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Modele rozwoju oraz agregacje larwalne chrząszcza nekrofagicznego z gatunku *Necrodes littoralis* L. (Staphylinidae: Silphinae)

Rozprawa doktorska

Poznań, 2023

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Development models and larval aggregations of necrophagous beetle *Necrodes littoralis* L. (Staphylinidae: Silphinae)

PhD thesis

Poznań, 2023

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Rozprawa doktorska została przygotowana

w Zakładzie Taksonomii i Ekologii Zwierząt, na Wydziale Biologii

Uniwersytetu im. Adama Mickiewicza w Poznaniu.

SPIS TREŚCI

STRESZCZENIE

Owady nekrofagiczne są wykorzystywane przez entomologów sądowych do szacowania okresu pośmiertnego (ang. post-mortem interval), czyli czasu jaki minął od śmierci do momentu ujawnienia zwłok. Najlepiej zbadanymi i najczęściej wykorzystywanymi w tym celu owadami są muchówki. Drugim, bardzo istotnym wśród nekrofauny rzędem owadów są chrząszcze. Jeden z przedstawicieli tego rzędu, *Necrodes littoralis* jest często odnotowywany na zwłokach ludzkich. W związku z tym posiada duży potencjał do wykorzystania w entomologii sądowej do szacowania okresu pośmiertnego. Dotychczas brakowało jednak odpowiednich modeli rozwoju dla tego gatunku, co ograniczało jego wykorzystanie w sprawach sądowych.

Larwy owadów nekrofagicznych często tworzą na zwłokach agregacje. Jest to zjawisko powszechnie występujące, szczególnie u larw muchówek z rodziny Calliphoridae. Tworzenie agregacji jest zjawiskiem bardzo korzystnym, gdyż wpływa pozytywnie na temperaturę oraz rozwój larw. Wcześniejsze badania na muchówkach wykazały, że zachowania agregacyjne mogą być wyzwalane przez bodźce chemiczne, termiczne lub być wynikiem tigmotaksji. Zaobserwowano, że *N. littoralis* również wykazuje tego typu zachowania. Dotychczas brakowało jednak informacji na temat mechanizmów leżących u podstaw takiego zachowania u badanego gatunku oraz jego wpływu na rozwój preimaginalny.

Pierwszym celem mojej pracy doktorskiej było zbadanie wzorców i mechanizmów agregacji larw z gatunku *N. littoralis.* Wraz z pozostałymi współautorami badania przeprowadziliśmy na larwach testy behawioralne w warunkach laboratoryjnych. Wykazały one, że larwy tworzą stabilne agregacje oraz wykazują zachowania termoregulacyjne, reagując na sygnały termiczne i akumulując się w najcieplejszych miejscach na powierzchni badawczej. Drugim celem było sprawdzenie jaki jest wpływ żerowania larw *N. littoralis* w agregacji na rozwój tych chrząszczy. W badaniach porównywałam rozwój larw hodowanych pojedynczo oraz larw hodowanych w agregacjach (50 larw). Wyniki wykazały, że zachowanie agregacyjne jest szczególnie korzystne dla owadów rozwijających się w niskich temperaturach, gdyż znacznie zmniejsza śmiertelność i ułatwia wzrost. Trzeci cel zakładał stworzenie temperaturowych modeli rozwoju *N. littoralis*, które będą mogły być wykorzystywane w praktyce przez entomologów sądowych do szacowania okresu pośmiertnego. Modele stworzyłam przy użyciu danych zebranych podczas badań rozwojowych, prowadzonych w 10 stałych temperaturach. Opracowałam modele sumowania cieplnego, diagramy izomorfeniczny i izomegaleniczny oraz krzywe wzrostu larw. Ostatnim celem była wstępna walidacja tych modeli. Wykazała ona, że modele sumowania cieplnego dają najdokładniejsze szacunki wieku fizjologicznego. Większość stworzonych przeze mnie modeli rozwoju *N. littoralis* pozwoliła na zadowalająco dokładne oszacowanie wieku chrząszczy w warunkach laboratoryjnych, co wstępnie potwierdza ich użyteczność dla praktyki entomologii sądowej.

Słowa kluczowe: entomologia sądowa, owady nekrofagiczne, rozwój preimaginalny, modele rozwoju, szacowanie PMI, agregacje larw

ABSTRACT

Necrophagous insects are used by forensic entomologists to estimate the post-mortem interval, i.e. the time that elapsed from death to the disclosure of the body. The most studied and most frequently used insects for this purpose are flies. Beetles are the second very important order of insects among the necrofauna. One of the representatives of this order, *Necrodes littoralis*, is often noted on human corpses. Therefore, it has great potential for use in forensic entomology to estimate the post-mortem interval. However, until now, there were no appropriate development models for this species, which limited its use in court cases.

Larvae of necrophagous insect often form aggregations on carrion. This is a common phenomenon, especially in Calliphoridae larvae. It can be very beneficial, as it has a positive effect on the temperature and development of the larvae. Previous studies in flies have shown that aggregation behaviour can be triggered by chemical cues, thermal stimulus, or thigmotaxis. *N. littoralis* has also been observed to exhibit this type of behaviour. However, until now, there was a lack of information on the mechanisms underlying this behaviour in the studied species and its impact on pre-imaginary development.

The first aim of my research was to investigate the patterns and mechanisms of aggregation of *N. littoralis* larvae. For this purpose, we conducted behavioural tests on the larvae in laboratory conditions. They showed that the larvae form stable aggregations and exhibit thermoregulatory behaviours, responding to thermal signals and accumulating in the warmest places on the research area. The second objective was to check the effect of feeding of larvae in aggregations on their development. In the study, I compared the development of larvae reared individually and larvae reared in aggregations (50 larvae). Results showed that aggregation behaviour is particularly beneficial for insects developing at low temperatures, significantly reducing mortality and facilitating growth. The third aim was to create temperature models of *N. littoralis* development, which can be used in practice by forensic entomologists to estimate the post-mortem interval. I created the models using data collected during extensive development studies conducted at 10 constant temperatures. I created thermal summation models, isomorphen and isomegalen diagrams and growth curves. The last aim was the initial validation of these models. It showed that thermal summation models give the most accurate estimates of physiological age. Most of the development models of *N. littoralis* allowed for a satisfactorily accurate estimation of the age of beetles in laboratory conditions, which initially confirms their usefulness for forensic entomology.

Keywords: forensic entomology, necrophagous insects, preimaginal development, developmental models, PMI estimation, larval aggregations

WPROWADZENIE

Owady są najliczniejszą w gatunki gromadą zwierząt. Reprezentują szeroki wachlarz przystosowań oraz preferencji siedliskowych. Jedną z istotnych grup troficznych są nekrofagi. Rozkład materii organicznej to jeden z najważniejszych procesów zachodzących w ekosystemach, gdyż umożliwia obieg pierwiastków w przyrodzie. Żywiąc się tkankami martwych zwierząt owady nekrofagiczne mają więc istotne znaczenie w środowisku. Pełnią funkcję „sanitarną", oczyszczając ekosystem z martwej materii ale również wpływają na chemiczną charakterystykę gleby, uwalniając do niej składniki odżywcze (Woelber-Kastner i in., 2021). Pożyteczność owadów nekrofagicznych nie ogranicza się jednak wyłącznie do ich roli w obiegu materii. Ze względu na zajmowane siedlisko oraz specyficzną fizjologię mogą być wykorzystywane przez ludzi w celach prawnych. Nekrofagi żywią się martwymi tkankami zwierzęcymi, są więc często znajdowane również na zwłokach ludzkich. W zależności od gatunku oraz stadium rozwojowego żerują na zwłokach objętych różnymi procesami rozkładu. Pierwsze osobniki dorosłe (zazwyczaj muchówki) pojawiają się na zwłokach od kilku minut do kilku godzin po śmierci. Następnie sukcesywnie przybywają kolejni przedstawiciele nekrofauny (Byrd i Castner, 2010). Dzięki zróżnicowanym preferencjom pokarmowym owady nekrofagiczne są w stanie rozłożyć wszystkie tkanki miękkie, aż do zupełnego zeszkieletowania zwłok (Schroeder i in., 2002). Owady są organizmami ektotermicznymi, co oznacza, że ich temperatura ciała, tempo metabolizmu oraz aktywność zależą w dużej mierze od temperatury otaczającego środowiska. W związku z tym również tempo rozwoju jest skorelowane z temperaturą. Zależność ta jest pozytywna – im wyższa temperatura, tym szybsze tempo rozwoju (Higley i Haskell, 2010). Wraz ze spadkiem temperatury tempo rozwoju również spada, aż do momentu osiągnięcia tzw. zera fizjologicznego lub dolnego progu rozwojowego (ang. lower developmental threshold), czyli temperatury, poniżej której rozwój zostaje zatrzymany (Amendt i in., 2007). Ta ścisła zależność sprawia, że znając wzorce rozwojowe dla danego gatunku oraz temperaturę w jakiej zachodził rozwój, można oszacować wiek owadów. Właśnie tę prawidłowość wykorzystuje się w entomologii sądowej – nauce łączącej entomologię z kryminalistyką i medycyną sądową w służbie wymiaru sprawiedliwości.

Pierwsze udokumentowane wzmianki o wykorzystaniu owadów w celach kryminalistycznych pochodzą z XIII-wiecznych Chin, gdzie nekrofilne muchówki przyczyniły się do identyfikacji sprawcy morderstwa, gromadząc się na narzędziu zbrodni, na którym pozostały ślady krwi ofiary (Greenberg, 1991). W miarę rozwoju entomologii sądowej pojawiały się kolejne zastosowania owadów do celów kryminalistycznych. Dzisiaj ślady entomologiczne pozwalają m.in. wykrywać toksyny, leki i narkotyki (Introna i in., 2001), a także ustalać w jakim środowisku nastąpił zgon i wnioskować, czy ciało było przenoszone po śmierci (Charabidze i in., 2017; Matuszewski i in., 2013). Nadal jednak najczęściej wykorzystuje się je do określania tzw. okresu pośmiertnego (ang. post-mortem interval, PMI), czyli czasu jaki minął od śmierci do momentu ujawnienia zwłok (Amendt i in., 2007). Czas aktywności owadów na zwłokach (ang. period of insect activity) zwykle nie jest jednak tożsamy z okresem pośmiertnym. Od momentu śmierci, do pojawienia się owadów na zwłokach upływa pewien okres

czasu, który może się różnić w zależności od gatunku. W związku z tym biegli entomolodzy sądowi wyznaczając wiek owadów, określają tym samym tzw. minimalny okres pośmiertny (PMI_{min}), czyli minimalny okres jaki według szacunków biegłego upłynął od momentu śmierci do momentu ujawnienia zwłok.

Ustalając PMI na podstawie śladów entomologicznych stosuje się dwa podejścia. Pierwsze z nich – podejście sukcesyjne wykorzystuje wzorce sukcesji stawonogów na zwłokach (Grassberger i Frank, 2004; Matuszewski i in., 2008). Drugie, to podejście rozwojowe, które wykorzystuje wspomniane wyżej prawidłowości rozwojowe owadów i ich zależność od temperatury (Higley i Haskell, 2010; Smith, 1986). W metodzie rozwojowej wykorzystuje się formy preimaginalne owadów, głównie larwy i poczwarki (albo w przypadku muchówek puparia). Wskaźnikami, które zmieniają się w przewidywalny sposób, a tym samym pozwalają w miarę dokładnie oszacować wiek danego osobnika są punkty orientacyjne rozwoju (np. wyklucie się larwy lub linienie) oraz rozmiar, najczęściej długość ciała larwy (Amendt i in. 2011). Podstawową jednostką w której wyrażany jest wiek fizjologiczny są tzw. skumulowane stopniodni (ang. accumulated degree-days, ADD) lub skumulowane stopniogodziny (ang. accumulated degree-hours, ADH), które określają ilość ciepła (powyżej dolnego progu rozwojowego), skumulowaną w danej jednostce czasu (Amendt i in., 2007). Znając wartość ADD lub ADH niezbędną do osiągnięcia danego wskaźnika rozwojowego oraz wiedząc w jakich temperaturach zachodził rozwój owadów na zwłokach, biegły dokonuje pewnego rodzaju retrospekcji - cofając się w czasie sumuje ilość otrzymywanego przez owada ciepła aż do momentu uzyskania pożądanej wartości ADH lub ADD. W tym momencie otrzymuje czas rozwoju danego osobnika na zwłokach, a tym samym minimalne PMI.

Wykonanie tego typu szacowania wymaga wykorzystania specyficznego gatunkowo modelu rozwoju. Aby go stworzyć prowadzi się badania nad rozwojem danego gatunku w warunkach laboratoryjnych. Muchówki, głównie z rodziny Calliphoridae, zwykle pojawiają się na zwłokach najszybciej i są najczęściej wykorzystywane przez entomologów sądowych do szacowania okresu pośmiertnego (Amendt i in., 2011; Byrd i Castner, 2010). Nie dziwi więc fakt, że większość prac badawczych w obszarze entomologii sądowej koncentruje się właśnie na tej grupie owadów. Chrząszcze, druga ważna grupa nekrofagów, jeszcze do niedawna były pod tym względem zaniedbywane. Niewielka liczba publikacji, a co za tym idzie brak użytecznych modeli rozwoju sprawiały, że ich potencjał kryminalistyczny nie mógł być w pełni wykorzystany. Pierwszy kompletny model rozwoju dla istotnego z punktu widzenia entomologii sądowej gatunku chrząszcza (*Thanatophilus micans*) został opublikowany w 2009 roku (Midgley i Villet, 2009) i od tego czasu zarówno liczba opublikowanych modeli jak i badań mających na celu dokładniejsze poznanie biologii i ekologii chrząszczy nekrofilnych sukcesywnie wzrastała.

Jednym z posposlitych chrząszczy nekrofagicznych w Polsce jest *Necrodes littoralis* (Linnaeus, 1758). Gatunek ten należy do chrząszczy omarlicowatych, klasyfikowanych do niedawna jako odrębna rodzina (Silphidae), a obecnie pozycjonowanych jako podrodzina Silphinae w ramach rodziny Staphylinidae (kusakowate) (m.in. Cai et al., 2022). *N. littoralis* występuje w całej Palearktyce, a w Europie jest jedynym przedstawicielem swojego rodzaju. Gatunek ten żeruje i rozmnaża

się na zwłokach dużych kręgowców (Matuszewski i in., 2014), zwykle w fazie aktywnego i zaawansowanego rozkładu (Anton i in., 2011; Charabidze i in., 2016; Matuszewski i in., 2008; Matuszewski i in., 2010). Zarówno imagines, jak i larwy tego gatunku były wielokrotnie wykazywane z ludzkich zwłok (Bajerlein i in., 2018; Bonacci i in., 2021; Charabidze i in., 2016; Dekeirsschieter i in., 2013; Lutz i in., 2021; Matuszewski i Mądra-Bielewicz, 2019; Saloña-Bordas i Perotti, 2014). W związku z tym jest to gatunek potencjalnie przydatny do szacowania okresu pośmiertnego. Do tej pory opublikowano jednak tylko częściowe dane rozwojowe dla *N. littoralis*. Pierwsza publikacja zawierała krzywe wzrostu larw i czas trwania rozwoju w dwóch temperaturach: 18°C i 23°C (Dekeirsschieter, 2012), co daje bardzo ograniczone możliwości do wykorzystania w praktyce. Druga praca zawierała modele sumowania cieplnego dla całkowitego rozwoju owadów (Gruszka i Matuszewski, 2020). W celu oszacowania czasu zgonu metodą rozwojową zwykle określa się jednak wiek najstarszych stadiów preimaginalnych, w związku z czym często potrzebne są modele rozwojowe dla punktów orientacyjnych następujących w trakcie rozwoju larwalnego oraz dla przepoczwarczenia.

Powszechnym i dosyć dobrze zbadanym zjawiskiem w przypadku muchówek nekrofagicznych jest tworzenie agregacji przez larwy żerujące na padlinie (Charabidze i in., 2011; Kotzé i in., 2016; Rivers i in., 2011; Turner i Howard, 1992). Jest to zachowanie, które może nieść znaczące korzyści dla przeżycia i rozwoju organizmów, np. zmniejszając ryzyko ataku drapieżnika (Prokopy i Roitberg, 2001; Wertheim i in., 2005). Zbiorowe żerowanie larw muchówek na zwłokach wywołuje tzw. efekt masy larwalnej (ang. larval-mass effect). Zjawisko to polega na podwyższeniu się temperatury w miejscu w którym skupia się duża liczba larw (Charabidze i in., 2011; Rivers i in., 2011; Slone i Gruner, 2007). Jednak przede wszystkim agregacja larw wpływa na zwiększenie efektywności żerowania. Dzieje się to na skutek zjawiska zbiorowego trawienia zewnętrznego. Larwy muchówek pokrywają padlinę enzymami, które wstępnie nadtrawiają martwe tkanki, zmieniając ich strukturę i ułatwiając tym samym pobieranie pokarmu. Zjawiska te przekładają się bezpośrednio na zwiększenie tempa rozwoju muchówek (Rivers i in., 2011; Scanvion i in., 2018). Tworzenie agregacji może być efektem reakcji na pozostawione na podłożu sygnały chemiczne lub wynikiem tigmotaksji, czyli dążenia do utrzymywania kontaktu dotykowego z elementami otoczenia (Boulay i in., 2013; Fouche i in. 2018), jednak niewątpliwie dużą rolę pełnią w tym przypadku zachowania termoregulacyjne i reakcje na bodźce termiczne (Aubernon i in., 2016; Slone i Gruner, 2007). Owady nie posiadają wewnętrznych mechanizmów pozwalających na regulację temperatury ciała, więc jednym ze sposobów na jej podwyższenie jest aktywne przemieszczanie się w miejsca o wyższej temperaturze, co zostało także wykazane w trakcie eksperymentów na larwach muchówek (Aubernon i in., 2016). Również larwy chrząszczy z rodzaju *Necrodes* w trakcie żerowania na padlinie tworzą duże agregacje (Matuszewski i in., 2010; Matuszewski i in., 2014). W ich przypadku mechanizmy leżące u podstaw tego zjawiska oraz jego wpływ na rozwój preimaginalny pozostawały dotychczas niewyjaśnione.

Główne cele mojej pracy doktorskiej obejmowały:

1. zbadanie wzorców czasowo-przestrzennych oraz mechanizmów agregacji larw chrząszczy z gatunku *Necrodes littoralis*,
2. sprawdzenie, czy agregacja larw tych chrząszczy wpływa na przeżywalność, tempo rozwoju i rozmiar chrząszczy, a przez to także na jakość pozyskanych w badaniach laboratoryjnych danych rozwojowych,
3. opracowanie temperaturowych modeli rozwoju dla chrząszczy z gatunku *N. littoralis,* użytecznych dla entomologii sądowej,
4. walidację stworzonych modeli rozwoju.

Skłonność larw *N. littoralis* do tworzenia dużych agregacji na zwłokach sprawia, że w specyficznych okolicznościach gatunek ten może być odpowiedzialny za aktywnego ich rozkład, czyli proces, za który zwykle odpowiedzialne są muchówki (Matuszewski i in., 2010; Matuszewski i in., 2014). Wzorce sukcesji owadów oraz opisy przypadków kolonizacji zwłok pokazują, że *Necrodes* zasiedla zwłoki na późniejszych etapach dekompozycji (Charabidze i in., 2016; Matuszewski i in., 2011; Matuszewski i Szafałowicz, 2013). W miarę postępowania rozkładu, zwłoki naturalnie charakteryzują się mozaikowatością rozkładu, co oznacza, że w tym samym czasie różne części ciała są rozłożone w różnym stopniu (Matuszewski i in., 2010). Zjawisko to może być jeszcze bardziej pogłębione wskutek oddziaływania zjawisk atmosferycznych (np. promieniowania słonecznego lub deszczu) w różnym stopniu na różne części ciała w zależności od ich usytuowania (Sharanowski i in., 2008). Metabolizm bakterii biorących udział w rozkładzie zwłok również może wpływać na podniesienie ich temperatury (Johnson i in., 2013). W związku z powyższym, można przypuszczać, że w przypadku *Necrodesa* bodźce termiczne będą miały większe znaczenie podczas agregacji larw, aniżeli wskazówki chemiczne. Sugerując się wynikami badań dotyczących zachowań termoregulacyjnych u innych owadów nekrofagicznych (Aubernon i in., 2016) postawiliśmy więc hipotezę, że agregowanie się larw chrząszczy z gatunku *N. littoralis* jest rezultatem aktywnego przemieszczania się poszczególnych larw w miejsca o wyższej temperaturze. Ponadto sprawdziliśmy, czy larwy badanego gatunku reagują na wskazówki chemiczne pozostawiane na podłożu oraz zbadaliśmy dynamikę agregacji we wszystkich stadiach larwalnych. Wyniki tej części badań opublikowano w artykule **Gruszka J, Krystkowiak-Kowalska M, Frątczak-Łagiewska K, Mądra-Bielewicz A, Charabidze D, Matuszewski S. 2020. Patterns and mechanisms for larval aggregation in carrion beetle *Necrodes littoralis* (Coleoptera: Silphidae). Animal Behaviour 162:1-10.**

Aby stworzyć użyteczne modele rozwoju dla danego gatunku owadów nekrofilnych, należy uprzednio przeprowadzić badania laboratoryjne nad jego rozwojem. Badania te obejmują zwykle monitorowanie tempa rozwoju oraz zmian wskaźników wieku owadów, takich jak punkty orientacyjne rozwoju i długość ciała larw (Amendt i in., 2011). W celu uzyskania dobrych modeli, powyższe dane powinny być pozyskiwane w odpowiedniej liczbie i jak największym zakresie temperatur tolerowanych przez dany gatunek (Richards i Villet, 2009). Najczęściej wykorzystywanymi w entomologii sądowej owadami są muchówki z rodziny Calliphoridae. W związku z tworzeniem przez

nie dużych mas larwalnych, również w rozwojowych badaniach laboratoryjnych hoduje się je w masach (Catts, 1992; Greenberg, 1991). W przypadku chrząszczy, wcześniej publikowane modele opierały się na danych pochodzących z eksperymentów w których owady rozwijały się osobno (Frątczak-Łagiewska i in., 2020; Lambiase i in., 2017; Midgley i Villet, 2009; Ridgeway i in., 2014; Zanetti i in., 2016). lub w niewielkich grupach, liczących od kilku do maksymalnie 30 osobników (Gengwang Hu i in., 2023; Guoliang Hu i in., 2020; Martín-Vega i in., 2017; Montoya-Molina i in., 2020; Velásquez i Viloria, 2009; Yinghui Wang i in., 2022; Yu Wang i in., 2020). Wiadomo jednak, że korzyści płynące z tworzenia agregacji u owadów mogą obejmować m. in. wzrost temperatury i efektywności żerowania (Rivers i in., 2011; Scanvion i in., 2018). Biorąc więc pod uwagę fakt, że pożywienie i temperatura są najważniejszymi czynnikami wpływającymi na wzrost i rozwój owadów, powstaje wątpliwość, czy w przypadku owadów wykazujących tendencje do agregacji, takich jak *N. littoralis*, hodowanie larw w odosobnieniu nie wpływa negatywnie na jakość uzyskiwanych wyników. W swoim drugim badaniu porównałam więc dane rozwojowe z hodowli pojedynczych larw z danymi uzyskanymi z hodowli larw w agregacjach. Postawiłam następujące hipotezy:

1) Hodowla larw *N. littoralis* w warunkach odosobnienia wpływa negatywnie na ich przeżywalność.
2) Agregacja larw *N. littoralis* powoduje przyspieszenie tempa rozwoju.
3) Agregacja larw *N. littoralis* wpływa pozytywnie na rozmiar osobników dorosłych.

Wyniki tej części badań zostały opublikowane w artykule **Gruszka J, Matuszewski S. 2021. Insect rearing protocols in forensic entomology: Benefits from collective rearing of larvae in a carrion beetle *Necrodes littoralis* L. (Silphidae). PLoS ONE 16(12): e0260680.**

Przydatność chrząszczy nekrofagicznych do szacowania okresu pośmiertnego została wielokrotnie wykazana zarówno podczas licznych badań sukcesji owadów na padlinie, w trakcie obserwacji prawdziwych przypadków kolonizacji zwłok ludzkich przez owady, jak również w przypadkach szacowania PMI na ich podstawie (Arnaldos, i in., 2005; Bajerlein i in., 2018; Bonacci i in., 2017; Charabidze i in., 2016; Grassberger i Frank, 2004; Kadej i in., 2020; Mashaly, i in. 2020; Matuszewski i Mądra-Bielewicz, 2019; Meira i in., 2020; Moemenbellah-Fard i in., 2018; Wang i in., 2019). Głównym czynnikiem ograniczającym wykorzystanie ich w praktyce entomologii sądowej jeszcze do niedawna był brak użytecznych modeli rozwoju. W ostatnich latach sukcesywnie publikowane były jednak wyniki badań rozwojowych chrząszczy nekrofilnych. Do tej pory opublikowano modele dla 17 gatunków chrząszczy ważnych z punktu widzenia medycyny sądowej. Mimo, że *N. littoralis* jest opisywany w wielu przypadkach jako jeden z gatunków często pojawiających się na ludzkich zwłokach, rzadko był używany do oszacowania okresu pośmiertnego (Bajerlein i in., 2018; Bonacci i in., 2021; Charabidze i in., 2016; Dekeirsschieter i in., 2013; Lutz i in., 2021; Matuszewski i Mądra-Bielewicz, 2019; Saloña-Bordas i Perotti, 2014). Jest to prawdopodobnie spowodowane faktem, że oprócz bardzo ograniczonych, częściowych danych rozwojowych dla tego gatunku (Dekeirsschieter, 2012; Gruszka i Matuszewski, 2020) nie opracowano dotychczas użytecznych modeli rozwoju, które byłyby oparte na pełnym zakresie temperatur optymalnych dla tego gatunku. Przeprowadzone przeze mnie badania

rozwojowe miały na celu stworzenie pierwszego kompleksowego zbioru modeli rozwojowych dla *N. littoralis*, w którego skład weszły:

1) modele sumowania cieplnego (ang. thermal summation models) dla pięciu punktów orientacyjnych rozwoju,
2) model izomorfeniczny,
3) model izomegaleniczny,
4) krzywe wzrostu larw w 10 temperaturach.

Zostały one opublikowane w pracy **Gruszka J, Matuszewski S. 2022. Temperature models of development for *Necrodes littoralis* L. (Coleoptera: Silphidae), a carrion beetle of forensic importance in the Palearctic region. Scientific Reports 12, 9689.**

Wnioski entomologów sądowych uzyskane na podstawie modeli rozwoju są często wykorzystywane jako dowody w sprawach karnych. Publikowane modele powinny więc być rzetelnie wykonane i naukowo uzasadnione (Hall, 2021). Wykorzystanie przez biegłego wadliwego modelu do oszacowania czasu śmierci może spowodować, że przedstawione dowody będą w sądzie kwestionowane, a w najgorszym przypadku mogą nawet przyczynić się do niesłusznych wyroków skazujących w procesach karnych (Peters i in., 2007; VanLaerhoven, 2008). Rzadko prowadzi się jednak badania walidacyjne, które weryfikowałyby dokładność entomologicznych metod szacowania PMI, czy jakość samych modeli rozwoju dla poszczególnych gatunków (Matuszewski, 2021). Przetestowanie modeli w trakcie badań walidacyjnych może dostarczyć informacji nie tylko o przydatności modelu do określonego celu (Peters i in., 2007), ale również o dokładności i precyzji oszacowania wieku owadów przy jego pomocy. Znajomość ewentualnego marginesu błędu szacowania pozwala biegłemu na rzetelne wykonanie ekspertyzy i wyciągnięcie najbardziej trafnych wniosków. Właśnie dlatego tworzenie modeli rozwoju dla nowego gatunku powinno każdorazowo iść w parze z ich walidacją. Przeprowadziłam więc dodatkowe laboratoryjne badania walidacyjne, które pozwoliły wstępnie zweryfikować użyteczność stworzonych przeze mnie modeli rozwojowych do szacowania wieku owadów. Wyniki tej walidacji zostały opublikowane w artykule **Gruszka J, Matuszewski S. 2023. Initial laboratory validation of temperature development models for *Necrodes littoralis* L. (Staphylinidae: Silphinae). International Journal of Legal Medicine 137, 903–911.**

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1. **Gruszka J**, Krystkowiak-Kowalska M, Frątczak-Łagiewska K, Mądra-Bielewicz A, Charabidze D, Matuszewski S. 2020. Patterns and mechanisms for larval aggregation in carrion beetle *Necrodes littoralis* (Coleoptera: Silphidae). Animal Behaviour 162:1-10. https://doi.org/10.1016/j.anbehav.2020.01.011

2. **Gruszka J**, Matuszewski S. 2021. Insect rearing protocols in forensic entomology: Benefits from collective rearing of larvae in a carrion beetle *Necrodes littoralis* L. (Silphidae). PLoS ONE 16(12): e0260680. https://doi.org/10.1371/journal.pone.0260680

3. **Gruszka J**, Matuszewski S. 2022. Temperature models of development for *Necrodes littoralis* L. (Coleoptera: Silphidae), a carrion beetle of forensic importance in the Palearctic region. Scientific Reports 12, 9689. https://doi.org/10.1038/s41598-022-13901-y

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Gruszka J, Krystkowiak-Kowalska M, Frątczak-Łagiewska K, Mądra-Bielewicz A, Charabidze D, Matuszewski S. 2020.

Patterns and mechanisms for larval aggregation in carrion beetle Necrodes littoralis (Coleoptera: Silphidae).

Animal Behaviour 162:1-10.

Animal Behaviour 162 (2020) 1–10

Contents lists available at ScienceDirect

Animal Behaviour

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

Patterns and mechanisms for larval aggregation in carrion beetle *Necrodes littoralis* (Coleoptera: Silphidae)

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ARTICLE INFO

Article history:
Received 14 July 2019
Initial acceptance 1 October 2019
Final acceptance 2 January 2020
Available online 23 February 2020
MS. number: 19-00475R

Keywords:
behavioural ecology
forensic entomology
gregariousness
necrophagy
thermoregulation

Carrion is a patchy, nutrient-rich and frequently bulky resource that hosts a complex community of competing organisms. Several insect species have specialized to use carrion for breeding, revealing a specific suite of behavioural adaptations. Larvae of carrion insects regularly aggregate while feeding, frequently forming spectacular larval masses. Here we analysed patterns and mechanisms of collective feeding by larvae of the communally breeding carrion beetle *Necrodes littoralis*. Using results of laboratory behavioural assays and findings from previous field experiments using pig carcasses we found that: (1) under field conditions *N. littoralis* larval aggregations were prevalent on large carcasses, during late decomposition stages; (2) under laboratory conditions larvae of all instars, when present on meat, formed stable aggregations; (3) larvae consistently responded to thermal characteristics of a feeding environment, aggregating in the hottest place and following changes in the heat source location, which suggests thermal cues are important for their aggregation; and (4) ground-deposited chemical cues elicited a minor response by the third-instar larvae, which indicates these cues are less important for the aggregation of larval *N. littoralis*. These findings highlight the prevalence of communal feeding among necrophagous insect larvae, revealing the interspecific and intraspecific diversity of patterns and mechanisms for larval aggregation on carrion.

Insects frequently form aggregations as adult or larval stages. Aggregated individuals benefit from more efficient use of food, faster growth, easier mate finding or protection against predators (Prokopy & Roitberg, 2001; Wertheim, van Baalen, Dicke, & Vet, 2005). Aggregation triggers costs as well, for example an increase in crowding, conspicuousness to predators and parasitoids, disease and parasite transmission or physiological costs to produce and receive aggregating stimuli (Prokopy & Roitberg, 2001; Wertheim et al., 2005). Aggregations can emerge in response to environmental or social cues and are often self-organized (Camazine et al., 2001; Krause & Ruxton, 2002; Parrish & Edelstein-Keshet, 1999; Sumpter, 2006; 2010). In this context, physical stimuli (e.g. temperature) or resource-originating chemicals are often involved, whereas olfactory (e.g. pheromones), auditory, visual or tactile stimuli are key social cues (Prokopy & Roitberg, 2001; Wertheim et al., 2005). Frequently, several types of cue contribute to the aggregation, as for example in the case of resting aggregations of cockroaches, which are formed in response to physical characteristics of a shelter and the presence of conspecifics (Ame, Halloy, Rivault, Detrain, & Deneubourg, 2006; Ame, Rivault, & Deneubourg, 2004; Canonge, Sempo, Jeanson, Detrain, & Deneubourg, 2009; Dambach & Goehlen, 1999; Jeanson & Deneubourg, 2007; Sempo, Canonge, Detrain, & Deneubourg, 2009), or those of woodlice, for which chemical cues, the density of conspecifics present in a shelter and shelter characteristics are important (Broly, Ectors, Decuyper, Nicolis, & Deneubourg, 2016; Broly, Mullier, Devigne, & Deneubourg, 2015; Devigne, Broly, & Deneubourg, 2011).

Carrion is a nutritionally rich resource, necessary for feeding and breeding of vertebrates, microbes and particularly insects (Barton, Cunningham, Lindenmayer, & Manning, 2013; Carter, Yellowlees,

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https://doi.org/10.1016/j.anbehav.2020.01.011
0003-3472/

& Tibbett, 2007; DeVault, Rhodes, & Shivik, 2003; Metcalf et al., 2016; Payne, 1965). Carrion insects have highly specific adaptations to locate, monopolize and consume carrion (Hobson, 1931, 1932a, 1932b, 1932c; Greenberg & Kunich, 2002; Hanski, 1987; Scott, 1998). Behavioural adaptations noticeably involve larval aggregation, a collective behaviour resulting in the formation of large and organized larval clusters on carrion (e.g. in blow flies: Boulay, Deneubourg, Hédouin, & Charabidze., 2016; Boulay, Devigne, Gosset, & Charabidze, 2013; Charabidze, Bourel, & Gosset, 2011; Fouche, Hedouin, & Charabidze, 2018; Gruner, Slone, Capinera, & Turco, 2016; Heaton, Moffatt, & Simmons, 2014; Heaton, Moffatt, & Simmons, 2018; Johnson & Wallman, 2014; Rivers, Ciarlo, Spelman, & Brogan, 2010; Rivers, Thompson, & Brogan, 2011; Scanvion, Hédouin, & Charabidzé, 2018; Slone & Gruner, 2007). Blow fly larvae (i.e. maggots) benefit from aggregating as this increases the temperature in their feeding microhabitat, an effect called the maggot mass effect (Aubernon, Boulay, Hédouin, & Charabidzé, 2016; Charabidze et al., 2011; Johnson, Wighton, & Wallman, 2014; Magni, Dhaliwal, & Dadour, 2016; Rivers et al., 2011). Aggregation of larval blow flies also facilitates food intake through the collective exodigestion of carrion (Scanvion et al., 2018). Social cues, that is, chemicals left on the substrate by larvae, have been reported to elicit larval aggregation (Boulay et al., 2013; Fouche et al., 2018); however, thermal orientation and thigmotaxis have also been suggested as mechanisms for aggregation (Aubernon et al., 2016). There is evidence that larvae of other insects, for example *Necrodes* or *Stearibia* (Diptera: Piophilidae), may aggregate on carrion similarly to blow flies, that is, in large larval feeding clusters (Matuszewski, Konwerski, Frątczak, & Szafałowicz, 2014), which suggests that collective feeding on carrion may be a widespread adaptation among necrophagous insects. The prevalence of larval aggregations, dynamics and mechanisms for this behaviour, however, have not been studied in any detail in carrion insects other than blow flies.

Species of *Necrodes* are regular components of insect fauna on large animal carcasses and human corpses (Charabidze, Vincent, Pasquerault, & Hedouin, 2016; Dekeirsschieter, Frederickx, Verheggen, Boxho, & Haubruge, 2013; Matuszewski et al., 2016; Ratcliffe, 1972). They start visiting carrion to breed several days post mortem (in summer conditions usually after 4–8 days, Matuszewski & Szafałowicz, 2013) and their larvae feed on carrion resources similarly to blow flies (Ratcliffe, 1972). They are used for post mortem interval estimation in forensic entomology (Bajerlein, Taberski, & Matuszewski, 2018; Matuszewski, 2017; Matuszewski & Mądra-Bielewicz, 2019).

Because *Necrodes* larvae colonize carcasses later than blow fly larvae, by more than 20 days in spring conditions and by about 10 days in summer conditions (Charabidze et al., 2016; Matuszewski, Bajerlein, Konwerski, & Szpila et al., 2011; Matuszewski & Szafałowicz, 2013), we assumed that aggregations of *Necrodes* larvae may have different causes and consequences from those of blow fly larvae. A typical carcass late in decomposition is a thermally heterogeneous environment, a consequence of carcass heterogeneity due to its mosaic decomposition (Matuszewski, Bajerlein, Konwerski, & Szpila, 2010a), different exposure of parts of carcasses to the sunlight, rainfall or wind (Sharanowski, Walker, & Anderson, 2008) or thermogenesis driven by carrion microbes (Johnson, Mikac, & Wallman, 2013; Korshunov Shved, Novikov, Vlasov, & Natcentov, 2003). Carrion insects, as typical ectotherms, thermoregulate mostly behaviourally. Thermoregulation plays a critical role during their development, promoting efficient growth in terms of development time, growth rate and size. Experiments in a thermally heterogeneous environment indicated that blow fly larvae generally move towards higher temperatures (Aubernon et al., 2016; Johnson et al., 2014). Moreover, while feeding in an aggregation they tend to position themselves in its hottest part (Johnson et al., 2014). Based on these patterns, we hypothesized that aggregation of *Necrodes* larvae (in this study *Necrodes littoralis*) is elicited by the thermoregulatory behaviour of individual larvae. Nonsocial environmental cues should, therefore, be responsible for larval aggregation, in contrast to blow fly social aggregations (Boulay et al., 2013; Fouche et al., 2018). To test this idea, we also investigated the effect on aggregation of cues deposited on the substrate by the larvae. Experiments with blow fly larvae provided evidence that cues left on a substrate by moving larvae attracted conspecifics (Boulay et al., 2013; Fouche et al., 2018). Although the nature of these cues was not specified, good candidates are chemicals on the surface of the larva that are deposited on the substrate while it is moving and larval excretions or secretions involved in exodigestion. Therefore, our main experiments aimed to compare the importance of thermal cues and ground-deposited chemical cues for the aggregation behaviour of *N. littoralis* larvae. Finally, because thermal requirements of insects may change as they develop (Richards, Price, & Villet, 2009), we tested the dynamics of aggregation and its mechanisms in all larval stages of the species.

METHODS

Field Data

To get insight into the prevalence of larval aggregations of *N. littoralis* in field conditions, we analysed the results of our previous experiments (Matuszewski, Bajerlein, Konwerski, & Szpila, 2010b; Matuszewski et al., 2016; Matuszewski, Szafałowicz, & Jarmusz, 2013). The sample comprised entomological data from 90 pig carcasses of various masses (6–64 kg) exposed in different seasons and years (2006, 2007, 2011 and 2012) and in various forest and open habitats of the Biedrusko military range (western Poland, 52°31′N, 16°54′ or 55′E). In all the studies beetles were sampled using the same techniques (i.e. pitfall traps and manual techniques) and similar frequency (i.e. at least once a day until the end of active decay and then less frequently; Matuszewski et al., 2010b; Matuszewski et al., 2016; Matuszewski et al., 2013). The presence of *N. littoralis* larval aggregations (i.e. larvae touching each other) was evaluated based on insect occurrence matrices. The analysis was complemented with observations and pictures taken during the inspections of the carcasses. From this data set, the occurrence of aggregations along the decomposition timeline was documented for 14 carcasses (with insect samples and onsite temperature records).

Laboratory Experiments

Insect colony establishment and maintenance

Adult *N. littoralis* were collected in alder forest of the Biedrusko military range (52°31′N, 16°54′E) from pig carcasses. They were kept in plastic insect containers (20–30 insects per container, sex ratio about 1:1) on a humid flower-growing substrate and were fed ad libitum with pork meat. The colony was maintained at room temperature (20–22 °C) and humidity (50–60%). Females usually started oviposition after 2–3 days (from colony establishment), laying batches of eggs in the soil. When larvae hatched, adult beetles were transferred to a new container, and larvae were kept in the same conditions as for adult insects. To reduce the number of mites inside the containers and prevent the growth of mould, soil and meat were replaced once a week. The lengths of larvae were as follows: first-instar larvae between 6.75 and 9.36 mm, second-instar larvae between 11.56 and 14.25 mm and third-instar larvae between 16.28 and 21.28 mm (Frątczak & Matuszewski, 2014).

Temporal and spatial patterns of aggregation

Patterns and dynamics of aggregation were studied in a meat set-up (about 0.5 cm layer of minced pork meat at the bottom of a plastic test container, 25 x 16 cm and 5 cm high, replaced after each trial) and a no-meat set-up. For both set-ups we used a 3 x 4 factorial design (larval stage: L1, L2, L3; larval number: 20, 40, 80 and 160), with time from the onset of a trial as a repeated measure factor (5, 15, 30, 60, 180 and 300 min). The meat set-up was replicated five times and the no-meat set-up three times. In each test, larvae of similar age were sampled from the colony and kept together in small containers with damp paper for about 10 min preceding the trial. Then, they were placed in the middle of a rectangular test container and kept in the dark. The number of aggregations (more than five larvae touching each other), the number of larvae within aggregations and the number of non-aggregated larvae were quantified over time as dependent variables. Differences between treatments were evaluated, separately in the meat and no-meat set-ups, with ANOVA for repeated measure designs using Statistica 13 (TIBCO Software Inc., Palo Alto, CA, U.S.A.).

Substrate-left chemical cues

The effect of chemical cues left by previously aggregated larvae was tested in a same-instar set-up (L1 after L1, L2 after L2, L3 after L3) and a cross-instar set-up (L1 after L3, L2 after L3). We used the same study protocol as in the temporal and spatial patterns tests with the following modifications. The container, filled with a 0.5 cm layer of minced pork, was divided into experimental (marked) and control parts of the same size by using a plastic barrier placed in the middle. Forty larvae were placed on the marked part. After 1 h, larvae and the barrier were removed, and 40 other larvae were placed in the middle of the container. After another 1 h, we counted the aggregations, larvae within an aggregation and nonaggregated larvae in each part of the container. Experiments were replicated 10 times in both set-ups. The significance of differences between marked and control parts of containers were tested with *t* tests using Statistica 13.

Thermoregulatory behaviour

Larval behaviour in a thermal gradient. To compare aggregations of different larval stages in a thermal gradient we used the same-instar set-up (40 L1, L2 or L3 per trial), with 10 replications. Additional experiments (replicated five times) were performed to compare aggregations in a thermal gradient of third-instar larvae of varying ages (1-, 3- and 5-day-old L3 reared at 20 °C, i.e. ages of 20, 60 and 100 accumulated degree-days above 0 °C starting from the second ecdysis). We used the protocol from patterns trials with several modifications. Hot water was poured under one side of the test container to increase the local temperature of the meat and create a gradient. The temperature on the surface of the meat was monitored using a thermal imaging camera (Testo 885–2, Testo, Alton, U.K.). The linear thermal gradient of 20–40 °C (± 3 °C) was chosen, as internal carcass temperatures in spring/summer conditions are rarely below 20 °C and regularly much above 20 °C (Johnson et al., 2013; Sharanowski et al., 2008). The gradient was maintained for 1 h, with continuous monitoring of conditions inside a container using a thermal imager and hot water refill when necessary. At the end of a trial, larvae in each aggregation were counted and the temperature within the aggregation was measured.

To test the behaviour of individual larvae in a thermal gradient we used the protocol described in the previous section. We tested 10 larvae of each stage in a gradient of 20–40 °C (±3 °C). The larva was positioned in the centre of a container and after 10 min we started to record its thermal conditions. Every 2 min for 12 min we recorded the meat surface temperature of the spot occupied by the larva using the thermal imaging camera. We used the grand mean from these six recordings in the analyses.

To test the importance of thermal versus thigmotactic cues, larval behaviour was also studied in a set-up with the heat source in a central position (40 L1, L2 or L3 per trial, 10 replications). The test container was placed on two pillars (22 cm high), with the heat source positioned below to make the hottest spot in the centre of the test container (40 ± 3 °C decreasing towards the walls of the container). Other elements of the protocol were as described in the previous section.

To test the importance of thermal versus food cues, we also observed the behaviour of larvae (40 L1, L2 or L3 per trial, five replications) in a thermal gradient of 20–40 °C (± 3 °C), with meat covering only the colder half of the test container. In this set-up, larvae had a choice between a colder spot with meat, a hotter spot without meat and middle-temperature spots with and without meat. The protocol for our basic thermal trials was used. Differences between larval stages were evaluated using MANOVA and *x,y* coordinates for centres of aggregations as response variables.

Aggregation in a thermally variable environment. To get insight into larval behaviour in a thermally variable environment, we ran experiments in which the heat source position changed during a trial (40 L1, L2 or L3 per trial). Two set-ups were used: one gradient of 20–40 °C (± 3 °C) and another of 20–30 °C (± 3 °C), with five replications per set-up. We employed the protocol from our basic thermal tests with the following modifications. The test container was placed on a pillar (22 cm high) with the heat source positioned under one side of the container. After 30 min the heat source was moved under the opposite side of the container and the trial was completed after another 30 min. Measurements (i.e. position of aggregations, number of larvae and temperature in aggregations) and thermal images were taken before changing the position of the heat source and at the end of each trial. Thermal conditions in a container were monitored continuously using the thermal imager. Differences between larval stages were evaluated using MANOVA and *x,y* coordinates for centres of aggregations as response variables.

Ethical Note

This study comprised laboratory experiments using the insect species *N. littoralis*, which is not under protection. No permission or approval from an Ethics Committee was needed. Larvae used in the experiments were not killed but were returned to the main colony. Excess insects (adult or larval stages) were released into their natural habitat.

RESULTS

Field Data

Larval aggregations of *N. littoralis* were recorded from April to August, regularly on carcasses larger than 30 kg and rarely on smaller ones (Fig. 1). Aggregations formed late in decomposition, usually starting between 220 and 320 post mortem accumulated degree-days (over 0 °C; Fig. 1).

Laboratory Experiments

Temporal and spatial patterns of aggregation

All stages formed stable aggregations on meat, usually at the wall of the container and sometimes under the layer of meat.

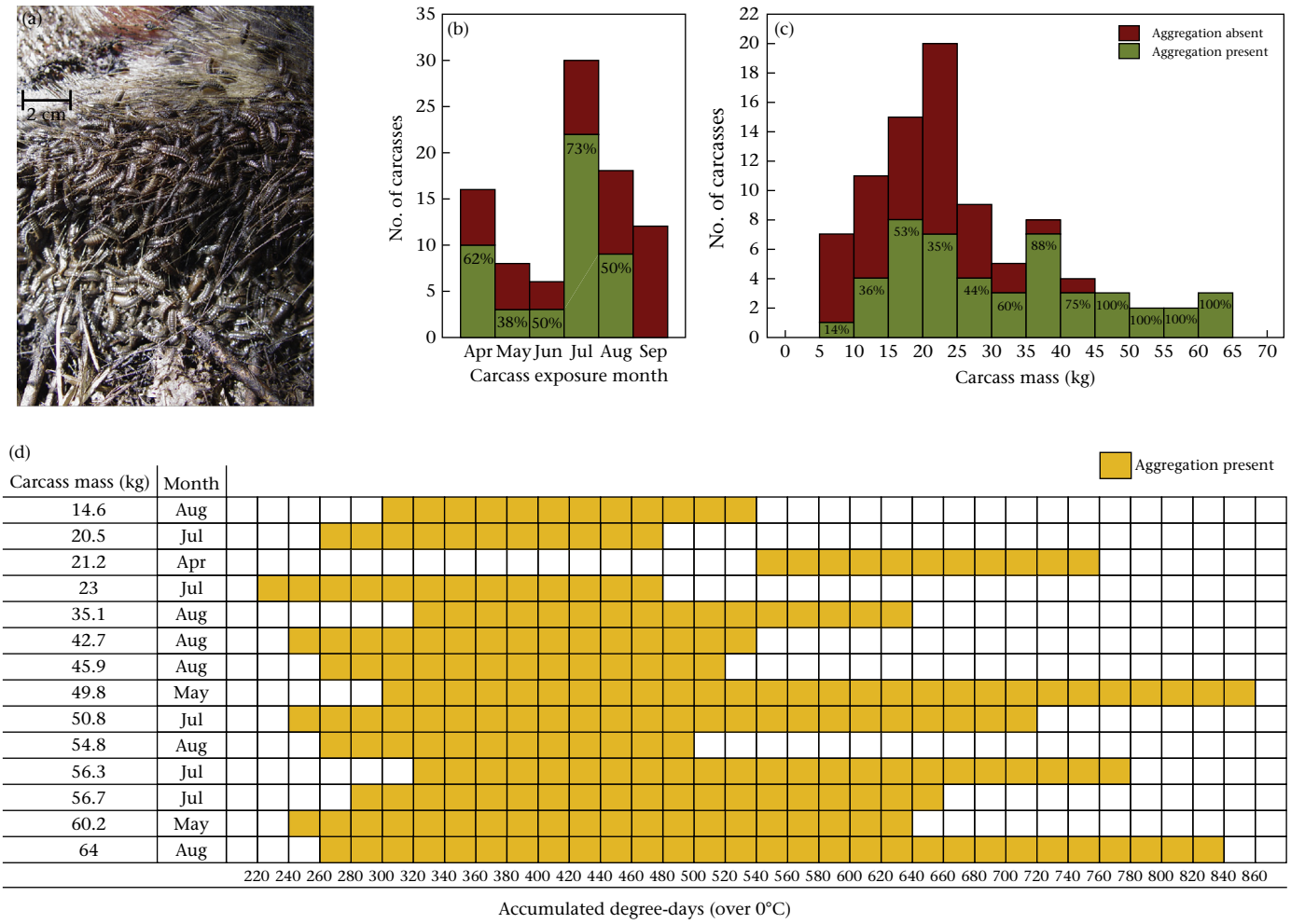

Figure 1. Larval aggregations of *Necrodes littoralis* on pig carcasses in field conditions. (a) A typical aggregation of third-instar larvae on a pig carcass, (b) the number of pig carcasses, from our previous field studies in spring–summer, on which larval aggregations of *N. littoralis* were either present or absent, (c) the number of pig carcasses on which larval aggregations of *N. littoralis* were either present or absent in relation to carcass mass, (d) the daily occurrence matrix showing presence of *N. littoralis* larval aggregations along the decomposition timeline (represented by accumulated degree-days): rows represent carcasses and cells represent carcass inspection days, starting from 220 accumulated degree-days (i.e. in summer temperatures after about 10 days).

Aggregations formed quickly (and were already present after 5 min; Fig. 2), enlarged with time and by the end of a trial, only one or two were usually present (Fig. 2). The percentage of aggregated larvae increased with time and number of larvae in a container, whereas the number of aggregations decreased with time but increased with the number of larvae (Fig. 2, Table 1). Aggregations of the second- and third-instar larvae were more stable and contained more larvae than those of the first-instar larvae (Fig. 2). In the no-meat set-up, larvae formed unstable aggregations, frequently oscillating between random dispersion and clear aggregation (Fig. 2, Table 1).

Mechanisms for aggregation

Substrate-left chemical cues. Larval stages responded differently to the food substrate on which other larvae were previously present (Fig. 3). Third-instar larvae were more abundant on the marked side (same-instar set-up, one-sample *t* test: $t_9 = 1.33$, $P = 0.22$; Fig. 3a) and a higher percentage aggregated there (paired *t* test: $t_9 = 2.90$, $P = 0.02$; Fig. 3c). Second-instar larvae revealed no preference/avoidance for the marked side (same instar set-up, one-sample *t* test: $t_{10} = -0.13$, $P = 0.90$; cross-instar set-up, one-sample *t* test: $t_9 = -0.44$, $P = 0.67$; Fig. 3). First-instar larvae were less abundant on the marked side (same instar set-up, one-sample *t* test: $t_9 = -2.41$, $P = 0.04$; Fig. 3a). When first-instar larvae received meat previously occupied by third-instar larvae, the avoidance effect was even larger (cross-instar set-up, one-sample *t* test: $t_8 = -7.22$, $P < 0.001$; Fig. 3d), with smaller aggregations on this substrate (cross-instar set-up, paired *t* test: $t_8 = -2.78$, $P = 0.02$; Fig. 3b).

Thermal gradient. Aggregations usually formed within the hotter part of the thermal gradient (Fig. 4). The temperature in the aggregation spot decreased with age of the larvae forming the aggregation (Fig. 4). First- and second-instar larvae usually aggregated within 35–39 °C (L1: median = 36.5 °C; L2: median = 36 °C; Fig. 4). Young third-instar larvae (i.e. 20 accumulated degree-days after the second ecdysis) formed aggregations between 22 and 36 °C, whereas older larvae (i.e. 60 and 100 accumulated degree-days after the second ecdysis) aggregated between 21 and 30 °C (young L3: median = 34 °C; old L3: median = 23.5 °C; Mann–Whitney test: $U = 51.5$, $P = 0.04$). In individual assays, larvae were very active but occupied spots of generally lower surface temperature than in the communal assays (L1: median = 23.55 °C; L2: median = 29.3 °C; L3: median = 24.35 °C; Fig. 5). When the heat source was positioned in

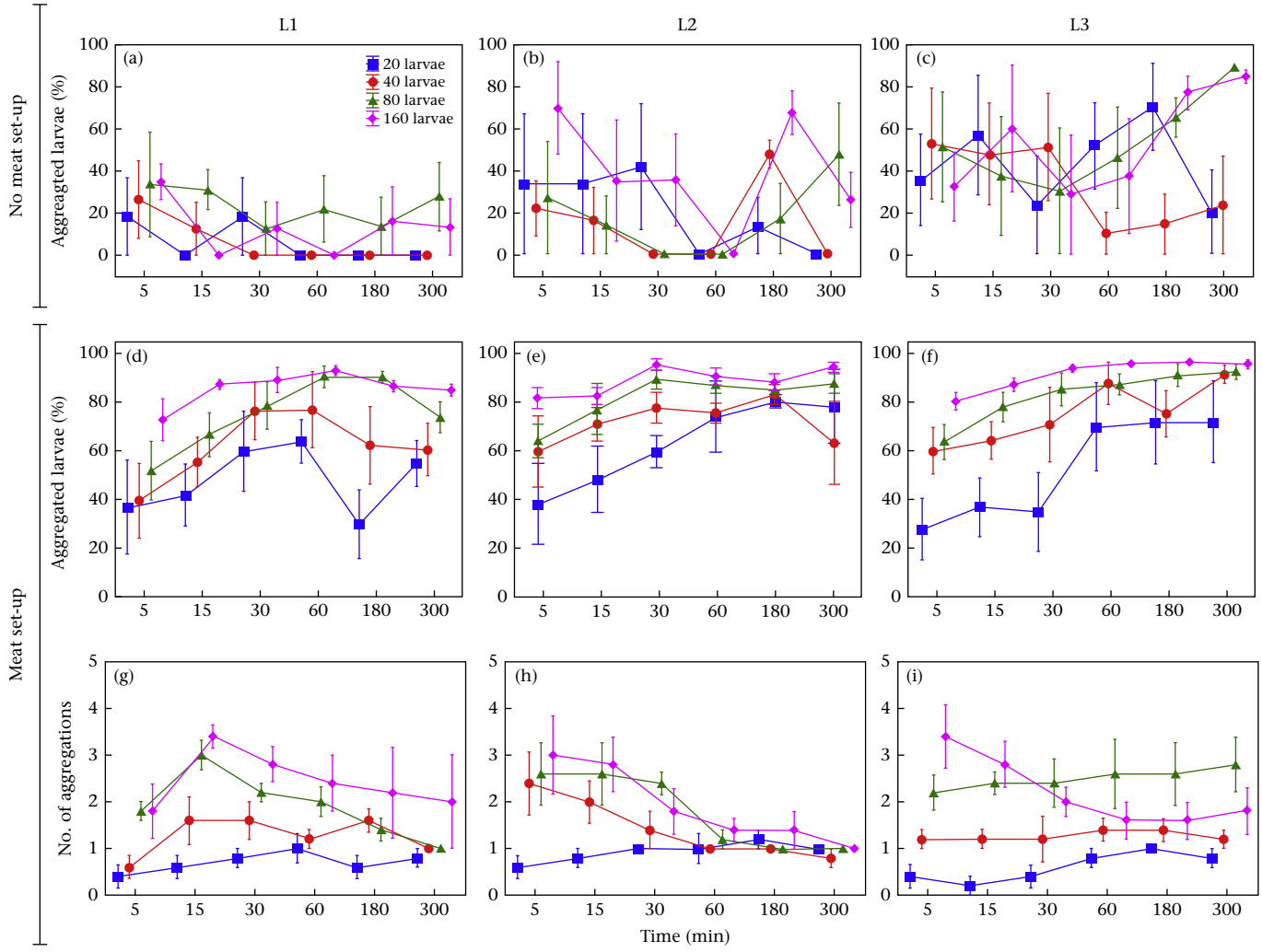

Figure 2. Temporal patterns of aggregation (mean ± SE) for larval stages of *Necrodes littoralis* in laboratory conditions. (a-c) No-meat set-up, i.e. without food in test containers. (d–i) Meat set-up, i.e. with food in test containers. (a, d, g) First-instar larvae, L1; (b, e, h) second-instar larvae, L2; (c, f, i) third-instar larvae, L3.

the centre of the container, about half of the aggregations were observed there (Fig. 6a). In the set-up with meat covering only the colder half of the container, 83% of aggregations were located on meat, but usually in the hottest area with meat, i.e. at the borderline between the meat and no-meat sides (67% of aggregations; Fig. 6b). All larval stages showed a similar response in this set-up (MANOVA: Wilks's lambda = 0.86, $F_{4,52} = 1.05$, $P = 0.39$).

Reversal of thermal gradient. The change in the heat source position, reversing the thermal gradient in the container, was regularly followed by relocation of an aggregation to the new hot spot (81% of cases in a gradient of 20–30 °C and 86% of cases in a gradient of 20–40 °C; Fig. 7). After gradient reversal, individual larvae separated one by one from the original aggregation and subsequently formed a new aggregation near the new location of the heat source. Larvae behaved similarly in both gradients and all larval stages responded in the same way to the gradient reversal (MANOVA: gradient of 20–30 °C: Wilks's lambda = 0.69, $F_{6,46} = 1.58$, $P = 0.18$; gradient of 20–40 °C: Wilks's lambda = 0.73, $F = 0.97$, $P = 0.46$).

DISCUSSION

Our results demonstrate that larvae of *N. littoralis* aggregate on carrion and aggregations regularly occur on large carcasses in particular. This behaviour is well known and has been thoroughly studied in blow flies (Charabidze et al., 2011; Slone & Gruner, 2007). Larval aggregations of the piophilid flies *Stearibia* (Matuszewski et al., 2014) and carrion beetles *Thanatophilus* (Bonacci, Greco, & Brandmayr, 2011; S. Matuszewski, personal observation) have also been recorded on pig carcasses under field conditions. Moreover, *Nicrophorus* larvae also feed in aggregations, although of smaller size owing to the small feeding substrate (Pukowski, 1933). This study demonstrates for the first time that larval aggregations are prevalent in beetles of *Necrodes*, suggesting that group feeding of larvae is widespread among necrophagous insects.

Recent studies of *Nicrophorus* beetles investigated competition between beetles and microbes (bacteria or fungi), highlighting the beetle's adaptations to preserve carrion, suppress competitors or favour symbionts (Duarte, Welch, Swannack, Wagner, & Kilner, 2018; Rozen, Engelmoer, & Smiseth, 2008; Shukla et al., 2018; Vogel et al., 2017). As there is evidence from other insects that larval aggregations have a positive effect on survival when the larvae are feeding on food contaminated with filamentous fungi (Rohlfs & Hoffmeister, 2003; Rohlfs, Obmann, & Petersen, 2005), we suspect that larval aggregation among necrophagous insects may be related to competition with microbes over carrion resources. Moreover, carrion is difficult for larval insects to feed on, and obligate necrophages have developed several mechanisms for its efficient use, for example through food provisioning to larvae by adult burying beetles (Capodeanu-Nägler et al., 2018) or

Table 1
Effects of larval instar, number of larvae in a container and time (from the onset of a trial) on the fraction of aggregated larvae and the number of aggregations in laboratory aggregation trials of *N. littoralis*

Experimental set-up	Dependent variable	Effect	*F*	*P*
No meat	% Aggregated larvae	Larval instar	**8.08**	**<0.01**
Larval instar * Number of larvae * Time (repeated measure factor); (*N*=3)		Number of larvae	1.24	0.32
		Time	**3.88**	**<0.01**
		Larval instar * Number of larvae	0.35	0.90
		Larval instar * Time	1.45	0.17
		Number of larvae * Time	1.71	0.06
		Larval instar * Number of larvae * Time	1.19	0.25
On meat	% Aggregated larvae	Larval instar	**3.39**	**0.04**
Larval instar * Number of larvae * Time (repeated measure factor); (*N*=5)		Number of larvae	**21.90**	**<0.01**
		Time	**14.85**	**<0.01**
		Larval instar * Number of larvae	0.53	0.79
		Larval instar * Time	1.24	0.27
		Number of larvae * Time	0.82	0.66
		Larval instar * Number of larvae * Time	0.74	0.83
	Number of aggregations	Larval instar	0.27	0.76
		Number of larvae	**23.67**	**<0.01**
		Time	**5.69**	**<0.01**
		Larval instar * Number of larvae	1.38	0.24
		Larval instar * Time	**3.84**	**<0.01**
		Number of larvae * Time	**2.77**	**<0.01**
		Larval instar * Number of larvae * Time	1.05	0.40

ANOVA for repeated measure designs was used with time as a repeated measure factor.
Bold indicates statistical significance at 0.05.

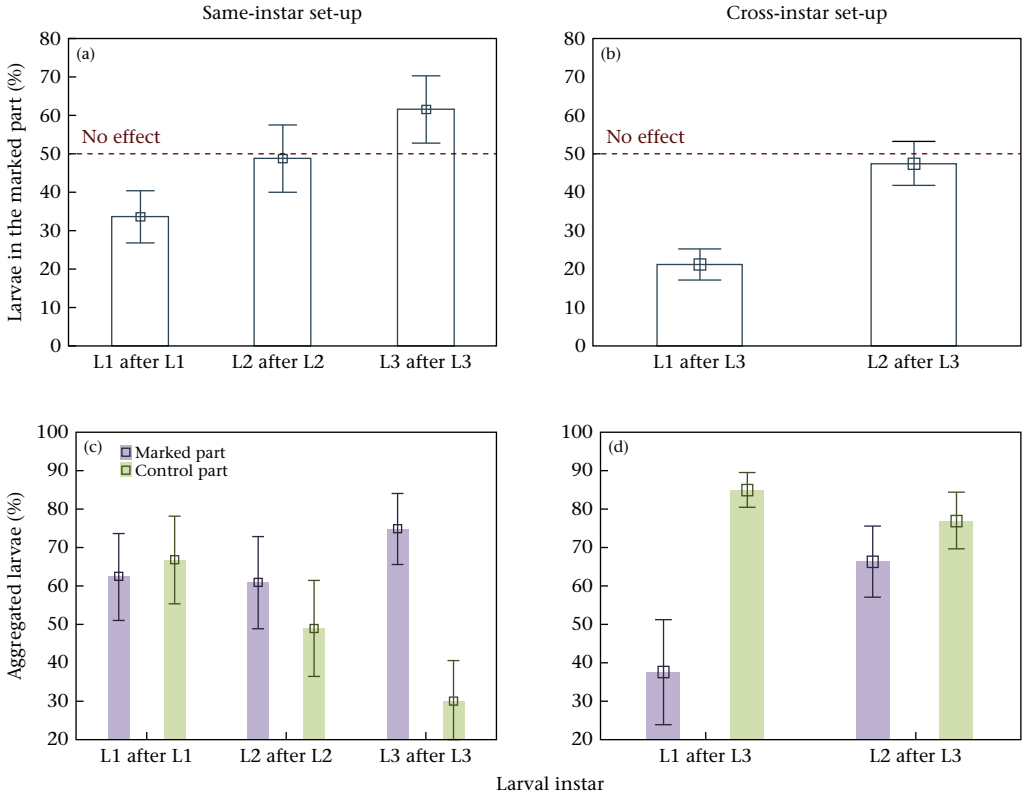

Figure 3. Effect of chemical cues left by previously aggregated larvae on the subsequent aggregation of different larval stages of *Necrodes littoralis*. Marked part: the part of a test container where larvae were previously present; control part: the part of a test container where no larvae were previously present. Larvae were placed in the marked part of the container at the start of a trial and new larvae, of the same or a different stage, were added to the middle of the container after 1 h. (a, c) In the same-instar set-up the same larval stage was used. (b, d) In the cross-instar set-up the added larvae were at a different stage. L1, L2, L3: first-, second- and third-instar larvae. Bars and boxes represent mean, vertical lines represent standard error of the mean.

exodigestion by larval blow flies (Scanvion et al., 2018). Communal feeding may therefore be viewed as an adaptation to outcompete microbes and feed on carrion more effectively. Unfortunately, mechanisms for cooperation and competition among carrion users and adaptations for efficient carrion use are poorly understood.

Our findings support the importance of thermal cues for larval aggregation. Larvae of *N. littoralis* consistently responded to thermal characteristics of their environment, formed aggregations near the heat source and regularly followed changes in its location. When the thermal profile of the environment changed,

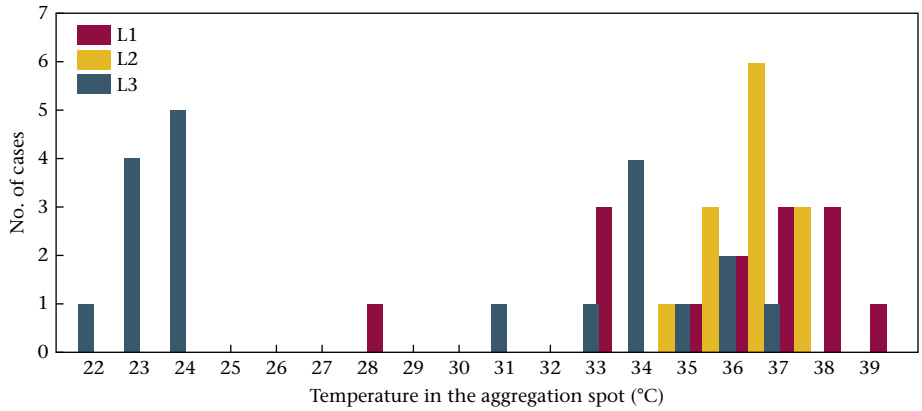

Figure 4. Aggregation of larval *Necrodes littoralis* along a thermal gradient of 20–40 °C (± -3 °C) in the same-instar set-up (i.e. only one larval stage in a container). L1, L2, L3: first-, second- and third-instar larvae.

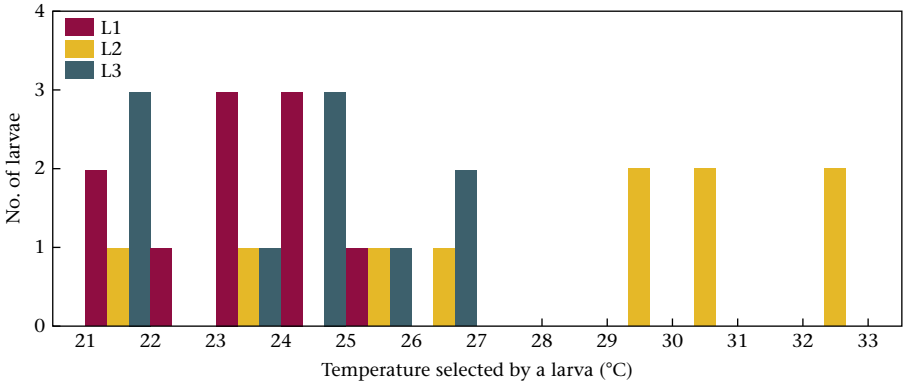

Figure 5. Temperatures selected by individual *Necrodes littoralis* larvae in a thermal gradient of 20–40 °C (± 3 °C). L1, L2, L3: first-, second- and third-instar larvae.

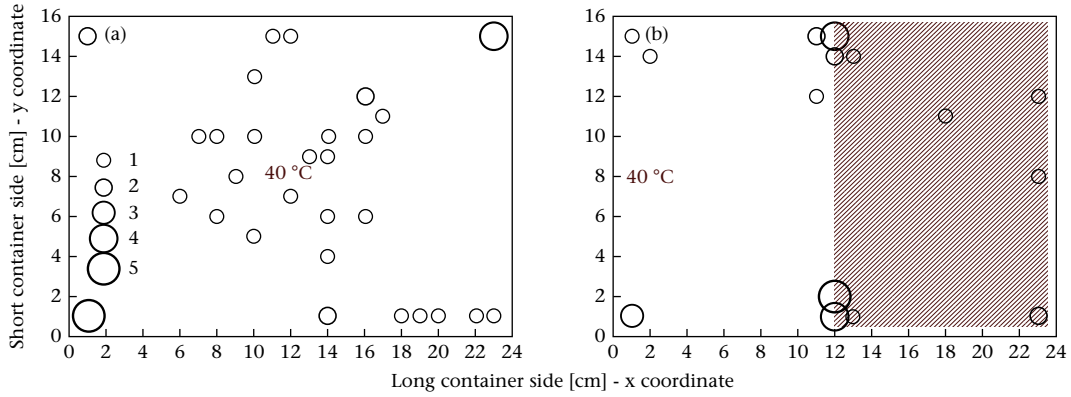

Figure 6. Location of *Necrodes littoralis* larval aggregations in a thermal gradient with (a) the heat source (of 40 °C) in the centre of a test container or (b) meat covering only half of a test container (red lines) and the heat source positioned at the opposite side of the container. Individual circles represent the location of aggregations (their central points, determined to the nearest centimetre) in the Cartesian coordinate system in which the bottom left corner of a test container has (0,0) coordinates. Circles are of various sizes to show the number of aggregations with the same position. Because different larval stages responded similarly in these set-ups, the stage-specific data were pooled, and the general spatial patterns are shown.

aggregations gradually disintegrated and relocated to the new heat source location. These patterns suggest that larval aggregation of *N. littoralis* may emerge as a collective effect of thermoregulatory behaviour of individual larvae. Carrion beetles, as typical ectotherms, need environmental heat to regulate their body temperature (Sanborn, 2008). They develop faster at higher temperatures, reducing time spent on carrion (Frątczak-Łagiewska & Matuszewski, 2018; Jakubec, 2016; Martín-Vega, Díaz-Aranda, Baz, & Cifrián, 2017; Midgley & Villet, 2009; Wang et al., 2017). This benefit potentially justifies several thermoregulatory adaptations in carrion insects (Hanski, 1987) and especially their natural orientation towards hotter places (Aubernon et al., 2016; Johnson et al., 2014). Moreover, larval aggregations on carrion are considered an efficient way to stabilize and elevate thermal conditions for

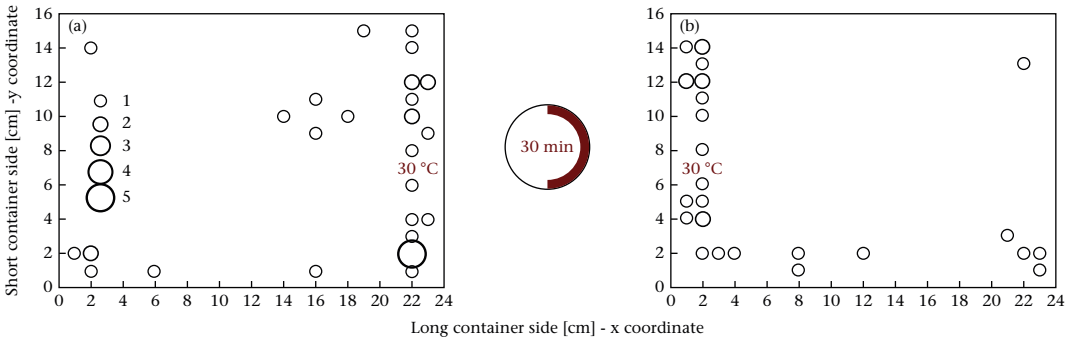

Figure 7. Location of *Necrodes littoralis* larval aggregations (a) before and (b) after reversal of the thermal gradient in a test container. The thermal gradient was 20–30 °C (± 3 °C). Individual circles represent the location of aggregations (their central points, determined to the nearest centimetre) in the Cartesian coordinate system in which the bottom left corner of a test container has (0,0) coordinates. Circles are of various sizes to show the number of aggregations with the same position. Because different larval stages responded similarly in this set-up, the stage-specific data were pooled, and the general spatial patterns are shown.

development (Charabidze et al., 2011; Rivers et al., 2011). It is, therefore, reasonable to assume that even in small, naturally developing aggregations, thermal conditions will be more stable and the temperature higher than outside the aggregation, resulting in the self-acceleration of aggregation formation. In combination with thermal heterogeneity of the carcass (Johnson et al., 2013; Korshunov, Shved, Novikov, Vlasov, & Natcentov, 2003; Matuszewski et al., 2010a; Sharanowski et al., 2008), it seems natural that stable larval aggregations may emerge on carrion as an effect of thermoregulatory behaviour of individual larvae. This thermal mechanism may be enhanced by thigmotactic or chemical cues. This is supported by our results from individual assays. When larvae were present individually on meat, they were very active. This hyperactivity may reflect searching for conspecifics. Accordingly, the presence of other larvae, possibly sensed by thigmotactic or chemical cues, may be considered important for aggregation behaviour.

In the experimental set-up without a food substrate, larvae formed aggregations that regularly disintegrated, indicating that the food substrate is necessary for aggregation persistence, but not for its formation. These findings support the view that stable aggregations of larval *N. littoralis* emerge on those parts of carrion that are suitable for feeding. Larval aggregation in this species may therefore be viewed as a result of searching for heat, conspecifics and food.

Thigmotactic and social cues have also been reported as mechanisms for aggregation in carrion insects, especially blow flies (Boulay et al., 2013; Fouche et al., 2018). Interestingly, the current study does not support the importance of ground-deposited chemical cues for the formation of larval aggregations of *N. littoralis*. Only third-instar larvae responded to marks (e.g. excretions or secretions) left by previously aggregated larvae. This pattern is different from that found in studies with larval blow flies *Lucilia sericata* and *Calliphora vomitoria*, which revealed a clear preference for sites previously occupied by conspecific or heterospecific larvae (Boulay et al., 2013; Fouche et al., 2018). Moreover, larvae of *N. littoralis* formed aggregations faster than blow fly larvae: on meat they were present after 5 min and most larvae (60–100%) were aggregated after about 30 min, whereas *L. sericata* aggregations began forming after 30 min, with the majority of larvae (80%) still not aggregated (Boulay et al., 2013). Aubernon, Hedouin, and Charabidze (2018) also found that aggregated *L. sericata* larvae responded irregularly to the change in the position of the heat source, with some larvae staying in the original place and others moving to the new hot spot. This is different to the regular response of larval *N. littoralis* to thermal characteristics of the feeding environment in the current study. These differences suggest that key mechanisms for the formation of larval aggregations may differ between species of carrion insects. Ground-deposited chemical cues are not, however, the only social cues used by insects while aggregating. Because the presence of other larvae is necessary to elicit normal aggregation behaviours, *N. littoralis* larvae may use other social cues during aggregation, for example thigmotactic stimuli from other larvae present in the feeding microhabitat.

We also found that older third-instar larvae preferred to aggregate at lower temperatures than younger larvae. A similar pattern has been recorded for larvae of *Chrysomya rufifacies* (Johnson et al., 2014). Selection for age-specific temperature indicates that thermal preferences and the resultant thermoregulatory behaviours of larval carrion insects may change during development. Perhaps older third-instar larvae prefer lower temperatures to gain mass more effectively. Ectotherms at lower temperatures grow more slowly but gain larger sizes, and at higher temperatures grow faster but are smaller at maturity, the pattern known as the temperature–size rule (Angilletta, Steury, & Sears, 2004; Atkinson, 1994). Based on this rule we think that older third-instar larvae of *N. littoralis* choose colder aggregation spots to slow down development and reach a larger body size. The shift towards lower temperatures may also be related to the transition of third-instar larvae into a postfeeding phase.

Silphidae are divided into Nicrophorinae, comprising obligate necrophages, and Silphinae, which groups insects with diverse food habits: necrophages (e.g. *Necrodes*), predators of soil invertebrates (e.g. *Dendroxena* or *Phosphuga*) and phytophages (i.e. *Aclypea*) (Heymons, Lengerken, & Bayer, 1927, 1928, 1929; Heymons & Lengerken, 1930; Ikeda, Kagaya, Kubota, & Abe, 2008; Sikes, 2005). The ancestor of Silphinae was probably a necrophage, as the *Ptomaphila* and *Oxelytrum* species grouped at the base of the subfamily are all necrophagous (Dobler & Muller, 2000; Ikeda et al., 2008; King, Riegler, Thomas, & Spooner-Hart et al., 2015). *Necrodes* and *Diamesus* are closely related to this basal Silphinae (Ikeda et al., 2008; King, Riegler, Thomas, & Spooner-Hart, 2015). Although there is no description of communal larval feeding in *Ptomaphila*, *Oxelytrum* and *Diamesus* (only indirect evidence exists), the prevalence of larval aggregations in the more thoroughly investigated genera of *Nicrophorus*, *Necrodes* and *Thanatophilus* suggests that communal feeding on carrion is probably the ancestral trait of Silphidae. Larval *Nicrophorus*, however, feed in small aggregations (Pukowski, 1933), and larval aggregations of *Thanatophilus* are less abundant and recurrent than aggregations of *Necrodes*. Species of *Necrodes* may, therefore, show the evolutionarily most advanced form of this behaviour among Silphidae.

Author Contributions

S.M. developed the concept for the study and the article, analysed the data and wrote the manuscript. J.G., M.K.K., K.F.Ł. and A.M.B. conducted experiments and prepared raw data for analyses. J.G. participated in writing the manuscript. All authors discussed the results and reviewed the manuscript.

Acknowledgments

We thank the anonymous referees for their comments, which helped to improve the manuscript. The study was funded by the National Science Center of Poland (grant no. 2016/21/B/NZ8/00788).

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Gruszka J, Matuszewski S. 2021.

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PLoS ONE 16(12): e0260680.

PLOS ONE

RESEARCH ARTICLE

Insect rearing protocols in forensic entomology: Benefits from collective rearing of larvae in a carrion beetle *Necrodes littoralis* L. (Silphidae)

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Citation: Gruszka J, Matuszewski S (2021) Insect rearing protocols in forensic entomology: Benefits from collective rearing of larvae in a carrion beetle *Necrodes littoralis* L. (Silphidae). PLoS ONE 16(12): e0260680. https://doi.org/10.1371/journal.pone.0260680

Editor: Maxwell J. Scott, North Carolina State University, UNITED STATES

Received: August 23, 2021

Accepted: November 12, 2021

Published: December 1, 2021

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The study was funded by the National Science Center of Poland (https://www.ncn.gov.pl), grant no. 2016/21/B/NZ8/00788. J.G. received financial support from the Dean of the Faculty of Biology Adam Mickiewicz University in Poznań (http://biologia.amu.edu.pl), grant no. GDWB-11/2019. The funders had no role in study design, data

Abstract

Forensic entomologists frequently use a developmental method to estimate a post-mortem interval (PMI). Such estimates are based usually on the blow fly larvae or puparia. Data on their development is obtained by rearing them in colonies. In the case of beetles, which can be also useful for PMI estimation, development data is frequently collected by rearing them individually. However, some carrion beetles are gregarious, for instance, *Necrodes littoralis* (Linnaeus, 1758) (Silphidae). We compared mortality, rate of development and body size of emerged adult beetles reared individually and in aggregations. Mortality was much higher for beetles reared individually, particularly at low temperatures. The rearing protocol affected the time of immature development and the size of adult insects. Individually reared specimens developed much longer at 16˚C, whereas at 20˚C and 26˚C development times of individually reared beetles were slightly shorter. Significant differences in the body size were observed only at 16˚C; beetles that developed in aggregations were larger at this temperature. These findings demonstrate that aggregating is particularly beneficial for larvae of *N. littoralis* at low temperatures, where it largely reduces mortality and facilitates growth. Moreover, these results indicate that in forensic entomology the protocol of individual rearing is unsuitable for gregarious beetles, as it produces reference developmental data of low quality.

Introduction

The developmental method is one of the tools used in forensic entomology to determine post-mortem interval (PMI), which is the time that elapsed from death to disclosure of the body. The foundation for its use is reference data from developmental studies carried out on species of carrion insects. The laboratory protocol for such studies is to monitor insect development under several constant temperatures. Then, the data collected is used to derive developmental models, for instance, the thermal summation model that uses accumulated degree days (ADD,

collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

the accumulation of heat above the threshold temperature over a certain number of days) [1]. ADD necessary for the development of insects found on a cadaver can be used to estimate the development interval or the minimum post mortem interval (PMI_{min}), which is the minimum time that elapsed from death [1,2]. Insects in forensic entomology are bred under a variety of conditions: individually or in aggregations consisting of various numbers of larvae. A rearing protocol may affect the quality of the data and in effect the development models, especially when insects are reared under different conditions than those occurring in nature. A literature review, outlined below, shows that there are no standards in forensic entomology in regard to the size of insect cultures that are used to collect development data.

Most frequently, blow flies are used for the estimation of PMI_{min} [3]. Data on larval development is obtained by rearing blow fly larvae in aggregations consisting of tens or hundreds of individuals. In the development studies on *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae), for instance, larvae were reared in aggregations consisting of 10 insects [4]. Larger aggregations were used in the research on *Phormia regina* (Meigen, 1826) (Diptera: Calliphoridae), in case of which there were 400 larvae per aggregation [5]. However, the most common size of the aggregation was 100 larvae [6–8].

In the case of beetles, rearing protocols frequently consisted in monitoring individual insects (both larvae and pupae) that developed in separate containers. For instance, *Creophilus maxillosus* (Linnaeus, 1758) (Coleoptera: Staphylinidae) larvae were reared separately in 80-ml (1^{st} instar larvae) and 120-ml (3^{rd} instar larvae) containers [9]. In the study of *Dermestes undulatus* Brahm, 1790 (Coleoptera: Dermestidae) and *D. frischii* Kugelann, 1792 (Coleoptera: Dermestidae), each larva was kept in a different jar [10]. In studies on the development of *Thanatophilus micans* (Fabricius 1794) and *T. capensis* (Wiedemann, 1821) (Coleoptera: Silphidae), individual larvae were kept in Petri dishes [11,12]. Similarly, in studies of *D. maculatus* De Geer, 1774 (Coleoptera: Dermestidae) larvae were reared in separate containers [13].

There were also studies in which beetle larvae were reared in aggregations. Their size, however, never exceeded 30 larvae. *Thanatophilus sinuatus* (Fabricius, 1775) (Coleoptera: Silphidae) were reared in Petri dishes in groups of up to 5 larvae [14]. In the case of *Oxelytrum discicolle* (Brullé, 1836) (Coleoptera: Silphidae), the number of larvae per jar varied between 10 and 30 [15]. In studies of *Omosita colon* (Linnaeus, 1758) (Coleoptera: Nitidulidae), there were about 22 larvae per Petri dish [16]. *Dermestes* species were bred in aggregations of 10 larvae [17]. Similarly, in the case of *Necrobia rufipes* (De Geer, 1775) (Coleoptera: Cleridae), there were 10 larvae per Petri dish [18].

Laboratory protocols, in which larvae are reared individually, are suitable for predators, species prone to cannibalism or solitary insects, but may not be suitable for gregarious species that in natural conditions feed in larval aggregations. Feeding in aggregations can be beneficial for insects due to many reasons [19]. This behaviour can make foraging more efficient, lowering mortality and decreasing the duration of development [20]. Moreover, it affects the temperature experienced by the larvae. Blow fly and *Necrodes* larvae can raise the temperature inside the feeding aggregation and thus minimize the negative effects of fluctuating ambient air temperatures [21,22]. Estimating the age of a species that naturally feed in aggregations, by using the data from a protocol in which larvae were reared individually, may lower the accuracy of estimation. Because the aggregation increases foraging efficiency and raises the temperature in the feeding microenvironment [22], individual rearing conditions may lower survival and extend the development. In consequence, by using such a biased data, insect age and eventually the minimum PMI may be overestimated. Depending on the density of larvae in an aggregation, massing can also affect the size of adult insects [23,24]. It may be important, as the size of adult *N. littoralis* may be used to improve the accuracy of PMI estimation [25].

Necrodes littoralis (Linnaeus, 1758) (Coleoptera: Silphidae: Silphinae) is a forensically important, necrophagous beetle [26–28], which is widely distributed in the Palearctic region. Females of *Necrodes* lay eggs in large batches into the soil near the corpse [29]. After hatching larvae go through three larval instars [30]. Postfeeding larvae bury into the soil to form a pupal chamber, where they pupate and eventually reach the adult stage [29]. In natural conditions these beetles feed on large cadavers, where they usually form large aggregations of larvae [31] and under favourable conditions can independently drive active decay [26]. A recent study showed that temperature plays an important role in the formation and maintenance of larval aggregations, as larvae reacted to changes in the temperature by relocating themselves [31]. It has been also demonstrated that *N. littoralis* form a feeding matrix on carrion that is the complex microenvironment where the larvae feed and warm-up, as it also generates heat [22]. To form the optimal matrix, activity of many larvae is necessary. Moreover, the earlier presence of adult beetles on the meat improved the quality of the matrix, with a decrease in mortality and development time of larvae that stayed in the matrix [22]. Therefore, we expect that under individual rearing conditions the larvae will not form a typical feeding matrix, which will negatively affect their survival and development.

Considering the ecology of *N. littoralis* and assuming that food intake and temperature are crucial factors that affect growth of these insects, the question arises, whether separating larvae of *N. littoralis* significantly affects results of developmental research? In this study, we tested whether rearing of immature *N. littoralis* individually influences the quality of resultant development data in comparison to the data obtained in the communal rearing conditions. We hypothesized that: 1) separating larvae lowers their survival; 2) rearing them in aggregations accelerates development and 3) collective rearing increases the size of the beetles at maturity.

Materials and methods

Adult beetles were taken from a laboratory colony established in 2017 using the beetles sampled in alder forest of Biedrusko military range (52˚31'N, 16˚54'E; Western Poland). The colony is maintained at Laboratory of Criminalistics at AMU (Poznań, Poland). Pairs of adult beetles were placed in 0.5 litre containers to allow them to lay eggs. They had pork *ad libitum* and constant access to cotton balls soaked with water. Containers were checked every 4 hours. The batches of eggs were transferred into 0.5 l containers filled with soil and placed in temperature chambers (ST 1/ 1 BASIC or ST 1+/ST 1+, POL-EKO, Poland) at five constant temperatures: 14, 15, 16, 20, and 26˚C. Containers were inspected for the presence of fresh first instar larvae (creamy white, not fully sclerotized) at time intervals of about 10% of the egg stage duration, which was calculated for each temperature based on the pilot study. The larvae were reared in incubators at the same constant temperatures according to two different protocols: individual rearing and rearing in aggregations.

Individual rearing

Thirty larvae per temperature were separated into 120 ml plastic containers with soil, cotton balls soaked with water (replenished during each inspection), and pieces (about 10 grams) of pork. New pieces of meat were added each time it had been found that the meat dried out or that there was less than half of its initial amount. Larvae were monitored in terms of transition to subsequent developmental stage (2nd instar larva, 3rd instar larva, post-feeding larva, pupa and adult beetle) at predetermined intervals, representing no more than 10% of the duration of the stage. Larval developmental stages were determined based on their colour, size and proportions of the body. Freshly moulted larvae are creamy-white and non-sclerotized. They darken with time, so the larvae that are shortly after ecdysis are easy to recognize. The size and

proportions of the larvae also change very clearly during ecdysis, so we used them as supplementary features to distinguish larval instars. When 3rd instar larvae ceased feeding (they start to bury themselves at this moment), remains of meat were removed and the soil in the containers was added to allow formation of pupal chambers. Beetles remained in the containers until the immature development was completed. Body length was measured for all of the emerged adult beetles. Insects were put into a transparent vial, placed against a geometrical micrometer [32] and their body length (from the clypeus to the end of the last abdominal segment) was read when they were fully erect and immobile. Their body was weighed using an analytical balance (AS 82/220.R2, Radwag, Poland; readability = 0.01/0.1 mg, repeatability [5% Max] = 0.015 mg, linearity = ±0.06/±0.2mg).

Rearing in aggregation

Four egg batches at each temperature were incubated. After larvae hatched, they were distributed at random into four small terrariums (30 x 20 x 20 cm) to allow them to form an aggregation (50 larvae per terrarium). In each terrarium, there was soil, cotton balls soaked with water, and pork *ad libitum* (pieces of about 80 grams, replenished if necessary). The larvae were inspected for developmental landmarks at the same time intervals as in the individual rearing protocol. The given landmark was reached when half of the larvae in the aggregation passed to the next developmental stage. The post-feeding larvae were transferred to 0.5 litre plastic containers (10 individuals per container) filled with soil to enable them to form pupal chambers. They remained there until emergence. Adult beetles were measured and weighed as in the individual protocol. Mortality of the post-feeding larvae and pupae was assessed at the end of the experiment. Containers were emptied and checked for the presence of dead beetles. Dead post-feeding larvae indicated that death occurred at the post-feeding stage, dead pupae indicated that death occurred at the pupal stage.

Data analyses

We calculated the percentage mortality for both protocols at each of the analysed temperatures. By counting beetles after each developmental landmark (e.g. first ecdysis or pupation), we collected data on mortality at particular life stages. We compared mortality between beetles bred individually and in aggregations using the Wilcoxon signed-rank test. To analyse the influence of the protocol (individual or in aggregations) and the temperature on the duration of development, body length and weight of adult beetles, we used the two-way ANOVA. The Tukey's HSD test was used for post hoc comparisons. All statistical analyses were made using Statistica 13 (TIBCO Software Inc.).

Ethical approval

The study comprised laboratory experiments using insect species *Necrodes littoralis* (Coleoptera: Silphidae). The species is not under protection. No permission or approval from Ethic Commission were needed.

Results

There were significant differences in mortality between the rearing protocols (the Wilcoxon signed-rank test; $Z = 2.02$, $p = 0.043$, N = 5). At all temperatures mortality was much higher for insects reared individually (Fig 1). There was 100% mortality of the beetles reared individually at 14˚C and 15˚C. Differences were also observed between the protocols in the distribution of deaths across developmental stages (Fig 1). Larvae reared individually died more often at the

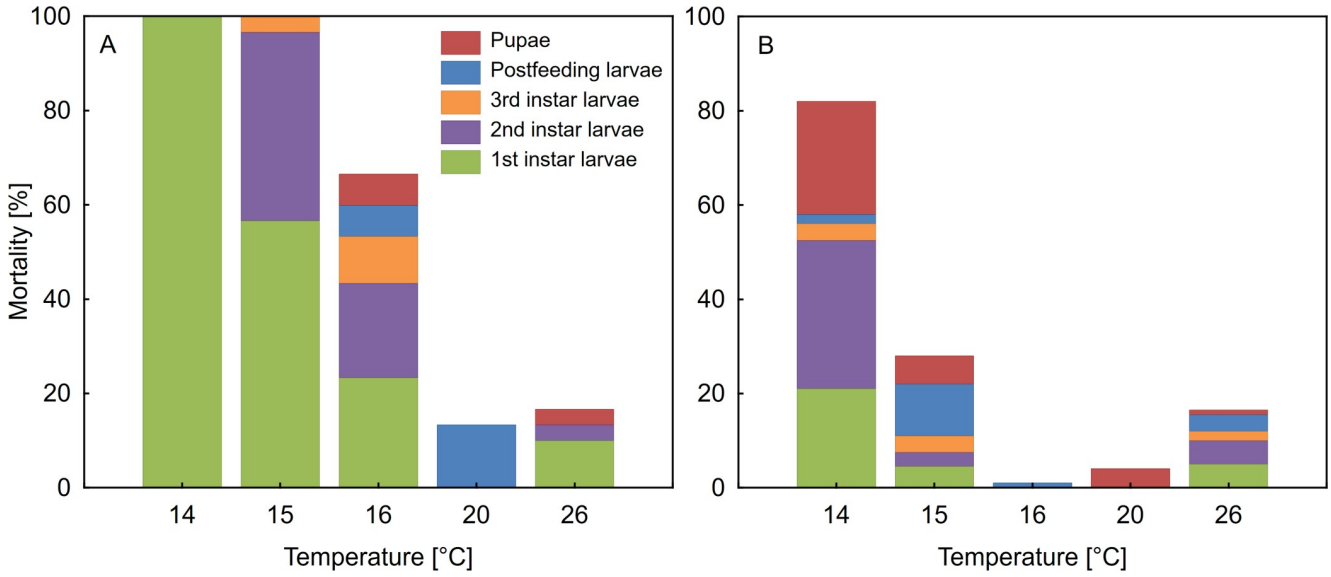

Fig 1. Mortality of *Necrodes littoralis* at preimaginal stages reared individually (A) and in aggregations (B).

https://doi.org/10.1371/journal.pone.0260680.g001

early stages of development (1st instar and 2nd instar larva), while mortality of the collectively reared larvae was much lower during these stages. At the highest tested temperature (26˚C), there was no influence of the laboratory protocol on the mortality (Fig 1).

Both the temperature and the protocol type affected the development time, length and weight of adult beetles (two-way ANOVA, $p<0.05$, Table 1). There was also a statistically significant interaction between the protocol type and the rearing temperature (Table 1).

Statistically significant differences between the protocols in the duration of development were recorded at 16˚C (Tukey's HSD: $p<0.001$) and 20˚C (Tukey's HSD: $p<0.001$) (Fig 2A). At 16˚C individually reared specimens developed 15.9% longer than those reared in aggregations (Fig 2A). At 20˚C, development of the beetles reared individually was shorter by 6.1% than in aggregations (Fig 2A). Beetles that developed in aggregations at 16˚C (Tukey's HSD: $p<0.001$) and 20˚C (Tukey's HSD: $p = 0.003$) were significantly longer than those reared individually (Fig 2B). Significant differences in body weight between the protocols were recorded only at 16˚C (Tukey's HSD: $p = 0.005$), where the mass of adult beetles reared in aggregations was much larger than under individual rearing conditions (Fig 2C).

Table 1. Results of ANOVA for effects of temperature and rearing method on the development duration, length and weight of adult *Necrodes littoralis*.

Dependent variable	Factors	df	F	p
Duration of development	Temperature	2	4780.66	< 0.001
	Protocol	1	41.41	< 0.001
	Temperature × Protocol	2	119.55	< 0.001
Body length	Temperature	2	45.96	< 0.001
	Protocol	1	61.71	< 0.001
	Temperature × Protocol	2	9.57	< 0.001
Body weight	Temperature	2	44.38	< 0.001
	Protocol	1	5.19	0.023
	Temperature × Protocol	2	13.78	< 0.001

https://doi.org/10.1371/journal.pone.0260680.t001

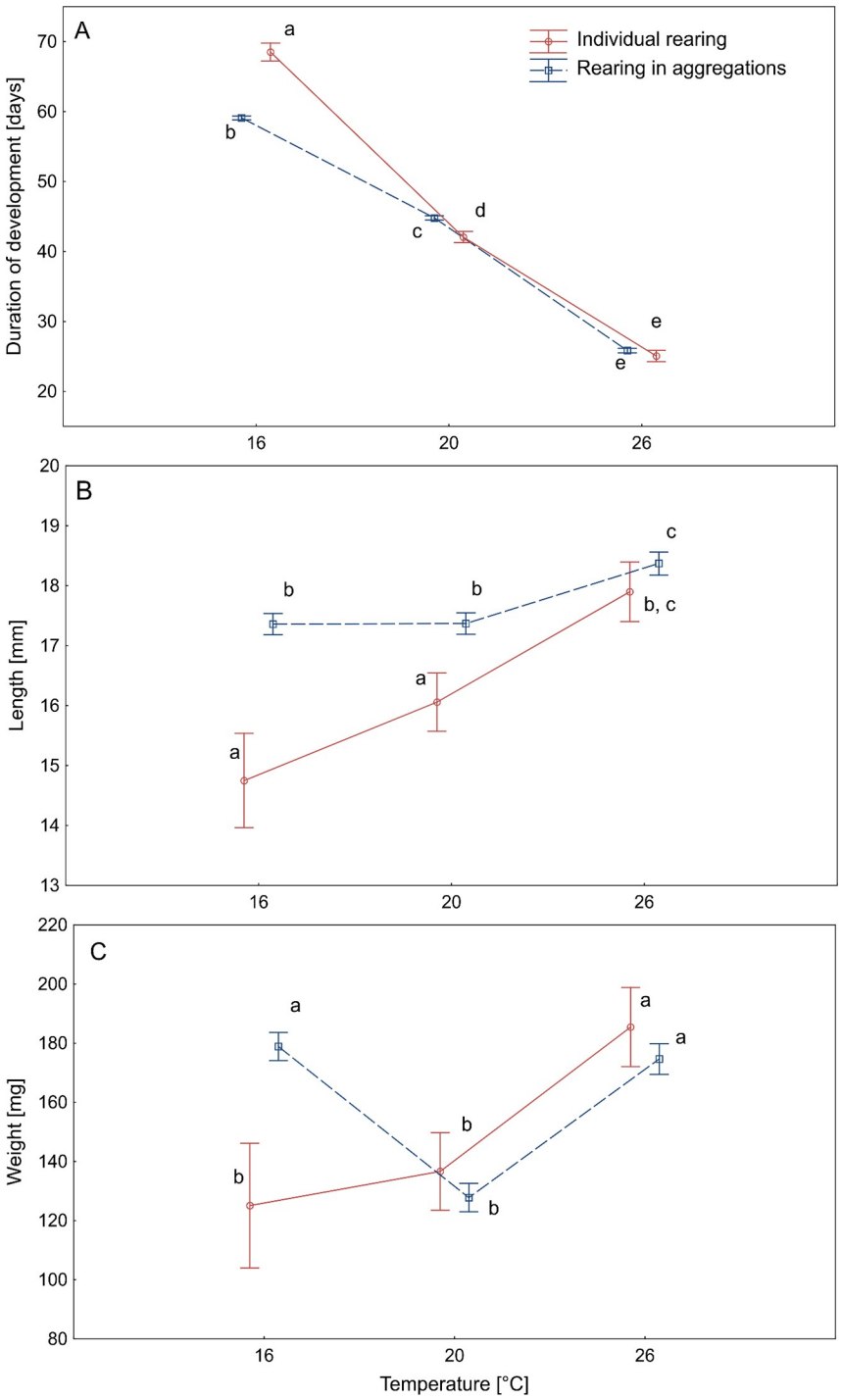

Fig 2. Mean duration of development (A), length (B) and weight (C) of adult *Necrodes littoralis* reared individually and in aggregations. Duration of development is time in days from the oviposition to the emergence of an adult beetle. Whiskers represent the 0.95 confidence intervals. Different letters denote significant differences in pairwise comparisons at $p = 0.05$ using Tukey's HSD test. We used all emerged individuals for the calculations. The total sample size at 16˚C, 20˚C and 26˚C was respectively: 198, 192 and 167 in the protocol with aggregations and 10, 26 and 25 in the protocol with individual rearing.

https://doi.org/10.1371/journal.pone.0260680.g002

Discussion

The individual rearing protocol excluded collection of full data on the duration and size of adult *N. littoralis* at low temperatures (14 and 15˚C). Rearing in aggregations at the lowest temperature (14˚C) was also associated with high mortality. However, it allowed for the collection of the complete data for a relatively large number of beetles. These results revealed a similar temperature pattern as in the previous studies of necrophilous beetles, i.e. a very high mortality at low temperatures [9,11,13]. This can be a problem when multiple temperatures need to be studied to create a developmental model. Rearing larvae in aggregations provided low-temperature data in case of *N. littoralis* and we believe that in other forensically-important gregarious beetles, this protocol may be similarly effective. Study by Scanvion et al. [20] showed a decrease in mortality with an increase in larval density of *Lucilia sericata* (Meigen, 1826) (Diptera: Calliphoridae). In *C. megacephala* and *C. rufifacies* (Macquart, 1842) (Diptera: Calliphoridae), an increase in density similarly reduced the mortality, but only up to a point, where the further increase in larval density had an adverse effect [24]. Current results indicate that this relationship may be true not only for blow flies but also for gregarious carrion beetles.

Results for the time of development at 16˚C suggest that the relationship between density and development of carrion beetles may be similar to the one found previously for carrion blow flies. In *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera: Calliphoridae), the greater the density of larvae in the aggregation, the faster the development [23]. Also in *C. megacephala* and *C. rufifaces*, density had a positive effect on the rate of development in the first and the second instar larvae [24]. In *P. regina*, rearing the larvae in aggregations of 10 insects shortened the development time [33]. In *L. sericata* development time also depended on density, but the decrease was observed only for densities from 50 to 250 larvae [20]. Sullivan and Sokal [34] studied the effect of seven rearing densities on the body mass of four *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) strains. Although the increase in density, in general, had a negative effect on body mass, this was only significant at high densities. Depending on the strain, at the lowest densities, the decrease in mass was insignificant or the body mass even increased. Only in groups larger than 160 individuals, there was a visible decrease in body mass. In *P. regina*, rearing the larvae in aggregations of 10 individuals had a significant, positive effect on the mass of the puparia [33].

The maximum body length in adult *Necrodes littoralis* collected from the field can reach around 26 mm [35]. Beetles in our study were systematically smaller than those developing under natural conditions. Probably the laboratory conditions used in this study were suboptimal for the species. The reason may be the absence of adult beetles on the meat in the pre-larval phase. Other studies have shown that the earlier presence of adult *Necrodes* beetles on meat can have a positive effect on the body mass of developing larvae [22]. It is also possible that other factors, such as humidity or the quality and quantity of larval diet may affect the final size of the beetles. Further studies are necessary on this topic. Moreover, at 20˚C, rearing of the larvae in aggregations resulted in a significant decrease in their mass, but not length compared to the other temperatures. This was probably due to the malnutrition of the third instar larvae at the end of the stage, when the body length does not change but the larvae are gaining mass. Due to unknown reasons these larvae might have ceased feeding too early.

Because at the highest temperature there were no significant differences in mortality, development time and size of adult beetles between the rearing protocols, it seems that high temperatures somehow eliminate negative effects of individual rearing. Bacterial degradation of meat may be of importance here. The activity of microbes causes meat to putrefy that partially disintegrates its structure and makes it easier for the beetle larvae to consume. High temperatures enhance the activity of microbes and putrefaction that makes it easier for the beetle larvae to

consume meat. At low temperatures, putrefaction is very limited and therefore the 'unprocessed' meat may be difficult for a single larva to consume. Collective feeding can facilitate food intake by the larvae under such temperatures. Previous studies on necrophagous flies supported the hypothesis of digestion benefits from the collective feeding by the larvae [19]. Results for *L. sericata* indicated that collective exodigestion can be beneficial for the larvae, as the experimental enrichment of the food with digestive enzymes reduced the time of development under low larval densities [20]. A similar mechanism may occur in *N. littoralis*. It was found that larvae of *N. littoralis* apply their exudates to the food substrate, forming a feeding matrix on carrion [22] This microenvironment may be formed only by an aggregation of larvae. As a result, meat does not dry and is more accessible for the larvae. Moreover, the feeding matrix produces heat that brings extra benefits for the aggregated larvae [22]. A similar aggregation of larvae and formation of the feeding matrix was observed in *Diamesus osculans* (Vigors, 1825), a closely related species, distributed from India to Australia (J. Růžička, personal communication). This is probably related to the similar ecology of the species and reproduction on large carrion. This indicates that results from this study may be important not only for *Necrodes* beetles but also for other closely related carrion beetles (e.g. *Diamesus* Hope, 1840; *Oxelytrum* Gistel, 1848 or *Ptomaphila* Kirby & Spence, 1828). Studies on other feeding protocols, e.g. with the presence of adult insects on the meat before providing it to the larvae, could further improve the rearing protocols for gregarious carrion beetles.

More research is also needed on the size of an aggregation and its impact on the development of *N. littoralis*. We examined the larval development in two densities only; therefore further studies are necessary to identify benefits of the larger aggregations. Nevertheless, even in small aggregations, as demonstrated in this study, the effect of aggregating on development of larvae was clear, especially at low temperatures. These effects are important not only for the development research in forensic entomology but also for the estimation of PMI in forensic investigations. If larvae of *N. littoralis* from a death scene are reared in the laboratory under individual conditions, the mortality may be high especially at low temperatures. Therefore, in a forensic practice we recommend rearing the larvae of *N. littoralis* in the aggregations. If the number of larvae collected on a death scene is low, they should be reared at high temperatures (preferably over 20˚C), as it may prevent the loss of insect evidence due to an increased mortality. Another way to facilitate rearing of small number of larvae from a death scene would be to use the previously prepared food substrate. Placing adult beetles or other *Necrodes* larvae on the meat to be used as a food substrate will provide a feeding matrix [22], which may enhance rearing success for the death scene insects. Unfortunately, the laboratory colony of *Necrodes* beetles is necessary for this purpose. Also, deriving thermal summation models using data from the individual rearing conditions can lead to errors in PMI estimation, as the physiological age (*K*) of death scene insects would be overestimated using such models. Consequently, the minimum PMI would be also overestimated, especially in spring and early summer cases, when the temperatures are frequently much below 20˚C [36]. A similar problem may occur when creating an isomegalen diagram (a graph modelling the size of the larvae in relation to their age and temperature) [37]. Individually reared larvae will be smaller than those developing in aggregations under natural conditions. Therefore, if one uses data from individual rearing protocol to create isomegalen diagram, and then on its basis to estimate the age of the larvae from a death scene (which are larger due to the benefits of aggregation), the result will be an overestimation of insect age. Similarly, the individual rearing protocol may limit the use of linear regression models between physiological age of insects and their size at maturity. Such models may improve the accuracy of PMI estimation as they allow for the calibration of physiological age for insects sampled on a death scene [25,38].

Conclusions

This study demonstrated that aggregation is beneficial for larvae of *N*. *littoralis*, resulting in lower mortality, shorter development time, and larger size. These effects depend on the temperature experienced by the larvae during development and occur primarily at low temperatures. Therefore, in gregarious carrion beetles they need to be considered, both while designing the developmental research for forensic applications and while rearing larvae sampled on a death scene in criminal investigations. Moreover, further research is necessary to fully understand the relation between the aggregation behaviour of carrion beetle larvae, its developmental benefits and the temperature.

Supporting information

S1 File. Mortality data. This file contains data on the mortality of *N*. *littoralis* at different developmental stages. Values are expressed as a percentage.
(XLSX)

S2 File. Development duration, length and weight data. This file contains data on the development time, body length and weight of adult *N*. *littoralis* within the two protocols.
(XLSX)

Author Contributions

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III

Gruszka J, Matuszewski S. 2022.

Temperature models of development for Necrodes littoralis L. (Coleoptera: Silphidae), a carrion beetle of forensic importance in the Palearctic region.

Scientific Reports 12, 9689.

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Temperature models of development for *Necrodes littoralis* L. (Coleoptera: Silphidae), a carrion beetle of forensic importance in the Palearctic region

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Analysis of insects can provide evidence in death cases, for example, by answering the question about the time of death. Apart from flies, beetles are the second most useful insect group in forensic entomology. To elucidate the time of death based on insect evidence, developmental models of a given species are necessary. In this study, we developed such models for *Necrodes littoralis*, a necrophagous beetle, which is common in the Palearctic region and has great potential in forensic entomology. We monitored the development at 10 constant temperatures (14–30 °C). Larvae were reared in aggregations. Thermal summation models, isomorphen and isomegalen diagrams and growth curves were derived using the data. Depending on the temperature, development lasted between about 23 and 89 days. Mortality was high at the extremes of the temperature range. The thermal summation constant for the total development was 434.7 ± 28.86 accumulated degree-days above a developmental threshold of 9.04 ± 0.55 °C. This is the first comprehensive dataset on the development of *N. littoralis*. Implications for its use in forensic casework are discussed.

Forensic entomology uses insects and other arthropods as evidence in legal investigations. Arthropods are used mainly in homicide, suicide and mysterious death cases, when insect evidence allows to establish some circumstances of death[1,2]. For instance, due to the ecological specialization of specific insect taxa, it is possible to determine the environment where death occurred and to answer the question whether the body was relocated after death[3,4]. Most often, however, insects are used to elucidate the time of death, usually through the estimation of minimum postmortem interval (PMI_{min})[5]. Two main methods can be used for this purpose. The first one uses the regularity of insect succession on carcasses[6,7]. Using models of insect succession on carrion for given environmental conditions and a set of insect taxa found on the death scene, we can try to estimate how much time has passed since death[8]. The second method uses the regularity of insect development, in particular a close relationship between temperature and the rate of development[1,9]. Developmental studies of necrophagous insects allow for the creation of developmental models that can be used to estimate PMI_{min}.

Forensic entomologists created various kinds of developmental models. Some of them, such as the isomegalen diagram or growth curve, represent the relationship between development time and size of the larvae at a given temperature. The isomorphen diagram shows the duration of developmental stages at given temperatures. The thermal summation model (TSM) assumes that within the certain temperature range, there is a linear relationship between the rate of development and temperature and that development stops below a certain temperature[10]. Accordingly, there is some constant amount of heat that needs to be accumulated by insects of a given species to reach certain developmental landmark (e.g. hatching, pupation or eclosion). This value is called a thermal summation constant (K) and is expressed in accumulated degree hours (ADH) or accumulated degree days (ADD). The temperature below which the insect species ceases to develop is termed a lower developmental threshold (D_0) and is expressed in temperature units[10]. One of the most frequently used methods to derive TSM, is the

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one proposed by Ikemoto and Takai[11]. It is a modification of the classic thermal summation method. Authors proposed an equation:

$$(DT) = K + D_0 * D$$

where D is the duration of development, D_0 is the lower developmental threshold, K is the thermal summation constant and T is the environmental temperature. The authors also suggested using the Reduced Major Axis (RMA) regression to derive TSM. Slope of the RMA model is the lower developmental threshold (D_0) and the y intercept is the thermal summation constant (K). Although TSMs simplify complexity of insect development and ignore its substantial intraspecific variation (e.g. variation in development time between the sexes[12,13] or between insects of different sizes[14,15]), their use enables satisfactorily accurate estimation of insect age and eventually PMI_{min}[8,16,17].

Flies are the most frequently used group of insects in forensic entomology. They usually appear on cadavers as first colonizers and age of their immature stages allow to estimate the PMI_{min} that is close to the true PMI[18]. Typically, the first colonizers are blow flies (Calliphoridae). They appear on the corpse within the first hours or even minutes after death[10,19]. During feeding, their larvae form large aggregations[20–22]. Beetles appear later on carcasses, and are often present there until the remains stage. Hence, they can be an important tool in estimating the PMI in the advanced stages of cadaver decomposition[23]. Numerous studies of insect succession on carrion and descriptions of specific forensic cases demonstrated that beetles may be useful for the estimation of PMI[6,17,24–34]. Among the forensically important families of beetles, the most frequently mentioned ones are carrion beetles (Silphidae), rove beetles (Staphylinidae), checkered beetles (Cleridae), skin beetles (Dermestidae), clown beetles (Histeridae) and sap beetles (Nitidulidae). The greatest limitation for the use of beetles in forensic entomology is the lack of developmental models.

Developmental models for only 15 species of forensically important beetles have been published so far. First models were created for *Thanatophilus micans* (Silphidae), population from South Africa[35]. The other carrion beetles with published models comprise: *Oxelytrum discicolle* population from northern part of South America[36], *Thanatophilus mutilatus* population from South Africa[37], *Necrodes littoralis*[15] population from Central Europe, *Necrophila (Calosilpha) brunnicollis* population from East Asia[38] and populations from Central Europe of *Thanatophilus sinuatus*[39] and *Thanatophilus rugosus*[40]. Models were created also for four species of *Dermestidae*: populations from south-western Europe of *Dermestes frischi, D. undulatus* and *D. maculatus* and Chinese population of *D. tessellatocollis*[41,42]. Developmental models were also published for central European population of *Sciodrepoides watsoni* (Leiodidae: Cholevinae)[43], Chinese[44] and central European[45] populations of *Creophilus maxillosus* (Staphylinidae) and Chinese populations of *Necrobia rufipes* (Cleridae)[46] and *Omosita colon* (Nitidulidae)[47]. Although there are beetle taxa that were extensively studied (e.g. *Thanatophilus* or *Creophilus*), still many forensically useful beetle species lack developmental models.

Necrodes littoralis (Silphidae) is widely distributed in the Palearctic region. It prefers open and forest natural habitats, but has also been recorded in urban open and quasi-indoor habitats[24,31,34,48,49]. Larvae feed on carrion mainly in spring and summer, adult beetles have also been recorded in the fall[24,48]. The species is associated with cadavers at active and advanced decay[7,31,48,50]. *N. littoralis* prefers large carrion, on which its larvae frequently form aggregations that allow them to drive active decay similarly to blow flies[50–52]. *N. littoralis* has been reported from many forensic cases, however it has infrequently been used to estimate PMI[17,31,34,49,53–55]. Comprehensive data on its occurrence on human cadavers in France was provided by Charabidze et al.[31]. According to their analysis, *N. littoralis* (larvae or adult beetles) were present in 154 cases (1 in 8 cases examined), with 91.6% of outdoor cases (mainly forests and bushes). Most of them occurred during spring or summer. In more than 85% of the cases *N. littoralis* was observed from the early to advanced decomposition stages. Moreover, the pre-appearance interval (PAI) of adult and larval *N. littoralis* was found to be strongly related to the preceding ambient temperature[56] and for this reason, it may easily be estimated using temperature methods for PAI[57]. Furthermore, the size of adult *N. littoralis* was found to be negatively correlated with its physiological age at maturity. Thus, such features as length or weight of adult *N. littoralis* can be useful to calibrate developmental constants for this species and to improve the accuracy of age estimation[15]. Some partial developmental datasets for *N. littoralis* were already published[15,58]. However, there is no comprehensive dataset providing all development models and based on the full temperature range for this species. This deficiency partially explains low frequency of *N. littoralis* use for the estimation of PMI. Current study aims to create the first comprehensive set of robust development models for *N. littoralis*. Some of the data used in this work was also used for previously published analyzes[15].

Results

Life cycle. Adult *Necrodes littoralis* is mainly active after dark. Mating occurs usually during the night. The female lays eggs in the soil, in batches, usually 50–70 eggs in each. The first instar larvae hatch creamy white and migrate quickly in search for food. It is the period when they are very fragile and particularly vulnerable to injuries. They darken, and their cuticle hardens with time. Likewise, the second and third instar larvae are white and non-sclerotized shortly after ecdysis. When feeding is complete, third instar larvae burrow into the ground. Then they form pupal chambers by thrashing the abdomen and thus compacting the soil around them. They go through the prepupal, pupal and teneral adult stages inside the chambers. The pupa is creamy white at the beginning and with time it gradually sclerotizes and darkens. The metamorphosis ends with the appearance of tenerals—creamy white and non-sclerotized adult beetles. Over time, their cuticle hardens and darkens, giving the insects their final black color. The beetles dig out of the pupal chamber after they became fully sclerotized and colored.

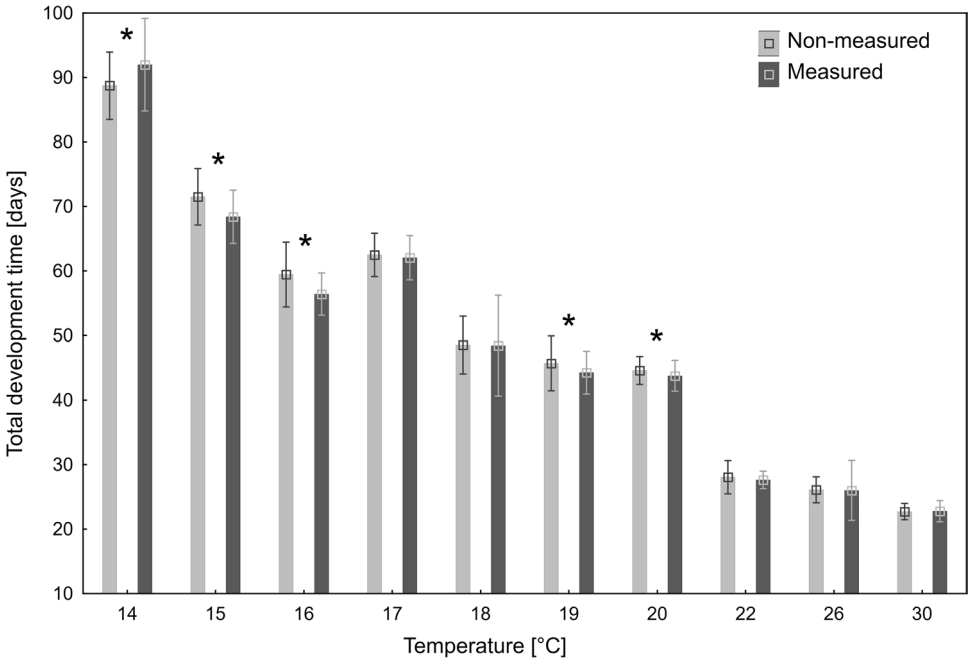

Figure 1. Differences in duration of total immature development between measured and non-measured specimens of *N. littoralis* at ten constant temperatures. *Statistically significant difference in Mann–Whitney *U* test at α = 0.001.

Model	Temperature range [°C]	Thermal summation constant—*K* (SE) [degree-days]	Developmental threshold—D_0 (SE) [°C]	r^2	N	p
Measured	14–30	421.09 (29.06)	9.251 (0.552)	0.968	9	< 0.001
Non-measured	14–30	436.90 (29.05)	9.051 (0.548)	0.967	9	< 0.001

Table 1. Thermal summation models for the total immature development of *N. littoralis*, calculated using measured or non-measured specimens.

Influence of in vivo measurements. In general, non-measured beetles developed longer (Fig. 1), with significant differences recorded at 15 °C (Mann–Whitney *U* test: $Z = 6.02$, $p < 0.001$), 16 °C (Mann–Whitney *U* test: $Z = 5.92$, $p < 0.001$), 19 °C (Mann–Whitney *U* test: $Z = 3.56$, $p < 0.001$) and 20 °C (Mann–Whitney *U* test: $Z = 3.20$, $p = 0.001$). The development time of non-measured beetles was shorter only at 14 °C (Mann–Whitney *U* test: $Z = -3.16$, $p = 0.001$). The largest difference was observed in 16 °C, where non-measured beetles developed 5.39% longer than measured beetles. However, in higher temperatures, starting from 17 °C, the differences between measured and non-measured beetles were very small (Fig. 1).

Developmental models calculated using measured and non-measured specimens were only slightly different (Table 1). There were, however no significant differences in the relative error of age estimation between the tested models (Wilcoxon signed-rank test: $Z = 0.96$, $p = 0.34$, $N = 110$). Both models yielded estimates of age with the average relative error below 0.08 (Fig. 2).

Mortality and development. The highest mortality was observed at extreme temperatures: 86.5% at 14 °C and 64.75% at 30 °C. At the other temperatures mortality was below 25%, except for 17 °C where it was 53% (Fig. 3). There were differences between the temperatures in the distribution of deaths across the life stages (Fig. 3.) At the lowest and the highest temperatures deaths occured during all developmental stages, while at the optimal temperatures beetles died mostly at postfeeding larval or pupal stages. At 17 °C deaths were also distributed across all developmental stages. The hypothesis as to why and consequences will be presented in the discussion section.

The development from egg to adult stage took between 22.84 days at 30 °C and 89.11 days at 14 °C (Table 2, Fig. 4.). Results from 17 °C deviated from the general pattern of decrease in development time with an increase in temperature. The first and second instar larvae at 17 °C developed much longer than larvae at 16 °C and 15 °C, and thus the total development time at 17 °C lengthened on average to 60.98 days. Due to this inconsistency and the exceptionally high mortality of larvae at 17 °C, we decided not to use the data from this temperature when developing the models (except for the model for hatching).

Immediately after hatching, the larvae had an average length of 6.91 ± 0.79 mm (Isomegalen diagram: Supplementary Fig. 1, Growth curves: Supplementary Figs. 2–10). At the growth peak, larvae were on average

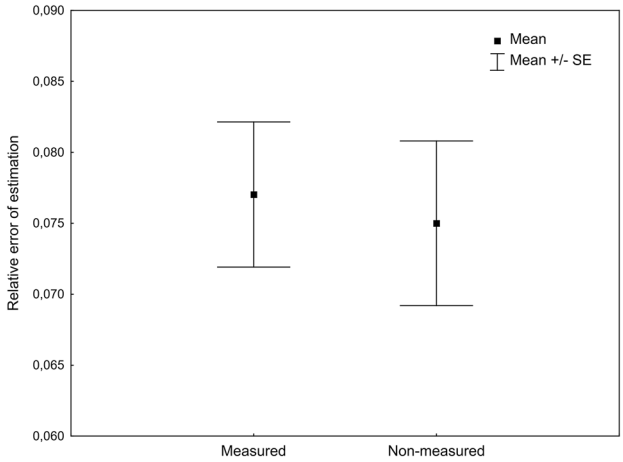

Figure 2. Relative error of age estimation using models calculated with measured or non-measured beetles.

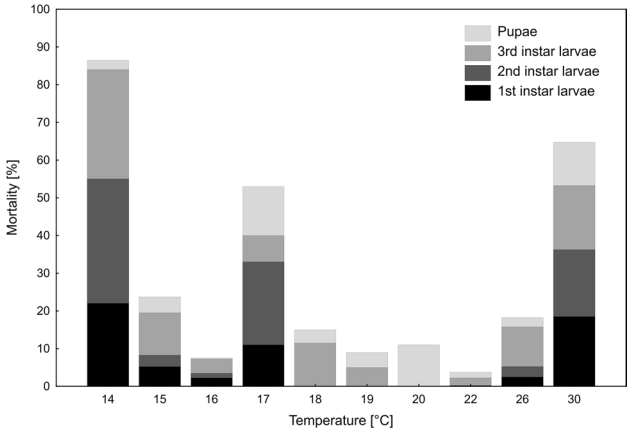

Figure 3. Mortality of *N. littoralis* at immature developmental stages in different rearing temperatures.

	Mean duration of development [days] (SE;N)					
Temp. [°C]	Egg	1st instar larva	2nd instar larva	3rd instar larva	Pupa	Total development
14	9.63 (0.15; 8)	10.33 (0.23; 8)	10.31 (0.41; 8)	41.35 (0.07; 63)	18.19 (0.42; 54)	89.11 (0.45; 54)
15	6.55 (0.22; 8)	7.65 (0.17; 8)	7.15 (0.35 ;8)	33.89 (0.15; 319)	16.34 (0.12; 302)	71.29 (0.17; 302)
16	5.95 (0.12; 8)	5.27 (0.11; 8)	4.83 (0.11; 8)	28.97 (0.16; 373)	13.35 (0.08; 370)	57.78 (0.14; 370)
17	5.53 (0.08; 8)	9.54 (1.31; 8)	8.55 (0.52; 8)	26.90 (0.16; 240)	12.10 (0.10; 188)	60.98 (0.21; 188)
18	4.93 (0.15; 8)	4.13 (0.16; 8)	4.01 (0.16; 8)	23.97 (0.11; 354)	11.85 (0.10; 341)	48.81 (0.20; 341)
19	4.02 (0.12; 8)	4.04 (0.18; 8)	3.92 (0.21; 8)	22.61 (0.13; 380)	11.80 (0.06; 364)	45.14 (0.13; 364)
20	3.67 (0.06; 8)	3.96 (0.10; 8)	3.77 (0.15; 8)	22.27 (0.11; 400)	10.35 (0.07; 357)	43.75 (0.08; 357)
22	2.73 (0.09; 8)	2.35 (0.08; 8)	2.29 (0.08; 8)	13.46 (0.05; 390)	7.43 (0.02; 385)	28.28 (0.06; 385)
26	2.66 (0.04; 8)	1.55 (0.03; 8)	1.68 (0.08; 8)	12.82 (0.07; 338)	7.03 (0.06; 327)	25.65 (0.08; 327)
30	2.38 (0.09; 8)	1.39 (0.07; 8)	1.45 (0.06; 8)	11.89 (0.05; 187)	5.93 (0.07; 141)	22.84 (0.10; 141)

Table. 2.. Duration of immature developmental stages of *N. littoralis* at ten constant temperatures.

25.14 ± 1.82 mm in length. Moulting usually occurred at a similar length of the larvae: 10.71 ± 0.79 mm for the first ecdysis and 16.10 ± 1.30 mm for the second ecdysis. Growth curves were distinctly sigmoidal (Supplementary Figs. 2–10).

All included temperature points were within 95% confidence interval for all reduced major axis regression models (Fig. 5). The coefficient of determination for each thermal summation model exceeded 0.96, indicating

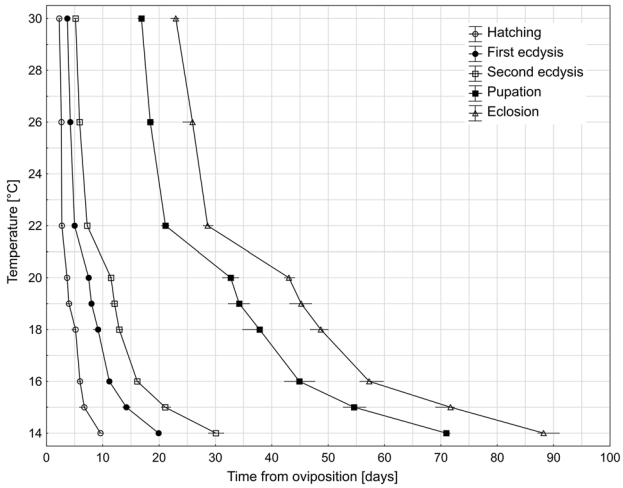

Figure 4. Isomorphen diagram for *N. littoralis* based on median times to reach developmental events at each of the nine temperatures tested (Supplementary Table 1). Horizontal bars represent interquartile ranges. Areas between lines represent developmental stages. Different symbols represent developmental events.

high fit of the models. Lower developmental thresholds (D_0) ranged from 9.04 °C for eclosion to 10.86 °C for second ecdysis. Thermal summation constants (K) were between 39.97 ADD for hatching and 434.705 ADD for eclosion (Table 3).

Discussion

Current results on the development time are consistent with the development times of *N. littoralis* obtained by Dekeirsschieter[58]. She studied the development at two temperatures only. The development (without the egg stage) took on average 42.79 days at 18 °C and 23.60 days at 23 °C. By subtracting the egg stage from the mean development time at equivalent temperature, in the current study, we get consistent values. TSM for the eclosion from this paper is different compared to the general model for the total immature development given in our previous paper (Table 1 in Gruszka & Matuszewski[15]). The previous model was created without data from 17 °C and 22 °C, as regression analysis showed that they were outside the 95% confidence interval. In the current study, we excluded the data from 17 °C before the analysis and therefore they were not used while calculating initial TSMs (we give the reasons for this in the results section and discuss proximate and distant causes later in this section). Omitting data from 17 °C changed the results of the regression analysis, and the data point from 22 °C was now within the 95% confidence interval, so it was not omitted while deriving the current final TSMs. Therefore, current article gives more accurate thermal summation values for the eclosion of *N. littoralis* and we encourage using the current TSM for the eclosion instead of the less accurate previous one.

The value of thermal summation constant (K) for the eclosion of *N. littoralis* is larger compared to those obtained for other silphid beetles (Table 4). Larger accumulation of thermal units needed to reach maturity by an insect species indicates its longer development time. The development of *N. littoralis* is the longest among studied silphid beetles. Furthermore, this species usually colonizes cadavers later than for instance *Thanatophilus* species[59,60]. Therefore, *Necrodes littoralis* expands the timeframe in which PMI may be estimated using carrion beetles. In other beetle families, the thermal summation values are larger than in carrion beetles (Table 4).

The pattern of mortality across temperatures is consistent with the patterns revealed for other forensically important beetles. In *T. micans*, *T sinuatus*, *D. tessellatocollis*, *C. maxillosus* and *N. rufipes* the highest mortality was observed at extreme temperatures[35,39,42,45,46]. The same pattern is reported in this study. At intermediate temperatures, mortality of *N. littoralis* was below 25%, which is also in line with mortality patterns reported for the other beetle species[42,45]. In the study of Dekeirsschieter at 23 °C, mortality of *N. littoralis* was surprisingly high. It amounted to 30%[58], whereas only 3.75% of the beetles died in 22 °C in the current study. Probably the higher mortality reported by Dekeirsschieter resulted from individual rearing of larvae, since mortality rate of individually reared larvae of *N littoralis* was found to be higher compared to the larvae that were reared in aggregations[61]. However, other effects might have been important, as well.

Mortality was exceptionally high at 17 °C in this study. More than half of the beetles died. Moreover, the development at this temperature deviated significantly from the general pattern, with the first and second instar larvae developing longer than at lower temperatures (16 °C and 15 °C). For this reason, we decided not to use data from 17 °C to calculate TSMs. Development extension and high mortality were probably caused here by rearing problems and were not directly related to the temperature itself. At 17 °C large numbers of nematodes were recorded in all containers. They were sticking to the larvae, which probably interrupted larval development and finally led to death of some larvae.

In order to create an isomegalen diagram and growth curves, it is necessary to measure larvae throughout their development. For this purpose, larvae can be killed and then measured. However, killing and storage methods may deform larvae[62]. Therefore, we decided to measure larvae in vivo. This method also has some

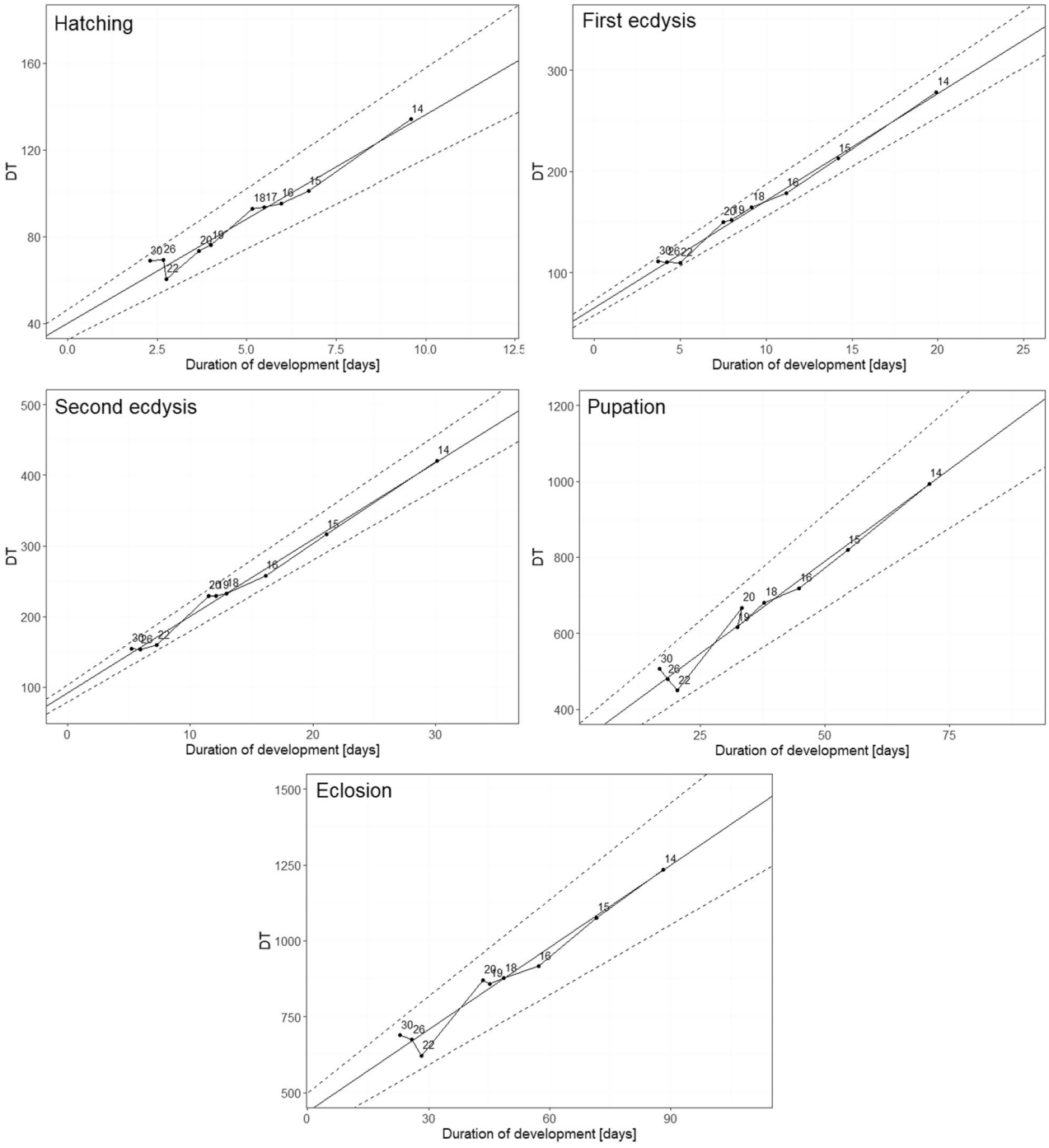

Figure 5. Reduced major axis (RMA) regression models for five developmental events of *N. littoralis*. DT is the time in days to reach developmental event multiplied by the constant rearing temperature. Temperature values are noted next to the points. Dashed lines represent 95% confidence intervals.

drawbacks. First, the temperature changes when larvae are removed from the temperature chambers for measurement. Second, by taking a measurement, stress is induced that in turn can cause an increase in juvenile hormone levels, which interfere with the development[63]. Previous study[64] revealed differences in development time between measured and non-measured *Creophilus maxillosus* beetles. However, after TSMs were built, it turned out that the estimation error did not differ significantly between the two models. The authors concluded that differences in development time were an effect of repeated stress rather than temperature changes during the measurements. Moreover, lack of significant differences between measured and non-measured TSMs was due to a small size of the differences and moderate size of the validation sample. In the current study, differences in the estimation error between the models were also insignificant. Based on this finding it can be concluded that in vivo measurements of forensically useful beetles have negligible impact on the resultant development models.

Developmental event	Temperature range [°C]	Model equation	Thermal summation constant—K (SE) [degree days]	Developmental threshold—D_0 (SE) [°C]	N	r^2	p
Hatching	14–30	$DT = 39.977 + 9.627 \times D$	39.977 (2.902)	9.627 (0.539)	10	0.968	< 0.001
First ecdysis	14–30	$DT = 65.747 + 10.583 \times D$	65.747 (3.235)	10.583 (0.315)	9	0.992	< 0.001
Second ecdysis	14–30	$DT = 92.060 + 10.861 \times D$	92.060 (5.096)	10.861 (0.326)	9	0.992	< 0.001
Pupation	14–30	$DT = 303.294 + 9.701 \times D$	303.294 (21.680)	9.701 (0.531)	9	0.973	< 0.001
Eclosion	14–30	$DT = 434.705 + 9.044 \times D$	434.705 (28.862)	9.044 (0.548)	9	0.967	< 0.001

Table 3. Thermal summation models for five developmental events of *N. littoralis* calculated using Ikemoto and Takai method.

Species	Family	Geographic population	Temp. range [°C]	Thermal summation constant [ADD]	Developmental threshold [°C]	References
Necrodes littoralis	**Silphidae**	**Central European**	**14–30**	**434.70 ± 28.86**	**9.04 ± 0.55**	**This study**
Thanatophilus micans	Silphidae	South African	17–20	197.97 ± 19.74	13.26 ± 0.58	Ridgeway et al.[37]
Thanatophilus mutilatus	Silphidae	South African	15–27.5	384.11 ± 16.99	9.04 ± 0.36	Ridgeway et al.[37]
Thanatophilus sinuatus	Silphidae	Central European	14–26	360.46 ± 10.75	9.85 ± 0.23	Montoya-Molina et al.[39]
Thanatophilus rugosus	Silphidae	Central European	12–22	362.76 ± 4.97	8.53 ± 0.08	Montoya-Molina et al.[40]
Dermestes tessellatocollis	Dermestidae	Chinese	16–25	664.39 ± 55.87	12.07 ± 0.53	Wang et al.[42]
Creophilus maxillosus	Staphylinidae	Chinese	17.5–30	492.06 ± 23.61	9.60 ± 0.58	Wang et al.[44]
		Central European	15–30	405.16 ± 14.63	11.66 ± 0.24	Frątczak-Łagiewska et al.[45]
Necrobia rufipes	Cleridae	Chinese	22–36	591.00 ± 39.53	16.62 ± 0.63	Hu et al.[46]
Omosita colon	Nitidulidae	Chinese	16–31	514.1 ± 8.7	10.65 ± 0.16	Wang et al.[47]

Table 4. Comparison of thermal summation models for the eclosion created for forensically important beetles.

Third instar larvae in this study had longer maximum body length than those in previous studies of *N. littoralis*[58,65]. In the studies of Dekeirsschieter[58], larvae were reared individually on small pieces of meat that could result in their smaller size. In the second study, larvae were collected from pig carcasses on the 17th, 20th and 24th days of decomposition[65]. The largest third instar larvae used in that study were therefore not the largest (longest) larvae present during decomposition, since after 24th day of decay larvae continued to feed and grow. In the current study, a substantial increase in larval length (above 22 mm) took place at the very end of the feeding phase (Supplementary Figs. 3–10). Fully mature Silphinae larvae range in size from 12 to 40 mm[66], current results on larval length are therefore consistent with this range.

When rearing larvae in aggregations, it is difficult to ensure that manual samples are taken at random. We tried to sample larvae irrespective of their position inside the aggregation and their size. However owing to a natural tendency to pick larger larvae, there may be a size bias in our analysis.

A transition of the third instar larva to the post-feeding phase is a critical moment in *N. littoralis* development. This is when the larva buries itself into the soil to form a pupal chamber. The transition was impossible in the containers in which larvae were reared in aggregations. Therefore, it was necessary to transfer them to separate, smaller containers after they ceased feeding. This transfer might slightly interfere with development. Identification of the exact moment of transition to the post-feeding phase is, however, difficult due to rather long duration and lack of unambiguous markers of transition. The same problem was reported in the study of *D. tessellatocollis* development, in case of which durations of the last larval stage and the prepupal stage were summed up due to the difficulties in identifying the transition point[42]. In the study on *T. sinuatus, T. rugosus* and *N. brunnicollis*, the post-feeding stage was distinguished[38–40]. Similarly, in the studies of *N. littoralis* by Dekeirsschieter, the post-feeding stage was identified, supposedly due to the rearing of larvae individually in Petri dishes that facilitated monitoring of the larvae[58].

Necrodes littoralis is a species with great forensic potential. However, the lack of development models has largely limited its use in forensic cases. By providing the first comprehensive developmental dataset for this species, current study makes a significant contribution to the advancement of forensic entomology, particularly in central and northern Europe.

Materials and methods

Laboratory colony. Adult beetles came from our laboratory colony established in 2017 using insects collected in the alder forest of Biedrusko military range (Western Poland, Central Europe, 52° 31′ N, 16° 54′ E). They were kept at room temperature in medium-sized terrariums, 20–30 adult beetles per box (2–3 boxes maintained simultaneously). Boxes contained soil and cotton wool soaked with water. Beetles were fed with pork ad libitum.

Experimental rearing. Laboratory rearing was conducted at ten constant temperatures: 14, 15, 16, 17, 18, 19, 20, 22, 26 and 30 °C. We followed the same protocol for each of the tested temperatures. Rearing was carried out inside the temperature chambers (type ST 1/1 BASIC or ST 1/ 1 +, POL-EKO, Poland). We controlled the chambers using temperature recorders. Deviations from the settings were consistent with the manufacturer's assurances (they did not exceed ± 0.5 °C). In order to induce oviposition adult beetles from the main colony were matched in pairs and placed in 0.5-L containers filled with soil. They had constant access to water and pork ad libitum. Containers were inspected every four hours for the presence of fresh eggs. If eggs were recorded, adult beetles were taken out from the container and eggs were left for hatching. Further inspections were carried at intervals representing no more than 10% of the egg stage duration. Inspection times were calculated based on the results of pilot studies. After hatching, first instar larvae were counted, and 50 larvae were placed in a small terrarium (18 cm × 11 cm × 14 cm) for further rearing. There were eight replicates per temperature (8 containers with 50 larvae each). Rearing boxes contained soil, cotton wool soaked with water, and pork meat ad libitum. Meat was covered with aluminum foil to avoid drying out. After feeding was completed and larvae started to bury themselves, they were transferred to new 0.5-L containers (8–10 larvae per container), filled with soil to allow them to form pupal chambers and complete their development.

Inspections and measurements. Immature beetles from every terrarium were checked for transition to the next developmental stage: second instar larva, third instar larva, post-feeding larva, pupa and adult beetle. Inspections were carried out at time intervals that were no longer than 10% of the stage duration. In the case of the second instar, third instar and post-feeding larvae data were collected per container. Transitions were identified when more than half of the larvae passed to the next stage. Data for pupae and adult beetles were collected per insect. Mortality was measured for each stage of development. For this purpose, live individuals were counted after transition to the next stage. In addition, 24 sampled larvae from four out of eight containers (six larvae per container) were measured in vivo during each inspection. Length of their body was measured from the anterior margin of clypeus to the posterior end of the last abdominal segment. Measurements were made using a geometrical micrometer[67]. Larvae in the other four containers were inspected for transitions and mortality only.

Data analysis. *Influence of in vivo measurements.* To test the influence of multiple in vivo measurements on the development of *N. littoralis*, we compared the total development time of measured and non-measured beetles. 100 randomly selected individuals were used per temperature (54 beetles at 14 °C due to high mortality). Non-parametric Mann–Whitney U test was used in this comparison. To account for the multiple tests we used Bonferonni correction (since there were 10 tests, we used 0.005 level of significance). Then, using data for measured and non-measured beetles, we created separate thermal summation models with the Ikemoto and Takai method[11]. In order to validate these models we used 110 non-measured beetles (15 beetles per temperature; insects from 17 °C were omitted; due to the high mortality, we had no specimens from 14 °C and only 5 beetles from 30 °C). The true physiological age of the insects (known based on laboratory rearing) was compared with the age estimated using the models. The differences between true and estimated age were used to quantify the estimation errors for measured and non-measured TSM. These errors were compared using the Wilcoxon signed-rank test. Analyzes were performed with Statistica 13 (TIBCO Software Inc.).

Mortality and development. We calculated the percentage mortality for each developmental stage and each of the temperatures tested. Due to the difficulty in accurate separation of the active feeding and post-feeding third instar larvae, we summed up the mortality for these stages. They were similarly combined for the purpose of all the subsequent analyzes. Using data for all individuals, we calculated the mean duration for each developmental stage at each temperature. Median times to reach developmental landmarks (i.e. hatching, first ecdysis, second ecdysis, pupation and eclosion) were calculated and used to build the isomorphen diagram. Using larval measurements, we built isomegalen diagram and growth curves. Thermal summation models were derived using the Ikemoto and Takai method[11]. For this purpose, we used measured and non-measured beetles. For hatching, first ecdysis and second ecdysis, the sample comprised eight observations per temperature, since we had eight containers for each temperature and could use only "container data" in these analyzes. For pupation and eclosion, the sample comprised 100 observations per temperature, since we could use "individual data" in these analyzes (for 14 °C the sample comprised 63 beetles upon pupation and 54 beetles upon eclosion). Growth curves and thermal summation models were created in R 3.5.2. The other analyzes were performed using Statistica 13 (TIBCO Software Inc.).

Ethical approval. The study comprised laboratory experiments using insect species *Necrodes littoralis* (Coleoptera: Silphidae). The species is not under protection. No permission or approval from Ethic Commission were needed.

Data availability

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

Received: 25 February 2022; Accepted: 30 May 2022
Published online: 11 June 2022

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Acknowledgements

The study was funded by the National Science Center of Poland (grant no. 2016/21/B/NZ8/00788).

Author contributions

J.G. performed laboratory rearing and data collection, analyzed the results and prepared figures. S.M. obtained funds and supervised the research. Both authors designed the study and wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-13901-y.

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Supplementary information for

Temperature models of development for *Necrodes littoralis* L. (Coleoptera: Silphidae), a carrion beetle of forensic importance in the Palearctic region

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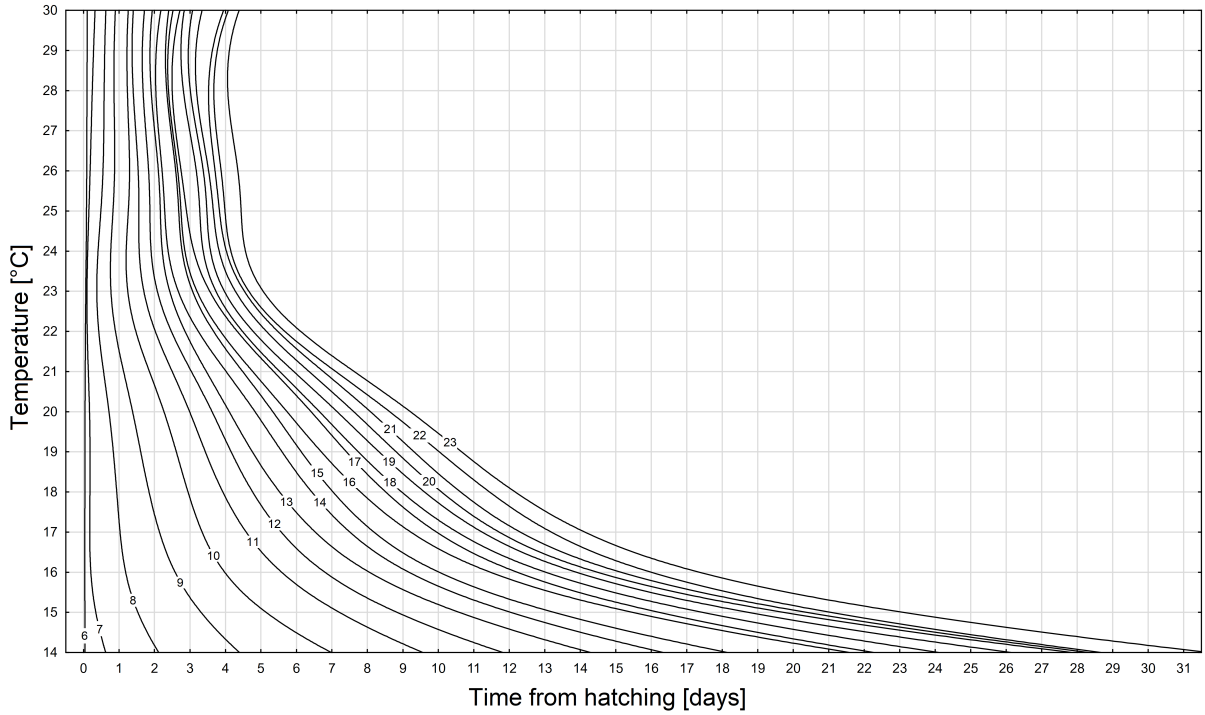
Supplementary tables

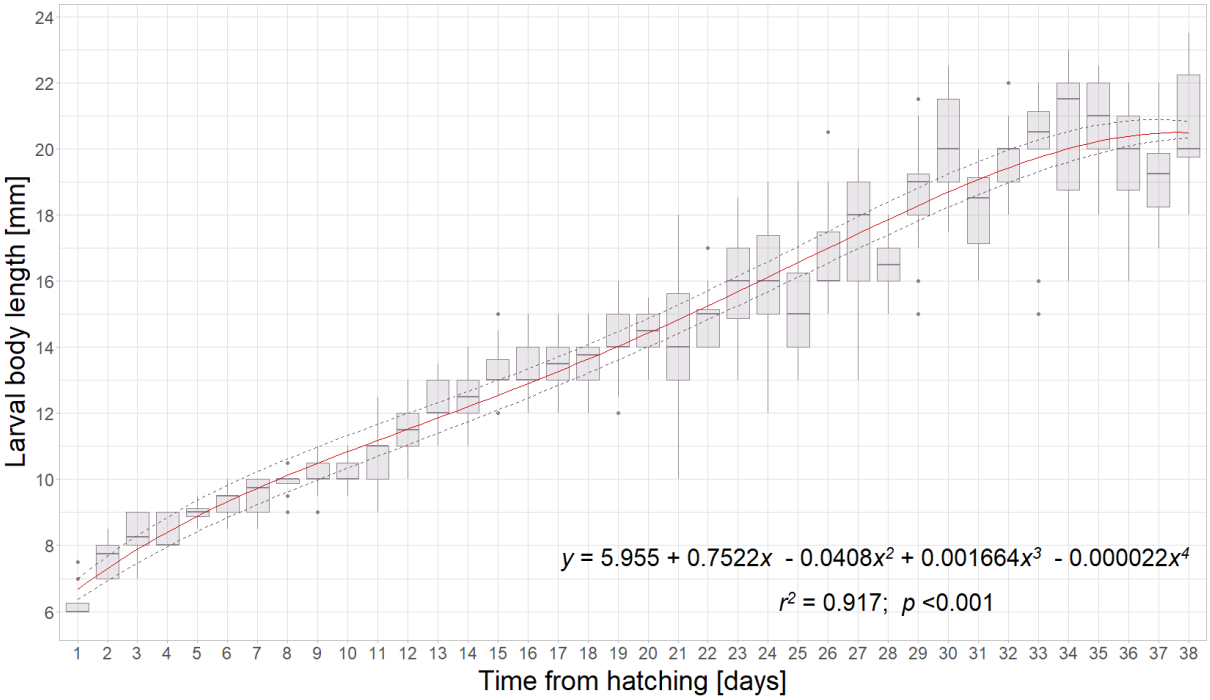
Supplementary Table 1. Median time to reach developmental events for *N. littoralis* at nine constant temperatures. IQR – interquartile range

Temperature [°C]	Median time to reach a developmental event [days] (IQR)				
	Hatching	First ecdysis	Second ecdysis	Pupation	Eclosion
14	9.58 (0.58)	19.90 (0.71)	30.06 (2.69)	70.96 (1.13)	88.21 (3.75)
15	6.73 (1.02)	14.19 (1.31)	21.06 (1.65)	54.58 (3.96)	71.71 (3.29)
16	5.96 (0.44)	11.17 (0.29)	16.13 (0.58)	44.88 (5.35)	57.27 (4.13)
18	5.17 (0.69)	9.17 (1.10)	12.92 (1.00)	37.83 (3.17)	48.71 (3.17)
19	4.00 (0.67)	8.00 (0.33)	12.08 (1.17)	34.21 (3.75)	45.21 (3.83)
20	3.67 (0.33)	7.50 (0.33)	11.46 (0.63)	32.71 (2.04)	43.04 (1.79)
22	2.75 (0.27)	5.00 (0.29)	7.25 (0.08)	21.13 (1.33)	28.60 (1.69)
26	2.67 (0.13)	4.25 (0.13)	5.90 (0.33)	18.42 (1.13)	25.92 (1.77)
30	2.29 (0.33)	3.71 (0.42)	5.17 (0.50)	16.88 (1.00)	22.96 (1.42)

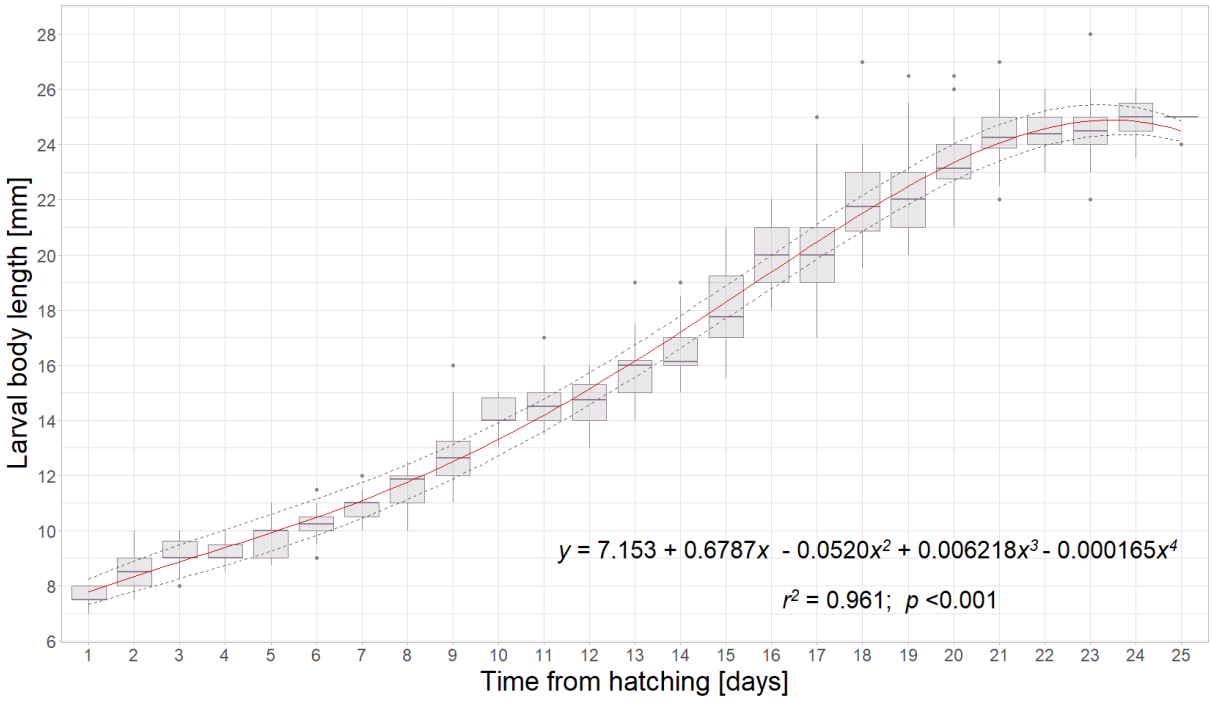
Supplementary figures



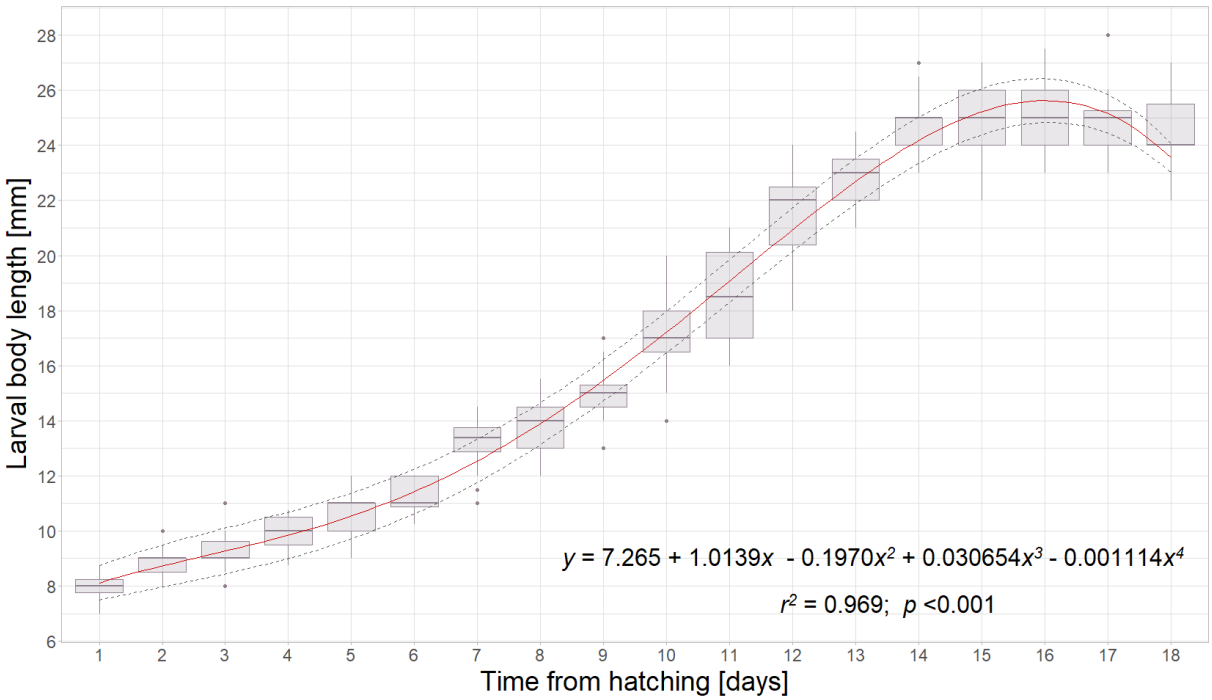
Supplementary Figure 1. Isomegalen diagram for *N. littoralis*. Each line represents larval body length.



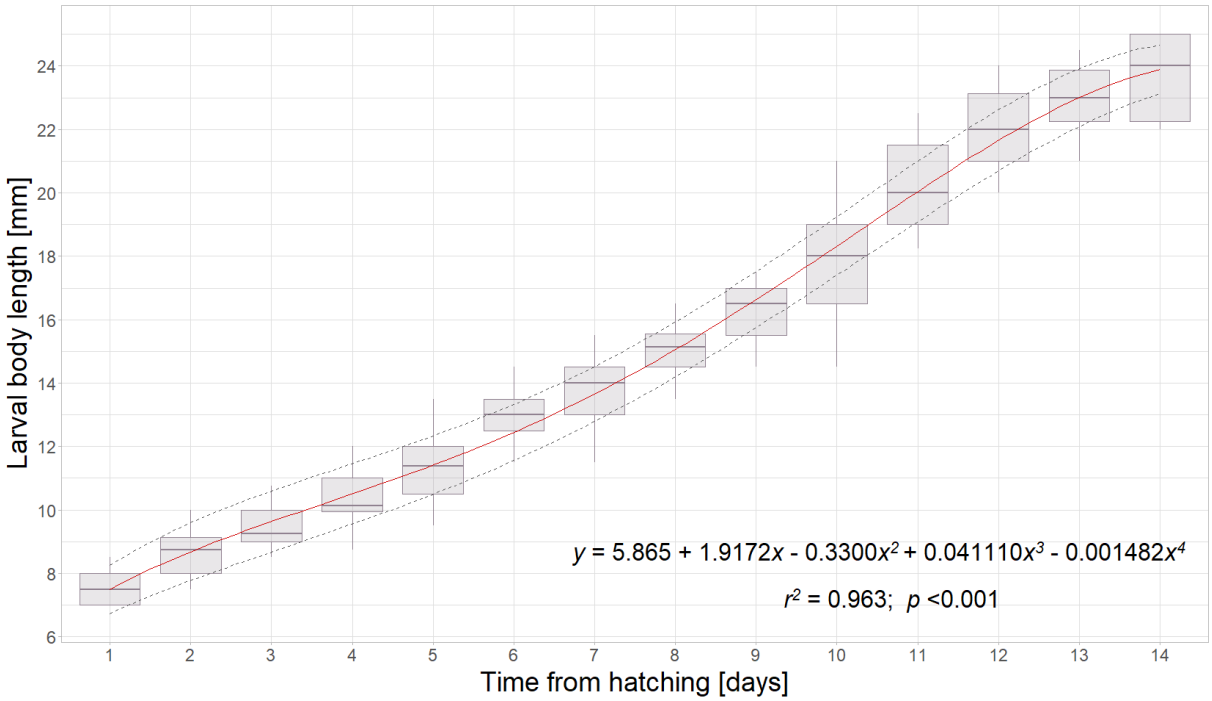
Supplementary Figure 2. Growth curve of *N. littoralis* larvae at 14°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



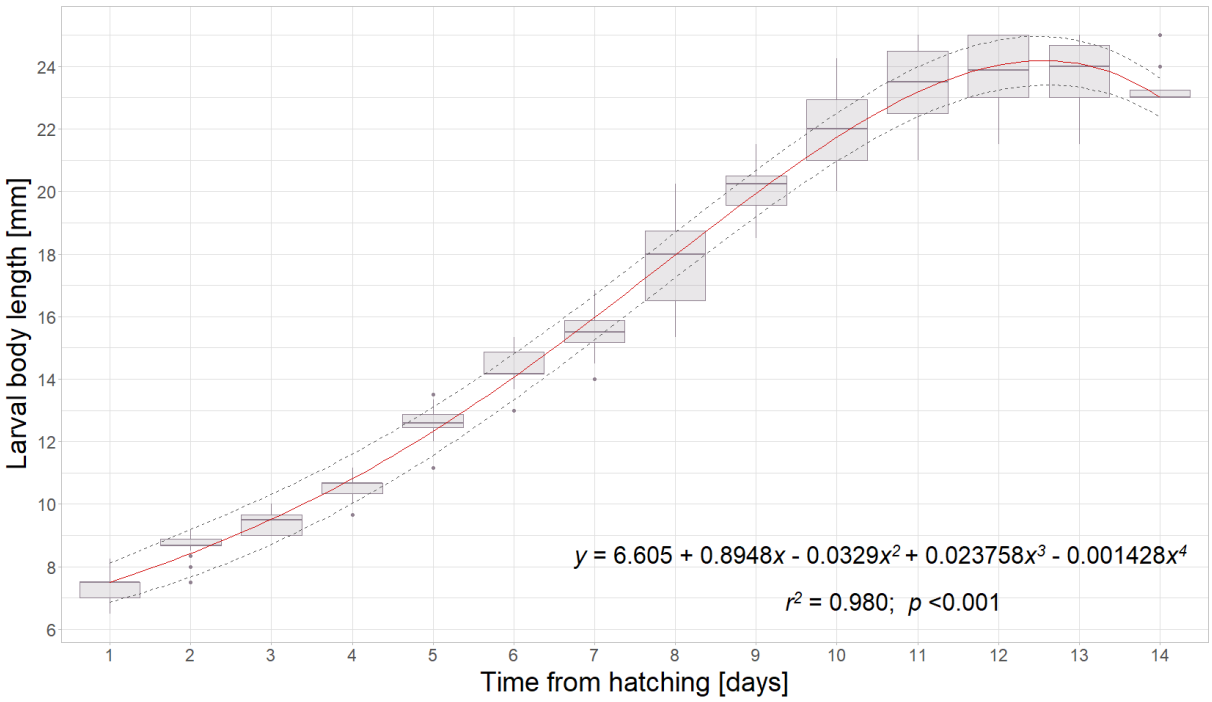
Supplementary Figure 3. Growth curve of *N. littoralis* larvae at 15°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



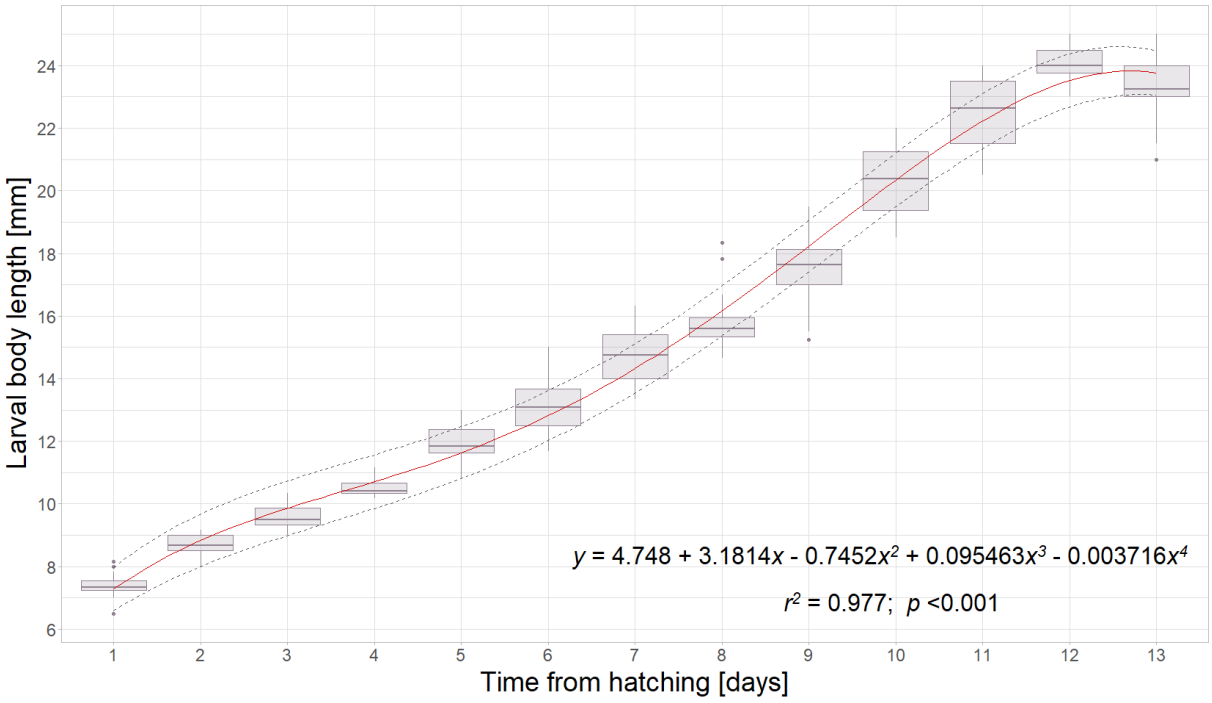
Supplementary Figure 4. Growth curve of *N. littoralis* larvae at 16°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



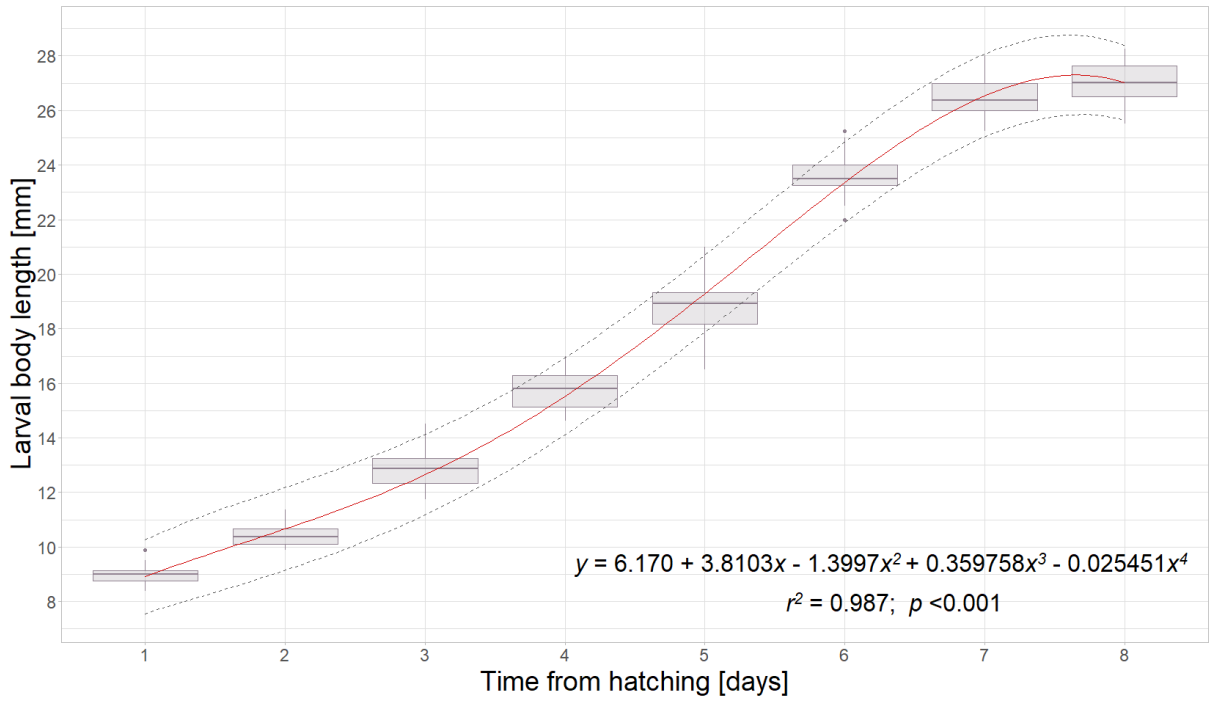
Supplementary Figure 5. Growth curve of *N. littoralis* larvae at 18°C. Boxplots show median, interquartile range, minimum and maximum body length. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



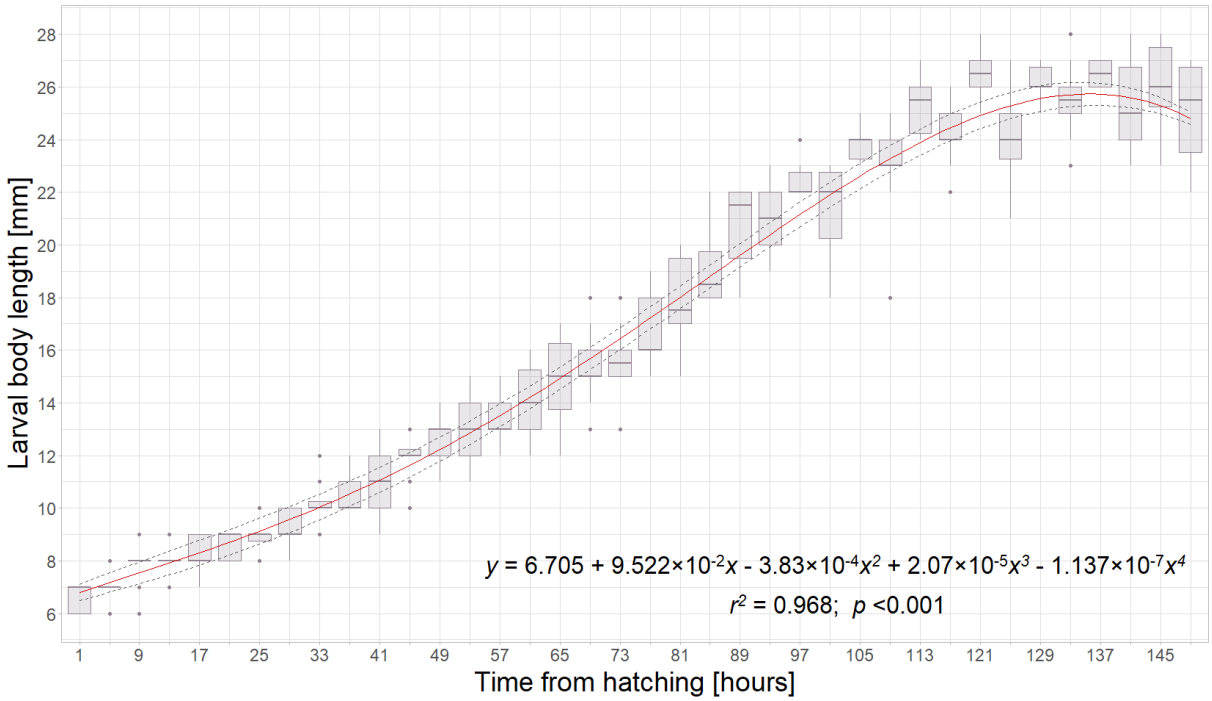
Supplementary Figure 6. Growth curve of *N. littoralis* larvae at 19°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



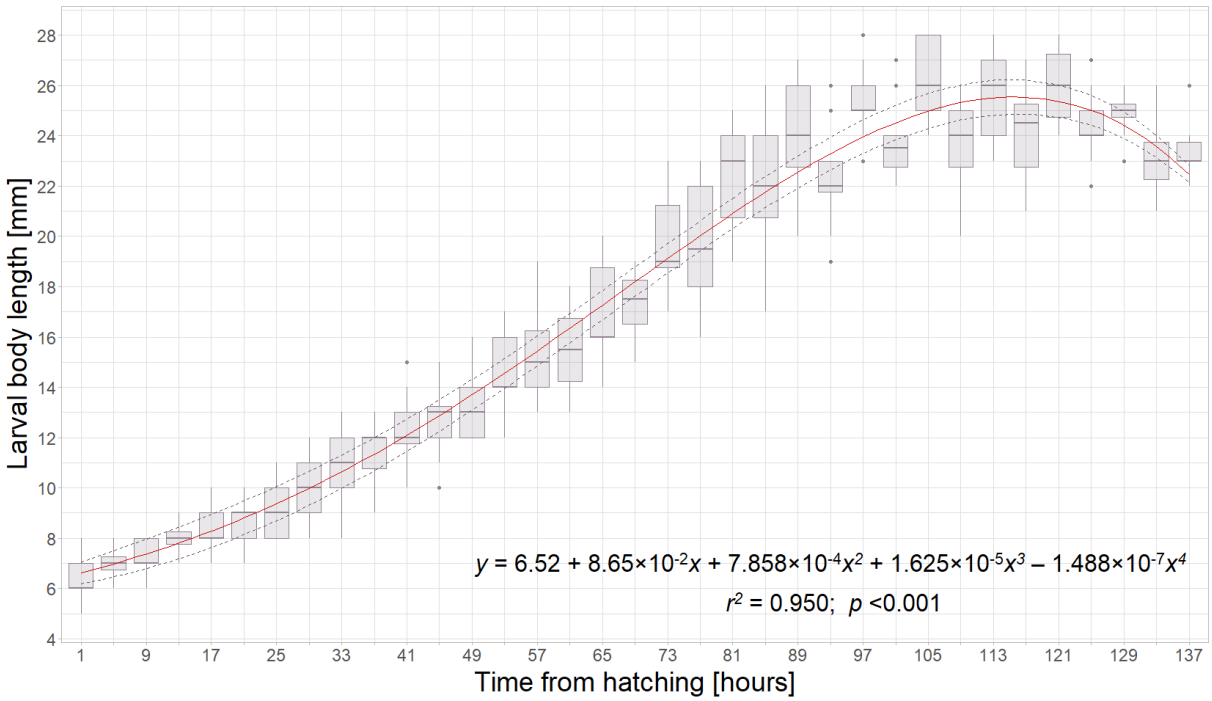
Supplementary Figure 7. Growth curve of *N. littoralis* larvae at 20°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



Supplementary Figure 8. Growth curve of *N. littoralis* larvae at 22°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



Supplementary Figure 9. Growth curve of *N. littoralis* larvae at 26°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.



Supplementary Figure 10. Growth curve of *N. littoralis* larvae at 30°C. Boxplots show median, interquartile range, minimum and maximum body length. Dots are outliers. Red line represents polynomial model of the larval body length over the time of development in the active feeding phase (equation and statistics are given in the plot). Dashed lines represent standard error bounds.

IV

Gruszka J, Matuszewski S. 2023.

Initial laboratory validation of temperature development models for Necrodes littoralis L. (Staphylinidae: Silphinae).

International Journal of Legal Medicine 137, 903–911.

International Journal of Legal Medicine
https://doi.org/10.1007/s00414-023-02969-4

ORIGINAL ARTICLE

Initial laboratory validation of temperature development models for *Necrodes littoralis* L. (Staphylinidae: Silphinae)

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Received: 21 November 2022 / Accepted: 8 February 2023

Abstract
Development models of necrophagous insects are applied in forensic entomology for post-mortem interval estimation. Such estimates may be used as scientific evidence in legal investigations. For this reason, it is important that the models are valid and that the expert witness is aware of their limitations. *Necrodes littoralis* L. (Staphylinidae: Silphinae) is a necrophagous beetle species that frequently colonizes human cadavers. Temperature models of development for the Central European population of these beetles were recently published. In this article, we present results of the laboratory validation study for these models. Errors of beetle age estimation differed significantly between the models. Thermal summation models yielded the most accurate estimates, and the isomegalen diagram least accurate estimates. Errors of the beetle age estimation varied across beetle developmental stages and rearing temperatures. In general, most development models of *N. littoralis* were satisfactorily accurate in estimating beetle age under laboratory conditions; therefore, the study provides initial evidence to support their validity in forensic cases.

Keywords Forensic entomology · Insect age estimation · Post-mortem interval · PMI · Validation study

Introduction

Development models of necrophagous insects are used in legal investigations to yield an age of insect evidence sampled from human cadavers and eventually to estimate the minimum post-mortem interval (PMI_{min}), which is the minimum time that elapsed from death to body disclosure [1]. Insect evidence can also be used to establish the circumstances of death in the case of endangered or protected wild animals [2]. Therefore, development models of forensically important species are used by forensic entomologists in their routine work. Since age estimates derived from these models are frequently used as scientific evidence in legal cases, the models should be valid [3]. Estimations based on unvalidated or flawed models may not only be challenged in court [4] but may also support unjust convictions in criminal trials [5]. Surprisingly, the validity of insect-based methods for the PMI estimation, and in particular the validity of development models for individual species, was rather poorly explored in validation studies [6].

PMI estimation protocols can be validated in several ways. The most common is the proof-of-assumptions study, which tests the basic assumptions of the protocol. The second type is the proof-of-concept study, where the protocol is tested in a simplified setup, usually under laboratory conditions. The least frequently used type is an experimental validation with human or non-human cadavers. It allows the protocol to be tested under experimental conditions, which can closely imitate conditions on a death scene. PMI estimation protocols can also be evaluated using forensic casework data [6]. Since development models of carrion insects are key elements of the protocols for the estimation of PMI based on insect development, validation types outlined above refer also to these models.

In recent years, efforts have been made to standardize the protocols used to sample and analyze insect evidence [7–10]. However, no attempts have been made to standardize

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Published online: 22 February 2023

methods used to create developmental models of necrophagous insects. Models are developed following various protocols, which in consequence can affect the accuracy and precision of age estimation using the models [4]. The creation of development models for a new species should be followed with their validation. The validation studies can objectively demonstrate the usefulness of a model for a specific purpose [5]. Knowing the limitations of the model, an expert may decide which method and model will be the most appropriate to the evidence at hand. In addition, validation studies frequently provide information on the precision and accuracy of age estimation using the model. Despite the awareness of the need to validate development models in forensic entomology [11–13], such studies are not common. Of the recently published models, only a few were validated (e.g., [14–18]).

Necrodes littoralis is a common necrophagous beetle with a Palearctic occurrence. Both larvae and adult insects are frequently found on human cadavers [19–24]. The first comprehensive development dataset for the Central European population of *N. littoralis* was recently published [25]. The dataset contains different types of temperature models that probably differ in the accuracy of insect age estimation. By analyzing the relative errors of age estimation using thermal summation models, isomorphen and isomegalen diagrams and growth curves, for beetles reared in the laboratory at five constant temperatures, we provide initial evidence to support the validity of these models. The current validation dataset exposes weaknesses and strengths of particular models when they are used to estimate the age of *N. littoralis*.

Materials and methods

Laboratory rearing and data collection

Data for the validation were collected from May 2021 to January 2022 using the same protocol as the one used for the modelling purposes [25]. However, we used a smaller number of rearing temperatures and smaller numbers of pupae and adult beetles. Moreover, the frequency of inspections (including measurements) was slightly lower.

To collect fresh eggs, adult beetles from our main colony were paired (two pairs per container) in plastic containers (18 cm × 11 cm × 14 cm) with soil, pork meat, and cotton wool with water. Containers were kept in temperature chambers (ST 1/1 BASIC or ST 1/1 +, POL-EKO, Poland) under five constant temperatures: 15, 18, 20, 22, and 26 °C. After oviposition, adult beetles were removed from the containers. Upon hatching, fifty fresh first instar larvae from each of the containers were transferred to new terrariums (the same size, soil, pork meat, and cotton wool with water). As a result, eight larval colonies (terrariums) were established per temperature. Once or twice a day (depending on larval stage and temperature), one larva from each of the eight containers was measured using a geometrical micrometer [26]. The larvae were measured in vivo and were returned to the container after the measurement. To ensure that a larva is fully extended, the measurement was taken in an Eppendorf tube while keeping it horizontally. Number of measurements were very small (12–20 measurements per colony during the whole study) to minimize the potential effects of in vivo measurements on the development. Post-feeding larvae were transferred to smaller containers (10 larvae per container) with soil for pupation. For this purpose, we looked for the moment when the larvae had finished feeding (in such case they are not present on the meat and do not walk on the soil surface, but bury themselves into the soil). Larvae placed in small, transparent containers usually form pupal chambers near the walls of the container, which allows for the monitoring of the further development and identification of the pupation and eclosion times.

Colonies were inspected for developmental landmarks (hatching, 1st and 2nd ecdysis, pupation, and eclosion) once, twice, or three times a day, depending on the temperature and the developmental stage. Since breeding was done in aggregations, the times to the first and the second ecdysis were determined for the colony. As there is variation in the timing of development within a colony, we recorded the beginning of the transition (when we observed the first post-ecdysis larva), the middle of the transition (when we observed that at least 50% of the larvae had moved to the next instar) and the end of the transition (when the last larva has moved to the next stage). These three timepoints were subsequently used in the analyses as the transition times for the colony. The times to the pupation and eclosion were monitored for individual beetles and therefore individual data were used in the analyses for these two landmarks.

Validation

To validate thermal summation models, we compared the physiological age from the models ("model" K) with the true physiological age ("true" K) calculated using current data, according to the equation:

$$K = D \times (T - t) \tag{1}$$

where D is the duration of development, T is the rearing temperature, and t is the lower developmental threshold.

To assess the isomorphen diagram, we compared medians for developmental landmarks extracted from the diagram against the true transition times recorded in the present study. Evaluation of the isomegalen diagram and growth curves was performed similarly. The length of the larvae from this study was used to derive the times to reach these lengths in a given temperature according to the isomegalen

diagram or a growth curve. Then, these times were compared with the true times recorded in this study.

For each model, we calculated relative errors of age estimation according to the formula:

$$\text{RELATIVE ERROR} = \frac{|\ \text{VALUE FROM THE MODEL} - \text{TRUE VALUE}\ |}{\text{TRUE VALUE}}, \quad (2)$$

where VALUE FROM THE MODEL is K from the thermal summation model or median time for a given developmental landmark (from isomorphen diagram) or the time to reach a given larval length (from isomegalen diagram or growth curves) and TRUE VALUES are the true equivalents of these values as recorded in this study.

We used Kruskal-Wallis ANOVA to compare errors between the models, development stages and rearing temperatures (at $\alpha = 0.05$, after Bonferroni correction 0.0056). Analyses were conducted in Statistica 13 (TIBCO Software Inc.).

Results

Errors of age estimation varied between the models, with the thermal summation model yielding the smallest errors and isomegalen diagram the largest errors (Kruskal–Wallis test: H(3) = 759.857, $p < 0.001$; Fig. 1; Supplementary Table 1).

Thermal summation models produced differently accurate estimates of age depending on the rearing temperature (Kruskal–Wallis test: H(4) = 373.592, $p < 0.001$; Fig. 2c). The smallest errors were observed at the highest temperature and the largest errors at the lowest temperature (Fig. 2c, Supplementary Table 3). There were no significant differences in the relative error of age estimation between models for different developmental landmarks (Kruskal–Wallis test: H(4) = 1.332, $p = 0.856$; Fig. 2b).

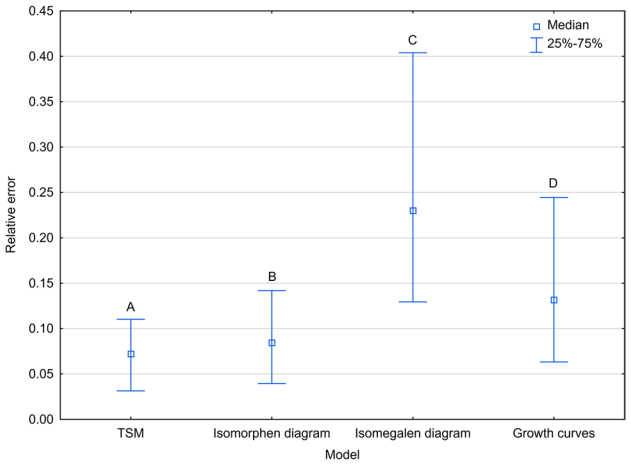

Fig. 1 Relative error of age estimation in *Necrodes littoralis* using different developmental models. The graph shows the medians for all developmental stages. TSM – thermal summation models; Different letters denote significant differences in pairwise comparisons for Kruskal-Wallis ANOVA at $p = 0.05$

The errors of age estimation using thermal summation models were in majority of cases below 0.1, regardless of the developmental event (70% of estimations for hatching, 68% for the first ecdysis, 69% for the second ecdysis, 65% for pupation, and 71% for eclosion). In the case of the thermal summation model for the eclosion, 93% of estimations had errors below 0.15 (Supplementary Fig. 1).

Isomorphen diagram gave different errors of age estimation depending on developmental landmark (Kruskal–Wallis test: H(4) = 14.871, $p < 0.001$, Fig. 3b) and rearing temperature (Kruskal–Wallis test: H(4) = 361,627, $p < 0.001$, Fig. 3c). Again, the smallest errors were recorded at the highest temperature (Fig. 3c). Most errors were below 0.1 (Supplementary Fig. 2).

Estimating the age of larvae using an isomegalen diagram gave more accurate results for the second and the third instar larvae compared to the first instar larvae (Kruskal–Wallis test: H(2) = 73.945, $p < 0.001$; Fig. 4b; Supplementary Table 6). There were also differences in errors of age estimation between temperatures (Kruskal–Wallis test: H(4) = 111.206, $p < 0.001$; Fig. 4c, Supplementary Table 5).

In the case of the first instar larvae, 57% of relative errors of age estimation using the isomegalen diagram were below 0.5 and 92% were below 1. At least half of the age estimates for the second and the third instar larvae had errors lower than 0.1 (Supplementary Fig. 3). There were mostly overestimations at 15 °C, and underestimations at 20 °C, 22 °C, and 26 °C (Fig. 5).

Errors of age estimation using growth curves varied between developmental stages (Kruskal–Wallis test: H(2) = 43.437, $p < 0.001$) and temperatures (Kruskal–Wallis test: H(4) = 45,267, $p < 0.001$). The largest errors occurred in the case of the first instar larvae (Fig. 6b, Supplementary Table 8) and at 15 °C and 18 °C (Fig. 6c, Supplementary Table 9). At lower temperatures, the errors were mainly overestimations, at higher temperatures they were mostly underestimations (Fig. 7).

Discussion

Our results indicate that the models based on the developmental landmarks, i.e., TSM and isomorphen diagram, allow for more accurate estimation of insect age than models based on the length of the larvae. The overall accuracy of the estimation is the result of a combination of precision and bias, and one of the sources of imprecision is the natural variation of the measured variable [11]. The variable relevant for the isomegalen diagrams and growth curves is the length of the larvae. It varies in larvae of the same age (i.e., larvae

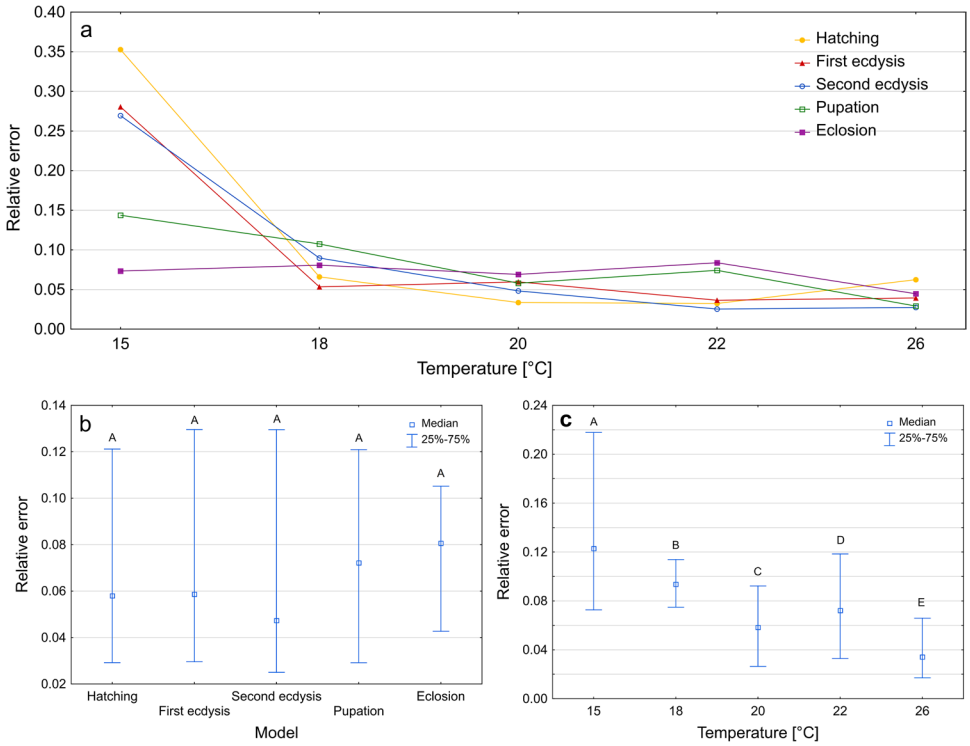

Fig. 2 Relative error of age estimation in *Necrodes littoralis* using thermal summation models. **a** Medians of errors for developmental landmarks at each temperature. **b** Medians of errors for developmental landmarks only. **c** Medians of errors for temperatures only. Different letters denote significant differences in pairwise comparisons for Kruskal-Wallis ANOVA at $p = 0.05$

of the same length may be in different age) [11]. Moreover, the change in larval length may be more or less dynamic depending on the larval instar. High natural variation of larval length and poor correlation between length and larval age during the initial and final phases of the larval stage lower the final accuracy of the age estimation. In the case of the models based on developmental landmarks, this type of variation does not occur or has smaller effects. There is little natural variation in transitions between developmental stages and these transitions are closely related to insect age.

However, it should be noted that the estimation errors in the case of TSM and isomorphen diagrams relate to the

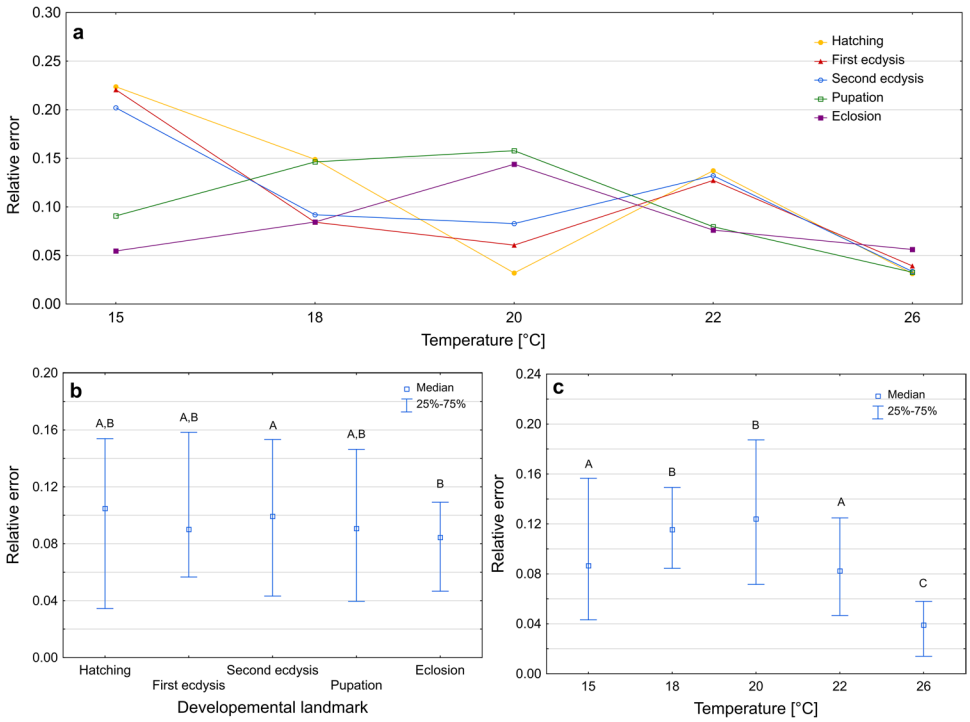

Fig. 3 Relative error of age estimation in *Necrodes littoralis* using isomorphen diagram. **a** Medians of errors for developmental landmarks at each temperature. **b** Medians of errors for developmental landmarks only. **c** Medians of errors for temperatures only. Different letters denote significant differences in pairwise comparisons for Kruskal-Wallis ANOVA at $p = 0.05$

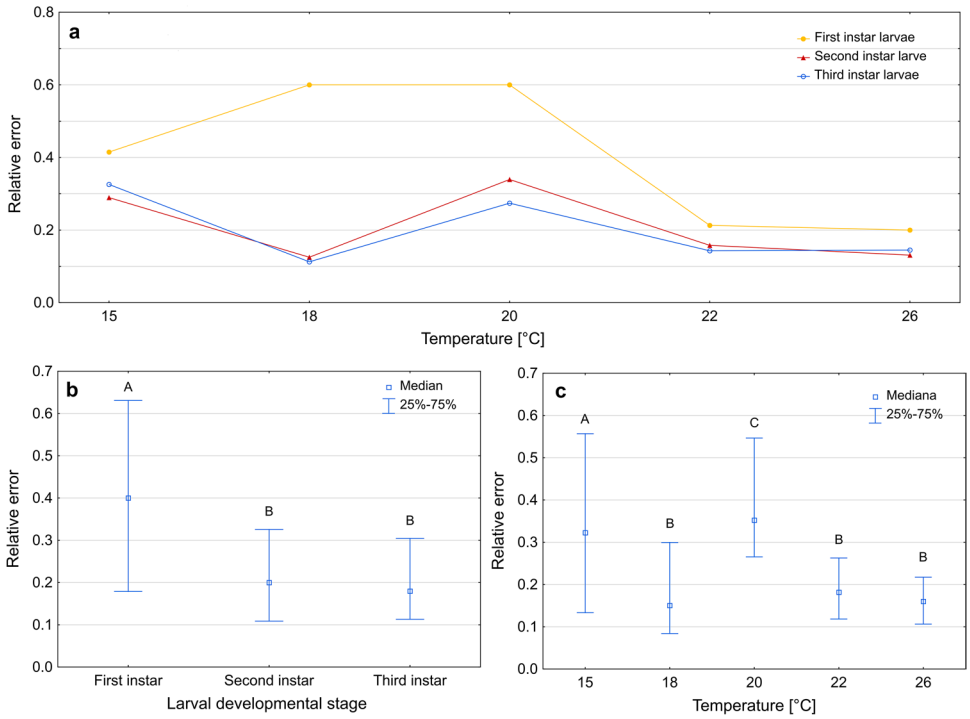

Fig. 4 Relative error of age estimation in *Necrodes littoralis* using isomegalen diagram. **a** Medians of errors for larval developmental stages at each temperature. **b** Medians of errors for larval developmental stages only. **c** Medians of errors for temperatures only. Different letters denote significant differences in pairwise comparisons for Kruskal–Wallis ANOVA at $p = 0.05$

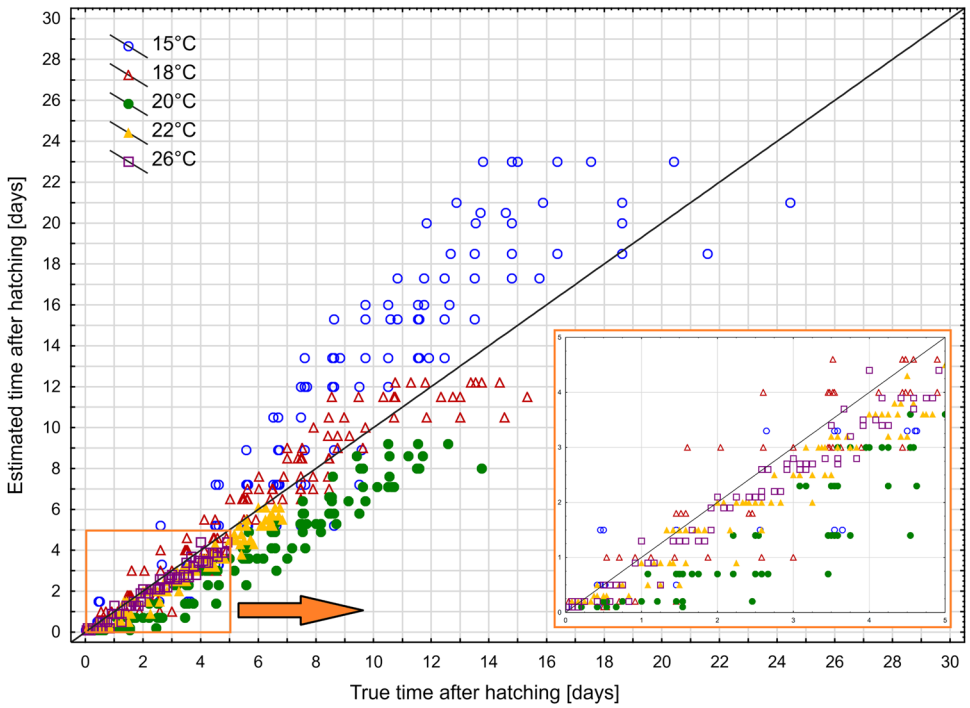

Fig. 5 The estimated time after hatching (using isomegalen diagram) plotted against the true time after hatching. Solid line represents perfect estimates

age of insects exactly at the point of transition to the next developmental stage. In forensic casework, insect evidence are rarely found shortly after this transition; usually, they are collected sometime later. At low temperatures, it can be up to several days even in the case of the short developmental stages. For this reason, the estimation of insect age up to the developmental landmark with high accuracy does not mean that the estimate of the entire age of an insect will be similarly accurate. In such cases, developmental models based on larval length can be helpful, despite relatively large estimation errors; particularly, when no living insects were sampled [9].

For all the models, the temperature at which insects were reared had a more pronounced impact on the accuracy of age

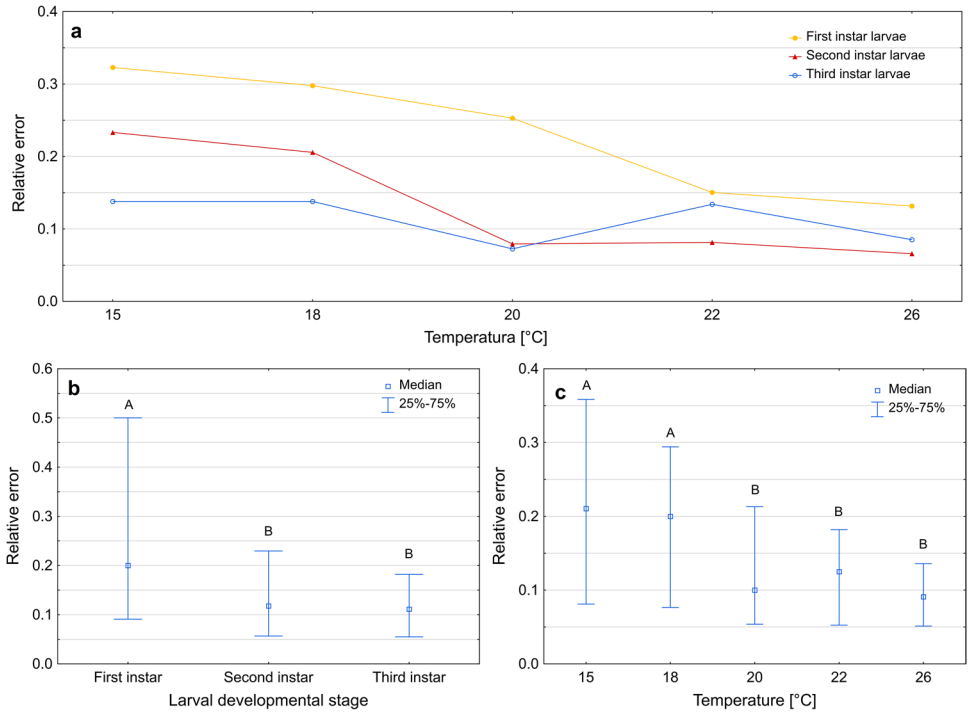

Fig. 6 Relative error of age estimation in *Necrodes littoralis* using growth curves. **a** Medians of errors for larval developmental stages at each temperature. **b** Medians of errors for larval developmental stages only. **c** Medians of errors for temperatures only. Different letters denote significant differences in pairwise comparisons for Kruskal-Wallis ANOVA at $p = 0.05$

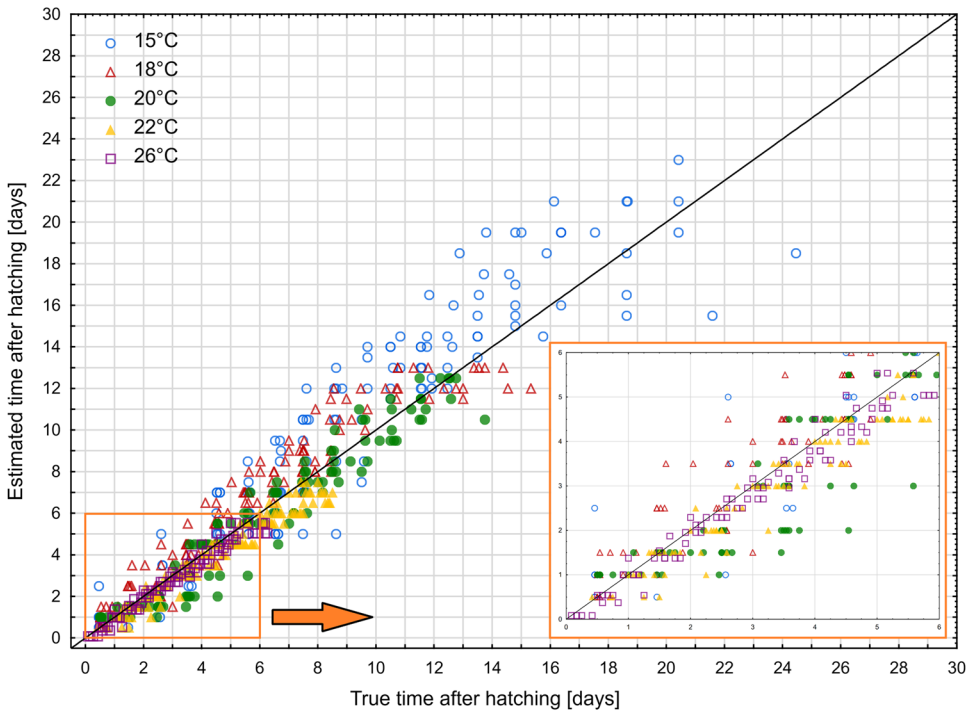

Fig. 7 The estimated time after hatching (using growth curves) plotted against the true time after hatching. Solid line represents perfect estimates

estimation than the developmental stage. Usually at higher temperatures, errors were smaller than at lower temperatures. The same pattern was observed in the case of *Creophilus maxillosus* [18]. These results suggest that the developmental models perform better at high temperatures. For TSM and isomorphen diagrams, this was likely because 15 °C was close to the temperatures at which the relationship between the rate of development and temperature is distinctly non-linear. On the other hand, 26 °C probably lies in the temperature range with a clearly linear relationship between the growth rate and the temperature [27]. In the case of growth curves and isomegalen diagrams, changes in larval length are simply more dynamic at high temperatures, and therefore, the accuracy of the estimation increases.

In the case of the isomegalen diagram and the growth curves, the age estimation errors of the first instar larvae were significantly larger than in the case of the second and the third instar larvae. First instar larvae grow at a lower rate than second and third instar larvae. Therefore, their length is less informative regarding larval age. Growth accelerates markedly in the second and particularly third larval stage, so in these stages, the length of a larva is a better predictor of its age than in the first larval stage. Moreover, the measurement errors, when using the Villet method [26] to measure larval length, are bigger for smaller larvae. Therefore, the first instar larvae are measured with less accuracy, which can lower the quality of a model and also the quality of validation data. In addition, the same absolute error (expressed in hours or days) when converted to a percentage value will constitute a larger relative error for the first instar larvae than for the second and third instar larvae [11].

Moreover, it should be remembered that development models are based on average values. Therefore, in both isomegalen diagrams and growth curves, there is a problem with the estimation of age for very small individuals. Either there will be a very large error in the estimation, or it will not be possible to estimate the age at all since extremely small larvae may be not included in the models. A similar limitation is with very large larvae which are significantly out of the largest average length (e.g., more than 23 mm for the isomegalen diagram and more than 25 mm for most growth curves). Although such long individuals are sometimes observed at peak growth, the average length for the population is smaller and the models (particularly isomegalen diagram) may not include such extreme lengths. Consequently, these models may be unsuitable for extremely small or large larvae. Therefore, models based on the length of *N. littoralis* larvae should be used with caution, especially when the insect evidence are the first instar larvae.

In addition, one of the problems in forensic entomology is that the studies leading to the construction of developmental models are laboratory studies at constant temperatures [28]. Under natural conditions, insects developing on cadavers experience fluctuations in temperature. The current validation dataset and the models that were initially validated here were also developed in the laboratory, and insects grew at constant temperatures. Therefore, further studies are necessary to give more insight into the validity and limitations of the models.

Another potential weakness of the development models for *N. littoralis* (and many other insects of forensic importance) is the use of the fresh meat as the food for the larvae. Recent studies indicated that *N. littoralis* reveal indirect forms of parental care. Adult beetles spread their exudates over carrion to form the feeding matrix that brings deferred thermal benefits for the larvae [29]. Moreover, adult *Necrodes* beetles were found to clear the carrion of the fly larvae to secure it for their offspring [30]. The latter behavior probably has no effect on the temporal patterns of larval development, but the preparation of the resource was found to shorten the development of the larvae [29]. At present, we are conducting a detailed study to quantify the differences in the timing of larval development between larvae fed with fresh meat and meat prepared by the adult beetles.

We are fully aware that the current study was only the first step in the validation process of the development models for N. *littoralis*. Ideally, validation using experimental data obtained from animal or human cadavers and validation based on case studies should be the next step to fully demonstrate the validity of the models. However, owing to the inherent difficulties, such studies were done only occasionally in forensic entomology [6]. Therefore, we encourage to validate the models at least in another study similar to the current one but performed in the different laboratory and using a different colony of *N. littoralis*.

While using thermal summation models for age estimation of *N. littoralis*, the relative error in most of the cases was below 10%. This suggests that the models allow for accurate estimation of beetle age and eventually PMI. However, *N. littoralis* is usually found on cadavers in an advanced stage of decomposition [19–21]. This means that insects of this species may colonize a cadaver many days after death. Their age, therefore, defines the minimum PMI. To get closer to the PMI, it is necessary in such cases to estimate the pre-appearance interval (PAI), which is the interval preceding appearance of an insect taxon on a cadaver [31]. The relative error of PAI estimation in *Necrodes littoralis* larvae using temperature methods is under 0.19 [32]. Accordingly, developmental models for *Necrodes littoralis* in combination with temperature methods for PAI should give a fairly accurate estimate of PMI.

It should also be remembered that the actual estimation of minimum PMI is based on several assumptions, and the errors inherent to these assumptions may affect the final accuracy of the minimum PMI [33]. The final result of the expert opinion will depend on the factors that directly affect the decomposition of a corpse and indirectly the development of insects, e.g., the temperature to which insects were exposed to [34], the type of habitat [35], body mass [36, 37], or drugs taken by the deceased [38].

Expert reports of forensic entomologists should contain a clear statement about variation in the estimation [27]. Hence, there is an urgent need to conduct validation studies and to publish case reports in this field, which may play an important role in learning more about the practical issues of forensic entomology [3]. It would also be useful to standardize methods for collecting laboratory development data and for deriving development models of forensically useful insects.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00414-023-02969-4.

Author contribution Joanna Gruszka performed laboratory rearing and data collection, analyzed the results and prepared figures. Szymon Matuszewski obtained funds and supervised the research. Both authors designed the study and wrote the manuscript.

Funding The study was funded by the National Science Center of Poland (grant no. 2016/21/B/NZ8/00788).

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

Declarations

Ethical approval The study comprised laboratory experiments using insect species *Necrodes littoralis* (Coleoptera: Silphidae). The species is not under protection. No permission or approval from Ethic Commission was needed.

Competing interests The authors declare no competing interests.

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Supplementary information for

Initial laboratory validation of temperature development models for *Necrodes littoralis* L. (Staphylinidae: Silphinae)

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Supplementary tables

Supplementary Table 1. Relative error of age estimation in *Necrodes littoralis* using different developmental models. IQR - interquartile range, SE - standard error

Developmental model	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
Thermal summation model	0.072 ± 0.079	0.085 ± 0.002	0.000	0.910	1704
Isomorphen diagram	0.084 ± 0.102	0.098 ± 0.002	0.000	1.068	1703
Isomegalen diagram	0.230 ± 0.274	0.339 ± 0.017	0.000	3.800	601
Growth curves	0.131 ± 0.181	0.206 ± 0.011	0.000	4.454	622

Supplementary Table 2. Relative error of physiological age (*K*) estimation using thermal summation models for five developmental landmarks. IQR - interquartile range, SE - standard error

Model	Relative error of *K* estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
Hatching	0.058 ± 0.092	0.113 ± 0.013	0.005	0.910	120
First ecdysis	0.059 ± 0.100	0.106 ± 0.011	0.001	0.499	120
Second ecdysis	0.047 ± 0.104	0.103 ± 0.011	0.000	0.521	120
Pupation	0.072 ± 0.092	0.083 ± 0.002	0.001	0.331	689
Eclosion	0.081 ± 0.062	0.076 ± 0.002	0.000	0.196	655

Supplementary Table 3. Relative error of physiological age (*K*) estimation using thermal summation models at five constant temperatures. IQR - interquartile range, SE - standard error

Temperature	Relative error of *K* estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
15°C	0.123 ± 0.145	0.151 ± 0.006	0.000	0.521	329
18°C	0.094 ± 0.039	0.108 ± 0.005	0.010	0.910	293
20°C	0.058 ± 0.066	0.060 ± 0.002	0.001	0.199	332
22°C	0.072 ± 0.085	0.073 ± 0.002	0.001	0.203	372
26°C	0.034 ± 0.049	0.045 ± 0.002	0.000	0.137	378

Supplementary Table 4. Relative error of age estimation using isomorphen diagram depending on developmental landmark. IQR - interquartile range, SE - standard error

Model	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
Hatching	0.105 ± 0.119	0.120 ± 0.013	0.001	1.068	120
First ecdysis	0.090 ± 0.102	0.118 ± 0.009	0.000	0.550	120
Second ecdysis	0.099 ± 0.110	0.117 ± 0.008	0.000	0.440	120
Pupation	0.091 ± 0.107	0.099 ± 0.003	0.001	0.333	689
Eclosion	0.084 ± 0.062	0.086 ± 0.002	0.003	0.257	654

Supplementary Table 5. Relative error of age estimation using isomorphen diagram at five constant temperatures. IQR - interquartile range, SE - standard error

Temperature	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
15°C	0.086 ± 0.113	0.108 ± 0.005	0.001	0.440	329
18°C	0.115 ± 0.065	0.133 ± 0.006	0.016	1.068	293
20°C	0.124 ± 0.116	0.127 ± 0.004	0.000	0.333	333
22°C	0.081 ± 0.077	0.089 ± 0.002	0.003	0.194	371
26°C	0.039 ± 0.044	0.047 ± 0.002	0.000	0.150	377

Supplementary Table 6. Relative error of age (time from hatching) estimation using isomegalen diagram depending on developmental stage. IQR - interquartile range, SE - standard error

Developmental stage	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
First instar larvae	0.400 ± 0.459	0.540 ± 0.041	0.000	3.800	221
Second instar larvae	0.200 ± 0.217	0.229 ± 0.011	0.000	0.774	214
Third instar larvae	0.180 ± 0.191	0.214 ± 0.011	0.005	0.690	166

Supplementary Table 7. Relative error of age (time from hatching) estimation using isomegalen diagram at five constant temperatures. IQR - interquartile range, SE - standard error

Temperature	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
15°C	0.323 ± 0.423	0.482 ± 0.049	0.007	3.800	147
18°C	0.151 ± 0.215	0.288 ± 0.041	0.000	3.800	140
20°C	0.352 ± 0.281	0.416 ± 0.018	0.087	0.935	128
22°C	0.182 ± 0.144	0.215 ± 0.017	0.000	1.400	109
26°C	0.160 ± 0.111	0.209 ± 0.027	0.000	1.400	77

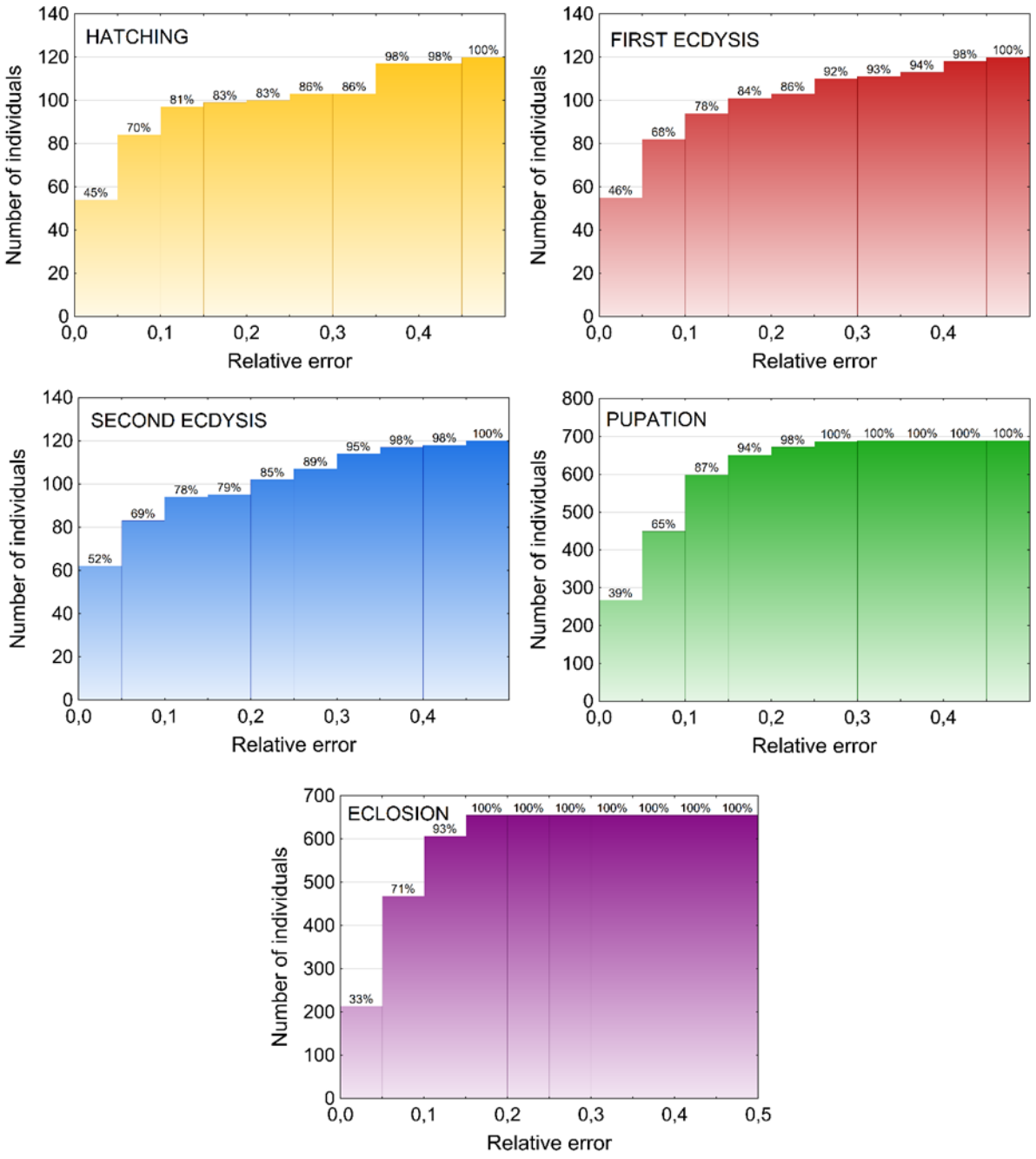
Supplementary Table 8. Relative error of age (time from hatching) estimation using growth curves depending on developmental stage. IQR - interquartile range, SE - standard error

Developmental stage	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
First instar larvae	0.200 ± 0.409	0.353 ± 0.032	0.000	4.454	189
Second instar larvae	0.118 ± 0.173	0.160 ± 0.009	0.000	0.578	214
Third instar larvae	0.111 ± 0.127	0.126 ± 0.006	0.000	0.442	219

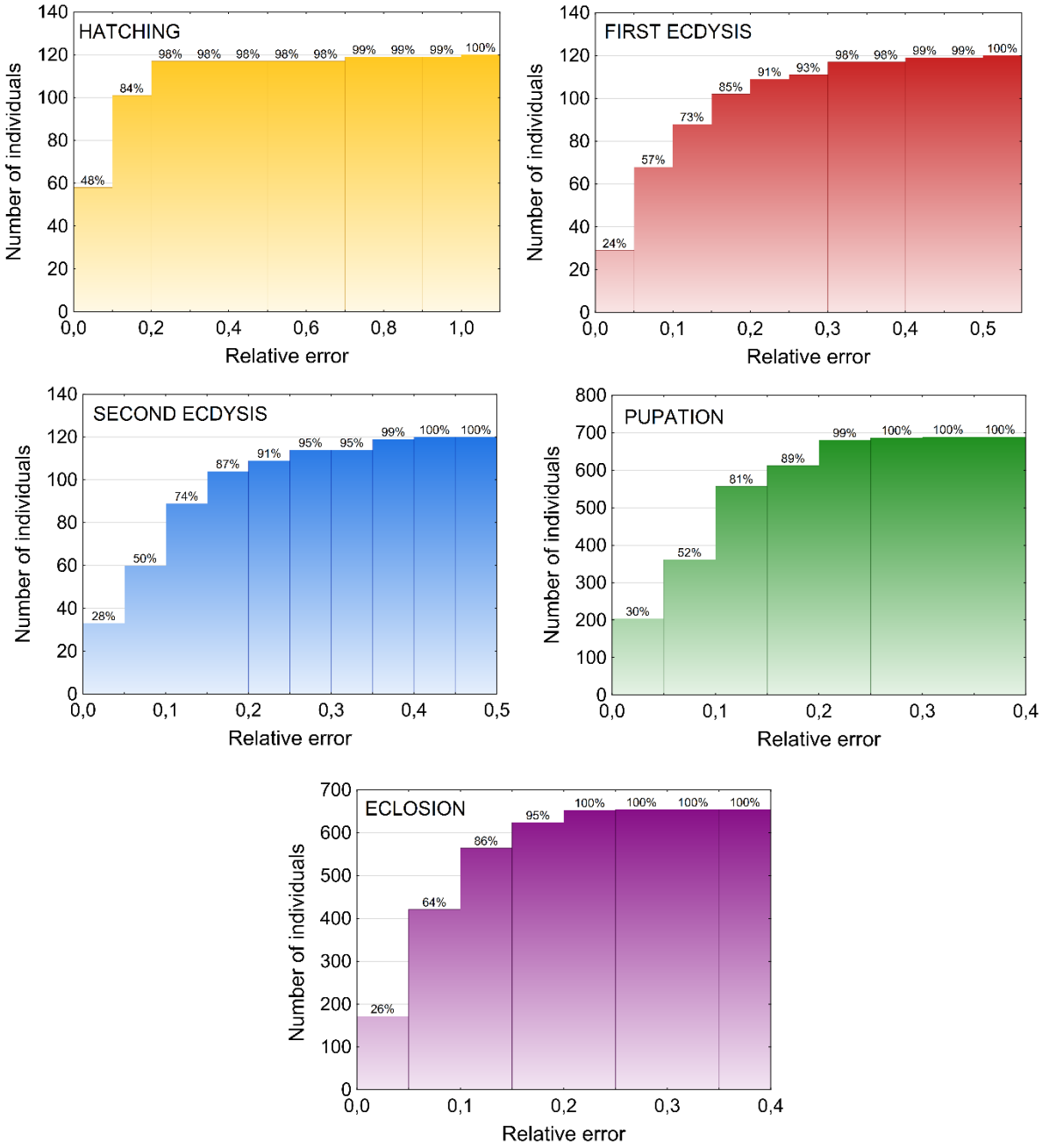
Supplementary Table 9. Relative error of age (time from hatching) estimation using growth curves at five constant temperatures. IQR - interquartile range, SE - standard error

Temperature	Relative error of age estimation				N
	Median ± IQR	Mean ± SE	Minimum	Maximum	
15°C	0.211 ± 0.277	0.301 ± 0.036	0.000	4.454	147
18°C	0.200 ± 0.218	0.258 ± 0.024	0.000	1.769	134
20°C	0.100 ± 0.159	0.171 ± 0.017	0.000	1.000	127
22°C	0.125 ± 0.129	0.135 ± 0.010	0.000	0.667	123
26°C	0.091 ± 0.084	0.126 ± 0.014	0.000	0.800	91

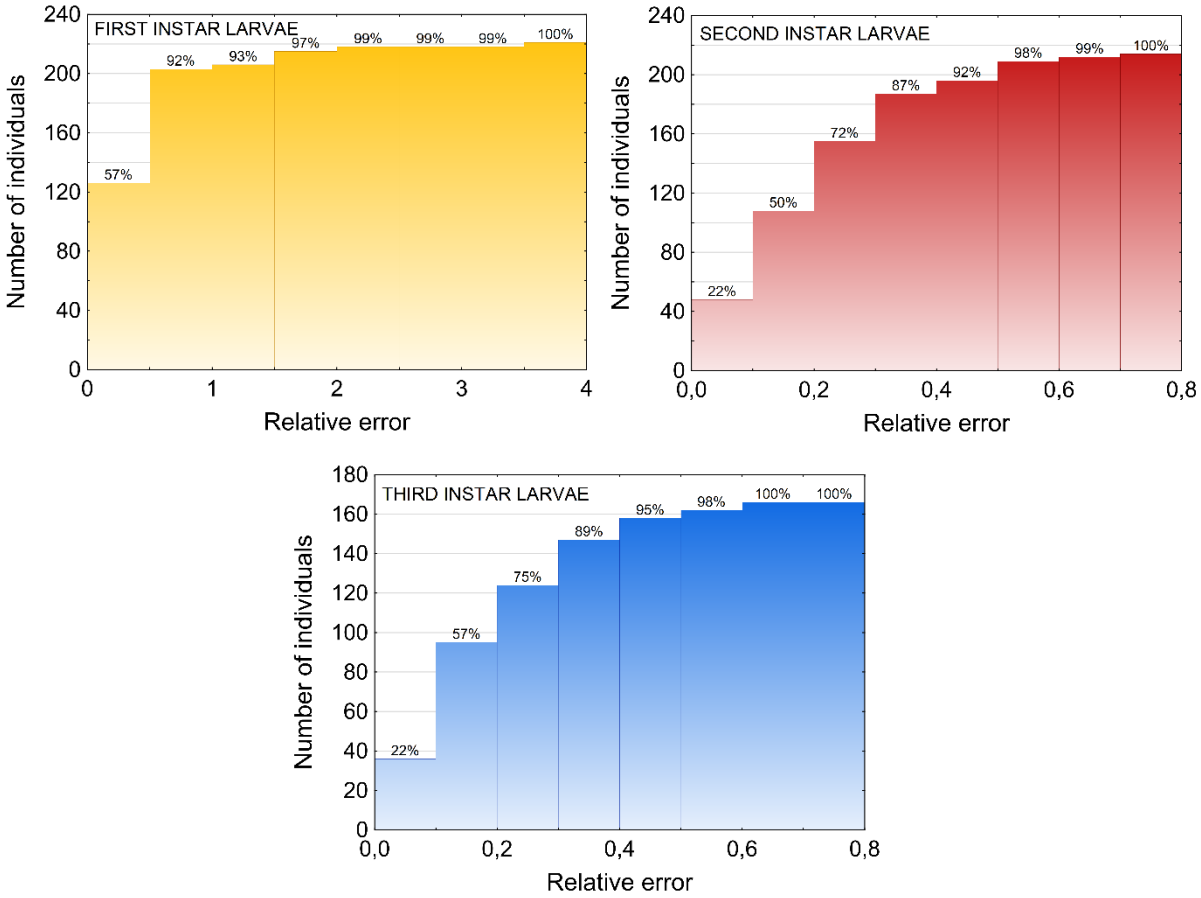
Supplementary figures



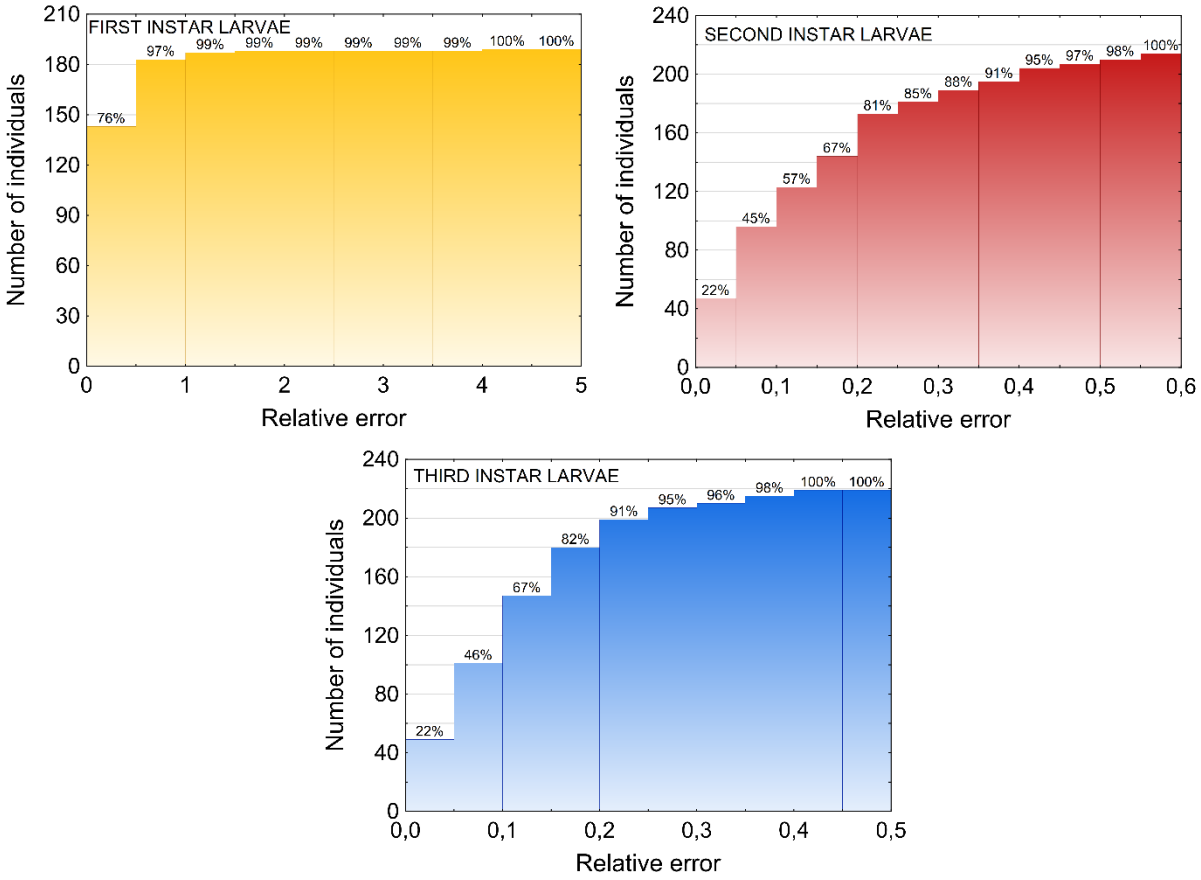
Supplementary Figure 1. Cumulative histograms for relative errors of physiological age (*K)* estimation, using thermal summation models for five developmental landmarks



Supplementary Figure 2. Cumulative histograms for relative errors of age estimation, using isomorphen diagram.



Supplementary Figure 3. Cumulative histogram for relative errors of age estimation (time from hatching) using isomegalen diagram, depending on the developmental stage.



Supplementary Figure 4. Cumulative histogram for relative errors of age estimation (time from hatching) using growth curves, depending on the developmental stage.

Oświadczenia doktoranta o wkładzie w powstanie artykułów

Poznań, 19.06.2023 r.

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Oświadczenie określające wkład w powstanie artykułu

Niniejszym oświadczam, że mój wkład w powstanie poniższego artykułu: Gruszka J, Krystkowiak-Kowalska M, Frątczak-Łagiewska K, Mądra-Bielewicz A, Charabidze D, Matuszewski S. 2020. Patterns and mechanisms for larval aggregation in carrion beetle *Necrodes littoralis* (Coleoptera: Silphidae). Animal Behaviour 162:1-10, polegał na: przeprowadzeniu eksperymentów, przygotowaniu surowych danych do analizy, interpretacji wyników, przygotowaniu rycin do publikacji, udziale w pisaniu manuskryptu oraz poprawie manuskryptu po ocenie recenzentów.

Mój całkowity wkład w pracę wynosi 25%.

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Mój całkowity wkład w pracę wynosi 15%.

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Authorship contribution statement

I hereby declare that my contribution to the following article: Gruszka J, Krystkowiak-Kowalska M, Frątczak-Łagiewska K, Mądra-Bielewicz A, Charabidze D, Matuszewski S. 2020. Patterns and mechanisms for larval aggregation in carrion beetle *Necrodes littoralis* (Coleoptera: Silphidae). Animal Behaviour 162:1-10, was the interpretation of the results and the revision of the manuscript after peer reviews.

My total contribution to the article is 5%.

Damien CHARABIDZE

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Mój całkowity wkład w pracę wynosi 20%.

FINANSOWANIE

Wszystkie badania zrealizowane w ramach mojej rozprawy doktorskiej zostały sfinansowane przez Narodowe Centrum Nauki (grant numer 2016/21/B/NZ8/00788).

Badania opisane w publikacji „Insect rearing protocols in forensic entomology: Benefits from collective rearing of larvae in a carrion beetle *Necrodes littoralis* L. (Silphidae)" otrzymały również dofinansowanie w ramach Grantu Dziekana Wydziału Biologii.

Koszty otwartego dostępu do publikacji zostały pokryte ze środków projektu ID-UB: Inicjatywa Doskonałości - Uczelnia Badawcza oraz częściowo z subwencji badawczej Wydziału Biologii UAM.

W trakcie studiów doktoranckich byłam również beneficjentką projektu POWER: Paszport do przyszłości - Interdyscyplinarne studia doktoranckie na Wydziale Biologii UAM (POWR.03.02.00-00-I006/17).

PODZIĘKOWANIA

Dziękuję mojemu promotorowi, Profesorowi Szymonowi Matuszewskiemu za możliwość udziału w interesujących projektach badawczych, cenne wskazówki oraz czas poświęcony na pomoc w realizacji mojej rozprawy doktorskiej.

Dziękuję również Ani Mądrej-Bielewicz za okazaną życzliwość, wszelką pomoc w kwestiach formalnych i technicznych oraz wsparcie w trakcie prowadzonych przeze mnie badań.