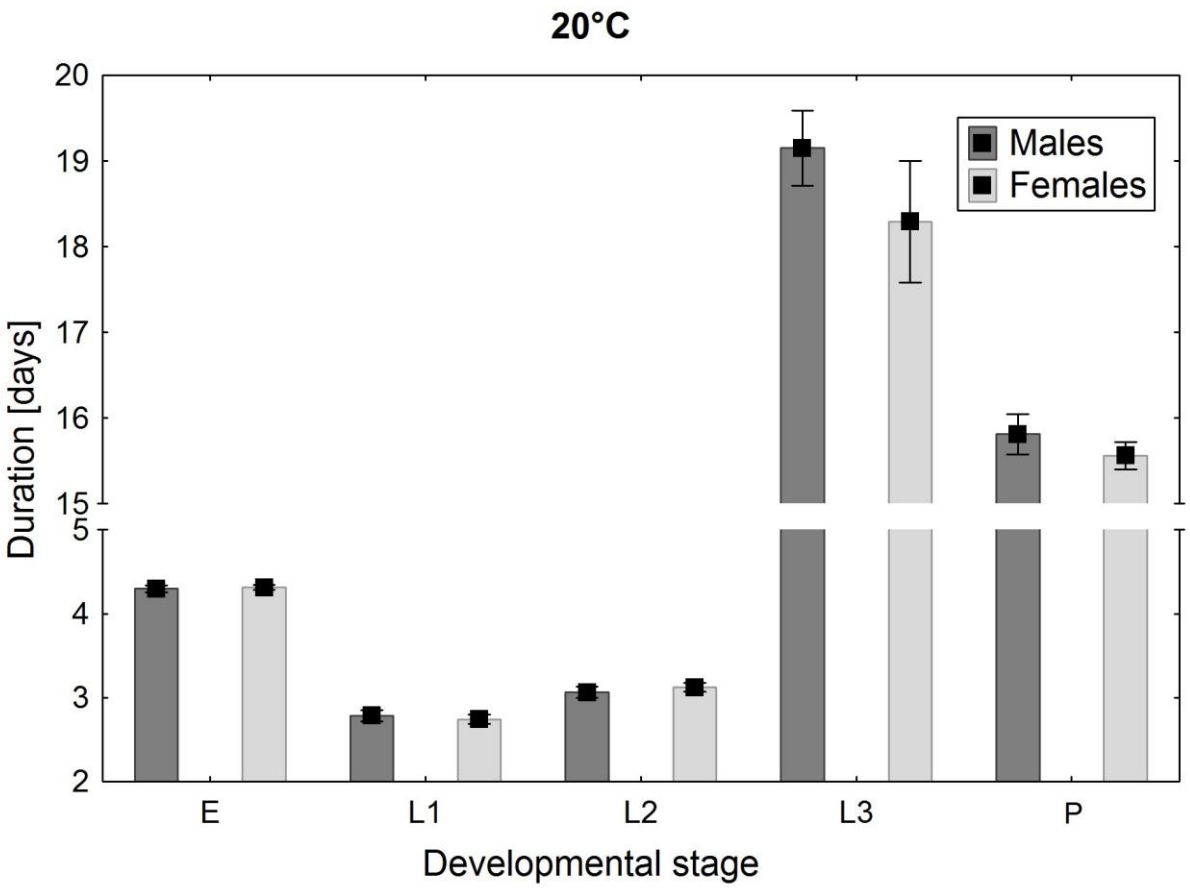
Content

Fig. 1. Differences between males and females of *C. maxillosus* in duration of life stages at 15°C, 17.5°C, 20°C, 22.5°C, 25°C, 27.5°C, 30°C; ■ – mean, whiskers – standard error of the mean; E – the egg stage, L1 – first larval stage, L2 – second larval stage, L3 – third larval stage, P – pupal stage.


15°C
Males
Females
Duration [days]
110
100
90
80
70
60
30
20
10
0
E
L1
L2
L3
P
Developmental stage


17.5°C
Males
Females
Duration [days]
45
40
35
30
20
15
5
0
E
L1
L2
L3
P
Developmental stage


20°C
Males
Females
Duration [days]
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19
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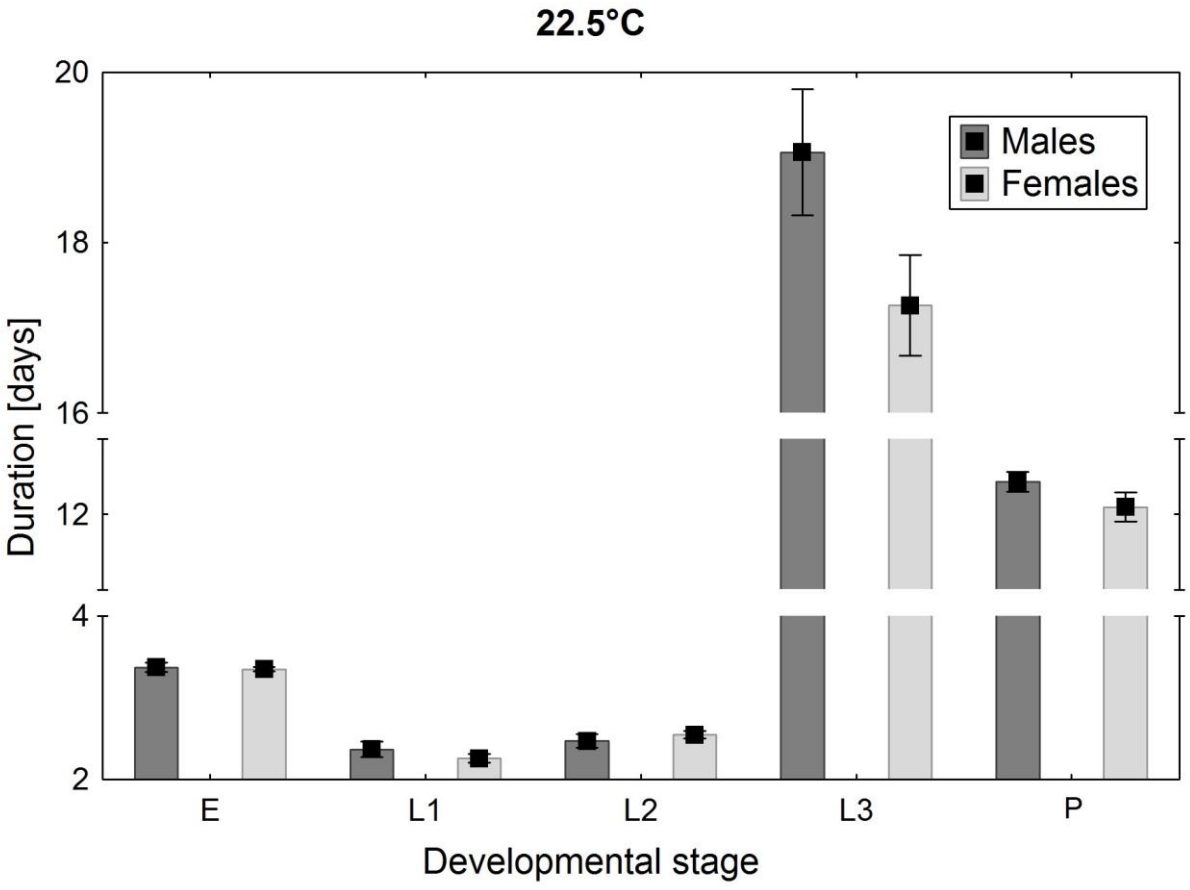
2
E
L1
L2
L3
P
Developmental stage


22.5°C
Males
Females
Duration [days]
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12
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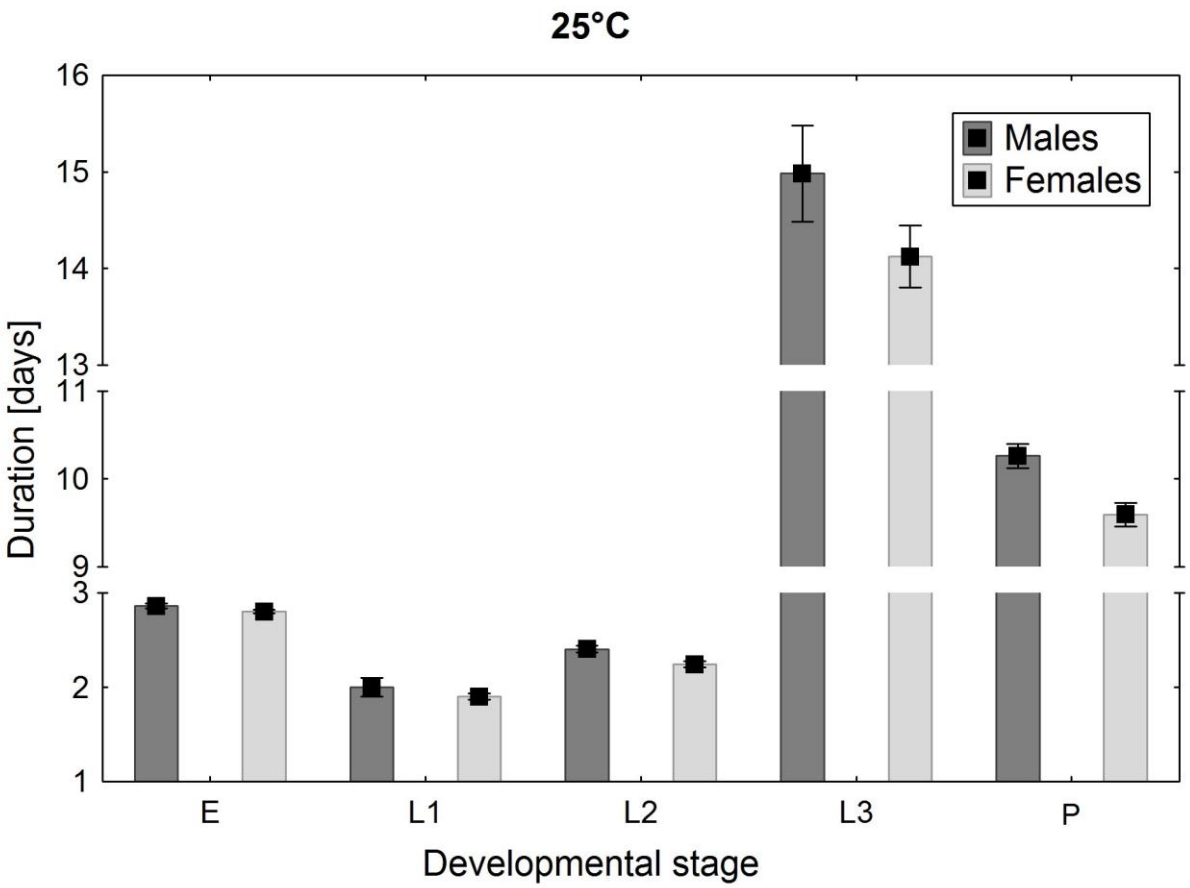
2
E
L1
L2
L3
P
Developmental stage


25°C
Males
Females
Duration [days]
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14
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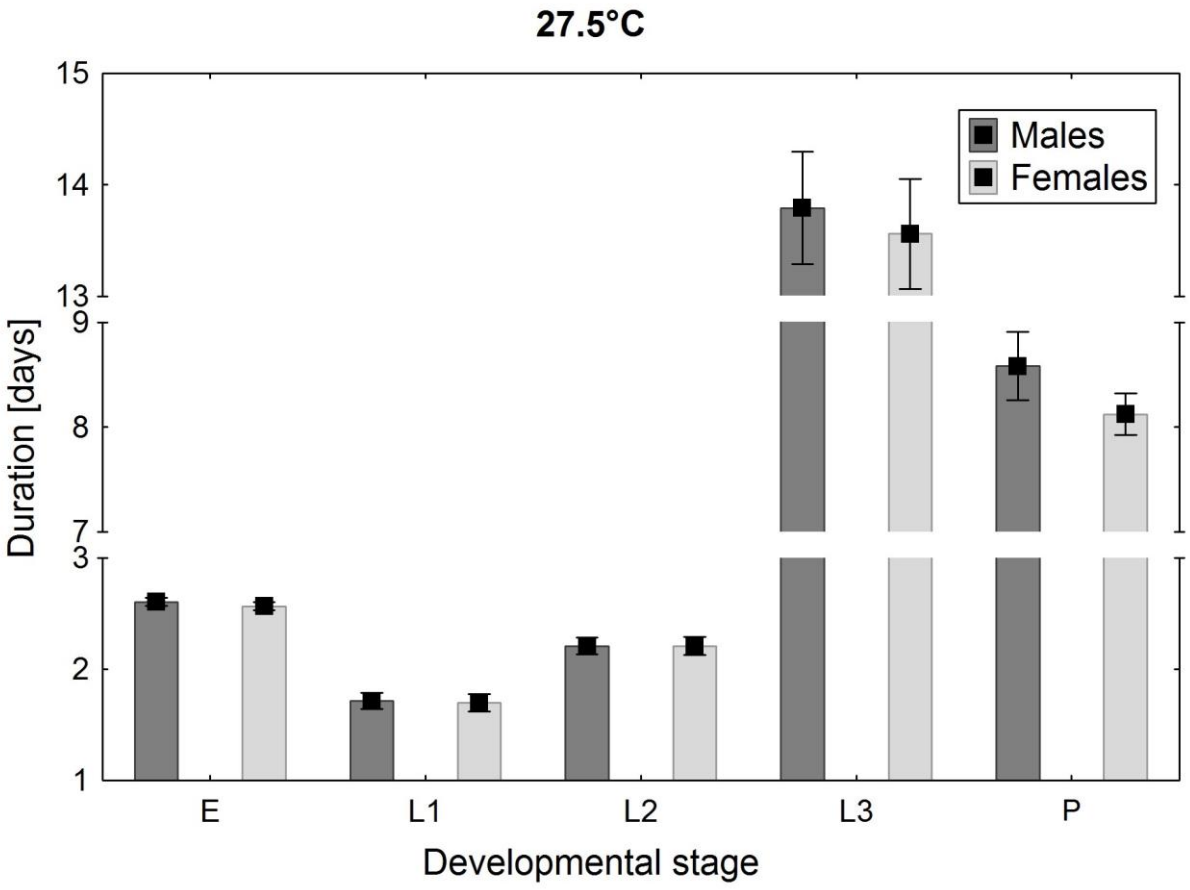
3
2
1
E
L1
L2
L3
P
Developmental stage


27.5°C
Males
Females
Duration [days]
15
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8
7
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1
E
L1
L2
L3
P
Developmental stage

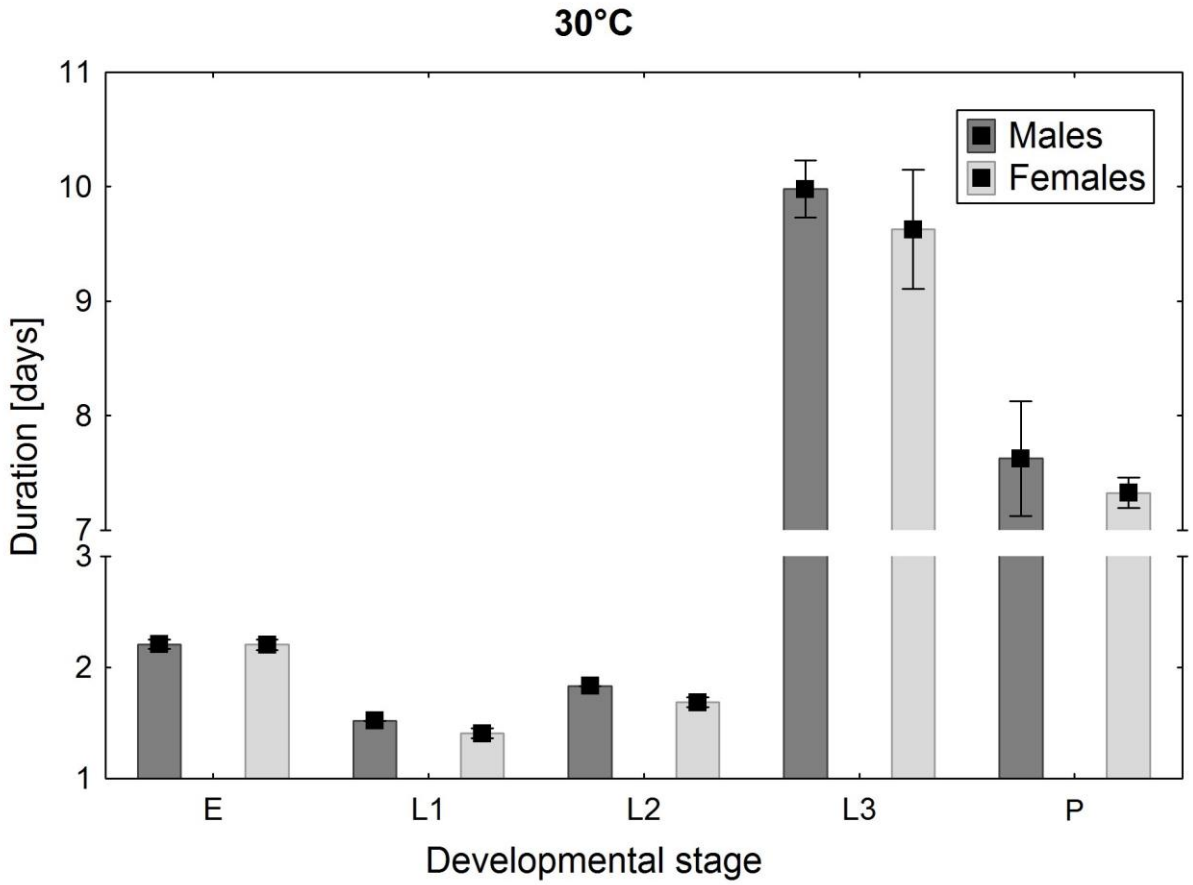

Fig. 1. Differences between males and females of *C. maxillosus* in duration of life stages at 15°C, 17.5°C, 20°C, 22.5°C, 25°C, 27.5°C, 30°C; ■ – mean, whiskers – standard error of the mean; E – the egg stage, L1 – first larval stage, L2 – second larval stage, L3 – third larval stage, P – pupal stage.

SCIENTIFIC REPORTS

OPEN

Size at emergence improves accuracy of age estimates in forensically-useful beetle *Creophilus maxillosus* L. (Staphylinidae)

Received: 18 September 2017
Accepted: 24 January 2018
Published online: 05 February 2018

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Insects colonizing human or animal cadavers may be used to estimate post-mortem interval (PMI) usually by aging larvae or pupae sampled on a crime scene. The accuracy of insect age estimates in a forensic context is reduced by large intraspecific variation in insect development time. Here we test the concept that insect size at emergence may be used to predict insect physiological age and accordingly to improve the accuracy of age estimates in forensic entomology. Using results of laboratory study on development of forensically-useful beetle *Creophilus maxillosus* (Linnaeus, 1758) (Staphylinidae) we demonstrate that its physiological age at emergence [i.e. thermal summation value (*K*) needed for emergence] fall with an increase of beetle size. In the validation study it was found that *K* estimated based on the adult insect size was significantly closer to the true *K* as compared to *K* from the general thermal summation model. Using beetle length at emergence as a predictor variable and male or female specific model regressing *K* against beetle length gave the most accurate predictions of age. These results demonstrate that size of *C. maxillosus* at emergence improves accuracy of age estimates in a forensic context.

There are several methods for post-mortem interval (PMI) estimation based on insect evidence. Most frequently, PMI is approximated based on the age of immature insects sampled from a cadaver. Usually the minimum PMI is being predicted, however the case circumstances (e.g. the probability of myiasis) may change the interpretation[1]. Insect age is estimated using laboratory-derived developmental data and temperature data specific for the decomposition site[2–4]. There are many factors affecting accuracy with which insect age is estimated in a forensic context[5]. Within species variation in development time is one of the largest importance[6,7]. Substantial intraspecific variation of development was revealed in many forensically useful insects[8–14]. Moreover, comparison of the same species studies demonstrated substantial between-study variation in thermal summation constant (K) and base temperature (T_b)[15–18].

Several sources of the intraspecific variation in development time were identified in forensically useful insects. Gallagher *et al.*[19] demonstrated for a blowfly *Lucilia sericata* (Meigen, 1826) that some part of this variation results from differences between local populations. Similar findings were reported for other blowflies, *Chrysomya megacephala* (Fabricius, 1794)[20] and *Cochliomyia macellaria* (Fabricius, 1775)[21]. Moreover, differences in development time between females and males were reported for *L. sericata*[22], a phorid fly *Megaselia scalaris* (Loew, 1866)[23] and a staphylinid beetle *C. maxillosus*[24]. Another source is the precocious egg development, resulting in some eggs from the batch hatching earlier, which occur commonly in sarcophagid flies and less frequently in calliphorid flies[5,25]. Several exogenous determinants of development time were identified as well; for example, quality and quantity of food[6,26–30] or intra and interspecific competition[31–33].

Although intraspecific variation in development time of forensically useful insects is usually large, there were just a few suggestions how these variation may be taken into account while estimating insect age (but see[34]).

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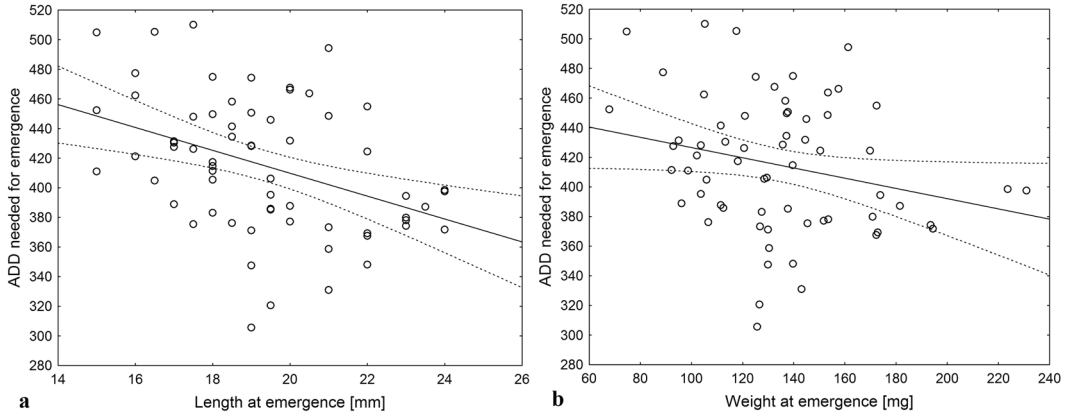

Figure 1. The relationship between adult *C. maxillosus* length (**a**) or weight (**b**) at emergence and thermal summation values needed for emergence (ADD over 11.58 °C). Solid line – linear regression model, dotted lines – confidence limits.

Regarding geographical variation several authors proposed to use, wherever possible, local developmental data[16,35]. It is, however, unclear what are the error rates resulting from geographical mismatch between laboratory and crime scene insects. Richards *et al.*[17] demonstrated that *K* for a blowfly *Chrysomya albiceps* (Wiedemann, 1819) was proportional to geographic latitude. Based on this finding they have suggested that it might be possible to develop a model useful for *K* and T_b estimation at any latitude[17]. Unfortunately, no such model has been derived for any forensically useful species. Another suggestion resulted from studies of sex-specific developmental patterns in forensically useful insects[22,24]. Picard *et al.*[22] suggested that sex-specific developmental data could be used to increase the accuracy of insect age estimates and reduce error rates in minimum PMI estimates. However, recent study with forensically-useful beetle *C. maxillosus* did not support the use of sex-specific developmental models in forensic entomology, as despite significant differences in development time between males and females of *C. maxillosus*, there was no gain in the accuracy of age estimates using sex-specific developmental models[24]. There is also a general recommendation that while estimating insect age, one should use developmental models derived in similar conditions compared to the case conditions[4,16]. Wells & LaMotte[36], however, indicated that not all differences between laboratory and crime scene conditions are of practical importance. They showed that despite highly significant effect of food type on larval growth rate, the developmental data for *C. megacephala* larvae grown on liver gave quite accurate age predictions for larvae grown on heart tissue[36]. Unfortunately, performance of the predictive model is usually unknown, the importance of the factor may still be evaluated based on its effect size on the relevant developmental parameter. Summarizing, none of the above suggestions is supported by the empirically demonstrated gain in the accuracy of insect age estimates.

Here we test the concept that insect size at emergence is a good predictor for development time and accordingly it may be used to improve the accuracy of insect age estimates in forensic entomology. A correlation between size at maturity and development time is widespread in insects[37,38]. Among herbivorous or predatory insects negative correlation was reported with high regularity, whereas the positive correlation was limited to parasitoids[38]. It is therefore reasonable to assume that insect size at emergence may be used to estimate its age and here this assumption is tested with forensically useful beetle *C. maxillosus*. We predict that 1) thermal summation value (*K*) needed by individual beetles of *C. maxillosus* to reach the adult stage is related to the size of beetles at emergence, 2) using the insect size to estimate *K* may significantly improve the accuracy of insect age estimates in forensic entomology, and 3) the relationship between insect size and *K* needed for emergence will be represented with higher accuracy by separate models for males and females than the pooled model. To test these predictions we used results of developmental experiment, in which immature *C. maxillosus* were reared using standardized laboratory protocol, at different constant temperatures and optimal food conditions, with monitoring of development time and determination of insect size and sex at emergence.

C. maxillosus is a predatory beetle regularly visiting and breeding in large vertebrate cadavers including humans[39–43]. Forensically useful developmental models were recently derived for this species[10,24]. Robust methods for classifying larval instars were developed as well[44]. It was also demonstrated that an interval preceding appearance of adult or larval stages of *C. maxillosus* on cadavers (i.e. the pre-appearance interval, PAI) is strongly related to temperature[45] and may be accurately estimated using temperature methods for PAI[46,47]. Accordingly, *C. maxillosus* may be regarded as useful for PMI estimation using entomological methods. As for the topic of this article, previous studies revealed substantial variation in size of adult *C. maxillosus*[48] and in the duration of third larval and pupal developmental stages[10,24].

Results

Relationship between physiological age and size at emergence in *C. maxillosus*. Thermal summation values (*K*) needed for emergence of *C. maxillosus* fell with an increase of beetle size at emergence (Figs 1, 2, Table 1). Depending on the model, beetle size explained from 6% to 28% of variation in *K* needed to reach the adult stage (Table 1). The relationship between *K* and insect size was represented with larger accuracy by models

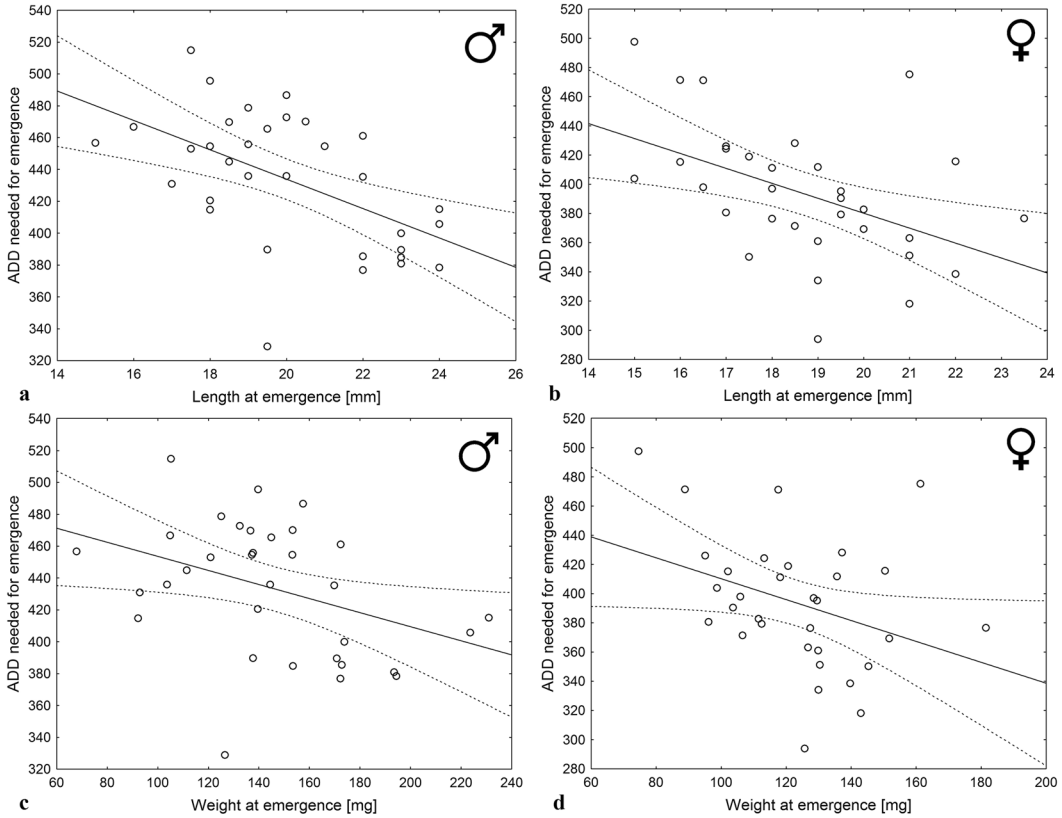

Figure 2. The relationship between adult *C. maxillosus* length (**a**,**b**) or weight (**c**,**d**) at emergence in male (**a**,**c**) or female (**b**,**d**) sample and thermal summation values needed for emergence (ADD over 11.43 °C for males and over 11.81 °C for females). Solid line – linear regression model, dotted lines – confidence limits.

Model	Response variable (*y*)	Predictor variable (*x*)	Model	*N*	*F*	*P*	r^2
Pooled	ADD over 11.58	Length	$y = 564.31 - 7.722*x$	65	12.08	0.0009	0.16
	ADD over 11.58	Weight	$y = 461.17 - 0.3456*x$	65	4.03	0.049	0.06
Male-specific	ADD over 11.43	Length	$y = 618.33 - 9.223*x$	33	12.34	0.0014	0.28
	ADD over 11.43	Weight	$y = 497.84 - 0.4423*x$	33	5.37	0.027	0.15
Female-specific	ADD over 11.81	Length	$y = 584.76 - 10.23*x$	32	8.41	0.0069	0.22
	ADD over 11.81	Weight	$y = 481.85 - 0.7165*x$	32	4.21	0.049	0.12

Table 1. Linear regression models for the relationship between length or weight of adult *C. maxillosus* at emergence and thermal summation values (accumulated degree days, ADD) needed to reach the adult stage

calculated separately for males (Fig. 2a,c) and females (Fig. 2b,d) than models calculated for the pooled sample (Fig. 1). Beetle length at emergence explained more variation in *K* than beetle weight at emergence (Table 1).

Estimation of physiological age based on insect size at emergence in *C. maxillosus*. There were significant differences in the relative error of estimation between methods used to estimate physiological age of *C. maxillosus* at emergence (Friedmann ANOVA, $\chi^2 = 17.33$, $P = 0.0017$, N = 108, 51 males and 57 females; Fig. 3). *K* from the general thermal summation model (i.e. 417.33 accumulated degree days [ADD] over 11.58 °C after [Frątczak-Łagiewska & Matuszewski[24]]) represented true *K* with the average difference of 9% (Fig. 3). Estimation of *K* based on *C. maxillosus* size, gave more accurate representation for the true *K* irrespective of the method used for the estimation (Fig. 3). Using beetle length at emergence as a predictor variable and sex-specific models resulted in the best representation of the true *K* (Fig. 3). While estimating *K* with this method, for the true *K* below 380 ADD, estimates were mostly overestimations, whereas for the true *K* above 440 ADD, estimates were mostly underestimations (Fig. 4). If the true *K* was below 380 ADD, error of estimation increased with the decrease of *K*, and for the true *K* above 440 ADD error increased with the increase of *K* (Fig. 5).

Discussion

As expected, physiological age at emergence of *C. maxillosus* was related to its size. The negative relationship is in line with the pattern revealed in the meta-analysis of Teder *et al.*[38], who demonstrated that it is prevalent in

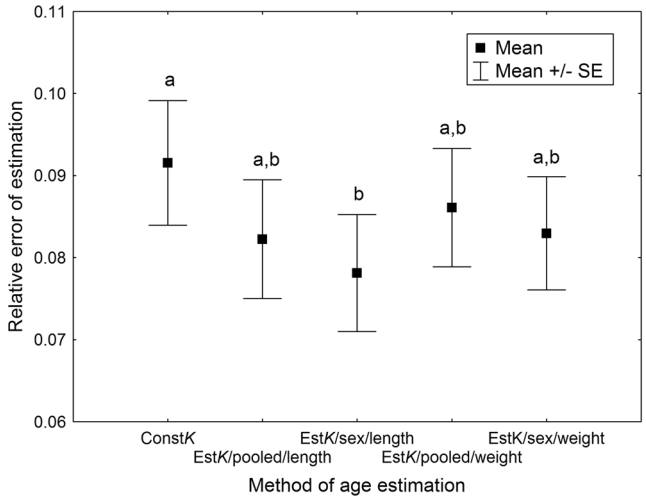

Figure 3. Relative error of *C. maxillosus* physiological age (*K*) at emergence estimation using different methods. Const*K* – the use of constant *K* (from the general thermal summation model, 417.33 ADD over 11.58 °C after Frątczak-Łagiewska & Matuszewski[24]); Est*K*/pooled/length – estimation of *K* using the model for the pooled sample and beetle length at emergence as the predictor variable; Est*K*/sex/length – estimation of *K* using models for females and males and beetle length at emergence as the predictor variable; Est*K*/pooled/weight – estimation of *K* using the model for the pooled sample and beetle weight at emergence as the predictor variable; Est*K*/sex/weight – estimation of *K* using models for females and males and beetle weight at emergence as the predictor variable. Different letters denote significant differences in pairwise comparisons (absolute differences between mean ranks were significant at 5% level of significance, if they were larger than 0.477).

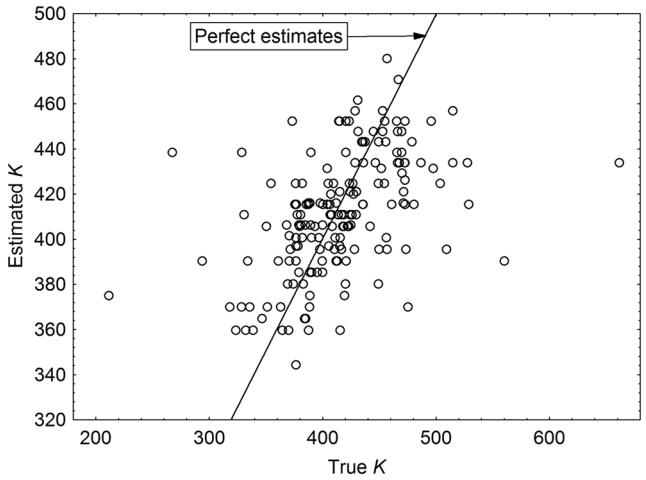

Figure 4. Estimates of physiological age (*K*) at emergence in *C. maxillosus* plotted against the true age. *K* was estimated using sex-specific models regressing *K* and beetle length at emergence. Solid line – hypothetical line representing perfect estimates.

predatory insects and *C. maxillosus* predatory feeding habits has been very well documented[49,50]. While validating our concept, no matter what method was used for the estimation, *K* predicted based on the adult insect size was significantly closer to the true *K* as compared to the *K* from the general thermal summation model. This finding demonstrates that size of *C. maxillosus* at emergence improves accuracy of physiological age estimates for this species. Because many other forensically-useful insects reveal large within-species variation of insect size, e.g. puparia of *L. sericata*[51], adult stages of larder beetle *Dermestes maculatus* (DeGeer, 1774)[52] or a silphid beetle *Necrodes littoralis* (Linnaeus, 1758)[48], the method described here may have much wider applicability. Our results indicate that length of adult *C. maxillosus* is more useful for prediction of age than weight. This finding reflects larger intra and inter individual variation in weight of adult insects.

Usage of the models representing association between *K* and insect size involves several basic concepts related to age and size. One of the most important is the temperature-size rule, the taxonomically widespread pattern of larger body size at lower developmental temperatures which holds for natural populations and laboratory reared ectotherms[53,54]. As a rule ectotherms (e.g. insects) in colder conditions grow slower but are larger at maturity,

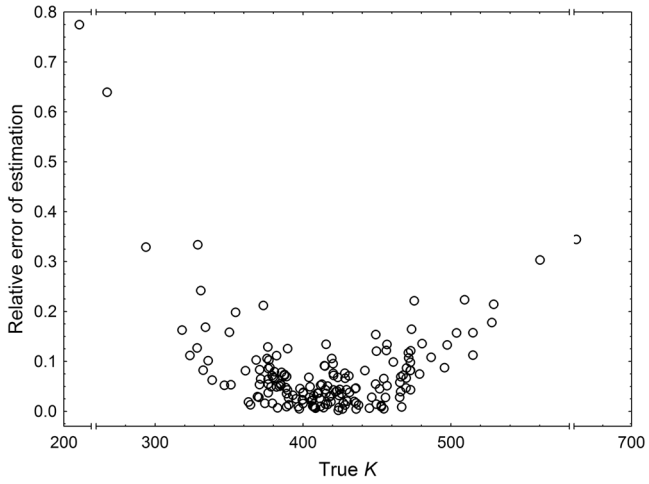

Figure 5. Relative error in estimates of physiological age (*K*) at emergence of *C. maxillosus* plotted against the true age. *K* was estimated using sex-specific models regressing *K* and beetle length at emergence.

whereas at higher temperatures they grow faster but are smaller[55,56]. The former conditions should result in proportionally more time needed for the emergence, whereas the latter conditions should be accompanied with proportionally less time. At lower temperatures one may expect that larger *K* will be associated with larger body size of an insect, and at higher temperatures smaller *K* with smaller body size. Current results contradict such a simple interpretation, as the relationship between *K* and size of *C. maxillosus*, analyzed in the whole temperature range, was clearly negative. Our results are however in line with predictions of optimality models[38,57–59], in case of which optimal conditions are associated with larger size and shorter development and non-optimal conditions with smaller size and longer development. It is thus possible that in the range of optimal temperatures the relationship between *K* and insect size is distinctly negative, whereas inclusion of non-optimal (lower and higher) temperatures may weaken the negative slope of the relationship. This interpretation suggests that the usage of separate models for optimal and non-optimal temperatures might further improve the accuracy of insect age estimates in forensic or other scenarios. To test this possibility further studies are necessary.

Optimality models indicate that poor resources cause insects to emerge at smaller sizes but after longer development and vice-versa at optimal resources. Such tradeoffs between age and size are common in insects[38] and were also reported for several forensically-useful species, with depletion of cadaver tissues or insect overcrowding on cadavers resulting regularly in stunted larvae[31,32]. Although we used uniform diet across the temperatures and therefore did not catched the diet-related variation in *K* and size, we feel that the models derived in the current study are reasonably universal and therefore may be used for prediction irrespective of the specimen feeding history. To justify this assertion further studies are however needed. Similar limitations may be posed by differences in development between local populations which are widespread in insects, forensically useful as well[19–21], and usually have some genetic component. In forensic entomology several approaches may tackle these complications. The most valid but at the same time the most laborious would be to diversify empirical foundations for the models, for instance by using insects from different populations, reared at different temperatures and various diets. Another solution is to test current and future models in prediction tasks with insects from different populations (with different geographic origins or different diets). While recently there has been substantial progress in forensic entomology, particularly in understanding foundations of the discipline[60], many of the findings have not translated into forensically useful techniques and we feel that there are still large areas of the field where substantial progress is needed and possible.

Some authors suggested that insect age estimates may be more accurate through the use of sex-specific developmental data[22]. Recent study revealed however no gain in the accuracy of age estimates while using separate thermal summation models for females and males[24]. Current results demonstrate that insect sex may be useful, yet it needs to be analyzed in conjunction with size. Different relationship between *K* and size in the case of males and females as well as the larger increase in the accuracy of *K* estimates while using sex-specific models indicate that sex may be regarded as useful co-predictor of insect age, at least in the case of *C. maxillosus* and in a forensic context.

Although there is a tendency in applied entomology to consider *K* as the species-specific constant, a large body of data contradicts this view. Current results support the notion of *K* as a characteristic of high intraspecific variability. Accordingly, we postulate that *K* should be predicted for an insect evidence and forensic entomologists should develop models useful for this purpose. Sex-specific models regressing *K* and insect length at emergence significantly improved the accuracy of age estimates in the case of *C. maxillosus*, however there is still the need to search for other traits useful for *K* prediction.

In order to implement the method, live immature beetles should be sampled at a crime scene and reared to the adult stage in the laboratory (Fig. 6). At emergence insect length should be measured and sex determined. They may be used to estimate *K* for the crime scene beetles using models from the current article. Then, estimated *K* should be used to approximate insect chronological age at the moment of sampling (and eventually minimum

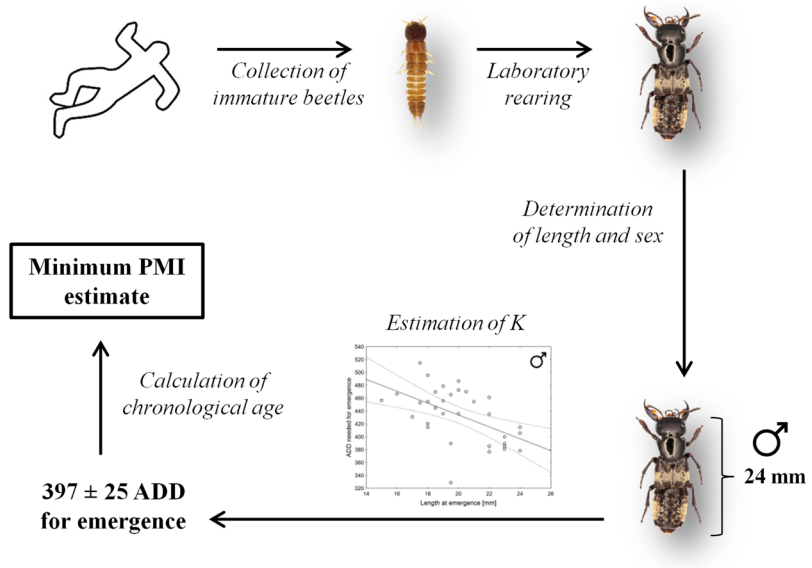

Figure 6. A schematic representation for the implementation of the method in forensic entomology. Anna Mądra-Bielewicz is the copyright holder of the *C. maxillosus* pictures used in this figure.

PMI) using protocols developed in forensic entomology. Because the method needs live specimens, it may be combined with the approach for aging insects developed by Marchenko[61].

Self-critique. *Strength of the relationship between physiological age and size of C. maxillosus.* R^2 derived for the best models indicate that for female *C. maxillosus* 22% of variation in *K* may be explained by variation in adult insect size, whereas in the case of male beetles it is 28%. Although for both sexes about a quarter of variation in *K* was due to the adult insect size, the models may and should be refined by future studies. Despite their weaknesses, in the validation part of the study the models outperformed the current routine of using constant *K*. This is the most important finding of the study. Current results demonstrate that simple models for physiological age and insect physical traits (here size) may significantly improve accuracy of insect age estimates in forensic or more general applied entomology.

The gain in the accuracy of K representation resulting from its estimation based on the adult insect size. Although estimation of *K* using our best model gave significantly more accurate representation for the true *K* as compared to the constant *K* from the thermal summation model, the gain in the accuracy was rather minor. The thermal summation constant represented the true K with the average error of 9.2% (Fig. 3), e.g. for the *K* of 417 ADD it gives an error of 38.4 ADD over 11,58 °C (e.g. about 6 days at average temperature of 18 °C). *K* estimated using adult insect length as the predictor variable and sex-specific models represented the true *K* with the average error of 7.8% (Fig. 3), e.g. for the *K* of 417 ADD it gives an error of about 32.5 ADD over 11,43 °C for a male beetle (e.g. about 5 days at average temperature of 18 °C). The gain of about 6 ADD (i.e. about a day at temperature of 18 °C) is not much (at 18 °C 417 ADD will accumulate over 11.58 °C after about 65 days). We think that this minor improvement was a result of large similarity between training and validation samples of insects used in the current study. Both samples originated from the same laboratory experiment and not surprisingly the baseline accuracy (the one associated with *K* from the general thermal summation model) was high leaving little area for improvement. We feel that validating our models with a more diverse insect sample (e.g. beetles with the diverse nutritional history) would reveal a larger gain in the accuracy of *K* estimates. Current method could be refined also by including other physical traits alongside insect size at maturity. This article therefore should be treated as the first step to build a multi-factor model for the estimation of *K*.

Materials and Methods

Rearing procedures. A colony of adult *C. maxillosus* was maintained in the laboratory at room temperature and humidity (20–22 °C, 50–60%). Insects were kept in plastic containers (30,4 × 20 × 20,1 cm) on a damp soil and were fed with puparia or third instar larvae of blowflies. Colonies were established in spring of 2015 and 2016 using beetles sampled from rabbit carcasses exposed in the Biedrusko military range (Western Poland, Europe; 52 31'N, 16 55'E). Throughout the study the colony consisted of 25–30 beetles, new beetles sampled in the field or reared in the laboratory were used to reinstate the colony. Between 10 and 20 new beetles were added to the colony per month. To minimize effect of laboratory inbreeding, we used eggs from field-captured insects or the first laboratory generation.

Immature beetles were reared individually (80 ml containers with 1,5 cm of soil for the 1st and the 2nd instar larvae, 120 ml containers with 5 cm of soil for the 3rd instar larvae and pupae) in temperature chambers (ST 1/1 BASIC or +, POL-EKO, Poland) at constant temperature and humidity (15, 17.5, 20, 22.5, 25, 27.5 and 30 °C; air humidity: 60–70%; photoperiod (h): 12:12 (L:D)). Individual rearing conditions are close to the natural

conditions for this species and make it possible to monitor individual insects throughout their development. Due to the difficulties in sampling eggs of *C. maxillosus*[24] and their high mortality in the laboratory conditions[10], beetles were kept in separate containers from the onset of larval stage. In order to obtain eggs the entire adult colony was transferred into a three liter container with soil and kept at 20–22 °C for four hours. Afterwards, containers (with no adult insects inside) were placed in incubators with the given temperature and were inspected for the presence of first instar larvae every 10% of the average egg stage duration (inspections started after 70% of the average egg stage duration). Forty freshly hatched larvae were used per temperature. Two or three temperatures were studied simultaneously with random assignment of insects to temperatures. Larvae were fed once a day with third instar larvae of blowflies.

Transitions between developmental stages (i.e. hatching, first ecdysis, second ecdysis, pupation and adult emergence) were monitored in all insects at intervals equal to 10% of the average stage duration. Half of the beetles were measured and weighed throughout larval and pupal development. At emergence beetle sex was identified based on the shape of the eighth abdominal sternite. Adult beetle length (from the anterior margin of the clypeus to the posterior margin of the last abdominal segment) was measured *in vivo* using geometrical micrometer[62] after beetle became fully erect in a 1.5 ml eppendorf tube. Weight of adult beetles was measured *in vivo* in an eppendorf tube using analytical balance (AS 82/220.R2, Radwag, Poland).

Data analyses. Base temperature (the temperature below which development stops[16]) for the total immature development in the case of the male-specific model is 11.43 °C, in the case of the female-specific model it is 11.81 °C and in the case of the pooled sample model it is 11.58 °C[24]. Thermal summation values (*K*) needed to reach the adult stage were calculated over these temperatures for individual beetles and then were regressed against adult beetle length or weight at emergence using linear regression. Insect length or weight at maturity were used as predictor variables and *K* as a response variable. Growth of insects slows (and eventually stops) after a larva surpasses a critical weight and several molecular mechanisms are responsible for insect size assessment and control at critical weight[63,64]. Therefore, the cessation of growth (and eventually *K*) depends on the insect size and not vice-versa. Regression analyses were used to test whether *K* and size are related to each other and what is the effect size of insect length or weight on *K*. Because we predicted that the relationship between *K* and size will be closer when analyzed separately for males and females as compared to the pooled sample, regression analyses were performed for the female sample, the male sample and the pooled sample. Models for females and males were based on the sample consisting of five randomly chosen males or females per temperature (in total 33 males and 32 females; at 15 °C there were just 2 females, at 30 °C there were just 3 males). Models for the pooled sample were based on 65 insects. Rest of the specimens (i.e. 51 males and 57 females) were used to test the concept (due to high mortality, 15 and 30 °C were underrepresented in the validation sample). The current regression models were used to predict *K* needed for emergence based on the length or weight at emergence of insects used in the validation. Then, estimated *K* were compared to the true *K*, and resultant error rates were analyzed across methods using the Friedman ANOVA. Analyses were made using Statistica 12 (Dell, Inc., 2013) at 5% level of significance.

Data availability. The datasets generated and/or analyzed during the study are available from the corresponding author on a reasonable request.

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Acknowledgements

We thank A. Mądra-Bielewicz (Poznań, Poland) for help in laboratory work and photographs of *C. maxillosus* used in Fig. 6, M. Nawrocka (Poznań, Poland) for help in field work and O. Łagiewski (Pobiedziska, Poland) for the Visual Basic scripts used in the analyses. The study was funded by the Ministry of Science and Higher Education (grant no. DI2013011043). Thanks are also extended to anonymous reviewers whose comments helped us to improve the manuscript.

Author Contributions

S.M. developed the concept for the study, analyzed the data and wrote the manuscript. K.F. conducted experiments, prepared raw data for analyses and participated in writing the manuscript. Both authors discussed the results and reviewed the manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Forensic Science International 298 (2019) 316–322

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

The quality of developmental reference data in forensic entomology: Detrimental effects of multiple, *in vivo* measurements in *Creophilus maxillosus* L. (Coleoptera: Staphylinidae)

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ARTICLE INFO

Article history:
Received 10 December 2018
Received in revised form 26 February 2019
Accepted 28 February 2019
Available online 9 March 2019

Keywords:
Forensic entomology
Post-mortem interval
Insect development
Laboratory protocols
Creophilus maxillosus

ABSTRACT

Although *in vivo* measurements of larval insects are often performed during developmental studies of necrophilous beetles, their impact on development has not been studied. During measurements insects are taken out of the incubator for a few minutes at room temperature, which may affect the development and eventually the quality of the developmental reference data. Additionally, while being measured larvae are under stress which may have an effect on their development. We conducted an experiment using predatory beetle species *Creophilus maxillosus* L. (Coleoptera: Staphylinidae) which often occurs and breeds on large vertebrate carcasses. We tested the hypothesis that multiple, *in vivo* measurements affect the development of *C. maxillosus* by increasing its duration and changing adult insect size at emergence. As a consequence, we predicted that the multiple insect measurement protocol will affect the accuracy of age estimates using the resultant reference developmental data. Development of *C. maxillosus* was studied at 7 constant temperatures. All individuals were inspected for developmental landmarks; half of them were also repeatedly measured and weighed. Measured beetles developed longer than non-measured beetles (e.g. 1.59 days longer at 22.5 °C) and at emergence were distinctly smaller (e.g. 1.5 mm shorter and 22 mg lighter at 22.5 °C). The accuracy of age estimates was greater while using the model for non-measured beetles. These results support the claim that multiple *in vivo* measurements of insects reduce the quality of resultant developmental data. The measurements were particularly detrimental for the adult insect size. Consequently, particular attention should be paid to isomegalen diagrams which are based on insect length. Our findings indicate that these diagrams, when based on multiple, *in vivo* measurements of larval beetles, will systematically overestimate their age.

1. Introduction

Forensic entomology is the branch of forensic science studying necrophilous insects usually in terms of their usefulness for estimating post-mortem interval (PMI) [1,2]. While estimating PMI entomologists usually focus on the age of immature insects sampled on a death scene [3–5]. Age of an insect is the time that elapsed between oviposition (or larviposition) and collection of the insect. Estimated insect age reflects the minimum time that has passed from death until the discovery of the cadaver, which is called the minimum PMI [3,6,7]. Both flies and beetles may be used for the estimation of minPMI based on their life cycles [2]. Although new genetic [8,9], chemical [10,11] and morphological [12,13] aging techniques are being developed, the ones which are still used most frequently are classical techniques based on developmental stage or larval size. Several larval size indicators have been proposed (e.g. weight or width), length is however still the most popular, due to its higher resolution compared to the other indicators [14]. Larval length may be used for the estimation of age by means of isomegalen diagrams [15], larval growth curves [16] or non-linear models [17–19].

Developmental data used by forensic entomologists are collected during experiments, which differ according to experimental design, insect rearing protocols, insect strains or the kind of data collected and the way of its interpretation or presentation. As a consequence, the data may differ in quality. In general, high quality data should accurately represent temporal patterns of

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https://doi.org/10.1016/j.forsciint.2019.02.059
0379-0738/

insect development on cadavers in natural conditions. Many factors are known to influence the growth rates of insects, e. g. food substrate [20,21] or larval density [22,23]. Having an impact on insect development, they may also affect the quality of the laboratory-derived developmental data and eventually the accuracy of the minPMI estimated using entomological techniques [5,24,25]. For example, in many cases larvae collected on a death scene were developing in large masses, whereas the majority of laboratory developmental data were obtained while rearing insects in small masses. In large aggregates insects experience stable temperature conditions and may use food more efficiently, developing faster and reaching larger sizes [26,27]. Several other factors related to the design of laboratory studies or insect rearing protocols may similarly lower the quality of developmental data, e.g. differences between temperatures experienced by insects and nominal chamber temperatures [28], small size of insect samples, small number of temperatures or low frequency of sampling [29].

A factor of possible importance for the quality of forensic developmental data is the protocol for measurement of larval insects. In case of flies, measurements of age indicators are taken on dead larvae preserved in an alcohol [24,30]. Necrophilous beetle larvae were measured after killing using an alcohol [31,32] or *in vivo* [16,33–36]. Unfortunately, there is no standard protocol for *in vivo* measurements of beetle larvae. We have also little knowledge on the influence of such measurements on the quality of resultant developmental data.

Midgley and Villet [37] while studying killing methods of beetle larvae, found that the methods may change insect specimens and for this reason they recommended *in vivo* measurements of beetle larvae in developmental studies. Such measurements are also useful for longitudinal monitoring of insect development. The protocol generates the full data set for each insect, enabling more sophisticated analyses. Moreover, laboratory experiments may be completed with smaller number of insects. For these reasons *in vivo* measurements have frequently been used in forensic studies of beetle development [16,33–36]. Some reasons however suggest that the protocol of *in vivo* measurements may impact the developmental pattern or rate. First, insects are taken out of the incubator at room temperature for a few minutes which may change the thermal profile of their development. Second, during the measurement larvae are very active and certainly under some stress which may have an effect on their development. Third, beetles are measured many times between hatching and pupation so the accumulation of the above effects may substantially change their size, mortality or development time. In this study these potential effects were tested in case of the predatory staphylinid *Creophilus maxillosus* L. (Coleoptera: Staphylinidae), which frequently visits and breeds in large vertebrate cadavers including humans [38–40]. In all forensic developmental studies of this species beetles were repeatedly measured *in vivo* [16,35,36]. The predictions for this study were as follows: (1) multiple *in vivo* measurements of a larva lengthen total developmental time; (2) the measurements affect the size of resultant adult beetle; (3) the protocol for multiple *in vivo* measurements diminishes the quality of developmental reference data.

2. Materials and methods

2.1. Collection of adult beetles and laboratory protocol

Approximately 50 adult beetles were collected manually from rabbit carcasses placed in a xerothermic grassland of the Biedrusko military range (Western Poland, Europe) during spring and summer of 2015 and 2016. New beetles sampled in the field and individuals bred in the laboratory were added to the main colony. Containers were kept at room temperature and humidity (20–22 °C, 50–60%). Beetles were fed once a day with blowfly pupae or third instar larvae.

In order to obtain eggs, adult insects from the main colony were put into a 3-l container filled halfway with soil (temperature 20–22 °C). After 4 h, adult beetles were removed and containers were placed inside an incubator (ST 1/1 BASIC or +, POL-EKO, Poland) at predefined temperature (7 constant temperatures: 15, 17.5, 20, 22.5, 25, 27.5, and 30 °C). After 70% of the average egg stage duration, containers were inspected for the presence of freshly hatched first instar larvae, at intervals equal to the 10% of the average egg stage duration. Larvae were sampled and transferred to separate cups. Forty larvae were usually used per temperature (32 at 17.5 °C). Larvae were fed once a day with third instar larvae of blowflies. Relative air humidity in incubators was maintained at 60–70%, and the photoperiod (h) was set on 12:12 (L/D).

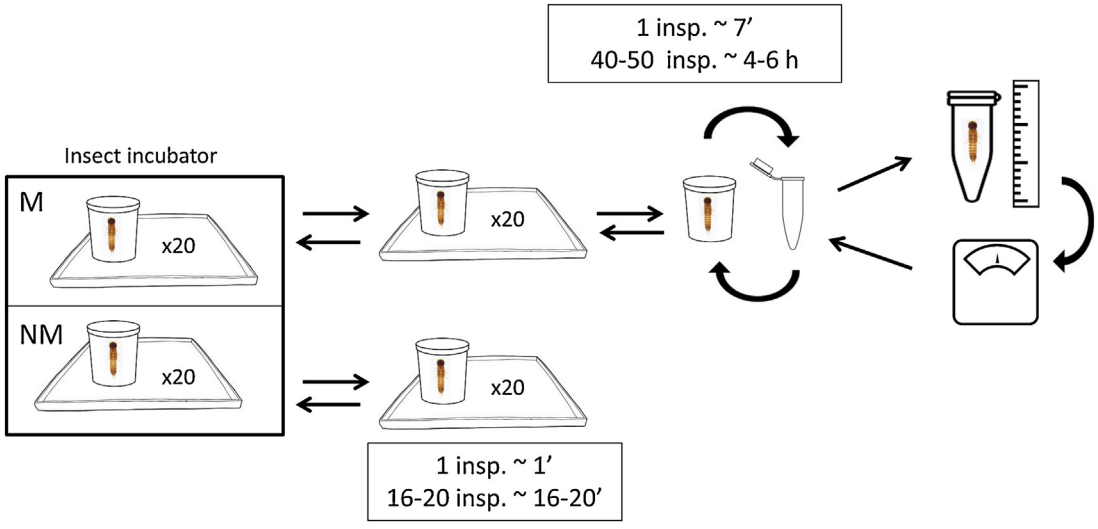

Fig. 1. Diagram showing the experiment's protocol; M – measured beetles, NM – non-measured beetles. Measured insects were removed from the insect incubator, each individual was placed in an Eppendorf tube and its length was measured using a geometric micrometer. Then each individual was weighed and it was transferred back to the container. Measurement of a single larva lasted about 20 s, so during single inspection beetles stayed outside of an incubator for about 7 min. Each insect was measured 40–50 times during its whole development, which gives a total of about 4–6 h outside of an incubator. In the case of non-measured beetles about 4–5 inspections were made in each stage, each about 1 min long. Therefore, non-measured individuals stayed outside of an incubator for 16-20 min during their whole development.

2.2. Inspections and measurements of larvae and pupae

All beetles were inspected for the following developmental landmarks: hatching, first and second ecdysis, pupation and adult emergence. Ecdyses were determined based on the color of a larva (creamy-white shortly after ecdysis) and the width of the mesonotum [41].

Non-measured individuals were inspected after 60% of the average duration of a stage, at frequency of 10% of the stage duration. Containers were taken out of the incubator and beetles were inspected for their developmental stage (Fig. 1). In each stage about 4–5 inspections were made, each about 1 min long. Therefore, non-measured individuals stayed outside of an incubator for 16–20 min during their whole development.

Measured individuals (chosen at random) were inspected as above and in parallel were repeatedly measured and weighed. Containers were taken out of an incubator, each larva (or pupa) was transferred into the 1.5-ml Eppendorf tube and after it had become immobile and fully erect, its length (from clypeus to the last abdominal segment) was measured with a geometrical micrometer described by Villet [42]. Then, the specimen was weighed using an analytical balance AS 82/220.R2 (Radwag, Poland) and it was transferred back to the container (Fig. 1). Measurement of a single specimen lasted about 20 s, so during single inspection beetles stayed outside of an incubator for about 7 minutes. Each insect was measured 40–50 times during its whole development, which gives a total of about 4–6 h outside of an incubator.

2.3. Statistical analyses

Significance of differences between measured and non-measured insects in length and weight at emergence was evaluated using the t test for independent samples. Thermal summation models for the total immature development were developed separately for measured and non-measured beetles using the equation proposed by Ikemoto and Takai [43]. Twelve insects per temperature (6 measured and 6 non-measured) were randomly selected to be used for the modeling purposes. In total 40 specimens were used to create each model.

Models for measured and non-measured insects were validated with the rest of non-measured beetles (i.e. 42 individuals) in the age estimation task. Due to the large mortality at extreme temperatures, 15 and 30 °C were poorly represented in the validation sample. Validation consisted of comparison of the true thermal units needed to reach the adult stage (calculated for each specimen over relevant

Table 1
Differences in the duration of development between measured and non-measured specimens of *C. maxillosus* at different temperatures and for different stages. Statistically significant differences are bolded.

Temperature (°C)	Developmental stage	Mean duration (days) (SD; N)		t	P
		Measured	Non-measured		
15	Egg	8.45 (0.32; 18)	8.39 (1.18; 18)	0.61	0.55
	1st instar larva	5.22 (0.86; 18)	5.03 (0.45; 18)	0.83	0.41
	2nd instar larva	5.45 (0.54; 18)	5.00 (0.44; 18)	2.70	0.01
	3rd instar larva	83.77 (12.6; 8)	77.7 (10,51; 4)	0.83	0.43
	Pupa	25.66 (1.76; 8)	26.15 (3.59; 4)	−0.32	0.75
	Total	128.89 (12.48; 8)	122.21 (13.26; 4)	0.86	0.41
17.5	Egg	6.00 (0.44; 15)	5.8 (0.37; 12)	1.45	0.16
	1st instar larva	4.26 (0.64; 15)	3.89 (0.29; 12)	1.85	0.08
	2nd instar larva	4.33 (0.48; 15)	4.45 (0.42; 12)	−0.72	0.48
	3rd instar larva	32.87 (11.26; 12)	36.62 (-0.89; 9)	−0.89	0.38
	Pupa	18.84 (1.04; 11)	19.12 (1.57; 8)	−0.45	0.66
	Total	66.16 (12.04; 11)	70.18 (7.37; 8)	−0.83	0.41
20	Egg	4.28 (0.16; 20)	4.29 (0.15; 20)	−0.29	0.77
	1st instar larva	**2.85 (0.26; 20)**	**2.69 (0.22; 20)**	**2.10**	**0.04**
	2nd instar larva	3.12 (0.26; 20)	3.1 (0.35; 20)	0.27	0.79
	3rd instar larva	19.39 (2.64; 16)	18.42 (2.19; 19)	1.19	0.24
	Pupa	15.87 (0.7; 15)	15.56 (0.88; 17)	1.11	0.28
	Total	45.33 (2.81; 15)	43.97 (2.28; 17)	1.51	0.14
22.5	Egg	3.39 (0.15; 20)	3.43 (0.15; 18)	−0.57	0.57
	1st instar larva	2.34 (0.27; 20)	2.29 (0.3; 18)	0.47	0.64
	2nd instar larva	2.61 (0.21; 20)	2.46 (0.34; 18)	1.64	0.11
	3rd instar larva	18.82 (2.84; 19)	17.66 (2.4; 17)	1.32	0.2
	Pupa	12.27 (0.7; 17)	12.38 (0.75; 13)	−0.42	0.68
	Total	39.22 (2.87; 17)	37.63 (2.09; 13)	1.68	0.1
25	Egg	2.8 (0.1; 19)	2.83 (0.1; 20)	−0.96	0.34
	1st instar larva	2.06 (0.26; 19	1.91 (0.3; 20)	1.63	0.11
	2nd instar larva	2.32 (0.17; 19)	2.26 (0.15; 20)	0.99	0.33
	3rd instar larva	14.86 (1.58; 19)	13.84 (1.72; 20)	1.92	0.06
	Pupa	**10.12 (0.48; 18)**	**9.59 (0.72; 18)**	**2.61**	**0.01**
	Total	**32.32 (1.76; 18)**	**30.44 (1.98; 18)**	**3.00**	**0.00**
27.5	Egg	2.58 (0.12; 20)	2.57 (0.13; 20)	0.35	0.73
	1st instar larva	**1.79 (0.28; 20)**	**1.62 (0.12; 20)**	**2.47**	**0.02**
	2nd instar larva	**2.26 (0.3; 20)**	**2.06 (0.21; 20)**	**2.44**	**0.02**
	3rd instar larva	14.51 (2.41; 17)	13.52 (2.08; 18)	1.31	0.2
	Pupa	8.56 (0.99; 14)	8.83 (3.15; 13)	−0.31	0.76
	Total	29.27 (2.38; 14)	27.64 (1.73; 13)	2.02	0.05
30	Egg	2.17 (0.13; 19)	2.2 (0.13; 17)	−0.7	0.49
	1st instar larva	**1.58 (0.16; 19)**	**1.35 (0.1; 17)**	**5.07**	**0.00**
	2nd instar larva	1.8 (0.2; 19)	1.69 (0.15; 17)	1.92	0.06
	3rd instar larva	11.18 (1.76; 15)	10.56 (1.87; 16)	0.95	0.35
	Pupa	7.24 (0.64; 5)	7.51 (0.46; 8)	−0.88	0.4
	Total	23.27 (1.67; 5)	21.96 (1.21; 8)	1.64	0.13

Table 2
Differences between measured and non-measured specimens of *C. maxillosus* in length and weight at emergence. Statistically significant differences are bolded.

Insect size	Temperature (°C)	Mean (SD; N)		t	P
		Measured	Non-measured		
Length (mm)	15	19.69 (1.85; 8)	21.25 (2.5; 4)	−1.23	0.25
	17.5	20.91 (1.32; 11)	21.00 (1.54; 8)	−0.14	0.89
	20	**20.9 (1.3; 15)**	**22.44 (1.16; 17)**	**−3.55**	**0.00**
	22.5	**19.65 (1.46; 17)**	**21.15 (1.55; 13)**	**−2.74**	**0.01**
	25	18.11 (1.09; 18)	17.92 (1.00; 18)	0.56	0.58
	27.5	17.61 (1.68; 14)	17.69 (1.70; 13)	−0.13	0.9
	30	17.5 (1.32; 5)	18.06 (1.18; 8)	−0.8	0.44
Weight (mg)	15	147.37 (13.73; 8)	175.1 (39.65; 4)	−1.84	0.1
	17.5	156.20 (20.02; 11)	151.5 (14.6; 8)	0.56	0.58
	20	**140.73 (16.02; 15)**	**170.3 (23.97; 17)**	**−4.04**	**0.00**
	22.5	**127.72 (16.3; 17)**	**149.73 (18.27; 13)**	**−3.48**	**0.00**
	25	124.93 (11.28; 18)	123.14 (10.5; 18)	0.49	0.63
	27.5	108.14 (19.14; 14)	115.77 (21.15; 13)	−0.98	0.33
	30	94.56 (5.54; 5)	99.59 (11.72; 8)	−0.89	0.39

developmental threshold) against thermal constants from the models. The accuracy with which models represented the true thermal units needed for the insect emergence was compared using the t test for dependent samples. All analyses were performed using Statistica 12 (StatSoft, Inc., 2014).

3. Results and discussion

3.1. Differences in development between measured and non-measured beetles

Measured individuals of *C. maxillosus* developed longer than non-measured beetles (Table 1). At the adult stage the differences were for some temperatures statistically significant or close to significant and of moderate size (at 20 °C – 1.36 days, at 22.5 °C – 1.59 days, at 25 °C – 1.92 days, the largest difference was 6.2% of the average development time for the non-measured beetles, Table 1). Although there are factors of larger effect on the development time and consequently accuracy of insect age estimation (e.g. crime scene temperatures and the accuracy of their reconstruction [44]), we think that small but systematic gains in the accuracy are worthwhile. Moreover, current differences concern the protocol for developmental studies in forensic entomology indicating the need for its change. If this change will be implemented and detrimental effects of multiple, *in vivo* measurements eliminated or reduced, resultant improvement in the accuracy of insect age estimation will be systematically present in each case.

Because the differences followed the same pattern at most temperatures (i.e. measured beetles developed longer, 17.5 °C was the only exception), we assumed that they were not related to the change in thermal profile of developing insects (as a result of multiple, short-time stays at room temperature) but were rather the consequence of repeated stress. Similar effect on the development has for example stress induced by cuticular wounding [45]. Exposure of insects to external stressors is known to affect their development by disrupting physiological homeostasis [46,47]. It increases level of the juvenile hormone which may prolong development. Once exposure to stressors is ceased, the juvenile hormone level decreases and normal development is continued [46].

At emergence, non-measured beetles were distinctly larger than measured beetles (e.g. at 20 °C – on average 1.5 mm longer and 30 mg heavier, and at 22.5 °C – on average 1.5 mm longer and 22 mg heavier) with statistically significant differences at 20 and 22.5 °C (Table 2). The largest difference was 7.6% (at 22.5 °C) of the average length and 21% (at 20 °C) of the average weight for measured beetles. At higher temperatures (25 °C, 27.5 °C, 30 °C) measured and non-measured beetles revealed no significant difference in size (Table 2) probably due to the generally smaller sizes of insects at high temperatures [48,49]. Because the size of an adult insect is already established at the onset of the pupal stage, we assume that the differences in size between measured and non-measured beetles appeared during the larval stage.

3.2. Developmental models of C. maxillosus

All temperature points were included while calculating model parameters (Fig. 2). Models have the same optimal temperature

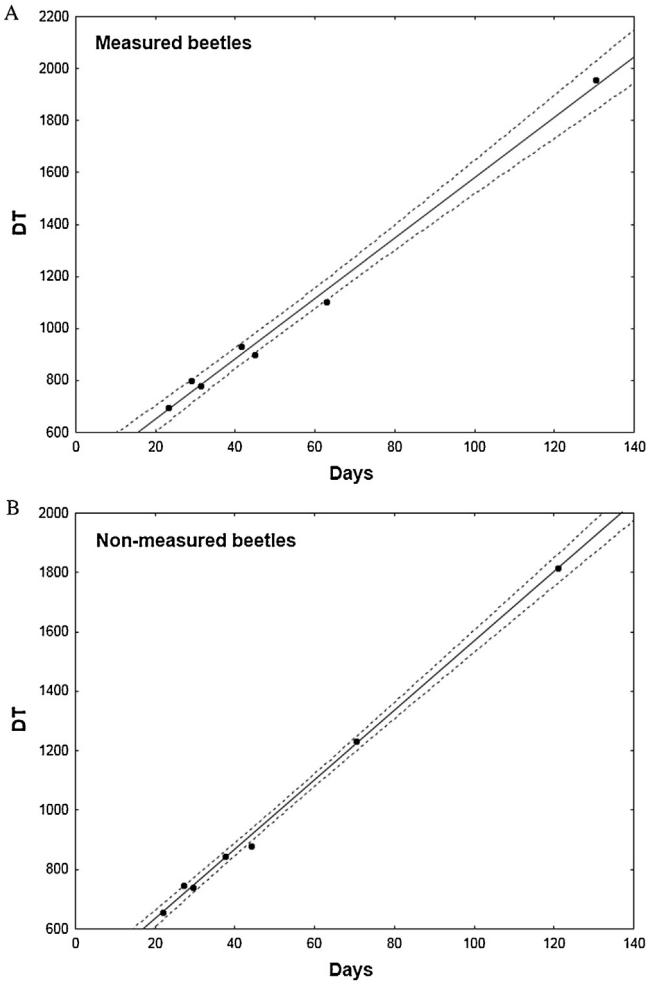

Fig. 2. Thermal summation models for the total immature development of measured (A) and non-measured (B) *C. maxillosus*.

range, similar developmental thresholds, but different thermal constant K, which was larger (by about 19 ADD, i.e. 4.6% of the K for non-measured beetles) in the case of the model for measured beetles (Table 3). The accuracy of age estimates was larger while using the model for non-measured beetles, however the differences did not reach significance probably due to their small size and moderate size of the validation sample (t test for correlated samples; t = 1,43, P = 0,16, N = 42, Figs. 3 and 4). The small size of these differences was a consequence of high similarity between beetles used for modelling and validating purposes. It may be assumed that the differences would be larger, if we had validated the models with a more diverse samples of insects, e.g. individuals sampled in the field or insects from another colony.

3.3. *Multiple* in vivo *measurements and the protocol for developmental studies in forensic entomology*

Our results support the claim that multiple *in vivo* measurements of insects reduce the quality of resultant developmental data. Because the differences in time of development between measured and non-measured beetles are already apparent at the larval stage (Table 1), the *in vivo* measurement protocol will reduce the quality of isomorphen diagrams and thermal summation models which are based on the stage duration times. Therefore, we recommend that these diagrams and models should be based on developmental data for non-measured beetles.

Detrimental effects of measurements for developmental time were however not as large as their effects for insect size (Table 2). Consequently, particular attention should be paid to isomegalen diagrams which are based on insect length. Our findings indicate that isomegalen diagrams based on multiple, *in vivo* measurements of larval beetles will systematically overestimate larval beetle age. Although the overestimation will not be very large, its elimination will improve the accuracy of age estimates in each case. Several solutions are possible. First, measurements of larval length may be abandoned at all. As a

Table 3
Thermal summation models for the total immature development of *C. maxillosus*.

Model	Temperature range (°C)	Thermal summation constant-K (SE) (days °C)	Developmental threshold-D_0 (SE) (°C)	r^2	N	P
Measured	15–30	420.84 (26.57)	11.61 (0.43)	0.993	7	<0.000
Non-measured	15–30	402.24 (14.72)	11.66 (0.25)	0.998	7	<0.000

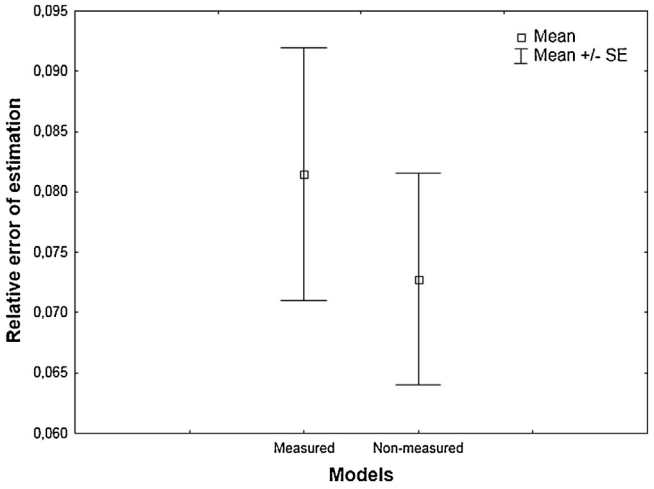

Fig. 3. Relative error in age estimation of non-measured *C. maxillosus* at emergence using models for measured and non-measured insects.

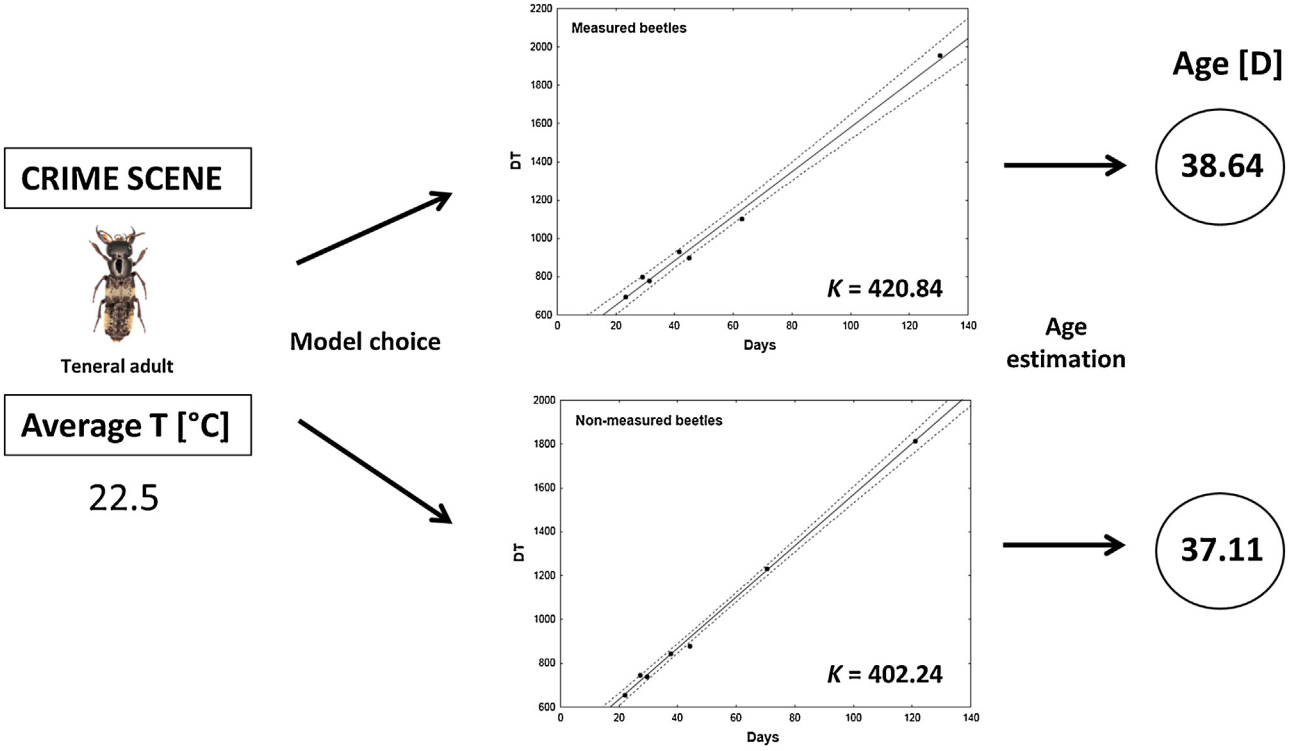

Fig. 4. The example of age estimation in *C. maxillosus* using models for measured and non-measured insects.

consequence, it would not be possible to develop isomegalen diagrams, and age of insects would have to be estimated using thermal summation models or isomorphen diagrams only. Second, insects could be measured less frequently. Although resolution of developmental data would be lower (for possible effects see [29]), the less frequent exposure to measurement-derived stress might diminish the detrimental effects of measurements. Third, a larger number of insects used in an experiment would allow for random selection of individuals for measurements, e.g. at each measurement point only 10% of insects would be measured. As a consequence, developmental data of high resolution would be collected but each insect would be measured less frequently and the protocol would not generate detrimental effects of *in vivo* measurements. Fourth, age estimates based on developmental models for measured insects could be corrected for the error resulting from *in vivo* measurement protocol. Finally, one might use the laboratory protocol similar to the one used for flies, i.e. measurements of killed insects and therefore maintenance of large insect colonies during the studies. Unfortunately, *C. maxillosus* and other predatory beetles which breed in carrion are difficult to rear in the laboratory, so the establishment of large laboratory colonies is very difficult for such insects.

CRediT authorship contribution statement

K. Frątczak-Łagiewska: Conceptualization, Investigation, Data curation, Formal analysis, Writing - original draft, Writing - review & editing. **S. Matuszewski**: Conceptualization, Supervision, Writing - original draft, Writing - review & editing.

Acknowledgements

The study was funded by the Ministry of Science and Higher Education (grant no. DI2013011043).

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Oświadczenia doktoranta o wkładzie w powstanie artykułów

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Poznań, 20.11.2020 r.

Oświadczenie określające wkład w powstanie artykułu

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W tym artykule jestem pierwszym i korespondencyjnym autorem.
Mój całkowity wkład w pracę wynosi 80%.

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Mój całkowity wkład w pracę wynosi 50%

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Mój całkowity wkład w pracę wynosi 80%.

Katarzyna Frątczak-Łagiewska

Oświadczenia współautorów o wkładzie w powstanie artykułów

Oświadczenia współautorów o wkładzie w powstanie pracy

Development and validation of forensically useful growth models for Central European population of *Creophilus maxillosus* L. (Coleoptera: Staphylinidae). International Journal of Legal Medicine, 134: 1531-1545

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Toruń, 20.11.2020 r.

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Mój całkowity wkład w pracę wynosi 5%.

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Mój całkowity wkład w pracę wynosi 15%.

Matuszewski

Oświadczenia współautorów o wkładzie w powstanie pracy

Sex-specific developmental models for *Creophilus maxillosus* (L.) (Coleoptera: Staphylinidae): searching for larger accuracy of insect age estimates. International Journal of Legal Medicine, 132 (3): 887-895

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Mój całkowity wkład w pracę wynosi 20%.

Matuszewski

Oświadczenia współautorów o wkładzie w powstanie pracy

Size at emergence improves accuracy of age estimates in forensically-useful beetle *Creophilus maxillosus* L. (Staphylinidae). Scientific Reports, 8: 2390

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Mój całkowity wkład w pracę wynosi 50%.

Oświadczenia współautorów o wkładzie w powstanie pracy

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Mój całkowity wkład w pracę wynosi 20%.

Matuszewski

PODZIĘKOWANIA

Pragnę na samym wstępie podziękować mgr Annie Mądrej-Bielewicz za nieocenioną pomoc merytoryczną, wielokrotną pomoc przy zbiorze materiału do badań oraz w pracach laboratoryjnych, a także za całe udzielone na różnych płaszczyznach wsparcie.

Serdeczne podziękowania kieruję do mojego Męża Oktawiana Łagiewskiego za opracowanie skryptów Visual Basic wykorzystanych podczas analizy danych surowych, a także za wyrozumiałość i cierpliwość.

Karolinie Kabzińskiej, Małgorzacie Kolickiej dziękuję za wsparcie i wiarę w moje możliwości.

Szczególne podziękowania kieruję do moich Rodziców za pomoc udzielaną w trakcie całych studiów.

Profesor UAM dr hab. Darii Bajerlein dziękuję za wszystkie uwagi i pomoc w końcowych pracach radakcyjnych.

Na koniec pragnę podziękować mojemu Promotorowi Prof. UAM dr. hab. Szymonowi Matuszewskiemu za wprowadzenie mnie do świata nauki, pomoc merytoryczną, zaangażowanie i wszystkie cenne wskazówki udzielane podczas powstawania tej pracy.

FINANSOWANIE BADAŃ

Badania, których wyniki przedstawiono w niniejszej rozprawie zostały sfinansowane w ramach programu Ministerstwa Nauki i Szkolnictwa Wyższego (DIAMENTOWY GRANT numer DI2013011043).