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**An overview of the thermal biology of the Central European
Thanatophilus (Coleoptera: Silphidae) species: carrion beetles as
an important tool for the forensic sciences**

DISSERTATION



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Ph.D. Thesis: An overview of the thermal biology of the Central European *Thanatophilus* (Coleoptera: Silphidae) species: carrion beetles as an important tool for the forensic sciences.

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To my beloved family. For your never-ending love and support, for that, I will be eternally grateful.

“If science was a constellation of facts, theories, and methods collected in current texts, then scientists are the men who, successfully or not, have striven to contribute one or another element to that particular constellation.”

Thomas S. Kuhn

Statement of originality

I certify and declare that this doctoral thesis has not been submitted or published elsewhere. My involvement in the manuscript and the research presented in this thesis is specified in the authors' contributions and implied by the authorship order of the published manuscripts. All relevant literature sources used while writing the following text in this thesis have been properly cited in accordance with the standard practice associated with publications in the biological sciences. Any thoughts by others or literal quotations are clearly referenced.

Santiago Montoya-Molina

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Summary

This thesis is the summary of the scientific work I have contributed to the knowledge of two important *Thanatophilus* (Coleoptera: Silphidae) species in Central Europe. All the chapters and articles aim to broaden the knowledge regarding the thermal biology (Thermal summation Model, Chill coma recovery time) for the selected species.

Physiology and subsequently ecology of Silphids are bounded to environmental temperature. The temperatures experienced by beetles, will significantly change the species developmental times and thermal performance parameters. Carrion beetles of the genus *Thanatophilus* are an important group of necrophagous insects, widely distributed and attracted to large carcasses. The combination of the previous characteristics matches the requirements for the species to be considered of great potential in the forensic entomology worldwide. Nevertheless, developmental information for most of the *Thanatophilus* species is still unknown. Changes in the developmental times as response to difference in diet quality and photoperiod regime is missing. Furthermore, the responses to short periods of low temperatures and the changes in their ecological functions were still unstudied. Consequently, in the next chapters, we will analyse the knowledge gaps above mentioned in two species of the genus *Thanatophilus*.

Chapter 1 gives an insight of the forensic entomology and the factors affecting ectotherms thermal biology. In Chapters 2 and 3 of this thesis, we provide the Thermal Summation Models for the two most common species of the genus *Thanatophilus* in Central Europe: *T. sinuatus* and *T. rugosus*. We bred the species at different ecological relevant temperatures and times of development and thermal summation parameters were calculated. In these manuscripts we present a novel methodology to breed Silphinae under constant laboratory conditions having into account previously suggested methodologies in the literature. As expected, *T. sinuatus* and *T. rugosus* development was faster at higher temperatures. In all the cases, development of both *Thanatophilus* species were faster than other forensically important species of Coleoptera.

Chapters 4 and 5 explores *T. rugosus* and *T. sinuatus* developmental responses to different diets and photoperiods (in *T. sinuatus*). To examine the effects of different diets on the development times, different types of diet for larvae were tested (Pork muscle, Pork liver and Chicken

muscle). Surprisingly, the impact of contrasting diets was different between the species. *Thanatophilus rugosus* times of development were faster when the species was fed under pork liver, followed by pork muscle. On the other hand, *T. sinuatus* developmental times were faster when larvae were fed with pork muscle followed by pork liver. Overall, chicken muscle prolonged the development in both species. Survival rate was also affected by the diet quality among the studied species. In *T. rugosus* survival rate decreased significantly when fed solely with chicken muscle (30.4%) compared to the highest pork liver (50.3%). In the case of *T. sinuatus*, survival rate was not affected by any of the studied diets. Also, no effect of the two tested photoperiods (16 h Light: 8h Dark and 12 h Light: 12h Dark) was found.

In Chapter 6, *Thanatophilus sinuatus* cold tolerance was tested measuring the Chill Coma Recovery times (CCRT). Cold response in ectotherms depends on species life history traits. In order to quantify the CCRT, adult beetles of three physiological ages (10, 20 and 30 days-old) were subject of two exposure times (2 hours and 16 hours) at zero degrees Celsius. Results showed that younger beetles recover faster than old ones. In general, females recovered slower than males. Unexpectedly, weight had no significant effect on the CCRT.

The results here presented provides an approximation on the species biology unveiling ecological and distributional temperature requirements. *Thanatophilus sinuatus* and *T. rugosus* developmental biology can be used during criminal investigations complementing the entomological evidence when collected with Diptera. Moreover, beetles might be beneficial when other forensically important species are absent due to successional (left the carcass during late stages of decomposition) or environmental factors.

Abstrakt

Tato dizertační práce shrnuje moji vědeckou práci, přispívající k poznání dvou významných středoevropských druhů rodu *Thanatophilus* (Coleoptera: Silphidae). Všechny kapitoly a zahrnuté články rozšiřují znalosti termální biologie (termálně sumační model, chill coma recovery time) dvou vybraných druhů.

Fyziologie a následně i ekologie mrchožroutovitých brouků jsou úzce svázány s teplotou prostředí. Teplota, které jsou brouci vystaveni, významně mění rychlost jejich vývoje. Mrchožroutoví brouci rodu *Thanatophilus* jsou významná skupina nekrofágního hmyzu, široce rozšířená a preferující velké mršiny. Kombinace těchto vlastností umožňuje považovat je za skupinu s velkým potenciálem využití ve forenzní entomologii. Bohužel, informace o rychlosti vývoje jsou pro většinu druhů rodu *Thanatophilus* stále neznámé. Změny v délce vývoje v závislosti na kvalitě potravy a délce dne nejsou k dispozici. Také změny jejich ekologických vlastností jako odpověď na krátkou expozici nízkým teplotám jsou jen nedokonale prostudované. V následujících kapitolách se budu snažit vyplnit tyto mezery ve znalostech u dvou vybraných druhů rodu *Thanatophilus*.

Kapitola 1 uvádí do problematiky forenzní entomologie a faktorů, ovlivňujících teplotní biologii ektotermů. V kapitolách 2 a 3 představuji termální sumační modely pro dva běžné středoevropské druhy rodu *Thanatophilus*: *T. sinuatus* a *T. rugosus*. Prováděli jsme chovy těchto dvou druhů v různých ekologicky relevantních teplotách, změřili jsme délky vývoje a odvodili termálně sumační modely. V těchto studiích jsme také uvedli novou metodu chovu zástupců podčeledi Silphinae za konstantních laboratorních podmínek, s přihlédnutím k metodám navrženým v literatuře. Podle očekávání byl vývoj *T. sinuatus* i *T. rugosus* rychlejší při vyšších teplotách. Ve všech případech byl vývoj obou druhů rodu *Thanatophilus* rychlejší než vývoj ostatních forezně významných druhů brouků (Coleoptera).

Kapitoly 4 a 5 studují změny rychlosti vývoje druhů *T. sinuatus* a *T. rugosus* v závislosti na různém typu potravy a fotoperiody (u *T. sinuatus*). Testovány byly tři typy potravy pro larvy (vepřové maso, vepřová játra a kuřecí maso). Je překvapivé, že vliv potravy na vývoj je u obou studovaných druhů různý. *Thanatophilus rugosus* se nejrychleji vyvíjel při krmení vepřovými játry, pomaleji při krmení vepřovým masem. Naopak, vývoj druhu *T. sinuatus* byl nejrychlejší

při krmení larev vepřovým masem, pomaleji se vyvíjel při krmení vepřovými játry. Celkově, kuřecí maso jako potrava prodloužila rychlost vývoje u obou druhů. Přežívání bylo také ovlivněno kvalitou potravy, rozdílně u obou druhů. U druhu *T. rugosus* významně kleslo přežívání při krmení kuřecím masem (bylo 30,4 %), nejvyšší bylo při krmení vepřovými játry (50,3 %). U druhu *T. sinuatus* nebylo přežívání ovlivněno žádným typem potravy. Nebyl také prokázán významný rozdíl při vývoji ve dvou různých světelných režimech (16 hod. světla a 8 hod. tmy, 12 hod. světla a 12 hod. tmy).

V kapitole 6 byla testována chladová tolerance druhu *Thanatophilus sinuatus* (chill coma recovery times, CCRt). Odpověď na chlad u ektotermů závisí na traitech (výběru vlastností) a životní historii druhu. Pro kvantifikaci CCRt byli dospělí brouci různého stáří (10, 20 a 30 dnů) vystaveni nulové teplotě do dobu 2 nebo 16 hodin. Výsledky ukazují, že mladší dospělci se zotavovali rychleji než ti starší. Dále, samice se zotavovaly pomaleji než samci. Váha jedinců neukázala překvapivě žádný průkazný efekt na CCRt.

Předložené výsledky popisují první přiblížení k ekologickým teplotním nárokům obou těchto druhů. Rychlost vývoje druhů *Thanatophilus sinuatus* a *T. rugosus* může být využita při kriminalistických aplikacích a doplnit forenzní entomologické stopy společně s dvoukřídlým hmyzem (Diptera). Brouci mohou být navíc použitelní i v situacích, kdy jiné forenzně významné skupiny hmyzu chybí, třeba u pozdějších sukcesních stádiích rozkladu mršín či v důsledku jiných faktorů prostředí.

1. Chapter 1: General Introduction to the thesis

1.1. Forensic Entomology overview

The forensic entomology is one part of the forensic sciences. In this particular science, insects and arthropods are studied in a legal context in order to assist the evidence in cases related to humans and wildlife (Amendt et al. 2010; 2006; Byrd and Castner 2010; Catts and Goff 1992; Gennard 2012). The forensic entomology is divided in three major areas: the urban, the stored products and the medico legal (Gennard 2012; Lord and Stevenson 1986).

The urban entomology is associated to problems related to urban plagues i.e., termites, (Isoptera), cockroaches (Blattodea) and flies (Diptera) occurring in human settlements (Byrd and Castner 2010; Gennard 2012). Meanwhile, in stored products context, the studied species are limited, including some already mentioned in the urban entomology such as flies, cockroaches, also being part of these beetles (Coleoptera), butterflies (Lepidoptera), and ants (Hymenoptera: Formicidae), inhabiting and infesting animal and plant products. In stored products and urban forensic entomology, the main role of the researcher is to determine the insect species and to understand the biology in each case of study (Gennard 2012).

The most outstanding application of the forensic entomology is in the medico-legal context. In this area, the forensic entomology uses the information on the life cycles, behaviour, and distribution of insects mainly, to provide insights to a death investigation (Amendt et al. 2010; Anderson 1995; 2011; Catts and Goff 1992; Joseph et al. 2011). Insect and arthropod communities in general, can help elucidate the cause of death, time of death, place of the murder and many other unique information (Catts and Goff 1992). It is important to mention that not long ago, entomological evidence was not recognized as true science (Amendt et al. 2004). Nowadays, immature, and adult insects found on and around corpses are considered as part of the physical evidence as much as other biological non-entomological physical evidence (e.g., fibers, blood stains); providing valuable information to the criminal investigation (Amendt et al. 2006; Joseph et al. 2011; García-Rojo et al. 2013; Anderson 1995). As such, methods and protocols are being published in a way that forensic entomologists, police, and technicians, follows appropriate standards when collecting this evidence and to be use in a legal trial (Amendt et al. 2006; Byrd et al. 2010; Matuszewski 2021).

Within the arthropods found on corpses, insects are clearly dominant, having at the same time two major groups preferably used for the forensic entomology: beetles (Coleoptera) and flies (Diptera), providing most of the evidence for forensic investigations (Byrd and Castner 2010). Much research has focused on understanding succession and colonization of carcass made by blowflies (Diptera: Calliphoridae), but little has been done respecting this process in Coleoptera despite the valuable information that this group can supply to criminal investigations (Dekeirsschieter et al. 2011b; Matuszewski et al. 2008; Midgley and Villet 2009; Ridgeway et al. 2014). Recently, due to their rapid localization and colonization of corpses soon after death, Carrion beetles (Silphidae) have shown to be as useful as blowflies providing accurate information to forensic investigation (Ridgeway et al. 2014).

As mentioned above, insects can provide information regarding the circumstances of the murder or the legal case. Due to specific ecological requirements, insects inhabiting carcasses are linked to this unique habitat to survive. This level of specialization causes adaptive advantages over the species to avoid or decrease competition. Some of these adaptive advantages are faster arrival to the carcass, accelerated development, predation over other species inhabiting the carcass, and location preference within the carcass (Byrd and Castner 2010). High competition is expected among the species able to colonize carcasses, as they are ephemeral resource in nature (Barton et al. 2013b). Adaptive advantages or responses can be used in favour of the forensic investigation when entomofauna is present and information regarding the species is available in the literature. Some of the latter are, Thermal Summation Model (TSM), the Pre-appearance interval (PAI), the Upper developmental threshold (UDT) (Amendt et al. 2006; Byrd and Castner 2010; Gennard 2012; Matuszewski 2021). A correct determination of the species is imperative as incorrect determination can lead to a wrong calculation of the previous values (Byrd and Castner, 2010).

Among the many information the entomofauna can provide to the legal investigation, some of the most relevant evidence/tool is the minimum post mortem estimation (PMI) or the minimum time between the death and the discovery of the corpse (Gennard 2012; Goff 2010; Anderson 2011; Midgley et al. 2010; Joseph et al. 2011; García-Rojo et al. 2013; Ridgeway et al. 2014). PMI estimation depends, to some extent, on the composition and dynamics of the local necrophagous arthropod communities (Pujol-Luz et al. 2008). Arthropods colonizes corpses in a predictable order, with some species attracted by fresh corpses, while others are attracted by different stages of the decaying corpse (Anderson 2010; Gennard 2012; Goff 2010).

Proper identification of the insect species is crucial job in the field of forensic entomology (Gennard 2012; Szpila 2010). It is the species identification that allows the proper developmental data and distribution ranges to be applied to an investigation (Szpila 2010). If the species determination is incorrect, estimation of postmortem interval is invalid; therefore, qualified forensic entomologist should always take care to verify species determinations (Byrd and Castner 2000).

1.1.1. Insects as evidence

The chronological and well-studied insect succession on the corpse, allows the forensic entomologist to estimate the colonization of the body by the different insect species (Amendt et al. 2010; Villet and Amendt 2011). The use of insects in the criminal investigation's dates to the early 13th century. The oldest record known of the use of forensic entomology is mentioned in the book by Sung Tzu's in the year 1247 AD (Jiangfeng 2015; Wallace 2017). The book presents more than 15 cases where insects evidence was used to clear the investigation (Jiangfeng 2015; Wallace 2017). The most cited record of the book is the description on how flies were used to identify the murderer (Catts and Goff 1992; Amendt et al. 2004; Benecke 2004, 2008; Wallace 2017). Later, studies of Bergeret (1855) and Megnin (1889; 1894; 1896) described arthropods succession carefully, including more than 400 papers related to the carrion decomposition and the arthropods associated to corpses (Catts and Goffs 1992). Nevertheless, just until the 19th century, the first case of forensic entomology in Europe analysed the use of blow flies pupae for the estimation of the postmortem interval (PMI) (Bergeret 1955 *in* Marchenko 2001).

Before the 1960's, forensic entomology was maintained as primarily medical matter, where a physician or a medical pathologist made the observations of the arthropods found in corpses. After reviews made by Keh (1985) "*Scope and applications of forensic entomology*" and Catts and Goff (1992) "*Forensic entomology in criminal investigations*", the study of this science advance in application and research.

Nowadays, the number of publications mentioning insects as evidence is constantly rising (Lei et al. 2019a; 2019b; Tomberlin and Benbow 2015). In present-day, forensic entomology is applied in criminal investigations in many countries, following strict protocols, and methods for post-mortem interval estimation are under continuous validation and improvement (Gruszka and Matuszewski 2022; 2023; Haskell et al. 2008; Matuszewski and Mądra-Bielewicz 2019; Matuszewski et al. 2020; Villet et al. 2010).

1.1.2. Insects and Forensics

1.1.2.1. Insects – and relation to the body

Insects are the major group of organisms directly participating in the decomposition of the carcasses in terrestrial ecosystems (Goff 2010). As mentioned before in chapter 1.1., due to ecological requirements, just a fraction of all insect's species is capable of exploiting this ephemeral resource (Barton et al. 2013a; 2013b). In the forensic sciences, insect diversity involved in the decomposition of the carcasses, can be divided based on the feeding strategy or guild (species using the same type of resource) making important consideration on whether they use the resource directly, opportunistically, or accidentally. Based on Amendt et al. (2004), Byrd and Castner (2010), Catts and Goff (1992), and Goff (2010) the categories are following.

The first group of insects are categorized as *Necrophagous*. These species which feed on the dead tissue includes many of the Diptera species involved in the decomposition. Some of the most important families of flies are the blowflies (Calliphoridae) and flesh flies (Sarcophagidae). Beetles (Coleoptera) as the Carrion (Silphidae: Silphinae), Burying (Silphidae: Nicrophorinae) and Skin beetles (Dermestidae) are included in this group. These species are known to be the most significant for PMI estimation during early stages of decomposition. Many of the species in within this group are important for the forensic sciences due to its close relationship with the decomposing body (many of the species feeds and develops in the corpse).

The second group, the *parasites and predators of the necrophagous species*, are the ones feeding mainly on the immature stages of the necrophagous insects. Comprises equally important families of Diptera (Calliphoridae and Stratiomyidae), Coleoptera (Silphidae, Staphylinidae and Histeridae) and Hymenoptera.

The three following groups may also be important for the forensic investigation as they may be associated to the first two groups. The *Omnivorous* species, not obligate necrophages, but can use the corpse as a food source if necessary. If their population is large, they can retard the rate of decomposition when predated on necrophagous species. Ants, wasps, and beetles are part of the latter. Both *Adventive* and *Accidental* species are specific to the habitat of the scene of a crime. Adventive may use the body as an extension of that habitat (e.g., Collembola, Aranea and Myriapoda). Accidental species have no real relationship with the corpse as they are “accidental”. These insects may have fallen by chance onto the corpse from vegetation and might give some information on whether the corpse was moved postmortem.

1.1.2.2. Insects – and association with post-mortem stages

Of all animal species, insects are the first arriving to the body soon after death, even some species can arrive and colonize within minutes (Anderson 2011; Catts and Goff 1992; Matuszewski et al. 2011; 2013; Michaud and Moreau 2009; Reibe and Madea 2010). Due to that trait, other conventional way to separate the species used in the forensic entomology is based on the stage of decomposition. The stages of decomposition are organized in a chronological sequence associating these to the insect community arriving to the corpse (Figure 1), known as insect succession (Gennard 2012; Joseph et al. 2011; Goff 2010). Forensic insect succession is the series of predictable changes in the body referenced to specific insect species (Amendt et al. 2006; Byrd and Castner 2010; Matuszewski et al. 2008) (Figure 1).

The number of stages may vary depending on the authors, and the geographic location (Goff 2010). Is important to mention that the stages of decomposition and insect succession are affected by the environmental conditions (e.g., temperature, relative humidity), carcass mass, chemical and physical barriers; therefore, changing the insect community (Goff, 2010; Matuszewski et al. 2016; Schröder et al. 2002; 2003). The stages of decomposition in terrestrial environment are basically five according to Gennard (2012), Goff (2010) and Tabor-Kreitlow (2010). The stages of decomposition are distinctive as the body is subject of physical and chemical changes, and insect groups are attracted differently to the corpse.

The first stage is called *Fresh stage*. Starts from the moment of death until the first signs of bloating. First colonizers such as blow flies (Calliphoridae) and flesh flies (Sarcophagidae) are the most common. *Bloated* is the second stage and marks the beginning of the breakdown of the body due to the bacterial activity, namely putrefaction. As the decomposition continues, gases released by the anaerobic bacterial activity bloats the body. In the Bloated (second stage) blow flies and flesh flies are attracted to the gases released from the body, with these two groups being the most abundant. Other group of insects as rove beetles (Staphylinidae) may be attracted to the body, predated on maggots and puparia of the fly species. Consequently, stage three is named *Active decay* or *Decay stage*, characterized by the opening of the body cavity due to the incessant activity of the maggots and gases escaping from the abdomen. Feeding masses of maggots are visible externally. Coleoptera becomes more evident during this stage, even though they have been present since the early stages of decomposition. Species of the families Histeridae and Silphidae are the most common.

INSECT FAMILY	STAGES OF DECOMPOSITION			
	FRESH	BLOATED	DECAY	DRY
CALLIPHORIDAE: (blow flies)	—	—	—	—
MUSCIDAE: (muscid flies)	—	—	—	—
SILPHIDAE: (carrion beetles)	—	—	—	—
SARCOPHAGIDAE: (flesh flies)	—	—	—	—
HISTERIDAE: (clown beetles)	—	—	—	—
STAPHYLINIDAE: (rove beetles)	—	—	—	—
NITIDULIDAE: (sap beetles)	—	—	—	—
CLERIDAE: (checkered beetles)	—	—	—	—
DERMESTIDAE: (dermestid beetles)	—	—	—	—
SCARABAEIDAE: (lamellicorn beetles)	—	—	—	—

* Each stage of decomposition is given the same amount of space in this table.

— Indicates a small number of individuals present.
 — Indicates a moderate number of individuals present.
 — Indicates a large number of individuals present.

(a)

INSECT FAMILY	STAGES OF DECOMPOSITION			
	FRESH	BLOATED	DECAY	DRY
CALLIPHORIDAE: (blow flies)	—	—	—	—
MUSCIDAE: (muscid flies)	—	—	—	—
SILPHIDAE: (carrion beetles)	—	—	—	—
SARCOPHAGIDAE: (flesh flies)	—	—	—	—
STAPHYLINIDAE: (rove beetles)	—	—	—	—
DERMESTIDAE: (dermestid beetles)	—	—	—	—
SCARABAEIDAE: (lamellicorn beetles)	—	—	—	—

* Each stage of decomposition is given the same amount of space in this table.

— Indicates a small number of individuals present.
 — Indicates a moderate number of individuals present.
 — Indicates a large number of individuals present.

(b)

Figure 1. Succession of insects (a) adult and (b) larvae on human cadavers during spring and summer in east Tennessee, USA. Taken from Byrd and Castner (2010).

Stage four or *Post-decay stage*, remains of the skin, cartilage, hair, bones, some leftovers of tissue and byproduct of decay (BOD) (Joseph et al. 2011). Diptera larvae leave the carcass to pupate. Skin beetles feed on the remains of dried flesh, cleaning the bones. The last of all decomposition stages is called the *Skeletal, Skeletonisation or Remains*. Bones and hair remain, mites and Collembola are useful taxa for forensic entomologist, other groups of insects previously present on the carrion disappeared. In special conditions, Nitidulidae beetles can be found (Gennard 2012).

The list of species used nowadays depends directly on the geographical location of the study and characteristics of the carrion (Marchenko 2001; Matuszewski 2021). Many are the insect species involved on carrion decomposition, Diptera, Coleoptera, Hymenoptera and Blattodea are among the most important orders (Byrd and Tomberlin 2020). Others not included on the

list (e.g., Lepidoptera, Orthoptera, Hemiptera), can also be important for forensic investigations.

Due to their prompt arrival and colonization of the body after death, flies are generally used for PMI estimations. Among Diptera, families such as Calliphoridae (blow flies), Sarcophagidae (flesh flies), Muscidae (Muscid flies), Piophilidae (Skipper flies), Phoridae (Humpback flies) and Stratiomyidae (Soldier flies) are the most used and developmental information of many of the species are available. Some of the most important representatives of the flies in Central Europe are: *Calliphora vicina* Robineau-Desvoidy, 1830, *C. vomitoria* (Linnaeus, 1758), *Chrysomya albiceps* (Wiedemann, 1819), *Lucilia caesar* (Linnaeus, 1758), *L. sericata* (Meigen, 1826) and *Phormia regina* (Meigen, 1826) (Matuszewski 2021).

The use of entomological evidence goes further apart from helping on the estimation of the PMI, it can aid linking a crime/body to specific locations (Byrd and Tomberlin 2020).

Historically, Coleoptera have always been part of the two major groups used for forensic science. But developmental biology of them has been overlooked (Midgley and Villet 2009; Midgley et al. 2010; Ridgeway et al. 2014). Therefore, currently several papers are connected to enlarge the understanding of the beetles arriving to the corpse that could be useful for PMI estimations, similar to blow flies (Charabidze et al. 2017; Frączak-Łagiewska et al. 2018; 2020; Gruszka and Matuszewski 2022; Jakubec 2016; Matuszewski and Madra-Bielewicz 2016; Midgley and Villet 2009; Midgley et al. 2010; Peck 2001; Qubaiová et al. 2021; 2022; 2023; Montoya-Molina et al. 2021a; 2021b; Velázquez and Vilorio 2009; Zhantiev and Volkova 1998; 1999).

1.2. Coleoptera in the forensic entomology

Coleoptera (or beetles) are one of the largest, if not the largest, orders containing about 387.000 to 400.000 described species (Cai et al. 2022; Stork 2018) (Figure 2a), approximately twenty-five percent of all known animal species (Cai et al. 2022). This order is of tremendous economic importance and constitutes forty percent of the whole insect diversity (Hunt et al. 2007; Lawrence et al. 2011; McKenna et al. 2015a; 2015b; Ślipiński et al. 2011). It is presumed that Coleoptera diversification was possible thanks to the development of sclerotized first pair of wings, allowing them to successfully defend from different environments and predation (Bouchard et al. 2017). The order's success indicates an early evolutionary history of having extensive adaptive radiation together with the angiosperms that may help the group's

diversification (Bouchard et al. 2017; Cai et al. 2022; McKenna et al. 2015a; 2015b; 2019; Zhang et al. 2018). Origin and diversification of beetles is still uncertain and controversial. The latest two estimations propose the origin of this mega-diverse order in the Permian (Beutel et al. 2019a; 2019b) and the second hypothesis dates it back to the Carboniferous (Cai et al. 2022). Differences in the estimations are based on the methods and models used by the authors to elucidate this question.

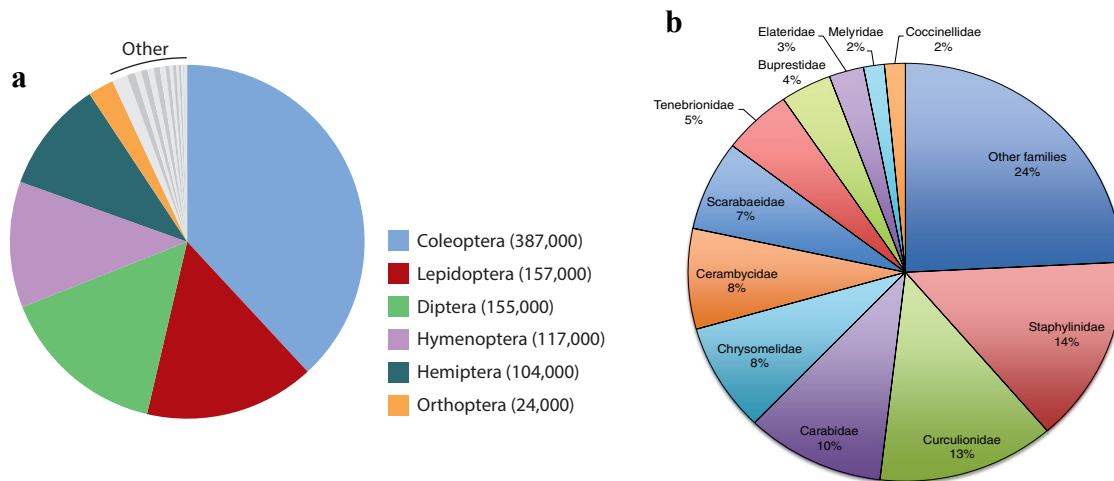


Figure 2. a. Orders of class Insecta with the approximate number of described species and, **b.** Diversity of Coleoptera per Family. Percentages are the estimated number of described species. Figures adapted from **a.** Stork et al. (2018) and **b.** from Bouchard et al., (2017).

Beetles occur both in terrestrial and aquatic environments. Sixty percent of all Coleoptera diversity is contained in six families, Staphylinidae, Curculionidae, Carabidae, Chrysomelidae, Cerambycidae, and Scarabaeidae (Bouchard et al. 2017) (Figure 2b). Beetles show a variety of body shapes and structures, granting a diversity of habitats. They can be threats to collections destroying preserved individuals (Skin beetles: Dermestidae), be pests in agricultural and forestry lands (Bark beetles: Curculionidae) and infest stored foods; at the same time being the ecosystem architects acting as, e.g., scavengers and decomposers helping in the organic matter cycle (Scarab beetles, Scarabaeidae: Scarabaeinae), plant pollinators (Chafer beetles, Scarabaeidae: Rutelinae) and through the soil burrowing (McKenna et al. 2015a; 2015b).

Necrophagous Coleoptera form a taxonomically and ecologically diverse part of the carrion insect community (Barton et al. 2013a; 2013b; Shea 2005; Tabor et al. 2004; 2010; von Hoermann et al. 2018; Watson and Carlton 2005) being responsible in the decomposition and dispersion of organic matter (Dekeirsschieter et al. 2011a; von Hoermann et al. 2018;

Matuszewski et al. 2018). Necrophagous entomofauna occupy diverse niches within the carrion (von Hoermann et al. 2018), that may cause changes in the dynamics of decomposition (von Hoermann et al. 2018; Matuszewski et al. 2008). A good example are the Carrion beetles, when having ideal environmental conditions (e.g., Temperature, humidity) they can accelerate the decomposition of a corpse (Schröder et al. 2002; 2003).

1.2.1. Forensic important Carrion beetles

Within the diversity of beetles visiting the carrion, Carrion beetles (Coleoptera: Silphidae) provide fundamental ecological functions in terrestrial ecosystems and assist in the breakdown and processing of organic matter (Barton et al. 2013a; Sikes 2008; von Hoermann et al. 2018). Adults and larvae of Silphids are attracted to feed on decaying organic matter, and the grand majority of the species are commonly occurring on animal and human corpses (Midgley et al. 2014; Ratcliffe 1996; Sikes 2005; 2008;). Although, Silphidae may exhibit predaceous and herbivorous feeding habits derived from a necrophagous ancestor (Ikeda et al. 2008). For three critical reasons, Carrion beetles are considered a reliable indicator of environmental disturbance. Firstly, the sensitivity to natural environmental gradients, presenting patterns of occurrence and beetle assemblage structure at regional and local spatial scales, displaying a response to climatic and edaphic preferences (Nichols and Gardner 2011; Benbow et al. 2016). Secondly, they exhibit different species-response patterns to land use change and disturbance. Third, carrion beetles are specialized scavengers sensitive to resource availability. They feed and reproduce primarily on birds and mammals carcasses (Gibbs and Stanton 2001; Benbow et al. 2016).

Classifications of beetles, based on the morphology of larvae and adults, consider Silphidae as sister lineage to rove beetles (Staphylinidae) (Lawrence et al. 2011; Grebennikov and Newton 2012). However, recent molecular phylogenies consider large carrion beetles as a monophyletic group, placed as an internal lineage within Staphylinidae, sister to the subfamily Tachyporinae or sister to a lineage combining the rove beetle subfamilies Apateticinae, Scaphidiinae and Osoriinae (Cai et al. 2022; McKenna et al. 2015a, 2015b; Zhang et al. 2018). Origin dating of the Silphidae go as early as to mid-Cretaceous, based on molecular clocks and fossil calibrations (McKenna et al. 2015b; Toussaint and Condamine 2016; Zhang et al. 2018). Within this lineage, Silphidae contains approximately 206 extant species (Bánki et al. 2023) distributed in two subfamilies: Nicrophorinae and Silphinae. **The phylogeny of Coleoptera is still a controversy and is under research (Boudinot et al. 2022). In this work, Silphidae**

will be considered as a family of Coleoptera and, Silphinae and Nicrophorinae subfamilies of the same.

Nicrophorinae, or Burying beetles, contains approximately 79 described species distributed primarily through the temperate zones (Bánki et al. 2023). Members of Nicrophorinae are predacious/necrophagous and some genera exhibit a unique breeding behaviour involving biparental care of the immature stages (Trumbo and Sikes 2021).

The second subfamily, Silphinae, also known as Carrion beetles, have so far 127 extant species with world-wide distribution (Bánki et al. 2023), having most of its diversity in Asia (Růžička 2015). Unlike Nicrophorinae, they contain diverse spectrum of feeding strategies. Some species of *Aclypea* Reitter, 1884 are considered phytophagous, whilst others are predacious such as *Dendroxena* Motschulsky, 1858 and *Silpha* Linnaeus, 1758 (Sikes 2008). Most of the species are considered unspecialized necrophages.

Silphidae show an amphitropical or amphipolar distribution (i.e., they are restricted to northern and southern temperate zones but generally absent from the intervening tropics except for tropical montane habitats) (Sikes 2008). The Nicrophorines are distributed mainly in the northern hemisphere, with species radiations occurring in various island montane habitats and into South America along the Andes. None are found in Africa south of the Sahara, in Australia, or in Antarctica (Sikes and Venables 2013). Silphines, however, are widely distributed, with greater representation in Gondwanan areas. The prior is related to their greater generic diversity (12 genera) and possible older age as a group. Unlike Nicrophorines, Silphines present four species in Australia and New Guinea (*Ptomaphila* Kirby and Spence, 1828, 3 endemic species; *Diamesus* Hope, 1840, 1 species) and show greater radiation in South America (*Oxelytrum* Gistel, 1848, 10 species). There are also seven silphine species in South Africa (*Thanatophilus* Leach, 1815, 3 species; *Silpha*, 4 species) and an entire silphine genus (*Heterotemna* Wollaston, 1864, 3 species) distributed in the Canary Islands off the northwest coast of Africa (Sikes 2008; Mahlerová et al. 2021; Newton 2022; J. Růžička et al. unpublished).

Midgley and Villet (2009) and Midgley et al. (2010) highlighted the importance of forensic entomologists and the accuracy of insect identification. Reliable identification methods for adults and immature stages of necrophagous such as silphids are required, as they are the most frequent evidence type encountered in forensic cases in temperate zones. In this sense, essential

morphological recognition of forensically important silphids among other beetles is the first step for studies in this area.

It is imperative for the understanding of the studied group to highlight some of the important characters for the delimitation of the group. Consequently, below is shown the main morphological characters (taken from Sikes 2008) for the delimitation of the adults and larvae of both Silphidae subfamilies.

Adults, ovate to moderately elongate, and slightly to strongly dorsoventrally flattened (Figure 3 a,b. *Thanatophilus*: Silphinae). Frontoclypeal suture absent, or present as fine line (Figure 4c. *Nicrophorus*: Nicrophorinae). Antennae are 11-segmented but appear as 10-segmented in Nicrophorinae due to reduced second segment fused to third segment; ending in 3-segmented club, usually preceded by two or three enlarged but sparsely setose segments (Silphinae and basal Nicrophorinae) or antennomeres 9–11 forming a large club (Figure 4c. *Nicrophorus*). Pronotum with lateral edges complete, sometimes explanate. Scutellum large, often as wide as head. Elytra truncate, exposing 1–5 abdominal tergites in *Diamesus*, *Necrodes*, and Nicrophorinae; not truncate in remaining Silphinae, covering abdomen; never striate; in Silphinae bearing 0–3 raised costae or carinae per elytron (present but indistinct in Nicrophorinae); with raised callus near posterior end of outermost costa; epipleura usually well developed and with ridge complete almost to apex.

The elytra of most species of *Nicrophorus* Fabricius, 1775, *Ptomascopus* Kraatz 1876, and *Diamesus* usually have broad coloured bands or spots (fascia and maculae) extending laterally to meet epipleura. Abdomen with sternite 2 not visible between hind coxae but visible laterally of metacoxae; sternites 3–8 visible in females, 3–9 visible in males. Legs with five tarsal segments per tarsus. Males usually with broadly expanded protarsal segments and longer protarsal setae (midtarsal also expanded in large males of *Diamesus*), pro- and mid tarsi of female similar.

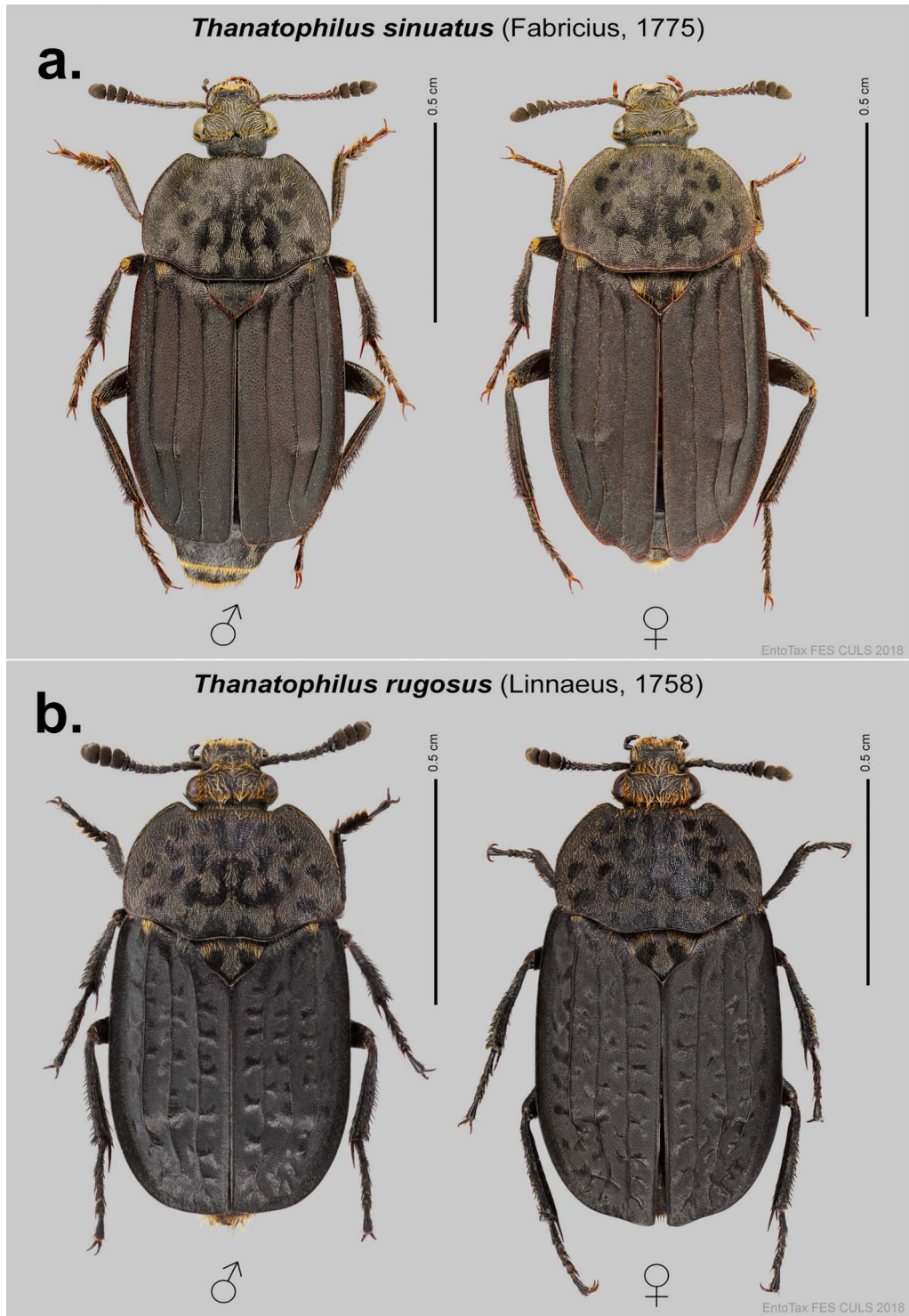


Figure 3. Dorsal habitus of **a.** *Thanatophilus sinuatus* (Fabricius, 1775) dorsal habitus. **b.** *Thanatophilus rugosus* (Linnaeus, 1758).

Larvae, campodeiform in most Silphinae (Figure 4a) or eruciform in Nicrophorinae (Figure 4b); elongate, more or less parallel-sided to ovate, slightly to strongly flattened, relatively straight or slightly curved ventrally. Body surfaces heavily pigmented and heavily sclerotized (Silphinae), or lightly pigmented and lightly sclerotized (Nicrophorinae). Stemmata 6 (Silphinae) or 1 (Nicrophorinae) on each side. Mandibles lacking mola. Thoracic terga and abdominal terga and sterna consisting of one or more sclerotized plates, without patches or rows of asperities, each tergum with 1 (Silphinae) or 2 (*Ptomascopus*) lateral tergal processes extending beyond edges of sterna or without such processes (*Nicrophorus*) but with four spinose projections along posterior margin of abdominal terga. One or two segmented, well developed urogomphi.

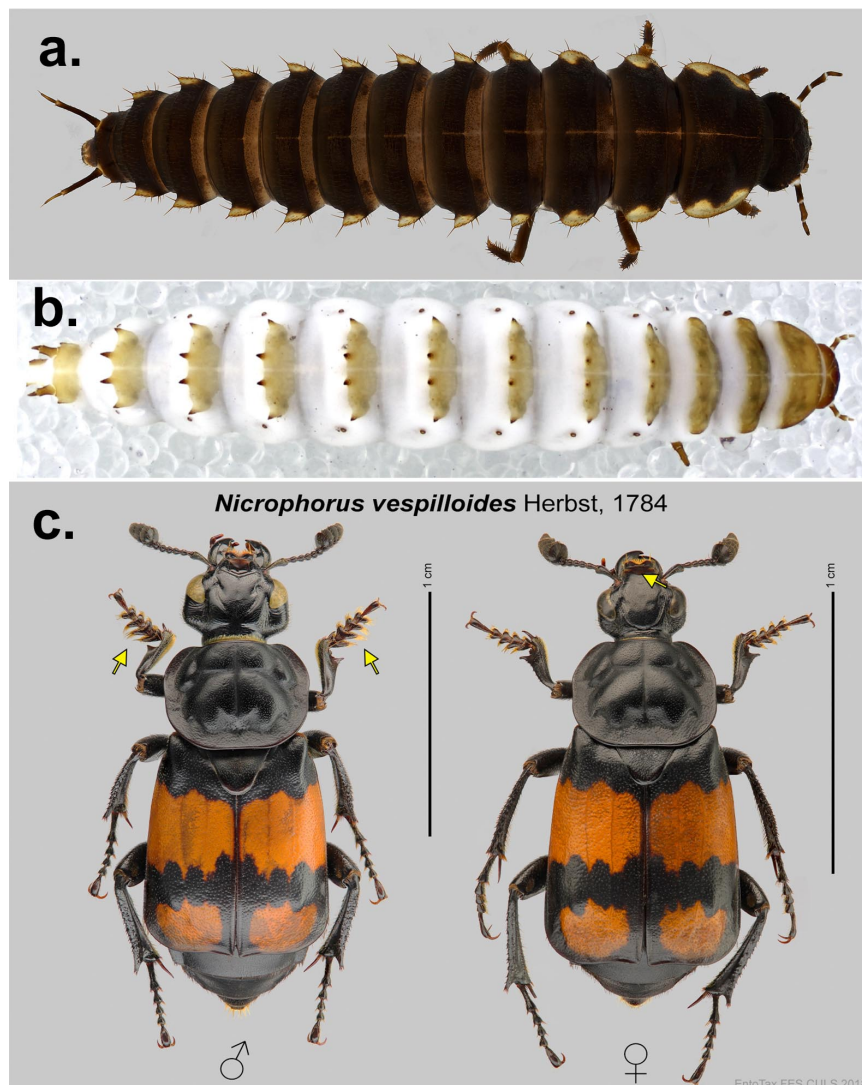


Figure 4. Dorsal habitus of **a.** *Thanatophilus sinuatus* (Silphinae) larvae; **b.** *Nicrophorus nepalensis* (Hope, 1831) (Nicrophorinae) larvae; **c.** *Nicrophorus vespilloides* Herbst, 1783 adult. Figure **b.** taken from Šípková and Růžička (2020).

1.2.1.1. *Thanatophilus*

The genus *Thanatophilus* (Silphidae: Silphinae) currently contains twenty-four described species occurring in Europe, Asia, North America, and Africa (Ratcliffe 1996; Newton 2022). They are attracted to large carrion inhabiting and feeding actively on them during most of the year (Dekeirsschieter et al. 2011; Matuszewski et al. 2013), shifting through seasons and habitat preferences (Bonacci et al. 2010; Dekeirsschieter et al. 2011a; 2011b; Kočárek 2001; Frątczak-Łagiewska and Matuszewski 2018). In many regions in the world, they constitute an essential part of the necrofauna found in corpses for criminal and legal investigations in these regions (Matuszewski 2021).

Adults and larvae of *Thanatophilus* species are frequent among the carrion community in temperate countries, associated to the active decay stage of decomposition (Matuszewski et al. 2008; 2011). Information regarding the biology and ecology of the species in Europe is available in Bonacci et al. (2010; 2011); Dekeirsschieter et al. (2011; 2013); Frątczak-Łagiewska and Matuszewski (2018); Kočárek (2001; 2002); Matuszewski et al. (2008; 2011; 2013); Prado e Castro et al. (2013). Morphological redescrptions providing diagnostic characters and new terminology used for instar determination of the immature stages were published by Frątczak-Łagiewska and Matuszewski (2016), Novák et al. (2018) and Jakubec et al. (2019) in Europe; Midgley and Villet (2009), and Daniel et al. (2017) in Africa, and further by Peck and Anderson (1985) and Anderson (1987) in North America. Until 1900, larval morphology of this genus was poorly known, containing formal and superficial descriptions of the immature stages (Xambeu 1900; von Lengerken 1937). However, Novák et al. (2018) and Jakubec et al. (2019) redescrbed the larval morphology for two of the three species occurring in Central Europe, *T. rugosus* and *T. sinuatus*. Taxonomical revisions of the genus based on adult morphology was published by Schawaller (1981), with extensions by Kozminykh (1994), Růžička (2002) and Ji (2012) based on the adult morphology.

Due to biological and ecological traits such as broad distribution, fast localization, and colonization of the corpses soon after death, some species of the genus *Thanatophilus* have become of high utility for the forensic entomology (Matuszewski 2021; Midgley and Villet 2009; Midgley et al. 2010; Ridgeway et al. 2014).

1.2.1.1.1. *Thanatophilus sinuatus* (Fabricius, 1775) – (Figure 3a; Figure 5a)

Thanatophilus sinuatus is a common carrion beetle frequently found on carcasses of large vertebrates in Europe (Dekeirsschieter et al. 2011a; 2011b; Jakubec and Růžička 2015; Jarmusz et al. 2020; Matuszewski et al. 2008; 2011; 2016). It is a broadly distributed Palearctic species (Figure 5a) (Růžička 2015). Seasonal activity of *T. sinuatus* comprises the warmer months of the year, April to September (Late spring – early autumn) (Frątczak-Łagiewska and Matuszewski 2018b). *Thanatophilus sinuatus* is a diurnal species, inhabiting open fields such as grasslands and meadows, preferring a particular type of soil (Dekeirsschieter et al. 2011a; Jakubec and Růžička 2015; Kočárek 2001). Males and females are attracted principally to cadaveric organic volatile compounds, such as the Dimethyldisulfide, butan-1-ol and *p*-cresol. (Dekeirsschieter et al. 2013; Kalinova et al. 2009). Morphology of the larval stages was recently redescribed by Jakubec et al. (2019). Here, developmental models for all larval stages are presented in Chapter 2. Diet and thermal preferences are presented in Chapters 5 and 6.

1.2.1.1.2. *Thanatophilus rugosus* (Linnaeus, 1758) – (Figure 3b; Figure 5b)

Thanatophilus rugosus is another potentially important species of forensic entomology in the temperate regions in Europe and along the Palearctic region (Figure 5b) (Růžička 2015); being frequently collected on carcasses during criminal investigations across Europe (Jakubec et al. 2019; Montoya-Molina et al. 2021b). The species seasonal activity is associated with the mild cold months of the year between spring and autumn (Matuszewski et al. 2011; Jakubec and Růžička 2015; Frątczak-Łagiewska and Matuszewski 2018). During winter months in southern Europe, mainly in Italy and Spain, the species is also active (Brandmayr et al. 2010; Bonacci et al. 2011; Díaz-Aranda et al. 2018). A complete description of all larval instars was recently published by Novák et al. (2018), hence making the species available for serious forensic interest in Europe. Developmental models for all larval stages are presented in Chapter 3 and the influence of the diet on the development is given in Chapter 4

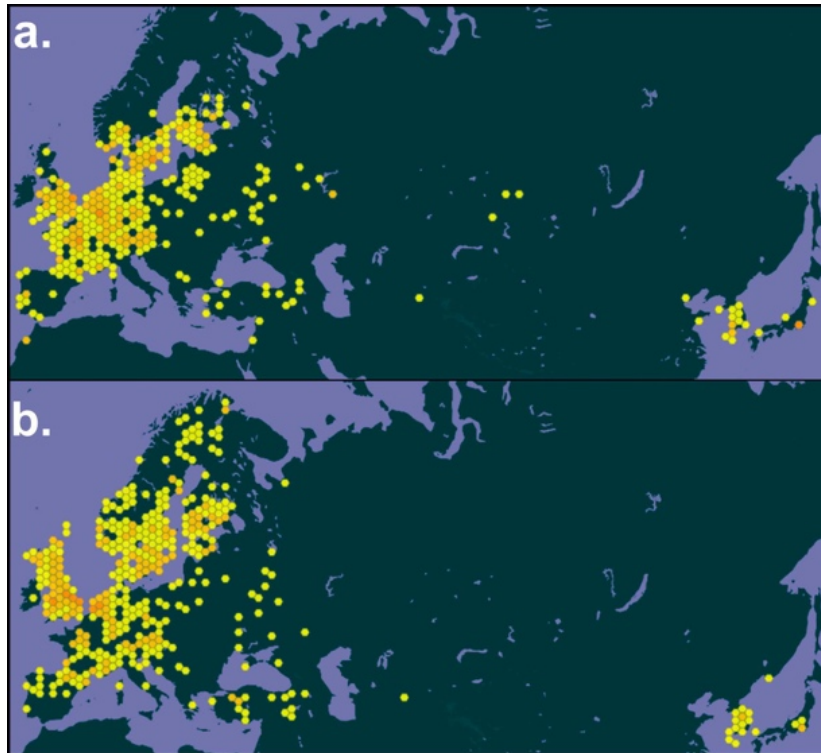


Figure 5. Distribution maps of **a.** *Thanatophilus sinuatus*. **b.** *Thanatophilus rufosus*. Source of the maps: <https://www.gbif.org/species/4448483>

1.3. Thermal biology of insects and applications in the Forensic science

In ectotherms such as insects, body temperature depends directly on the environmental temperature (Dixon et al. 2009; Trudgill et al. 2005). Thus, their physiological functions (e.g., metabolism, development, growth, movement, reproduction), behaviour and ecology are determined by the temperature (Gotcha et al. 2021; Sinclair et al. 2012; 2016; Trudgill et al. 2005). In theory, intraspecific values of thermal biology across different locations may vary as an adaptive response to local thermal conditions experienced (Masaki 1966; Ghalambor et al. 2007; Sinclair et al. 2012; Honěk et al. 2014; Sunday et al. 2011; 2019). The latter can affect the accuracy of phenological calculations (life cycle timing throughout season) on ectothermic species of critical importance in applied fields of entomology (e.g., Agronomy or Forensic). Furthermore, the values used to estimate generation cycles for pests or establishing colonization time in medico-legal cases (murders, abuse, neglect, etc.) can be significantly biased (Byrd and Castner 2001; Gennard 2012).

Thermal adaptation studies provide valuable information on the mechanisms of how the environmental temperature regulates the ecology, distributions, and evolution of the species

(Ghalambor et al. 2007; Kingsolver and Umbanhoar 2018; Sunday et al. 2011; 2019). Theoretical work in thermal biology often uses two kinds of measurements: Measurements of Performance (MP) and Measurements of Failure Temperatures (MFT) (Sinclair et al. 2015). In the MP, thermal constants such as Lower Developmental Threshold (LDT also referred as T_o) and Accumulated Degree-Days (ADD also referred as K). Often these measurements are used to interpret the possible interpopulation variation (Addo-Bediako et al. 2000; Bennet et al. 2021; Huey et al. 2012; Saska et al. 2014). In practice, the individuals are usually exposed to different non-lethal environmental temperatures over time and certain traits are measured (growth or development) as used in the forensic sciences for calculating the PMI and TSM (Frątczak–Łagiewska et al. 2020; Gruszka and Matuszewski 2022; 2023; Huey et al. 2012).

Contrarily, studies focused on MFT use critical thermal limits (Upper and Lower = CTmax and CTmin) as explanatory values for variation. MFT are particularly based on individual's locomotive response to extreme temperatures for a period of time. CTmax and CTmin are the extreme limits in which the movement (or coordinated movement) ceases (Figure 6). Both measurements of MP and MFT can give us an insight to intraspecific response of the underlying thermal biology across particular range of temperatures and show us clearly if the populations exhibit an adaptation or just a phenotypic plasticity (Ghalambor et al. 2007; Sinclair et al. 2012; Sunday et al. 2011; 2019).

Cold tolerance an MFT, can be analysed in different ways, e.g., Critical Thermal minima (CTmin) and Chill mortality. Other simple way to measure the cold tolerance is by investigating the recovery of the organisms after short periods of cold stress, also known as Chill Coma Recovery Time (CCRT). It is important to understand that Chill Coma is the comatose state induced by the low temperatures on insects and, is associated to the loss of muscle action potential amplitude (Hosler et al. 2000; Overgaard and MacMillan 2017). This state can be lethal to insects or reversible based on both magnitude of the variation and the duration of the exposure (Angilletta 2009; Denlinger and Lee 2010; Kostal et al. 2004; 2006; 2011). The measurements of CCRT are used as proxy of the response of the insects to cold stress exposing the specimens to periods of cold stress and measuring the recovery time after transferring into room temperature (David et al. 1998). These measurements are useful as they can provide an approximation on the species plastic and evolutionary responses to environmental changes (Ghalambor et al. 2007; Hoffmann et al. 2013; Sunday et al. 2014; 2019).

1.3.1. Insects thermal biology and use of insect development for forensic sciences

Measurements of Performance (MP) are continuously used in legal sciences when calculating the developmental times for a forensically important species (Amendt et al. 2006). Observations regarding to insect development states that the development depends on temperature. This was first recognized by Reaumur in 1700s, but methods for using this understanding to describe or predict insect development mostly date from the 1900 to the present (Honěk 1996; Ikemoto and Takai 2000; Shi et al. 2015; Mirhosseini et al. 2017; 2018; Rebaudo and Rabhi 2018). Growth and development of all organisms is clearly temperature dependent. For organisms with constant body temperature (Endotherms), we can affirm that, development is also dependent of the individual's metabolism (Angilletta 2009; Flouris and Piantoni 2015). However, organisms such as insects who cannot maintain constant body temperature (Ectotherms), development rates depend almost completely on external temperature (Angilletta 2009; Mirhosseini et al. 2017). Consequently, several mathematical models are being developed nowadays describing the influence of temperature on insect growth rates (Ikemoto and Takai 2000; Mirhosseini et al. 2017).

Relationship between time and temperature is curvilinear at low and high temperatures (Figure 7A and 7C) and linear in between (Figure 7B). The lowest temperature at which development can proceed is called minimum threshold (Figure 7), and the highest temperature is called Maximum threshold (Angilletta 2009; Hoffmann et al. 2013). Threshold temperatures typically occurs near the upper and lower lethal temperature for a species. The thermal development curve (or Thermal performance curve) exists for all insect species, although the specifics of the curve will vary by species (Huey et al. 2012; Sunday et al. 2019; Villeneuve et al. 2021). Lamb (1992) demonstrated that the curvilinear relationship between temperature and development rate reflects the underlying physiology of development and not genetic variation among individuals. Temperature dependent development is represented using the inverse of the development time, in other words, developmental rate. The development rate in insects starts from a critical thermal minimum and increases slowly as temperature increases (Figure 6). It reaches a temperature range where development rate is almost linear, then continues growing up to a thermal optima, to finally decrease rapidly to a critical thermal maximum (Figure 6) (Rebaudo and Rabhi 2018). Describing thermal development curve for an insect species is difficult, in that it requires substantial replication of development with many individual insects at multiple temperatures. Also, obtaining reliable development rates at low and high

temperatures is particularly challenging. These, are principally near to the lethal temperatures for the species (upper and lower extremes of the linearity zone), impacting negatively specimens survival (Angilletta 2009; Kingsolver and Buckley 2017).

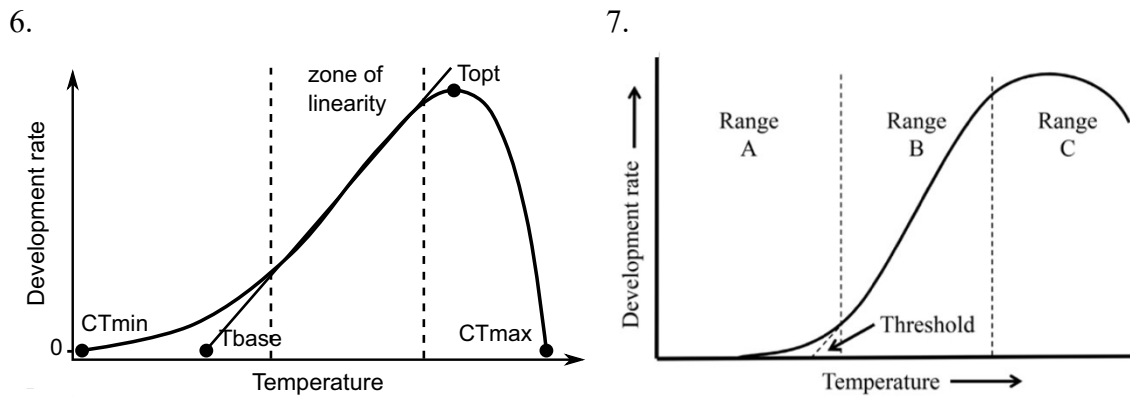


Figure 6. and 7. Relationship between temperature and insect development rate (Thermal development curves). 6A and 6C are nonlinear portions and B the linear part. Figure 5 is taken from Rebaudo and Rabhi (2018) and Figure 6. is taken from Mirhosseini et al. (2017).

Insect development can be represented using the following developmental models. The first, the Isomorphen diagrams, models the duration of development for each instar (stage of development) against temperature. The second, more sophisticated than the previous, the Isomegalen diagrams, models the size of the larvae as a measure of age against the temperature and the time. Developmental data of insects could also be described using temperature summation approaches such as the Thermal Summation model (TSM) (Amendt et al. 2010; 2011).

Thermal Summation Models parameters (LDT and ADD) can be used to calculate the age of the insect developmental stage using the temperature where the development took place (crime scene/corpse). The TSM being the most accurate one (Ikemoto and Takai 2000; Villet et al. 2010; Amendt et al. 2011), essentially analyses the response of development rate against experimental temperatures (often constant temperatures), resulting in two thermal parameters (LDT and ADD) that can be used to estimate the age of the insects species to accurately calculate the PMI (Ikemoto and Takai 2000; Gennard 2012; Richards et al. 2008; Richards and Villet 2008). The PMI is an interval which elapsed from time of death (Byrd and Castner 2001; Villet et al. 2010). Estimation of PMI can be influenced by biotic and abiotic factors of the

environment (e.g. relative humidity, light cycle, resource, temperature), nevertheless, as shown by many authors, temperature has a stronger relation in larval development (Beck 1983; Howe 1967; Caneparo et al. 2017; Lambiase et al. 2018 Zanetti et al. 2015). This is true also for species of Silphids (Merrick and Smith 2004; Midgley and Villet 2009; Matuszewski 2011); in which the temperature that the larvae were bred will affect directly the duration of immature stages.

It is important to mention that methods and models how to estimate PMI are being developed in very rapid pace (Ikemoto and Takai, 2000; Byrd and Allen, 2001; Dadour et al. 2001; Higley and Haskell 2010; Ieno et al. 2010). And methods of collecting the data on cadavers and its surroundings, together with analysis of reliable environmental data from the location of body, are enabling the entomologist to determine precisely the age of the immature insects and, consequently, estimate with great precision the PMI (Lutz and Amendt 2020).

1.3.2. Factors affecting Insects development times.

As aforementioned, information about species' thermal biology, especially their thermal summation models parameters (LDT and ADD), are used in forensic entomology to approximate the time of death or postmortem interval (PMI). Thermal summation model (TSM) parameters of forensically important insects are often calculated from colonies bred in the laboratory under constant temperature, relative humidity, and light regime conditions. Many of these TSM for forensically important beetles are continuously being published (Ridgeway et al. 2014; Frątczak-Łagiewska et al. 2018; 2020; Gruszka and Matuszewski 2022; Jakubec 2016; Montoya-Molina et al. 2021a; 2021b; Qubaiová et al. 2021; 2022). However, LDT and ADD parameters may be bias when modelling insect development under laboratory conditions due to disregarded factors. These may influence insects life history traits such as development, growth, and survival rate (Chown and Nicolson 2004). Food quality, dietary habits, and photoperiod are among the many factors that can influence the latter (Day and Wallman 2006a). Diet and photoperiod are poorly understood, and insect development times are considerably affected (Lopatina et al. 2007; Qubaiová et al. 2021; 2022).

Diet is essential for insect physiology and performance and depends on the source from which the larvae are fed (Scriber and Slansky, 1981). The organic composition of the natural food source and diets used for rearing do not always meet the nutritional requirements of the developing insects (House, 1969; Nation, 2015). Understanding the nutritional requirements

and the optimal food sources for the different necrophagous insects will positively impact the reference data from the species being collected in crimes and used for the PMI estimations (Clark et al. 2006; Day and Wallman 2006a; 2006b). Many studies focus on forensically important Diptera (Bambaradeniya et al. 2019; Bernhardt et al. 2017; Grassberger and Frank 2004; Grassberger and Reiter 2002; Kordshouli et al. 2021; Nabity et al. 2007; Niederegger et al. 2010; Rogers et al. 2021 Thyssen et al. 2014), and relatively a few ones on beetles (Frątczak-Łagiewska et al. 2020; Jakubec et al. 2021; Qubaiová et al. 2021; 2022).

Photoperiod is another factor affecting insect development (Higley and Haskell 2010; Lopatina et al. 2007). Photoperiod is associated to insect metabolism, physiology and behavior (Dolezel 2015). In temperate latitudes, photoperiod and temperature are the principal environmental factors regulating insect development (Saunders 2014). Shifts on the photoperiod may not only be the cause of changes on insect diapause, developmental times, growth and fecundity but could also provoke changes on insects body size (Beck 1980; Tachibana and 2004; Hodek 2012).

Many forensic entomology-related studies have been made using Diptera as taxa of interest. The effect of photoperiodism on development has been studied using flies, mainly blow flies (Calliphoridae). For example, Bala and Singh (2011) use *Chrysomya regina*, *C. rufifacies*, and Nabity et al. (2007) *Phormia regina* to obtain a delayed development when exposing the pupae to a constant light regime (24 h L: 0 h D). Opposite results were observed by Da Silva Mello et al. (2012) when exposing *C. albiceps* to a constant light regime. However, results of Fisher et al. (2015) suggested that the development of three species of blowflies was faster under cyclic light regime (12 h L: 12 h D or 16 h L: 8 h D). As previously stated, much of the work has been made on Diptera. Regarding this, few investigations in the scientific literature have been performed with important forensic Coleoptera. Longer days can delay the sexual maturity in females of *Nicrophorus nepalensis* (Hwang and Shiao 2011), or the reproduction and ovarian development of *N. quadripunctatus* (Nisimura et al. 2002). Importance of temperature on ectotherms development has long been accepted (Beck 1983; Howe 1967; Rebaudo and Rabhi 2018). Nevertheless, species-specific responses to other important factors such as photoperiod and diet preference on insects' development is until now limited.

1.4. Prologue

All the literature cited above attempts to show the importance of beetles, specifically *Thanatophilus*, for forensic entomology and, in general, to the forensic sciences. Nevertheless, much information regarding *Thanatophilus* thermal biology is still unknown. Moreover, information related to the effect of intrinsic and extrinsic factors on the species (*Thanatophilus sinuatus* and *T. rugosus*) thermal performance and thermal sensitivity (CCRT) is poorly studied. The following chapters of the thesis aim to broaden the understanding of *Thanatophilus sinuatus* and *T. rugosus* thermal performance. Chapters 2 and 3 provide the thermal summation models for *Thanatophilus sinuatus* and *T. rugosus*. Chapters 4 and 5 are associated with extrinsic factors affecting the development of *T. rugosus* and *T. sinuatus*. Finally, Chapter 6 provides insight into the Chill Coma recovery times (CCRT) for the species *T. sinuatus*. In the meantime, many more questions will arise from the previous work and may remain unsolved until more research is done. In general, any factual information provided here will contribute to understanding the species' biology (*Thanatophilus sinuatus* and *T. rugosus*) and is an approach to use the group as solid evidence in forensic investigations.

2. Chapter 2: Developmental Models of the Forensically Important Carrion Beetle, *Thanatophilus sinuatus* (Coleoptera: Silphidae)

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This first chapter of the thesis provides the thermal summation models for all the developmental stages of the forensically important carrion beetle *Thanatophilus sinuatus* (Fabricius, 1775). As mentioned in the introduction, developmental models still need to be calculated for many species belonging to the *Thanatophilus* genus, even though the group has great potential for forensic entomology. Thus, the results presented here will aid forensic entomologists in estimating the PMI when the species are collected on corpses in temperate regions.

Authors contributions: **SMM:** Concept, field work, lab work, data analysis and writing. **PJ:** Concept, lab work, data analysis and writing. **JQ:** field work, lab work, and writing. **MN:** field work, lab work, and writing. **HŠ:** writing. **JR:** field work and writing.

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Developmental Models of the Forensically Important Carrion Beetle, *Thanatophilus sinuatus* (Coleoptera: Silphidae)

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Abstract

Carrion beetles of genus *Thanatophilus* (Leach, 1815) are an important group of necrophagous insects, with great potential for forensic entomology in temperate zones of Africa, America, Asia, and Europe. Developmental models for majority of *Thanatophilus* species remain unknown. In this study, we will provide new thermal summation models for all the developmental stages of *Thanatophilus sinuatus* (Fabricius, 1775), one of the most abundant and widespread species of the genus. The beetles were bred at seven different constant temperatures, and developmental time was measured for each developmental stage (egg, three larval instars, postfeeding stage, and pupa). Temperature–sex influence was tested, and thermal summation constants were calculated to be used for postmortem interval estimation during criminal investigations.

Key words: Silphinae, developmental biology, thermal summations model, forensic entomology

Forensic entomology studies arthropods related to criminal investigation (Catts and Goff 1992, Anderson 1995, Byrd and Castner 2010, Joseph et al. 2012). The insect community associated with corpses provides an important tool for estimating the length of time since death, expressed as the postmortem interval (PMI; Anderson 2010, Goff 2010, Amendt et al. 2011). A common method for PMI estimations gathered from insect evidence is based on the age calculation of the oldest immature stages collected from the cadaver (Higley and Haskell 2010, Amendt et al. 2011). Within insects, two major groups are generally used: beetles (Coleoptera) and flies (Diptera), together providing the majority of evidence for forensic investigations (Byrd and Castner 2010). Much of the research has focused on understanding the succession and colonization of cadavers by blowflies (Diptera: Calliphoridae), but there has been much less research of this process in Coleoptera, underutilizing the valuable information that this group can provide to criminal investigations (Matuszewski et al. 2008, Midgley and Villet 2009, Dekeirsschieter et al. 2011b, Ridgeway et al. 2014). Only recently, studies have shown that Silphid beetles could be as useful for criminal investigations (Midgley et al. 2010, Ridgeway et al. 2014). Two characteristics are crucial in that regards, namely swift colonization of cadavers soon after death (particularly in species of genera *Thanatophilus* Leach, 1815 and *Necrodes* Leach, 1815) and low development rate

of the majority of known species (see Ratcliffe and Luedtke 1969, Midgley and Villet 2009, Ridgeway et al. 2014, Jakubec 2016, Frątczak-Łagiewska and Matuszewski 2018a, Jakubec et al. 2019).

PMI can be calculated based on the age of the oldest developmental stage of an insect collected from a corpse using mathematical models. These, among others, include the thermal summation model (TSM), which is the most widely used among forensic entomologists (Amendt et al. 2011, Villet 2011). TSM models are species and stage specific (Ridgeway et al. 2014); thus, accurate PMI estimation depends on the availability of these models for species found at the crime scene.

The family Silphidae comprises 187 species distributed almost worldwide (Sikes 2008, Newton 2020), with the highest diversity in the temperate zone of Eastern Palearctic Region (Růžička 2015). Adults and larvae are attracted by decaying organic matter, and majority of the species can be found on animal and human cadavers (Sikes 2008, Ridgeway et al. 2014). Developmental stages of silphids are nowadays recognized as an indicator of PMI in medico-legal studies (Midgley and Villet 2009, Joseph et al. 2012, Ridgeway et al. 2014).

Among Silphidae, genus *Thanatophilus* contains 24 valid extant species, distributed in Europe, Asia, North America, and Africa (Ratcliffe 1996, Newton 2020). Due to its wide distribution, fast

localization, and colonization of carrion within 24 h of death, some species of the genus have high applicability in forensic studies (Midgley and Villet 2009, Ridgeway et al. 2014). *Thanatophilus sinuatus* is a common carrion beetle frequently found on carcasses of large vertebrates in Europe (Matuszewski et al. 2008, 2010; Dekeirsschieter et al. 2011a,b; Jakubec and Růžička 2017; Jarmusz et al. 2020). Furthermore, the beetle was recorded on 13.27% of human remains, investigated by forensic entomologists in the Czech Republic (Jakubec et al. 2019). Based on its life cycle and natural history, the species meets most of the criteria to be used for PMI estimation as defined by Matuszewski et al. (2010). Recent studies conducted on *T. sinuatus* were related to its seasonal and daily rhythms (Kočárek 1997, 2002), preference of particular type of soil (Jakubec and Růžička 2015), behavioral responses (Dekeirsschieter et al. 2013), and larval morphology (Jakubec et al. 2019). Jakubec et al. (2019) additionally reared the species under artificial conditions and gathered preliminary data that have been supplemented and re-analyzed in the present work.

In this study, we present the first TSM based on the developmental cycle of the widely distributed trans-Palaearctic species, *T. sinuatus*. To achieve this, we reared *T. sinuatus* at seven different temperatures measuring the development rate for each of the temperatures and stages. Additionally, we tested whether sex influences the developmental length and whether the temperature affects the time proportions among the stages. Finally, we categorize the species as forensically relevant and applicable in accurate calculations of the PMI.

Materials and Methods

Laboratory Colony Establishment

Adult beetles of *T. sinuatus* were collected by pitfall traps baited with pork muscle tissues (*Sus scrofa* Linnaeus, 1758) and beef tissue (*Bos taurus* Linnaeus, 1758). The sampling was conducted in the Czech Republic during 2 yr. Between May and July of 2016, we sampled at three localities: Albeř (49°01'37"N, 15°08'43"E), Borová (49°44'30.05"N, 16°09'44.03"E), and Prague-Petřín (50°5'6.86"N, 14°23'11.60"E). During May and August of 2019, the collection took place around Kostelec nad Černými lesy (50°00'01"N, 14°51'33"E), Kolín-Štítary (50°0'49.78"N, 15°10'9.54"E), and Prague-Lysolaje (50°07'33.0"N, 14°21'44.0"E). Due to the ecological preferences of the species, the traps were placed in open areas, in habitats such as harvested agricultural fields or grasslands.

All beetle specimens collected were transported into the laboratory, identified, and sexed with the help of an identification key (Šustek 1981, Schawaller 1981, 1987). Individuals were separated into breeding colonies (maximum 10 per box), with an equal number of males and females. Each breeding colony came from the same locality avoiding cross-breeding and placed into a breeding box (Exo Terra: Art. #PT2250, dimensions 180 × 110 × 125 mm) filled with ca. 50 mm of sterilized moist gardening soil, used as the oviposition substrate. Beetles were provided with a piece of pork muscle tissue ad libitum. Additionally, a water source was provided in a form of 5-ml micro Eppendorf tube filled with water and stoppered by a piece of cotton.

Colonies were kept inside climatic chambers (custom made by CIRIS s.r.o.) at seven constant temperatures (14, 16, 18, 20, 21, 24, and 26°C) and 16:8 (L:D) h of dark photoperiod regime, maintained by fluorescent light (Osram L 8W/640) to simulate the light conditions during the species breeding season. Used temperatures and photoperiod were based on the previous rearing of this species by

Jakubec et al. (2019). Breeding colonies were inspected once a day to remove newly laid eggs. Egg batches were placed into separate Petri dishes (100 × 15 mm) and kept under the same conditions as their parents. Each Petri dish was filled with a thin layer of moist gardening soil, and the eggs were placed on the surface. Approximately 5 g of pork meat was also placed inside the dish and that was then secured with an elastic rubber band. The setup described here permits the larvae to start feeding immediately after hatching.

Once the first-instar larvae hatched, they were separated into smaller Petri dishes (60 × 15 mm). This layout followed the methodology suggested by Ridgeway et al. (2014). Each dish was filled up with moist gardening substrate to two thirds, placed on its side, and held closed by rubber band. Furthermore, the larvae were provided with a piece of pork meat, and the substrate was moistened regularly by submerging the bottom third of the dish into water for a few seconds. Unconsumed and moldy meat was regularly removed and replaced with fresh pieces. This layout enables observation of various life stages without unnecessary disturbances to the specimens. Newly emerged first-instar larvae hatched from the same clutch of eggs were placed in the Petri dish in groups of up to five specimens maximum. Developmental milestones (egg, first-instar larva [L1], second-instar larva [L2], third-instar larva [L3], postfeeding stage [PF], and pupae) were distinguished based on morphological features described by Jakubec et al. (2019). The whole breeding process was monitored once a day at 14, 16, 18, 20, 21°C and twice a day at 24 and 26°C.

Larvae were kept together in the same dish when their development rate in terms of instar level was the same or until reaching the final third larval instar (L3). This way, we minimized cannibalism and mortality that individual larvae often experience due to inefficient feeding. All newly emerged L3 were individually separated into Petri dishes (60 × 15 mm), so they would not be disturbed by other larvae in their postfeeding stage and especially once they created a pupation chamber. When a specimen reached pupal stage, the food was removed, and the Petri dish remained closed until its development to adult stage was completed.

Thermal Summation Model

To show the relationship between the beetles' developmental times and the accumulated degree days (DD) for all the developmental stages, we applied Ikemoto and Takai (2000) linear regression model ($DT = k + tD$) for calculating thermal summation parameters (k and t). In the equation, D is the duration of development, T is the environmental temperature, t is the lower developmental threshold, and k is the thermal summation constant. The main advantage of using this model is its robustness and simplicity. Both parameters of thermal summation are defined as a slope (t) and intercept (k) of the linear regression, respectively. Therefore, both t and k can be easily calculated along with their respective SE (Ikemoto and Takai 2000).

Effect of Sex on Development Length

A group of specimens from 14, 18, 20, 24, and 26°C that have completed development until adult stage was selected for the study of the effect of sex on development length, as only these specimens could be sexed. A binomial test was conducted to determine whether the observed sex ratio for the species is significantly different from the expected one (1:1).

To assess the potential differences in developmental time between males and females, two linear mixed effect regression models were fitted (null and 'sex' model). Response variables of both models were the developmental lengths of the six stages (egg, L1, L2, L3, PF, and

pupae). Null and alternative model also shared two fixed effect explanatory variables: temperature (14, 18, 20, 24, or 26°C) and developmental stage. Because the measurements of development times were done repeatedly on the same individual, the identity of that specimen had to be incorporated into the analysis; therefore, we used it as a random effect in both models. The only difference between the alternative and the null models was fitting the latter with sex as a fixed effect explanatory variable. The fit of these two models was compared via Akaike information criteria (AIC) to find out if the information about sex does significantly improve it.

Developmental Rate Isomorphy

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

Statistical Analysis

Data management and analyses were carried out using the R program (version 3.5.1; R Core Team 2016). Additionally, we used lme4 package for fitting mixed effect models, and visual outputs were processed via packages ggplot2 and sjPlot (Bates et al. 2015, Wickham 2016, Lüdtke 2018).

Results

Development of *T. sinuatus* at Seven Constant Temperatures

In this study, 918 individuals of *T. sinuatus* were used to gather information regarding to the developmental length at seven constant temperatures (14, 16, 18, 20, 21, 24, and 26°C). All tested temperatures, except for 21°C (see Discussion), allowed the completion of the life cycle from egg until adulthood. In total, 155 specimens reached adulthood. Observed developmental lengths differ between

temperatures. The duration of the development at the lowest temperature (14°C) was 78.24 d (SD = 6.31; $n = 23$), while at the highest temperature of (26°C) was only 22.23 d (SD = 1.20; $n = 16$; Table 1). The greatest proportion of *T. sinuatus* development cycle was spent during the postfeeding (25%) and the pupal stage (33%), whereas the shortest time was spent in the L1 (6–7%) and L2 (8–10%) stages in all studied temperatures.

Mortality rate was calculated using a subset of specimens that were obtained during 2019 only, as the mortality was not tracked in the previous years. Obtained data spanned five temperatures (14, 16, 18, 24, and 26°C) and covered all of the developmental stages. The overall mortality rate was mostly associated with extreme temperatures. Higher mortality was found on both ends of the extreme temperatures tested: 14°C (73.19%) and 26°C (78.37%). Mortality rate across intermediate temperatures (16–24°C) oscillated between 35.0 and 50.0%. Mortality rate also varied between the developmental stages. The highest mortality was found during the egg development at all temperatures, in which 60.11% of the eggs did not hatch. In the follow-up stages, the mortality increased very little. Being 54.92% for L1; 53.27% for L2; 43.98% for L3; and 42.62% for pupae.

Thermal Summation Model

Thermal summation models were established for all developmental stages (egg, L1, L2, L3, PF, and pupae; Fig. 1, Table 2). The (k) constant and (t) values were calculated with expected errors (Table 2). To complete the whole development from start to finish the individual needs to accumulate on average 360.46 DD with lower developmental threshold set at 9.85°C. Females needed only 330.24 DD, whereas males needed 375.36 DD and their respective t values were 10.21 and 9.35°C. Values of determination coefficient (R^2) for most of the models were above $R^2 > 0.91$, indicating good fit of the models on the data (Table 2). For egg and L2 stages, the determination coefficient was <0.91 ($R^2 = 0.857$ and $R^2 = 0.889$).

Effect of Sex on Development Length

Effect of sex on the developmental length was calculated using a group of beetles that were able to complete the development until the adult stage, from the temperatures of 14, 16, 18, 20, 24, and 26°C. Sex ratio (number of males related to number of females) was on average 1.15 in favor of males; however, the probability of males and females in the sample did not differ significantly from equality (exact binomial test, $n = 168$, 95% confidence interval [0.37, 0.53], $P = 0.247$). Sex ratio varied substantially between temperatures. The minimum observed at 16°C = 0.69 ($n = 54$) and 18°C = 0.67 ($n = 5$) and maximum at 20°C = 2.33 ($n = 10$) and 24°C = 2.20 ($n = 32$).

Development length was similar for both males and females, throughout all of the developmental stages and even as a total (see

Table 1. Mean developmental times (SD; N) of *Thanatophilus sinuatus* at seven constant temperatures for each developmental instar (d, °C)

Temperature	Egg	First instar	Second instar	Third instar	Post-feeding	Pupae	Total development cycle
14	7.51 (1.28; 47)	5.39 (0.73; 44)	5.77 (1.08; 37)	13.67 (3.38; 35)	20.60 (3.86; 25)	28.90 (3.73; 23)	78.23 (6.31; 23)
16	—	6.50 (1.85; 58)	6.16 (1.37; 58)	14.31 (2.50; 62)	19.64 (4.46; 56)	28.70 (4.38; 54)	NA
18	5.36 (0.9; 132)	3.89 (1.18; 128)	4.93 (1.80; 127)	9.29 (2.77; 121)	11.10 (4.10; 49)	14.84 (2.64; 38)	43.44 (7.40; 38)
20	3.47 (0.54; 82)	2.73 (0.73; 115)	4.21 (1.30; 92)	8.56 (1.55; 50)	11.17 (2.57; 29)	13.56 (2.44; 20)	42.04 (3.12; 21)
21	3.44 (0.66; 100)	2.61 (0.57; 91)	3.57 (0.85; 85)	6.18 (1.46; 65)	8.87 (1.82; 7)	NA	NA
24	2.96 (0.33; 37)	1.74 (0.44; 32)	1.86 (0.20; 24)	4.77 (0.90; 24)	5.94 (2.11; 32)	7.50 (2.89; 32)	24.77 (2.40; 32)
26	2.61 (0.43; 32)	1.50 (0.49; 22)	2.00 (0.73; 17)	4.52 (1.82; 17)	4.99 (0.87; 16)	7.03 (0.90; 16)	22.22 (1.20; 16)

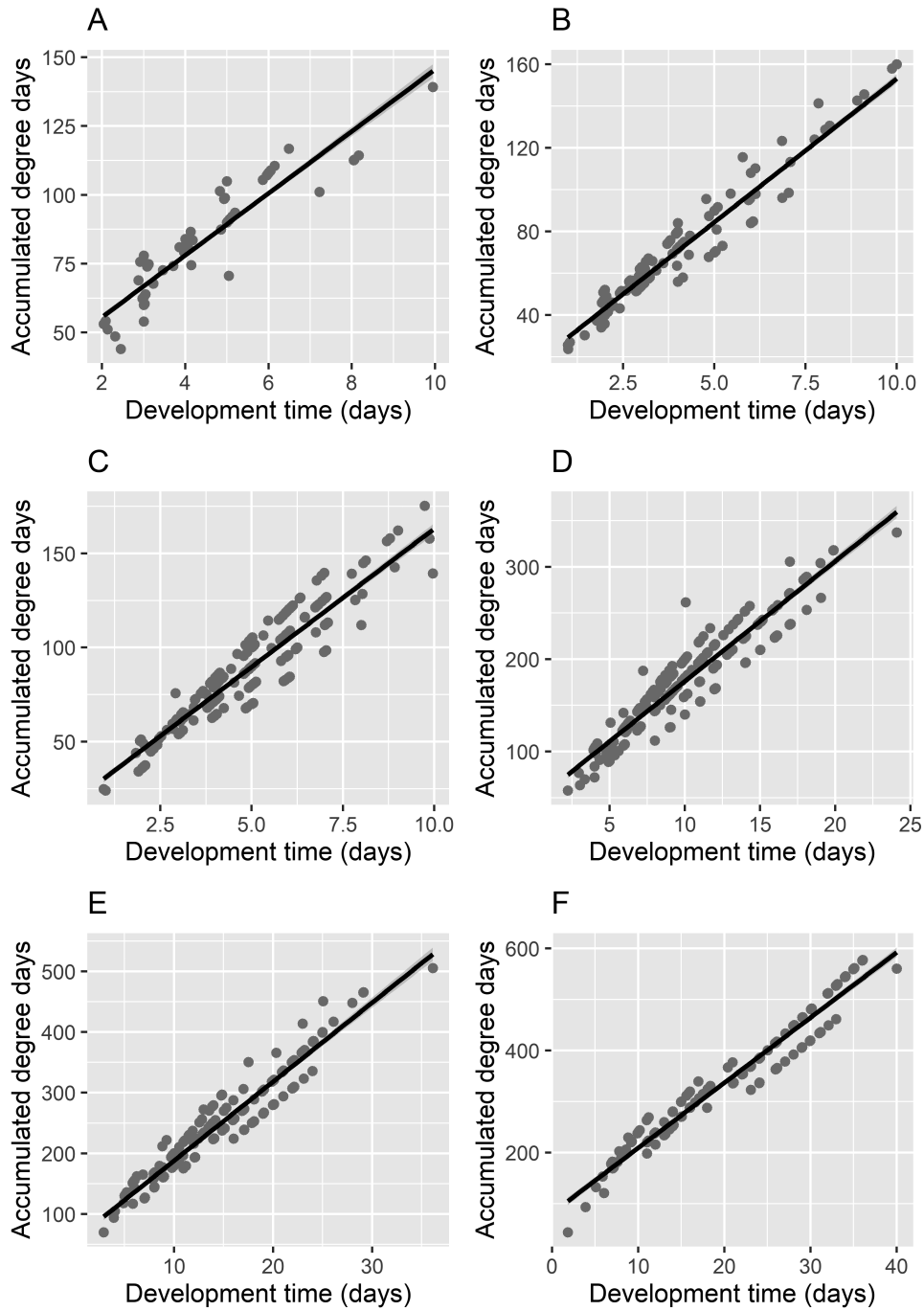


Fig. 1. Ikemoto and Takai's (2000) thermal summation model for all developmental stages of *Thanatophilus sinuatus*: (A) Egg, (B) first larval instar, (C) second larval instar, (D) third larval instar, (E) post-feeding, (F) pupae. The points indicate used data for the regression analysis.

Table 2. Overview of thermal summation models for six developmental stages of *Thanatophilus sinuatus*

Developmental stage	Temperature range of model (°C)	R ²	df	P-value	K (°C)	T ₀ (°C)
Egg	14–26	0.857	428	>0.001	33.097 (1.03)	11.25 (0.22)
First-instar larva	14–26	0.933	488	>0.001	15.785 (0.66)	13.727 (0.16)
Second-instar larva	14–26	0.884	138	>0.001	16.171 (1.22)	14.719 (0.25)
Third-instar larva	14–26	0.903	372	>0.001	45.893 (2.24)	13.021 (0.22)
Post-feeding	14–26	0.936	212	>0.001	57.701 (3.44)	13.03 (0.23)
Pupa	14–26	0.957	181	>0.001	81.637 (4.18)	12.764 (0.19)
Complete development	14–26	0.933	128	>0.001	360.46 (10.75)	9.85 (0.23)

Number in parenthesis are the SE.

Fig. 2). Comparison of the null and sex model by AIC values showed that the null model had lower AIC values (null AIC = 2,785.5, sex AIC = 2,788.2); consequently, we can imply that the information about the sex of immature stages did not improve the fit of the model significantly.

Developmental Rate Isomorphy

Dirichlet regression was applied to individual development data for the egg, L1, L2, L3, PF, and pupae of *T. sinuatus* reared under the temperatures of 14, 18, 20, 24, and 26°C. When comparing all three models (mod0, mod1, and mod2), the model with quadratic effect of temperature had the lowest value of AIC (AIC = -2,334.8), followed by the model with temperature as an explanatory variable (AIC = -2,304.2). On the opposite side was the model implying DRI, which was accompanied by the highest value of AIC (AIC = -2,272.19), suggesting the worst fit to the data. These results imply that the proportion of the time spent in each developmental stage is not constant but has a quadratic relationship with the temperature.

Discussion

The effects of different temperatures on developmental times in Silphinae have been investigated only recently, and the number of works related to the topic is now increasing, thanks to the use of the taxa in the forensic investigations (Velásquez and Vilorio 2009, Midgley et al. 2010, Matuszewski et al. 2013, Ridgeway et al. 2014, Novák et al. 2018, Jakubec et al. 2020).

In comparison with other species of genus *Thanatophilus*, our study shows that *T. sinuatus* spends similar or longer time on the corpses compared with Afrotropical species [*T. micans* (Fabricius, 1794) and *T. capensis* (Wiedemann, 1821)] (Ridgeway et al. 2014). More specifically, at 20°C, *T. sinuatus* takes 42.04 d to complete development from egg to adult. Under the same conditions, *T. capensis* takes 39.6 d and *T. micans* takes 22.64 d (Ridgeway et al. 2014). These species also differ in the lower developmental threshold values: *T. sinuatus* ($t = 9.85^{\circ}\text{C}$), *T. capensis* ($t = 9.04^{\circ}\text{C}$), and *T. micans* ($t = 13.26^{\circ}\text{C}$; Ridgeway et al. 2014). In this case, it is clear that phylogenetical relatedness does not guarantee similarity in developmental characteristics.

Differences in developmental time between Afrotropical species of genus *Thanatophilus* and *T. sinuatus* could be attributed to

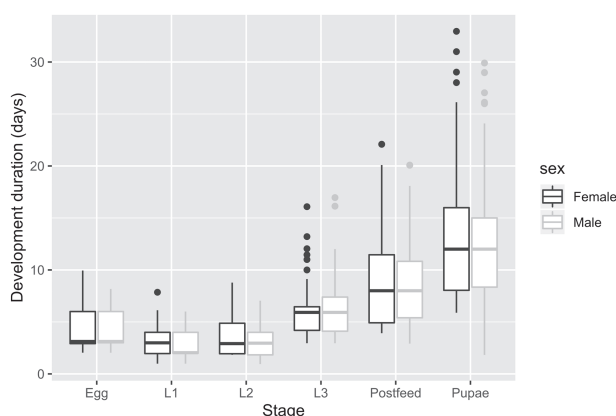


Fig. 2. Developmental length of males and females at each developmental stage (from egg until adulthood). Horizontal lines within the boxes indicate median values; upper and lower boxes indicate the 75th and 25th percentiles, respectively. Whiskers indicate the values within the 1.5 interquartile ranges. Small dots are outliers.

differences in their areas of distribution and probably adaptation to local climatic conditions. However, comparison could be made between *T. sinuatus* and *T. rugosus* as these two species overlap in their occurrence patterns especially in Europe (Růžička 2015). These two species also share very similar habitat preferences (Kočárek 2001, Jakubec and Růžička 2015, Frączak-Łagiewska and Matuszewski 2018b). Development length at 20°C seems to be slightly faster in *T. sinuatus*, as the development of *T. rugosus* takes on average 45.48 d (Novák et al. 2018). Deeper comparison between these two species is unfortunately not possible as TSMs for *T. rugosus* are not available.

Our results agree with the general concept that development of beetles from genus *Thanatophilus* takes longer than development of forensically important blow flies (Diptera: Calliphoridae) (Ridgeway et al. 2014). Development of common European species of blow flies, such as *Calliphora vicina* Robineau-Desvoidy, 1830, *Phormia regina* Meigen, 1826, and *Lucilia sericata* (Meigen, 1826), can take approximately 21.45, 20.5, and 24.75 d, respectively, at 20°C (Anderson 2000, Nability et al. 2006, Roe and Higley 2015), which is significantly faster than *T. sinuatus*. Therefore, in cases when the victim is found after more than 25 d, *T. sinuatus* can add additional information to the PMI estimates.

To our knowledge, this study represents the second TSM of any member of the family Silphidae from Europe (see Gruszka and Matuszewski 2020), although TSMs of several nonsilphid necrobiont beetle species from the region were recently reported (e.g., *Sciodrepoides watsoni* (Spence, 1813) (Coleoptera: Leiodidae), *Creophilus maxillosus* (Linnaeus, 1758) (Coleoptera: Staphylinidae), and *Aleochara nigra* Kraatz 1859 (Coleoptera: Staphylinidae); Lin and Shiao 2013, Jakubec 2016, Frączak-Łagiewska and Matuszewski 2018a, Wang et al. 2018).

Thanatophilus sinuatus oviposition was carried out mostly at night and into the substrate, thus preventing desiccation of the viscous layer that covers the eggs clusters. It differs from the closely related *T. capensis* that oviposits on the surface allowing the eggs to have contact with external air and light, influencing the activity hours of the species (Ridgeway et al. 2014). Nocturnal oviposition in *T. sinuatus* is not exceptional due to the fact that the adults of the species are also active during night, and explanation of the aforementioned authors has some logic, influencing the activity levels of the future larvae during the night.

The methodology suggested by Midgley and Villet (2009) and Ridgeway et al. (2014) has been successfully applied in this work with some variations. Main difference was that freshly hatched L1 was separated into groups of up to five individuals per one small Petri dishes. This approach was based on the premise that larvae up to L2 stage need to be among siblings for the successful development and survival as the aggregation may enable more efficient feeding (Scanvion et al. 2018). The communal feeding of *T. sinuatus* was observed under the field condition by Bonacci et al. (2011). Furthermore, Gruszka and Matuszewski (2020) hypothesized that this could be an ancestral trait of Silphidae. Our observations of high mortality of individually raised L1 larvae of *T. sinuatus* seemed to be independent of the environmental factors and further supports the need for aggregation in early developmental stages in this age group.

During the first year of the breeding (2016), we observed that no larvae were able to finish the development at 21°C. This could have been caused by severe infestation of the experimental animals at this temperature with mites of the genus *Poecilochirus* G. Canestrini et R. Canestrini, 1882 (Acari: Parasitidae), or by spread of a disease we were unable to identify. We did not observe this phenomenon in the

chambers with lower and higher temperatures, thus excluding temperature as the primary cause for the mortality.

The highest mortality was found in the egg stage. Often, the eggs did not hatch and died due to what was probably a bacterial or fungal infection. In addition, we suspect that some eggs may not have been fertilized. We also observed low mortality in early larval stages; nonetheless, L3 larvae often died during the process of building of pupation chamber while in postfeeding stage. In all of the cases where L3 interrupted the PF stage, due to a disturbance or without apparent reason, the L3 was unable to continue the normal developmental cycle and consequently died. Furthermore, we observed that specimens reared at extreme temperatures 14 and 26°C experienced complications during ecdysis from L3 to pupa (see Fig. 3B and C) or had malformations after emerging into adult stage (e.g., deformed wings and vestiges of pupae stage such as the bristles in the pronotum and visible thoracic sclerites (see Fig. 3A).

We did not find any statistically significant difference of developmental parameters between males and females of *T. sinuatus*, which is in line with our previous study of *Necrophila (Calosilpha) brunnicollis* (Kraatz, 1877) (Jakubec et al. 2020) and also conclusions of Gruszka and Matuszewski (2020) for *Necrodes littoralis* (Linnaeus, 1758; both Coleoptera: Silphidae). We were unable to confirm the presence of developmental rate isomorphy as well, following the methodology of Boukal et al. (2015). This also agrees with previously published conclusions for carrion beetles (Jakubec et al. 2020).



Fig. 3. Developmental malformation of *Thanatophilus sinuatus*: (A) Ventral and dorsal view of the deformed adult. (B) Lateral view of the larvae unable to leave the third-instar exuvia for pupation. (C) Pupa unable to complete the developmental cycle into adult; larval appendages and mouth parts are still attached to the pupa.

This study presents the first thermal summation models for all developmental stages of the species. The results here provided could enable forensic entomologists to calculate PMI in cases when the species is found on victim's cadaver. Using *T. sinuatus* for PMI calculations becomes especially useful in cases when the body is found in advanced stage of decay and other species with faster developmental rate already left the corpse. Our results are relevant mainly in the context of the Central Europe as our samples cover a limited geographic range, but they can be used in the future studies of geographic variability of developmental characteristics.

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3. Chapter 3: Developmental models of the carrion beetle *Thanatophilus rugosus* (Linnaeus, 1758) (Coleoptera: Silphidae)

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The second manuscript of the thesis (Chapter 3) continues broadening the knowledge regarding the developmental models for the species belonging to the *Thanatophilus* genus. The manuscript below presents for the first time the Thermal Summation Models for the carrion beetle *Thanatophilus rugosus*. Due to similar ecological requirements, Together *T. rugosus* and *T. sinuatus* are distributed along the Palearctic region, being collected in large carcasses in a variety of open land habitats. Different from *T. sinuatus*, *T. rugosus* seasonal activity is during early spring and beginning of the summer, utilizing carrion resource only during short periods of time during the year.

Authors contributions: **SMM:** Concept, field work, lab work, data analysis and writing. **PJ:** Concept, lab work, data analysis and writing. **JQ:** field work, lab work, and writing. **MN:** field work, lab work, and writing. **HŠ:** writing. **JR:** field work and writing.

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OPEN Developmental models of the carrion beetle *Thanatophilus rugosus* (Linnaeus, 1758) (Coleoptera: Silphidae)

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Coleoptera are currently considered a fundamental tool to help solve criminal investigations, allowing forensic entomologists to estimate post-mortem intervals and obtain other ecology-related information. *Thanatophilus rugosus* (Linnaeus, 1758) is an important necrophagous beetle distributed through most of the Palaearctic region, where it is readily found on human bodies and animal carcasses. In this study, the new thermal summation models for all the developmental stages of *Thanatophilus rugosus* are provided. Beetles were reared at six different constant and ecologically relevant temperatures (12, 14, 16, 18, 20, and 22 °C), and their developmental times were measured. Thermal summation constants were calculated for each developmental stage (egg, three larval instars, post-feeding stage, and pupa).

Insect specimens found on a carrion during criminal investigations are a valuable piece of evidence when investigating time intervals relevant for the forensic sciences, e.g., Postmortem interval (PMI) and Pre-appearance interval (PAI)^{1,2}. Using developmental information of Silphinae in medico-legal cases has nowadays proven to have many advantages when calculating the post-mortem interval³.

Thanatophilus Leach, 1815 (Silphidae: Silphinae) is distributed in Europe, Asia, North America, and Africa⁴. Forensic entomologists took interest in the genus only recently as they recognized its value for the field^{5,6}. Many aspects of its ecology and morphology have already been studied such as the seasonal and daily rhythms^{7,8}, preference to a particular soil type⁹, chemical ecology¹⁰, larval morphology and instar identification¹¹. Recently, developmental models have been proposed for some of the *Thanatophilus* species e.g., *T. micans* (Fabricius, 1794)⁵, *T. capensis* (Wiedemann, 1821)³ and *T. sinuatus* (Fabricius, 1775)¹². However, many more have yet to be studied, as this genus is one of the most diverse in the subfamily Silphinae with 24 described members to date⁴.

Along with other species colonizing carrion, *Thanatophilus rugosus* (Linnaeus, 1758) is frequently found on carcasses during early spring in central Europe^{13,14} and throughout most of the year also in Southern Europe¹⁵. Despite the species being extensively collected on carcasses during criminal investigations across Europe, no developmental studies have been published so far.

In this article we present thermal summation models (TSM) based on the full developmental cycle of *T. rugosus*. Furthermore, we analyze whether the sex of an individual influences the developmental length, and whether the time proportions among certain instars are constant and independent. Additionally, survival rates among temperatures and developmental stages are evaluated.

Results

Development of *T. rugosus*. Overall, 713 individuals of *T. rugosus* were analyzed at six constant temperatures (12, 14, 16, 18, 20 and 22 °C). In all tested temperatures, individuals were able to complete the life cycle from egg to adulthood. In total, 526 out of 713 specimens reached adulthood.

The observed developmental lengths differed among temperatures. The duration of the development at the lowest temperature (12 °C) was 88.36 days (SD = 8.70; N = 88) while at the highest temperature (22 °C) was only 25.66 days (SD = 1.48; N = 70) (Table 2). The greatest proportion of the *T. rugosus* development cycle was spent in the Post-feeding (24%) and the pupal (33%) stages, while the shortest time was spent in the L1 and L2 stages (7–8%) in all studied temperatures.

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Developmental stage	Survival rate	Lower and upper CI (95%)
Egg	0.94 (37; 0.0098)	0.920–0.958
1st instar larva	0.89 (52; 0.0128)	0.864–0.914
2nd instar larva	0.85 (25; 0.0145)	0.823–0.880
3rd instar larva	0.84 (8; 0.0150)	0.809–0.868
Post-feeding	0.83 (13; 0.0155)	0.795–0.855
Pupae	0.81 (11; 0.0159)	0.782–0.845

Table 1. Survival rates for all developmental stages of *T. rugosus* at six constant temperatures. The number of deaths followed by standard error shown in parentheses (*N*; *SE*).

Survival rate and mortality. In total, the survival rate was above 70% in all the tested temperatures. Among all of the developmental stages, first larval instar (L1) and egg stage (Egg) had the highest in mortality rates, specifically 5% (*N* = 52) of deaths for L1 and 6.1% (*N* = 37) of deaths for Egg. The lowest mortality rate values were observed at the third larval instar (L3), post-feeding stage (PF) and pupal stage (Pupae), specifically 1.3% (*N* = 8) of deaths, 1.3% (*N* = 8) and 1.2% (*N* = 11) respectively. Second larval instar (L2) mortality rate was 3.8%. Survival rates values for each developmental stage are given in Table 1. Regarding the tested temperatures, the highest mortality rate was found at the highest temperature of 22 °C, where 25.5% of individuals did not complete their development. Increased mortality was found at 14 °C (19.2%) and 18 °C (19.5%) as well. Cannibalistic behavior in L3 larvae was observed towards the younger developmental stages of L2. Larvae cannibalized by the L3 were recognized due to the feeding marks on the ventral side, with their carcasses mostly comprising of the remnants of dorsal plates.

Thermal summation model. Thermal summation models were established for all developmental stages (Fig. 1, Table 2). The accumulated degree days (ADD) and lower developmental threshold (LDT) values were calculated with expected errors (Table 3). To complete the whole development from the egg to the emergence of imago the individual needs to accumulate 362.75 ADD with a LDT set at 8.52 °C. Values coefficient of determination (R^2), for all inspected models were above 0.85, indicating good fit on the data (Table 2).

Effect of sex on developmental length. The effect of sex on the developmental length was calculated using a group of beetles that were able to complete development until the adult stage, from across all of the studied temperatures. The sex ratio (the number of males related to the number of females) was on average 1.067 (i.e. in favor of males); however, the probability of males and females in the sample did not differ significantly from equality (Exact binomial test, *N* = 253, 95% CI [0.44, 0.52], *p* value = 0.4842).

The developmental length was similar for both sexes throughout all of the developmental stages and even as a total (see Fig. 2). Comparison of the null and sex model by AIC values showed that the null model had a significantly lower AIC value (null AIC = 15,460.29, sex AIC = 15,464.73), therefore the information about the sex did not significantly improve the fit of the model.

Developmental rate isomorphy—DRI. Dirichlet regression was applied to the individual development data for the Egg, L1, L2, L3, PF and the Pupae of *T. rugosus* reared under all studied temperatures. When comparing all three models (mod0, mod1 and mod2), the mod2 with quadratic effect of temperature had the lowest value of AIC (AIC = −13,909.4), followed by the mod1 with temperature as the explanatory variable (AIC = −13,839.0). On the opposite side was the mod0 implying DRI, which was accompanied with the highest value of AIC (AIC = −13,638.2), suggesting the worst fit to the data. Therefore, we can conclude that the proportion of time spent at the individual developmental stages is not constant but has a quadratic effect with temperature.

Discussion

This manuscript proposes the first thermal summation model for all the developmental stages of *T. rugosus*. This work complements the recent research on the Central European carrion beetles of forensic importance (see^{11,12,16–19}). Moreover, the results provided are also applicable in countries outside Europe where *T. rugosus* occurs²⁰.

Overall, the survival rate was above 70% (*N* = 526) in all the studied temperatures. These results suggest that the tested temperatures were not stressful or unfavorable. On the contrary, the survival rates indicate that all the tested temperatures were optimal for the beetle's successful development, ensuring a more extensive data set for the TSM. We found that *T. rugosus* is likely to survive and perform slightly better at the lower intermediate temperatures (16–18 °C) rather than the high ones (> 20 °C). This could be associated to its natural seasonal activity in Europe^{7,13,15}.

Developmental data at two constant temperatures were previously published by Ref.²¹, for species referred to as *Silpha rugosa* Linnaeus, 1758, which is an invalid taxonomic name for *T. rugosus*. However, *T. rugosus* has never been reported from Algeria where the study took place, nor northern Africa in general²⁰, and its occurrence here is unlikely. We believe that the developmental information presented by Guerroudj and Berchi is actually referring to *T. ruficornis* (Kuster, 1851). *T. ruficornis* does occur in Algeria and can be misidentified as *T. rugosus*,

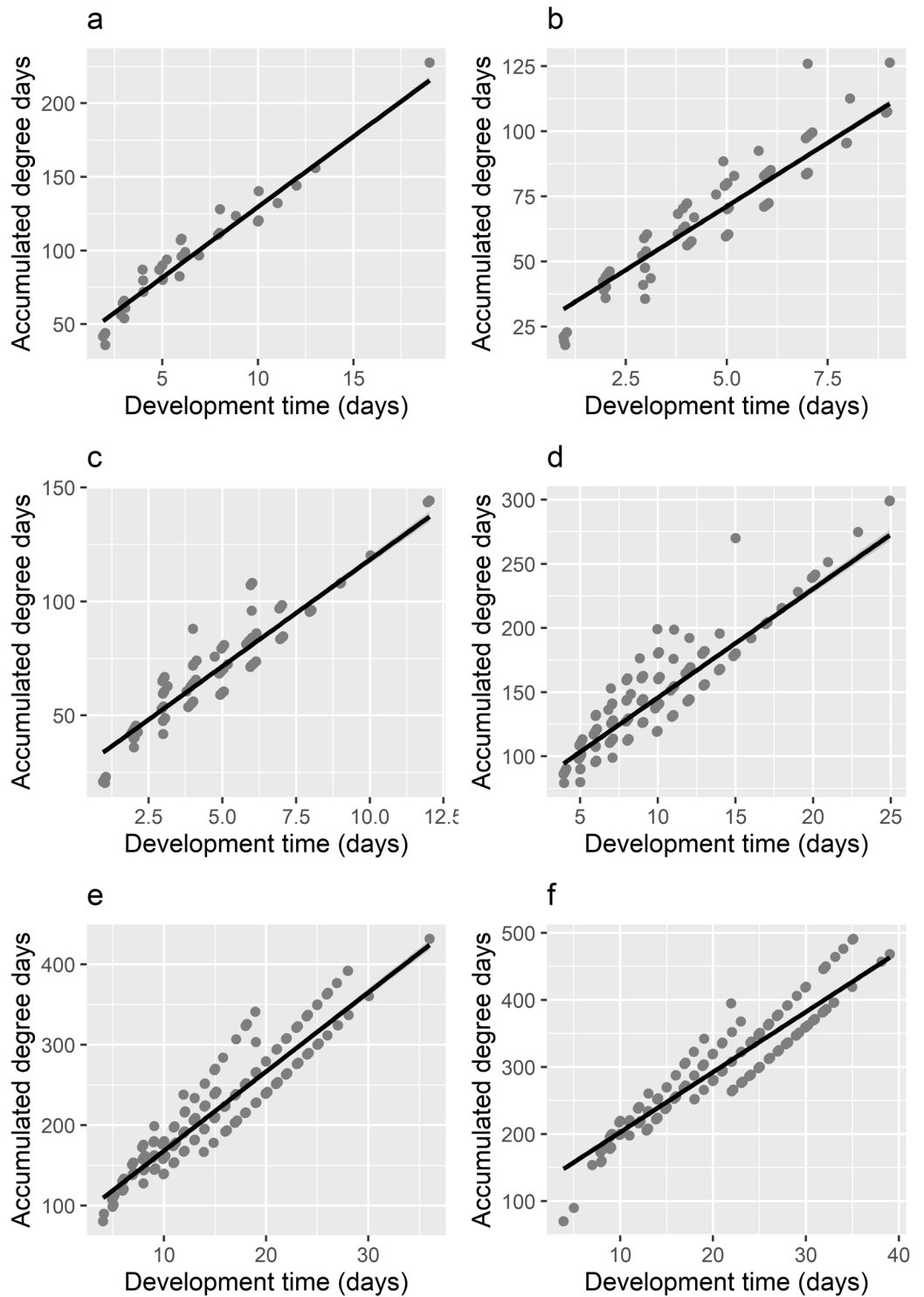


Figure 1. Ikemoto and Takai³⁰ thermal summation model for all developmental stages of *T. rugosus*: (a) egg, (b) 1st larval instar, (c) 2nd larval instar, (d) 3rd larval instar, (e) post-feeding, (f) pupae. The points indicate the data used for the regression analysis.

due to the similar shape and texture of elytra²². Both species can be further separated morphologically, e.g., by the color of the antennomeres (combination of black and red in *T. ruficornis*, uniformly black in *T. rugosus*) and by the differences in shape of the posterior margin in the female’s abdominal tergum VIII (lateral emargination being deeper than the middle one in *T. ruficornis*, while equally emarginated in *T. rugosus*^{22,23}). This means that

Temperature	Egg	1st instar larva	2nd instar larva	3rd instar larva	Post-feeding	Pupae	Total
12	11.60 (1.35; 105)	6.40 (1.11; 98)	6.82 (1.22; 98)	14.70 (3.10; 95)	20.80 (3.50; 92)	28.36 (3.58; 88)	88.03 (8.70; 88)
14	7.60 (1.07; 120)	5.30 (1.04; 105)	5.30 (0.86; 102)	10.48 (1.54; 99)	18.08 (4.65; 98)	25.17 (3.73; 97)	72.09 (8.02; 97)
16	5.81 (0.96; 107)	4.20 (0.70; 107)	4.16 (0.58; 106)	7.76 (1.14; 106)	11.52 (1.72; 105)	16.41 (1.96; 105)	49.85 (3.93; 105)
18	4.22 (1.12; 139)	3.17 (0.82; 115)	3.13 (0.78; 113)	7.14 (1.40; 112)	10.59 (2.40; 106)	13.65 (2.22; 101)	41.88 (4.82; 101)
20	3.40 (0.56; 14)	2.14 (0.38; 109)	2.40 (0.56; 100)	5.71 (0.95; 100)	6.70 (1.04; 99)	10.13 (1.02; 98)	30.47 (2.00; 98)
22	2.62 (0.63; 83)	1.81 (0.43; 82)	2.02 (0.65; 72)	4.75 (0.70; 71)	6.00 (0.83; 70)	8.60 (0.65; 70)	25.66 (1.48; 70)

Table 2. Mean developmental times of *T. rugosus* at six constant temperatures for each developmental stage (days, °C). Standard deviation followed by the number of observed specimens shown in parentheses (SD; N).

Developmental stage	Temperature range of model (°C)	R2	df	p-value	ADD (°C)	LDT (°C)
Egg	12–22	0.942	663	>0.001	34.191 (0.60)	9.532 (0.91)
1st instar larva	12–22	0.89	611	>0.001	22.417 (0.60)	9.762 (0.13)
2nd instar larva	12–22	0.875	586	>0.001	24.902 (0.64)	9.320 (0.14)
3rd instar larva	12–22	0.871	579	>0.001	61.117 (1.25)	8.465 (0.13)
Post-feeding	12–22	0.91	566	>0.001	70.080 (1.79)	9.820 (0.12)
Pupae	12–22	0.924	555	>0.001	114.166 (2.03)	8.923 (0.10)
Complete development	12–22	0.944	555	>0.001	362.758 (4.97)	8.528 (0.08)

Table 3. Summary of the developmental constants for *T. rugosus* at six developmental stages. Standard errors shown in parentheses.

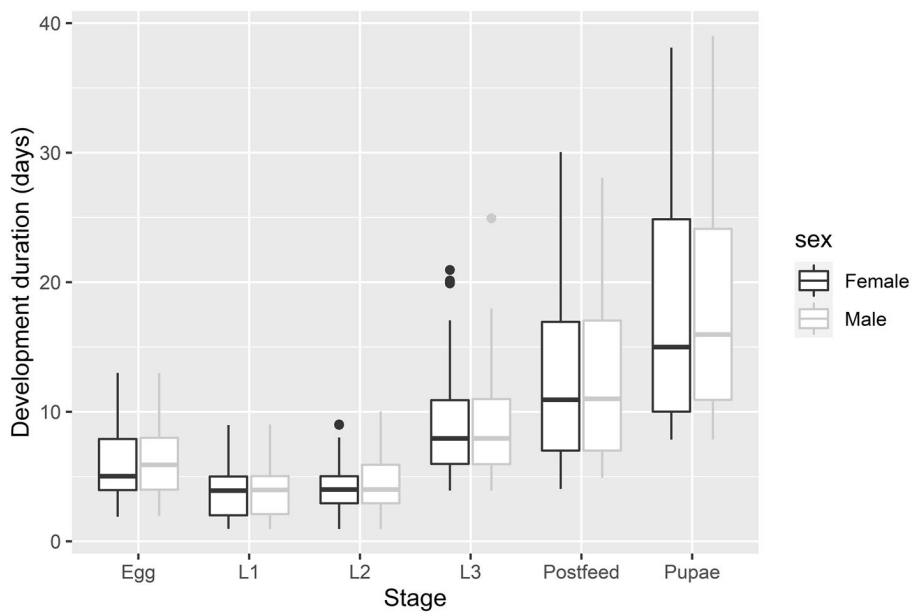


Figure 2. Developmental length differences between sexes. Horizontal lines within the boxes indicate median values; upper and lower boxes indicate the 75th and 25th percentiles, respectively. Whiskers indicate the values within the 1.5 interquartile ranges. Small dots are outliers. Females indicated in clear black boxes and Males in faded gray color boxes.

	<i>T. rugosus</i>	<i>T. sinuatus</i> ¹²	<i>T. micans</i> ³	<i>T. capensis</i> ³	<i>N. brunnicollis</i> ²⁶	<i>N. littoralis</i> ¹⁸
14	72.09	78.23	110.68	77.11	–	85.09
18	41.88	43.44	44.90	46.14	74.09	49.30
22	25.66	29.67	28.16	32.92	33.43	34.70

Table 4. Comparison among the estimated developmental times of *T. rugosus* at three constant temperatures for five forensically important Silphid species.

the species was most likely misidentified and the developmental data in fact referred to *T. ruficornis* instead of *T. rugosus*. This would also explain the difference in our developmental data and those reported by²¹. They recorded that the development at 23 °C took 32 days, whereas our data suggests that the development at a lower temperature (22 °C) took on average 25.66 days, which represents a significant difference. This shows that correct identification of the insect species investigated in criminal sciences is critical for the precise calculation of the PMI because for even a closely related species, the physiological requirements may vary greatly.

The information provided by our TSM models and their parameters allow us to compare the thermal requirements of *T. rugosus* with other studied species from the genus and put them into perspective with their underlying ecology. It seems that even species that occur together do not share a similar lower developmental threshold. The LDT of *T. rugosus* (LDTT.rugosus = 8.528) differs considerably from its closest European sister species, *T. sinuatus* (LDTT.sinuatus = 9.85)¹². However, total ADD are very similar for both species (ADDT.rugosus = 362.7 and ADDT.sinuatus = 360.4)¹². When compared to the Afrotropical species, the thermal constants vary significantly. For *T. capensis*, the LDT and ADD values (9.04; 384.1)³ were relatively close to the ones of both *T. rugosus* and *T. sinuatus*¹². Interestingly, *T. micans* represents a peculiar case, where its TSM values of LDT are the highest (LDTT.micans = 13.26) and ADD is the lowest (ADDT.micans = 197.97)³, when compared to the *Thanatophilus* species aforementioned (*T. rugosus*, *T. sinuatus* and *T. capensis*)^{3,12}.

Honěk²⁴ mentions that the species thermal constants show a negative relationship between LDT and ADD, therefore the species with the higher LDT requires a lower amount of ADD than the species with lower LDT. LDT and ADD values can be linked to environmental adaptations such as seasonality and temperature depending on the species distribution^{24,25}. In practice, it is expected that the lower LDT values can be found in a species occurring in temperate regions such as *T. rugosus*, *T. sinuatus* and *T. capensis*. On the other hand, higher values of LDT can be expected for the species distributed throughout the tropical areas such as *T. micans*. The different values of LDT and ADD between *T. rugosus* and *T. sinuatus* demonstrate their differences in seasonal activity, that regulate competition between these two closely related sympatric species²⁵.

The LDT and ADD values also differ among other silphid species outside the genus *Thanatophilus*. In *Necrodes littoralis* (Linnaeus 1758) the LDT value (LDTN.littoralis = 8.49) is closer to the one of *T. rugosus*, however the ADD is much higher (ADDN.littoralis = 468.89), probably due to the larger size of the species. On contrary, in *Necrophila brunnicollis* (Kraatz 1877) both parameters vary significantly (LDTN.brunnicollis = 14.71) (ADDN.brunnicollis = 243.74) when compared to *T. rugosus*. In this case we could attribute the discrepancy to the adaptation to different climatic conditions as *N. brunnicollis* is a tropical species while *T. rugosus* is a temperate species.

Regarding the developmental times, differences were found between *T. rugosus* and other *Thanatophilus* species (Table 4). *T. rugosus* seems to develop faster at all temperatures compared to *T. sinuatus* as well as Afrotropical species *T. micans* and *T. capensis*. In addition, *T. rugosus* needs less time to develop also compared to other silphid species, like *Necrophila brunnicollis* (Kraatz 1877)²⁶ and for *Necrodes littoralis* (Linnaeus 1758)¹⁸. In addition, our results confirm that the proportion of time spent in a particular developmental stage is dependent on the temperature. A faster development rate and the violation of the DRI could be suggested for species distributed in the temperate region as its developmental rate and growth can be adjusted based on the seasonal limitations and photoperiods²⁷. Our findings are thus in accordance with the results already published in other forensically important species of Silphidae^{12,26}.

Here we present the first thermal summation models for all developmental stages of *T. rugosus* reared at six constant temperatures (12, 14, 16, 18, 20 and 22 °C) under controlled laboratory conditions. The models allow *T. rugosus* to become another important tool for forensic entomology. The added benefit of including this species is its utility during the cold season when many other forensically important species are absent. Moreover, the data provided here can be used to validate PMI calculations based on other forensically relevant insects.

Methods

Laboratory colony establishment. Adult beetles of *T. rugosus* were collected using pitfall traps baited with pork muscle tissue (*Sus scrofa* Linnaeus, 1758) (Mammalia: Suidae) and beef muscle tissue (*Bos taurus* Linnaeus, 1758) (Mammalia: Bovidae). The sampling was conducted in the Czech Republic in 2020. Specimens were sampled at three localities: Stará Lysá (50° 13' 08.0" N, 14° 48' 25.5" E), Slapy (49° 47' 31.9" N, 14° 23' 52.7" E) and Prague—Lysolaje (50° 07' 33.0" N 14° 21' 44.0" E). Due to the seasonal and ecological preferences of the species, individuals were collected between April and May by placing the traps in open areas, in habitats such as harvested agricultural fields or grasslands^{15,28}.

The methodology used for larval breeding in this study follows Montoya-Molina et al.¹² and was originally based on Ridgeway et al.³, with modifications described in detail there. The larvae of the first and second instar from the same clutch of eggs were kept together under the condition that they were at the same developmental stage. If some larvae molted before the rest of the group, they were moved to separate Petri dish. Developmental

milestones (egg, 1st instar larva, 2nd instar larva, 3rd instar larva, Post-feeding stage) and pupae) were identified based on morphological features described by Novák et al.¹⁶. The whole breeding process was monitored daily.

Colonies were kept inside climatic chambers (custom made by CIRIS s.r.o.) at six constant temperatures (12, 14, 16, 18, 20 and 22 °C) and 16 h of light and 8 h of dark photoperiod regime, maintained by a fluorescent light (Osram L 8W/640) to simulate the light conditions during the species breeding season. The temperatures used were based on previous rearing of this beetle by Jakubec et al.¹¹.

Mortality rate analysis. Survival data were evaluated by a nonparametrical log rank test using the function `survdiff` from the R package `survival`²⁹. The effect of temperature on mortality was investigated at all experimental temperatures (12, 14, 16, 18, 20 and 22 °C). We measured the mortality of immature individuals starting with the egg and the data were right hand censored at the moment of adult beetle emergence. Bonferonni correction was used for post-hoc comparisons of the treatments.

Thermal summation model. To model the relationships between the developmental time and the temperature for all the developmental stages, a linear regression model defined by Ikemoto and Takai³⁰ was applied. Both estimated parameters of the TSM (LDT and ADD) are defined as a slope and intercept of the linear regression respectively. Therefore, both LDT and ADD can be easily calculated along with their respective standard errors³⁰.

Effect of sex on development length. Across all temperatures, only the individuals that have completed the development until the adult stage were selected for the study, as only these specimens could be sexed.

To assess the potential differences in developmental time between males and females, two linear mixed effect regression models were fitted (null and “sex” model). Response variables of both models were the developmental lengths of the six stages (Egg, L1, L2, L3, PF and Pupae) and their total. Null and the alternative model also shared two fixed effect explanatory variables: temperature (12, 14, 16, 18, 20 and 22 °C) and developmental stage. Because the measurements of the development times were done repeatedly on the same individual, the identity of that specimen had to be incorporated into the analysis, therefore we used it as a random effect in both models. The only difference between the alternative and the null models was fitting the latter with sex as a fixed effect explanatory variable. The fit of these two models was compared via AIC criteria to find out if the information about sex does improve it significantly.

Developmental rate isomorphy (DRI) and sex ratio. The presence of developmental rate isomorphy (DRI) was tested using Dirichlet regression as suggested by Boukal et al.²⁷. The isomorphy hypothesis suggests that the development rates do not depend on the temperature. For the analysis, a proposed null model (mod0) was tested without the explanatory variables. The mod0 implies that the proportions of time spent in individual instars are constant and independent of other factors (true presence of the DRI in the species). Two alternative models were fitted, one with temperature as the explanatory variable (mod1) and the other with expected quadratic effect of temperature (mod2). Both alternative models suggest that the DRI is not present in the species. Relative quality of each model was evaluated by Akaike information criteria (AIC), to allow comparison. The one with the lowest value was considered as the most appropriate description of the underlying relationship.

A Binomial test was conducted to determine whether the observed sex ratio for the species is significantly different from the expected one (1:1).

Data management and analysis. All data management and analyses were carried out using the R statistical program³¹. Additionally, we used a “lme4” package for fitting mixed effect models and visual outputs were processed via packages “ggplot2” and “sjPlot”^{32–34}.

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Author contributions

S.M.M. and P.J. designed the study, collected material in the field, bred the beetles in the laboratory, analyzed the data and wrote the manuscript. J.Q. and M.N. collected material in the field, bred the beetles and reviewed the manuscript. J.R. and H.Š. collected material in the field and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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4. Chapter 4: Influence of Diet on Development and Survival of *Thanatophilus rugosus* (Coleoptera: Silphidae)

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In this chapter (Chapter 4), we tested the effect of different diet types on the developmental times of *Thanatophilus rugosus*. We demonstrate that some diet types provide better results when breeding the carrion beetles at laboratory conditions. In this particular case, we demonstrated that pork liver and pork muscle are the best diets for breeding *T. rugosus* in laboratory conditions. Our findings showed that pork liver and pork muscle are optimal dietary resources based on developmental times results.

Authors contributions: **JQ**: Concept, field work, lab work, data analysis and writing. **PJ**: Concept, data analysis and writing. **SMM**: field work, lab work and writing. **MN**: lab work and writing. **HŠ**: writing.

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Development, Life History

Influence of Diet on Development and Survival of *Thanatophilus rugosus* (Coleoptera: Silphidae)

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Abstract

This study examined the effects of various diets on the development time and survival of the carrion beetle *Thanatophilus rugosus* (Fabricius, 1775). We attempted to find the best diet for rearing the species in laboratory conditions for entomological research and forensic purposes, and to further understand its feeding habits. The larval stages were monitored while feeding on three types of meat under a constant temperature. We discovered that the shortest development time in total was achieved in larvae fed with pork liver, followed closely by pork muscle. The longest development and lowest survival rates were observed when introducing a chicken diet. We were also able to identify specific stages during which the diet significantly affected the development.

Key words: Necrophagous, development time, mortality, nutritional, larval instar, forensic entomology

Many species of carrion beetles (Coleoptera: Silphidae) play a key role in medico-legal investigations, mainly in the postmortem interval (PMI) estimations during early or late decomposition stages, or possibly even in the detection of postmortem body manipulation (Byrd and Castner 2009, Charabidze et al. 2017). Therefore, studying life histories, colonization processes, and ecology of the different species in this group are important and can be valuable for forensic sciences.

Effective methods for PMI estimation using entomological evidence are based on measured development time (DT) of the larval stages of forensically important insects (Higley and Haskell 2009). In order to attain legitimate developmental data, it is necessary to establish laboratory rearing protocols for proper management of the insect evidence (Matuszewski 2021). Rearing larval stages on various types of meat and observing their DT and food preferences, have been mainly studied in Diptera (Smith 1986, Haskell et al. 1997). For many years now, the focus has shifted to carrion beetles as they have an extended developmental time compared to flies, thus enabling PMI estimation during all stages of body decomposition (Midgley et al. 2009).

Extrinsic factors such as temperature, photoperiod, moisture, or humidity may influence the development, growth, or survival of the

necrophagous larvae (Chown and Nicolson 2004). Other influencing factors can be food quality and dietary habits. Diet is essential for the general insect performance depending on which nutrients the larvae acquire from the food (Scriber and Slansky 1981). The organic composition of the natural food source (cadaver) as well as diets used for rearing may not always meet the nutritional requirements of the developing insect (House 1969, Nation 2015). This could impact the data obtained from species collected on different parts of the carcass and consequently alter the PMI results (Clark et al. 2006, Day and Wallman 2006a).

Genus *Thanatophilus* Leach, 1815 (Coleoptera: Silphidae) exhibits a wide distribution in the Holarctic and Afrotropical realms (Schawaller 1981). It contains 24 species (Newton 2020) that recurrently appear on many types of cadavers of every size including human corpses (e.g., Anderson 1995, Adair and Kondratieff 2006, Cherix et al. 2012, Lira et al. 2020). Some *Thanatophilus* species can colonize carrion early on; within 34 h such as *T. micans* (Fabricius, 1794), *T. sinuatus* (Fabricius, 1775) and *T. rugosus* (Linnaeus, 1758) (Midgley and Villet 2009, Matuszewski and Szafałowicz 2013). Therefore, species of this genus could be considered forensically relevant and noteworthy.

We focused on *T. rugosus*, which is one of the potentially important indicator species for forensic entomology in the Palearctic

region (Růžička 2015). The species was detected on 8.16% of the forensic cases in the Czech Republic, when sampling the entomological evidence ($N = 28$ out of 343 cases between 2003 and 2020) (Šuláková, unpublished data). It inhabits open landscape as well as alder forests, and was found active not only during spring and autumn (Matuszewski et al. 2010, Jakubec and Růžička 2015, Frączak-Łagiewska and Matuszewski 2018), but even during the winter months in Italy and Spain (Brandmayr et al. 2010, Bonacci et al. 2011, Díaz-Aranda et al. 2018). The pre-appearance interval (PAI) of *T. rugosus* may be estimated from preceding temperatures as demonstrated by Matuszewski and Szafałowicz (2013). The development at two temperatures of *T. rugosus* was also published by Guerroudj & Berchi (2016) from Algeria, although this is probably a misidentification as *T. rugosus* is not present in Africa (Newton 2020). Also, the complete description of all larval instars was recently established by Novák et al. (2018), hence making the species of serious forensic interest in Europe.

Larval feeding habits of *Thanatophilus* have not been investigated nor linked to the developmental time or to survival. To address this knowledge gap we reared the larvae of *T. rugosus* under constant laboratory conditions on three diets from two animal species (domestic pig and chicken). Based on the daily observation of the developmental stages from egg to the adult, and quantifying survival rates (%), we aimed to determine the possible effects of diet on the larval development time and possibly their size. In addition, we intended to find a suitable diet for rearing in laboratory conditions, not merely regarding economical objectives, but also and foremost, to achieve the best survival results and fastest development. Our findings can be valuable and applicable in experimental research and casework involving *T. rugosus* and possibly other species of the genus.

Materials and Methods

T. rugosus Rearing and Experiment

Adult beetles were collected from carrion placed in two localities in the Czech Republic: sika deer carcass in Stará Lysá (50°13'08.0"N 14°48'25.5"E) and domestic pig carcass in Slapy (49°47'31.9"N 14°23'52.7"E) during April 2020. The beetles were then identified and transferred to the laboratory at the Czech University of Life Sciences Prague, where they were sexed (using identification key (Jakubec et al. 2018)) and set inside breeding boxes (Hagen Exo Terra Faunarium mini 3 L; dimensions 18 × 11.6 × 14.5 cm) containing at least 10 cm of gardening soil. The substrate was sprinkled with water when required, and a supplementary water-tube sealed with cotton was inserted as a water source and to increase humidity. Adults from each locality were placed in separate breeding boxes. The boxes were kept in climatic chambers (custom made by CIRIS s.r.o.) under constant temperature 18°C ± 1°C and photoperiod 16 h of day light and 8 h of darkness. The beetles were fed ad libitum with beef muscle (*Bos taurus* Linnaeus, 1758) which is different from the diets tested. A tin foil, shaped as a triangular prism, was placed over the meat to prevent desiccation.

Breeding boxes were examined daily for egg batches, each contained approximately 15 eggs. Every batch was divided into three parts across each treatment in order to remove hereditary effects, and then placed into separate Petri dishes (10 cm in diameter). The Petri dishes were filled to three-quarters with moist gardening soil and the eggs were placed in a small depression made in the center of the surface. Each of the three Petri dishes was then supplied with the specific diet (about 5 g). The diets were selected based on their source (species) and nutritional values. From the domestic pig (*Sus scrofa* Linnaeus, 1758), we used the liver (internal organ) that is generally

high in nutrients, and lean leg muscle (referred to as pork muscle), as an intermediate meat that contains lower concentrations of some nutrients. From chicken (*Gallus gallus domesticus* (Linnaeus, 1758)) we used the breast muscle (referred to as chicken) which has lower values of many essential growth nutrients compared to the previous two meats. This selection permitted the assessment of the diets based on their source, and the comparison between organ and muscle from the pig source. We considered the availability and the economical values of the diets for rearing purposes, in addition to the closest substitute to human tissue (the pork muscle) (Byrd and Tomberlin 2009, Matuszewski et al. 2020).

All dishes were then properly labeled and rubber banded to prevent newly emerged larvae from escaping, and kept under the same conditions as the main breeding boxes. Eggs were examined in case of fungal growth and the affected ones were consequently removed. Moisture in the dishes was consistently maintained by gently sprinkling the surface using a wash bottle. The developmental progress was monitored daily, and all changes were recorded.

Developmental stages were separated as follows: egg stage, first-instar larva (L1), second-instar larva (L2), third-instar larva (L3), post-feeding stage, and pupal stage. Larvae in all stages were provided with supplemental food ad libitum to minimize cannibalism. Fresh food was added if considerably consumed, dried, or molded. Newly hatched L1 larvae were counted and kept together to increase their survival rates as we observed increased mortality of individually raised specimens during our previous attempts to breed *T. rugosus* and its close relative *T. sinuatus* (see Novák et al. 2018 and Montoya-Molina et al. 2021). The particular reason is still unclear but might be connected to group feeding efficiency as suggested by (Scanvion et al. 2018). The L2 larvae were moved into a separate Petri dish and also kept in groups. Later all emerging L3 larvae were individually separated into smaller Petri dishes (6 cm in diameter) provided with food, properly labeled and placed in an upright position (following the methodology of Jakubec et al. 2021, Montoya-Molina et al. 2021). This separation makes monitoring of the remaining developmental stages more efficient.

Subsequently, we photographed the L3 larvae and measured their pronotal width, which provided size information. L3 larvae were chosen for two reasons: first, their pronotum allows stable measurements unlike their body length; and second, to avoid the risk of losing the size data in case the larva did not remove hereditary effects, and then since L3 is the last feeding stage. In order to prevent the larva from moving while taking the photograph and without harming it, we carefully placed it on a stack of round cotton pads that filled the bottom part of a small Petri dish (6 cm in diameter) and covered it gently with the upper part of the dish. Photographs were taken using Canon macro photo lens MP-E 65 mm on a Canon 550D body, the camera was controlled using the 'Automated Macro-rail Ford Focus Stacking StackShot' (COGNISYS.INC). After reaching the post-feeding stage and onward, the Petri dishes were opened only to moisten the substrate or to remove molded food. Newly emerged adults were sexed and moved to new breeding boxes.

Statistical Analysis

The recorded pronotal width measurements of the L3 were used to test the size disparity between males and females reared on different diets. We used the linear mixed effect model (LMM) (R package lmerTest (Kuznetsova et al. 2017)) with the response variable being pronotal width, and the explanatory fixed effect variables being sex, the diet and their interaction. The random effects of the individual's origin (identity of egg batch) and the breeding condition (identity of its Petri dish) were introduced to the model to limit the influence of the random effects of

the heritability and breeding condition. These two variables were nested as each batch of eggs was distributed across three Petri dishes, and each presented with one of the three diets tested.

We also investigated the relationship between the total DT and our two main explanatory variables, the sex and the diet. However, we also introduced the pronotal width to control the effect of size on the DT. This relationship was explored using the LMM with the same two random explanatory variables as in the previous analysis. The fit of the model was evaluated based on the visual evaluation of the diagnostic plots (R package sjPlot [Lüdecke 2020]). The post-hoc analysis based on the Holm correction (R package multcomp [Hothorn et al. 2008]) was used.

The relationship between DT and the diet can be further explored in terms of determining which developmental stages are significantly affected. We again applied the LMM approach as described above. Generally, developmental stages of holometabolous insects differ in their duration, and we expected that each stage will be affected differently by diet. This is especially important as the larvae do not consume any food at some stages (post-feeding and pupal stages). To test the relationship, we created two models, one with interaction between diet and the developmental stage (int.mod) and one simplified additive model (add.mod) without interaction. The data were confounded by three random nested effects; identity of the individual's original egg batch (parents), correspondence of an individual to a particular Petri dish (where the development of the first two instars took place) and identity of the individual itself. An additional random explanatory nested factor of the individual's identity was introduced in order to consider the repeated measurements of DT at multiple developmental stages, as these measurements are not independent. These random factors were used in both models (with the interaction and without it). The fit of the two models was evaluated using the Bayesian information criterion metric (BIC). The post-hoc analysis with multiple comparisons between variables of interest was calculated using the Tukey method from R package lsmeans (Lenth 2016).

Survival data were evaluated by the nonparametrical log rank test using the function survdiff from the R package 'survival' (Therneau 2021). The effect of diet on mortality was investigated at three levels (pork liver, pork muscle, and chicken). We measured the mortality of immature individuals starting at the point of eclosion from egg with right hand censoring, at the moment of the adult beetle's emergence. The Bonferroni correction was used for post-hoc comparisons of the diets on survival.

Results

Overall, we were able to successfully record the development of 270 L1 larvae of *T. rugosus*, of which 94 individuals were raised on pork liver, 88 on pork muscle, and 88 on chicken. The difference in the total numbers per treatment was caused by the random mortality of eggs designated to each group. In total, 200 individuals were able to complete their development to adulthood, of which 81 were raised on pork liver, 68 on pork muscle, and 51 on chicken. We were able to determine the sex of only 119 individuals, 68 females and 51 males.

Size Differences

The size difference between the sexes was significant; males had smaller pronotal width (males = 3.36 ± 0.027 mm vs females = 3.46 ± 0.039 mm; T test value = -3.457 , $df = 96.951$, $P < 0.001$). However, size was unaffected by the proposed diet (pork liver vs pork muscle: T test value = 1.306 , $df = 22.014$, $P = 0.205$), (pork

muscle vs chicken: T test value = -0.313 , $df = 20.716$, $P = 0.757$). Further, the post-hoc test did not show any significant difference in pronotal size between the pork liver and chicken treatments (T ratio = -1.599 , $df = 22.5$, $P = 0.267$).

Development Time

Despite the difference between the size of males and females expressed as the pronotal width, we did not find any evidence of discrepancies between the sexes in the total DT (T test value = 0.004 , $df = 90.428$, $P = 0.997$). Also, size as a whole, did not significantly affect the total time of development (T test value = -0.491 , $df = 104.71$, $P = 0.625$). The only significant difference was found among the three diets. The total development took on average $36.41 (\pm 5.21)$ days for the pork liver treatment. In comparison, it was prolonged in the pork muscle and chicken treatments; $38.54 (\pm 6.00)$ and $40.13 (\pm 6.00)$ days, respectively (pork liver vs pork muscle: T test value = 2.699 , $df = 16.70$, $P = 0.015$; pork liver vs chicken: T test value = 4.686 , $df = 16.69$, $P < 0.001$). Subsequent post-hoc comparison indicated that the difference between pork muscle and chicken (estimated at 1.59 ± 0.81 d) was statistically significant as well (Z test value = 1.957 , $P = 0.05$).

Statistical analysis of the two competing models (int.mod and add.mod) of DT and how it is affected by the stage and diet, revealed that the int.mod described the data significantly better (BIC = 3815.51) than its additive counterpart (BIC = 3830.55). The int.mod identified several stages where the diet might cause a significant difference in the developmental time (see [Supp Material \[online only\]](#) for complete summary of results). The L3 larvae fed with pork muscle developed faster than those fed with chicken (T test value = 2.436 , $df = 1076.818$, $P = 0.015$). Most notably, we found that during the post-feeding stage DT differed between pork liver and pork muscle (T test value = -3.415 , $df = 1074.366$, $P < 0.001$), with the development faster on pork liver. Moreover, the variation was notable between the pork muscle and chicken (T test value = 4.926 , $df = 1087.831$, $P < 0.001$) where the chicken seemed to hinder the development.

The only other combination of developmental stage and diet that came quite close to be identified as significant was found in the L2, where the chicken showed signs of hindering the development compared to the pork muscle (T test value = 1.855 , $df = 1072.835$, $P = 0.0639$). The post-hoc testing further revealed significant differences in the DT of L2, L3 and the post-feeding stage, between the pork liver and chicken (the L2: T ratio = 4.211 , $df = 351$, $P = 0.003$, the L3: T ratio = 4.809 , $df = 358$, $P = 0.0002$, the post-feeding stage: T ratio = 11.237 , $df = 442$, $P < 0.0001$). In all cases, chicken diet delayed the development (the pattern can be seen clearly in [Fig. 1](#)). We did not observe any significant influence of the diet on the L1 and pupal stages.

Survival

The highest survival rate to adulthood, when comparing all three diets together, was observed among individuals fed with pork liver ($50.3 \pm 3.94\%$). A lower survival rate was observed in pork muscle ($41.2 \pm 3.83\%$) and the lowest in the chicken ($30.4 \pm 3.56\%$; [Table 1](#)). The difference in the survival of the specimens throughout their development was statistically significant when comparing pork liver versus chicken (log rank test with Bonferroni correction, $P = 0.0072$), but not for pork muscle versus chicken (log rank test with Bonferroni correction, $P = 0.373$), nor for pork liver versus pork muscle (log rank test with Bonferroni correction, $P = 0.469$).

Discussion

We evaluated the effect of three diets (pork liver, pork muscle, and chicken) from two sources (domestic pig and chicken) on the DT and the survival of the larval stages of *T. rugosus*. We confirmed that diet had a direct influence on the larval development in general and can be further traced in specific stages (L2, L3, and post-feeding stages, see Results). This is the first study that was able to verify this effect empirically in the larval stages of *Thanatophilus* sp., and the second, to our knowledge, in necrophagous beetles in general; the previous being the work of Watahiki and Sasakawa (2019). Although, their work was not directly relevant to forensic entomology as it mainly focused on the ability of the species to shift between predatory and necrophagous diets.

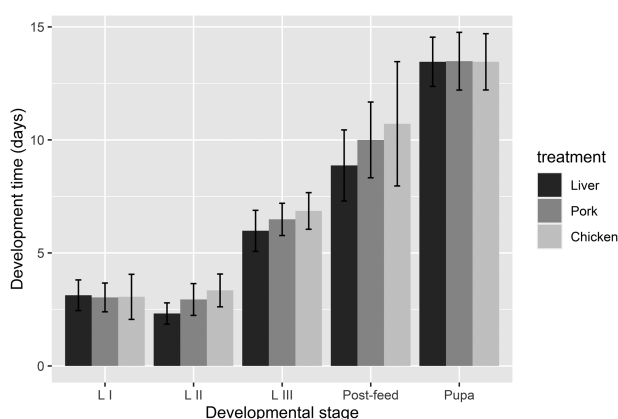


Fig. 1. Comparison of the development time between different diets at five developmental stages (L1 – L3, post-feeding and pupal stages). The height of the bars represents the mean development time and the error bars are the standard deviation of the sample.

Most diet studies of necrophagous species were performed on Diptera. These occasionally demonstrated discrepancies in growth and/or DT in association with diet suitability and quality or even structure. Even though the comparison of necrophagous fly and beetle larvae regarding their developmental needs is probably not quite accurate, these studies could still shed light on the effects of diet sources and their quality on the necrophagous beetle larvae.

Recently, Bernhardt et al. (2017) proposed the beneficial influence of fat content and attributed it to the highest growth rate in the larvae of the necrophagous fly *Calliphora vicina* Robineau-Desvoidy, 1830 (Diptera: Calliphoridae). However, they did not find any correlation between fat content and the developmental time. Our results conversely indicated that size (a representative of growth) was not affected by any of the diets. Nevertheless, the influence on the development time was apparent. It was faster on pork liver which contains higher amounts of total fat (3.65 g), according to the USDA (2019), compared to chicken with lower fat content (2.62 g). The development was still the fastest on the pork liver, although its fat content is lower than that of pork muscle (5.41 g). This suggests that the total fat content may affect development but probably only up to a certain level. Possibly other specific components of the total fat may play a role such as cholesterol.

Cholesterol is indispensable for the synthesis of the molting hormone in larvae (Hobson 1935, Nation 2015). Its content in pork muscle is lower (68 mg) than in chicken (73 mg). Yet, both the DT and the survival rate were higher in the former. In that regard, pork liver has much higher amounts of cholesterol (301 mg) and could be responsible for the faster development and lower mortality among the three diets. Furthermore, proteins are compulsory for growth, and larvae can transform them into fat bodies, which are later utilized as an energy source during metamorphosis (Chapman 2012). However, proteins did not accelerate the development with chicken diet, even though their value is somewhat higher (22.5 g), compared to pork liver (21.39 g) and pork muscle (20.48 g). These discrepancies in the development are almost certainly instigated by other

Table 1. Summary of the survival model for *T. rugosus* across six developmental stages

Pork liver					
Stage	N. event	Survival	Std. err.	Lower 95% CI	Upper 95% CI
L1	22	0.863	0.0271	0.812	0.918
L2	45	0.584	0.0388	0.512	0.665
L3	2	0.571	0.0390	0.500	0.653
Post-feeding	3	0.553	0.0392	0.481	0.635
Pupa	4	0.528	0.0393	0.456	0.611
Adult	4	0.503	0.0394	0.432	0.587
Pork muscle					
Stage	N. event	Survival	Std. err.	Lower 95% CI	Upper 95% CI
L1	20	0.879	0.0254	0.830	0.930
L2	57	0.533	0.0388	0.462	0.615
L3	9	0.479	0.0389	0.408	0.561
Post-feeding	2	0.467	0.0388	0.396	0.549
Pupa	5	0.436	0.0386	0.367	0.519
Adult	4	0.412	0.0383	0.343	0.495
Chicken					
Stage	N. event	Survival	Std. err.	Lower 95% CI	Upper 95% CI
L1	19	0.887	0.0245	0.840	0.936
L2	60	0.530	0.0387	0.459	0.611
L3	11	0.464	0.0387	0.394	0.547
Post-feeding	2	0.452	0.0387	0.383	0.535
Pupa	15	0.363	0.0369	0.298	0.443
Adult	10	0.304	0.0356	0.241	0.382

N. event = number of censoring events, Survival = proportion of surviving individuals at the beginning of the stage.

factors that are not very clear but are most likely associated with the lack of some other nutrients or an imbalance among them. The importance of optimum levels and proper balance between the main micro- and macro-nutrients is necessary for the most favorable life histories (House 1974, Nation 2015).

Based on our results, we may argue, that poor diet quality can be linked with the lack of specific nutrients that limit the development and increase mortality, as demonstrated in the chicken treatment. We also observed higher frequency of morphological abnormalities and lighter coloration of their integument among individuals raised on chicken. It may be assumed that the more nourishing pork liver with its much higher content of some essential nutrients (e.g., cholesterol, minerals, and vitamins) could better enable survival and shorten development time in *T. rugosus*. Pork muscle is also rich in various nutrients and indicated good results for rearing. Hence, both pork liver and muscle diets can be considered better quality food sources from the perspective of life histories.

We argue that the differences in DT in the post-feeding stage, where the larvae no longer feed, could be caused by the time demanded for processing the food accumulated in the gut. Thus, a highly nutritious or a well-balanced diet (the pork liver) could take less time to process. The pupal stage, on the other hand, showed no significant difference in DT among the treatments, as there is no further feeding or growth but rather a highly energetically demanding transformation. The larva must have accumulated all the necessary nutritional elements of an adequate composition in the previous stages to have the ability of undergoing a successful metamorphosis. This is closely linked with attaining the critical weight, which is the point where the larva stops producing juvenile hormones (Nijhout and Williams 1974). If the composition of the diet is not suitable, then compensatory feeding occurs which further prolongs the development (Lavoie and Oberhauser 2004).

Many studies have revealed that the influencing factors could be tissue-specific rather than structural. For example, rearing blow fly larvae (Diptera: Calliphoridae) on liver from several sources (cow, domestic pig, or sheep) was unfavorable for their development and/or growth (e.g., *Lucilia sericata* (Meigen, 1826) (Clark et al. 2006, El-Moaty and Kheirallah 2013), *Calliphora augur* Fabricius, 1775, and *Lucilia cuprina* (Wiedemann, 1830) (Day and Wallman 2006a, b), and three *Chrysomya* species (Thyssen et al. 2014)). Only one study by Ireland and Turner (2006) on *Calliphora vomitoria* (Linnaeus, 1758) had similar results to ours, where pork liver facilitated the shortest developmental time followed closely by pork muscle. These results suggest species-specific nutritional requirements that are not shared among the necrophagous taxa.

Kaneshraja and Turner (2004), Clark et al. (2006), and Day and Wallman (2006a) warned, based on the results from their research on dipteran species, that the forensic entomologist should be alert while gathering forensic material (larvae or pupae) from various positions on the dead body. This is attributed to the fact that DT and the growth rate of the dipteran stages can be greatly influenced depending on the specific tissues they were found on, and thus, they recommended adjusting the PMI calculations accordingly. However, Bernhardt et al. (2018) contend, that these recommendations may not apply in actual forensic cases, since the larvae sampled on specific organs may have been displaced from their original feeding sites due to the transportation of the dead body or the high mobility of the larvae. Also, they make a point that internal organs (except for the brain) were seldom infested in their study involving domestic pigs. Although we agree with all of the arguments provided above, the behavior of carrion beetle larvae, seem to contradict the internal organ hypothesis of Bernhardt et al. (2018), whether in field experiments or in actual forensic cases (Šuláková, unpublished

observations). The larvae were frequently found inside the animal carrion or human corpses and on the internal organs in all phases of decomposition. Accordingly, it does seem sensible to obtain data from DT studies conducted on both muscle and organ tissues.

In order to provide a rather conservative PMI calculation, we should strive to eliminate its overestimation (calculating time of death to the point when the individual was still alive). Underestimation (calculating time of death to the point when the individual was already deceased) is usually not as problematic according to the judicial system. Even if such estimate is too conservative, it would not invalidate the expert testimony of the forensic entomologist. We recommend using developmental reference data from a diet that enables the shortest DT throughout rearing. Though this may not improve the accuracy, it will reduce the probability of overestimation of the PMI.

In conclusion, we demonstrated that some of the best diets for rearing the larvae of *T. rugosus* were pork liver and pork muscle. These inexpensive food sources represent optimal diets to achieve the shortest developmental time and the highest survival rates. Our findings can be useful in both theoretical entomological research and in practical forensic investigations. In future studies, we intend to perform similar experiments and include varying factors that might facilitate feeding such as temperature, size of larval aggregation, and the presence of adults. These factors may elucidate the feeding habits in relation to the idea development, but were beyond the scope of this article.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

Acknowledgments

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5. Chapter 5: The Impact of Diet and Photoperiodism on the Life History of *Thanatophilus sinuatus* (Coleoptera: Silphidae)

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In Chapter 5 we demonstrate that the temperature is not the only extrinsic factors influencing the developmental times on the forensically important species *Thanatophilus sinuatus*. Our results showed that the species developmental times are not only subject of the temperature in which they are bred, but also, diet and the locality/origin from where the parental population is collected affecting significantly results of developmental times and survival rate. On the other hand, larvae of *T. sinuatus* was insensitive to two tested photoperiods.

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Development, Life History

The Impact of Diet and Photoperiodism on the Life History of *Thanatophilus sinuatus* (Coleoptera: Silphidae)

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Abstract

The forensically important genus *Thanatophilus* Leach, 1815 (Coleoptera: Silphidae) is a widespread group of carrion beetles that occupies the Holarctic and Afrotropical realms. It has recently received more recognition, as its species are frequently detected on large carrion, including humans, and can be useful in estimating the time of colonization (TOC) and the post-mortem interval (PMI). The immature stages of *Thanatophilus sinuatus* (Fabricius, 1775) were reared on three different meat diets (pork liver, pork muscle, and chicken muscle). Diet influence on some of the essential life history traits of the species was examined for different populations. Our findings indicated an accelerated development on pork muscle followed closely by pork liver and a prolonged development on chicken muscle. The diet significantly affected the size, and the body condition (weight/size) as both pork muscle and liver produced individuals in better physical condition (larger and heavier). The impact of two cyclic photoperiod regimes (16 h Light (L): 8 h Dark (D) and 12 h L: 12 h D) was further evaluated and indicated no significant variability.

Key words: necrophagous, development time, survival, nutritional ecology, photoperiod

Forensic entomology is a continuously expanding field that substantially advances medico-legal investigations when associated with insects as a source of evidence in criminal cases (Amendt et al. 2011, Lutz et al. 2021). Recently more studies are dedicated to forensically relevant carrion beetles (Coleoptera: Silphidae), which are considered reliable indicators that aid in more accurate estimation of the post-mortem interval (PMI) (Midgley et al. 2009, Byrd and Tomberlin 2019a). This may be attributed to the fact that beetles have a longer life cycle that can extend the estimation period, primarily when the commonly used forensic insects (mainly Diptera: Calliphoridae and Sarcophagidae) have completed their life cycle and left the cadaver (Haskell et al. 1997).

Laboratory rearing of carrion insects collected from death scenes is useful for properly identifying the species occupying the dead body and specifying the time of colonization (TOC) more accurately. Rearing also delivers developmental information under regulated conditions that may further improve future TOC and PMI estimations (Byrd and Tomberlin 2019b). Therefore, it is important to create laboratory rearing protocols and methods to gain a clearer understanding of the main biotic and abiotic factors that

may affect the development, and further enhance the quality of the developmental data obtained (Ridgeway et al. 2014, Gruszka and Matuszewski 2021, Jakubec et al. 2021a, b; Matuszewski 2021).

One vital influencing factor is diet, which can impact many aspects of the individual's life history and performance traits (e.g., time of development, survival, and morphological features) (Rabêlo et al. 2011, Li et al. 2014, Watahiki and Sasakawa 2019). It was previously established that diet affects the development inconsistently, even in closely related species (e.g., Day and Wallman 2006, Thyssen et al. 2014). These effects usually vary immensely depending on the quality of the tissue type (organ/muscle) and animal or human source consumed, as each diet does not provide equal nutritional benefits (Slansky and Scriber 1985, Bernhardt et al. 2017, Hooper and Bonduriansky 2019, Frączak-Łagiewska et al. 2020; Qubaiová et al. 2021, 2022). Selecting an optimal high-quality diet for the developing necrophagous beetles not only produces a successful breeding with low mortality, but it also allows reaching the shortest development time. Such acquired developmental data in turn avert the PMI overestimation which is necessary to attain during medico-legal investigation or forensic research in general.

Another abiotic factor with a profound influence is the photoperiod, where varying periods of daylight enable insects to adapt for seasonal changes (Bradshaw and Holzapfel 2007). The photoperiod length can trigger changes in diapause, development time, growth, and fecundity, as well as the phenotypic traits of the insect (Beck 1980, Tachibana and Numata 2004, Hodek 2012). Research on the effects of photoperiodism on the development time was primarily dedicated to forensically important Diptera, which indicated different results depending on the species. For instance, full light photoperiod regime (24 h L: 0 h D) may either extend the larval or pupal development time of different calliphorid fly species (Nabity et al. 2007, Bala and Singh 2011), or in contrast shorten it (da Silva Mello et al. 2012), when compared to cyclic light (12 h L: 12 h D) or full darkness (0 h L: 24 h D). However, Fisher et al. (2015) suggested that full light or darkness experiments are extremes, as their results showed that larval developmental rates of three species of blow flies were faster under cyclic light (whether 12 h L: 12 h D or 16 h L: 8 h D). Photoperiod can also affect other characteristics that may be vital for forensic research, such as the burrowing depth, which appears to deepen with the increased light (highest at 24 h L: 0 h D) (Gomes et al. 2006, Bala and Singh 2011).

Unfortunately, only minor investigations were performed on necrophagous beetles. For example, it was discovered that a longer day regime (increased light) delayed sexual maturity in the females of *Nicrophorus nepalensis* Hope, 1831 (Hwang and Shiao 2011), or the reproduction and ovarian development in *Nicrophorus quadripunctatus* Kraatz, 1877 (Nisimura et al. 2002). Regarding other coleopteran taxa, the photoperiod also seemed to impact their development quite variably (e.g., Berkvens et al. 2007, Lopatina et al. 2011, Kutcherov et al. 2018). Overall, such differences among the taxa illustrate the importance of correctly selecting the photoperiod regime for rearing each species. Hence, it is only appropriate to perform more experimental research regarding the effects of both the diet and photoperiodism, which can reveal the best development, survival, and reproduction conditions, as both factors may influence the developmental documentation used for constructing the TOC/PMI estimations in forensic analyses.

We conducted our experiments on the species *Thanatophilus sinuatus*, which is distributed throughout the trans-Palaeartic and mainly occupies open biotopes, though forest habitats are not an exception (Matuszewski et al. 2010, Dekeirsschieter et al. 2011, Růžička 2015, Frączak-Łagiewska and Matuszewski 2018). It has an extended seasonality, from spring to autumn, with two activity peaks (Kočárek and Benko 1997, Esh and Oxbrough 2021). In the Czech Republic *T. sinuatus* was detected on human remains of 31 out of 443 forensic cases, between 2003 and 2022 (Šuláková, unpublished data). The abundance of this species, wide dispersal, its common occurrence on large carrion, and the rapid colonization period (1.4 days) make it an essential asset for the forensic sciences (Matuszewski and Szafałowicz 2013; Frączak-Łagiewska and Matuszewski 2018).

Few researchers studied the immature stages of *T. sinuatus*; Jakubec et al. (2019) established the complete description of all larval instars, their morphological characters, identification key, and development time. The ecology of the species, colonization behavior, carrion preference, activity, and seasonality were presented by Frączak-Łagiewska and Matuszewski (2018), and Montoya-Molina et al. (2021) designed the thermal summation models at six different temperatures. However, the nutritional habits of the immature stages were not explored thus far.

In this article we aim to further expand the ecological knowledge of the feeding behavior of *T. sinuatus*, as well as reveal the potential

impacts of different diets and photoperiod regimes on its development time, survival, and size. Also, determine the possible disparity between the dietary habits of different populations of the species. An additional objective was, to establish the appropriate nutritional requirements and photoperiod regimes for rearing *T. sinuatus* in a laboratory setting.

Materials and Methods

T. sinuatus Collecting and Breeding

During the summer of 2021, adult *T. sinuatus* beetles were captured using baited traps with ripened cheese and fresh shrimps, set in two Czech Republic localities: Včelná pod Boubínem (49° 1' 23" N, 13° 51' 29" E), altitude: 850 m, and Ploužnice – Lomnice nad Popelkou (50° 30' 39.571" N, 15° 23' 4.138" E), altitude: 540 m. For the photoperiod experiment, a second collection took place during the spring of 2022 using baited traps with beef meat in Prague-Lysolaje (50° 07' 33.0" N, 14° 21' 44.0" E), altitude: 260 m – Czech Republic. Adult beetles were transported to the Czech University of Life Sciences Prague laboratory, where they were identified and sexed using the identification key of Jakubec et al. (2018). Adults from the localities were placed in separate breeding boxes (Hagen Exo Terra Faunarium mini 3 L; dimensions 18 × 11.6 × 14.5 cm) containing at least 10 cm of gardening soil. Fifteen boxes with approximately 20 collected adults in each (almost the same number of both sexes) were prepared for both following experiments. Humidity in the boxes was maintained by sprinkling water on the soil (once every three days) and adding a small centrifuge-tube (10 ml) filled with water and sealed with cotton. The boxes were kept in climatic chambers (custom-made by CIRIS s.r.o.) under a constant temperature of 20°C and photoperiod of 16 h L: 8 h D. This temperature was selected, as it resembles favorable reproductive and survival conditions. The beetles were fed ad libitum with pork muscle (*Sus scrofa* Linnaeus, 1758), that was covered with tin foil to delay drying. Daily observation of the breeding boxes was necessary for collecting egg batches.

Diet Experiment

The food source (animal species), meat availability, and cost were regarded when selecting the diets for our experiment. Each of the chosen diets, whether the different animal source and/or tissue type, has its specific nutritional composition; thus, we expected possible disparities during the larval development. From the domestic pig (*Sus scrofa* Linnaeus, 1758), we chose the pork back-leg muscle (here referred to as pork muscle) and pork liver (internal organ). From the chicken [*Gallus gallus domesticus* (Linnaeus, 1758)], we chose the breast muscle (here referred to as chicken muscle).

This experiment was replicated over 92 collected egg batches raised in two climatic chambers. Individual batches (consisting of approximately 11–16 eggs) were divided among the three diet treatment and placed in separate Petri dishes (6 cm in diameter). Eggs were positioned on the surface of the moistened gardening soil, which filled three-quarters of the Petri dish. A specific diet (about 5 grams) was added to each dish, which was labeled, rubber-banded, and kept under the same conditions as the primary breeding boxes. The dishes were sprinkled with water whenever the soil was dry.

Developmental milestones were monitored daily and divided as follows: first instar larva (L1), second instar larva (L2), third instar larva (L3), post-feeding stage, and pupal stage. The L1 larvae were kept in one group until L2 larvae appeared. The L2 larvae were moved into a separate Petri dish and kept together as well. All emerging L3 larvae were individually separated into new Petri dishes

(6 cm in diameter), provided with a specific diet, and placed upright to facilitate monitoring of further development. All larval stages were fed ad libitum to minimize competition and cannibalism, and fresh pieces of food were added if markedly eaten, dry, or infested by mold.

Size and Weight Information

The newly emerged adults were weighted using a precision balance (Adam Equipment/Highland HCB 123). To acquire the size data from the pronotal width, we photographed adult beetles using a Canon macro photo lens MP-E 65 mm on a Canon 750D body. We followed our proposed methodology (Qubaiová et al. 2021, 2022), where the individual was momentarily immobilized by softly enclosing it inside a small Petri dish (6 cm in diameter) that contained a stack of round cotton pads on the bottom side. The pronotal width was measured from the produced photographs using the 'EidosMicro' software.

Photoperiod Experiment

Two cyclic photoperiod regimes were tested, long-day (16 h L: 8 h D) and short-day (12 h L: 12 h D), provided by fluorescent light (Osram L 8 W/640) under a constant temperature of 20°C. For this experiment we collected 82 egg batches, where each was separated into two parts (one for each photoperiod regime). The experiment was repeated in two separate runs. Each run contained four climatic chambers (replications), and every two chambers were designated for a specific photoperiod regime. This regime was interchanged among the chambers in the second run. All climatic chambers were equipped with dataloggers to record the temperature. The developing larvae were fed ad libitum with pork muscle, which showed the best results from the *Diet experiment* part of this study. The same rearing protocols for observing the developmental stages and the handling of food, and Petri dishes as in the *Diet experiment* were applied here as well.

Statistical Analysis

We examined how the total development time of the beetles was affected by their sex, diet used, and locality of parental origin (here referred to as locality). We created four generalized mixed linear models (GLMM) with three fixed effect explanatory variables that differed in complexity. The least complex model had only additive effects among the variables, and the most complex one allowed varying slopes of each of them (see Table 1 for general form). The L1 or L2 stages were each raised in their own group and Petri dish, which might have a confounding effect. Hence, we used this variable across all models as a random effect. The differences in the fit of the models were analyzed using 'Akaike' information criterion with a correction for small sample size (AICc) (package 'gamlr' (Taddy 2017)). The difference among the diets tested was established based on a post-hoc test, using Tukey's contrast

Table 1. An overview of delta AICc (the difference between the best model and all others) response values for models of the size and total development time of reared *T. sinuatus* beetles

Model	df	Size AICc	Development AICc
~diet + sex + locality	7	0	0
~diet + sex * locality	8	2.569728	1.935444
~diet * sex + locality	9	12.40787	3.84966
~diet * sex * locality	14	23.44684	7.344117

with adjusted p values by Benjamini and Hochberg method (BH method) (Benjamini and Hochberg 1995) using 'multcomp' package (Hothorn et al. 2008).

Additional analysis was conducted for the development time of geographically different populations (from three localities). It was possible to merge data from the *Photoperiod experiment* (fed with pork muscle) and the *Diet experiment* (only individuals fed with pork muscle), as the breeding methodology was identical in both. We used the normal linear model to investigate the relationship between the total development time and the locality of the given population.

Further, the log-rank test was used to evaluate the survival probability of the immature stages among the tested diets (function 'survdiff', package 'survival' (Therneau 2021)). The egg stage was not included in the analysis as its survival could not be influenced by diet. The BH correction method was used for multiple comparisons (Benjamini and Hochberg 1995).

We also investigated how the size of the reared beetles was affected by their sex, diet, and locality. The same GLMM model method was used here, including the same explanatory variables of fixed and random type. The body condition (weight/size) of the beetles was further examined for the possible effects of diet and the locality. The body condition value was expressed by body weight divided by pronotal width. This value was subsequently standardized for each sex to create the standardized body condition score (SBCS). Two generalized mixed linear models (GLMM) were made with two fixed effect explanatory variables that differed in their complexity. One with additive effects between the diet and the locality, and a subsequent model that allowed the interaction between these two terms. The differences in the fit of the models were analyzed using 'Akaike', and the disparity among the tested diets was proved based on post-hoc test as stated above.

The relationship between the photoperiod and the total development time from L1 until adulthood was examined using the GLMM model. The explanatory part of the model contained the photoperiod regime as a fixed effect and the climatic chamber as a random effect.

Results

We tracked the development of 686 *T. sinuatus* immature stages in the *Diet experiment*, of which 543 reached adulthood successfully (277 males and 266 females). Of those, 225 individuals were reared on pork liver, 239 on pork muscle, and 222 on chicken muscle. Regarding the *Photoperiod experiment*, we observed 298 immature stages under the long-day photoperiod regime, of which 267 individuals reached adulthood. On the other hand, for the short-day regime, it was 302 immature stages, of which 266 were able to reach adulthood. For the statistical analysis, 275 males and 258 females were used from both regimes.

Development Time and Survival

The best model that fitted the data was the simplest, with additive effects between the variables (AICc = 2273.671). The residuals of the model did not violate normality or homoscedasticity. Population differences were noticed in individuals from parents collected at Včelná pod Boubínem as they developed slower compared to those from Ploužnice (t value = 2.496, degrees of freedom (df): 83.62, p value = 0.0145), they were delayed for 1.2135 d (standard error [SE] = 0.4861) (See Fig. 1).

When testing the diet effect, a faster development time was observed in larvae fed with pork muscle (27.98 days (d), SD = 2.28 d) compared to pork liver (28.31 d, SD = 2.29 d) (z value = -2.402,

p value = 0.0163) although the difference was small ($-0.4279 + -0.1781$ days). The longest development time was with chicken muscle (29.05 d, SD = 2.89 d), and when compared to both pork muscle and liver the difference was significant (Pork: t value = -6.518 , df: 476.12, p value < 0.001; Pork liver: t value = -4.102 , df: 476.99, p value < 0.001). In contrast, the effect of sex was not significant (t value = -0.816 , df: 469.31, p value = 0.415).

The analysis of both diet (only pork muscle) and photoperiod data of 741 individuals from three localities (Lysolaje = 543, Ploužnice = 148, and Včelná pod Boubínem = 50) revealed significant variability in the developmental time among them. Beetles from Ploužnice showed shorter development time than Lysolaje (est. difference = -0.66816 days, t value = -3.534 , df: 738, p value < 0.0001), while beetles from Včelná pod Boubínem developed longer than those from Lysolaje (est. difference = 0.94427 d, t value = -3.134 , df: 738, p value = 0.0018).

The survival analysis did not indicate any difference in the survival probability of the developing stages among the tested diets (Chi-square = 2.6, df: 2, p value = 0.3).

Size

We found that the best model in this instance was the simplest (AICc = -285.2993) (see Table 1 for delta AICc values of other models). The residuals of the model did not violate the normality or homoscedasticity. In general, males were significantly smaller than females (t value = -15.447 , df: 487.69, p value < 0.001) (See Fig. 2). Further, the developed adult beetles from Včelná pod Boubínem were smaller than those from Ploužnice (t value = -2.558 , df: 80.32, p value = 0.0124).

The possible differences in the size of beetles reared on chicken muscle compared to pork muscle and liver were also assessed. The latter two positively and significantly affected the final size (Pork: t value = 6.336, df: 496.34, p value < 0.001; Pork liver: t value = 5.613, df: 498.61, p value < 0.001) (See Fig. 2) and produced larger individuals. The post-hoc test did not find any difference in size between beetles reared on pork muscle and pork liver (z value = 0.599, p value = 0.549).

Body Condition (Weight/Size)

The best model here was again the simplest with additive effects between the variables (AICc = 1774.036). The AICc value of the more complex model was not significantly worse (1775.867), but we selected the simpler one due to its higher complexity for better

data fitting. The residuals of the model did not violate normality or homoscedasticity. The model evaluated the differences among the standardized body conditions (SBC) of beetles reared on chicken muscle compared to pork muscle and liver. Both pork muscle and pork liver influenced positively and significantly the final SBC (Pork: t value = 6.597, df: 511.92, p value < 0.001; Pork liver: t value = 4.952, df: 515.42, p value < 0.001) (See Fig. 3). The post-hoc test did not find any difference in SBC between beetles fed with pork muscle and liver (z value = 1.572, p value = 0.232). Furthermore, reared beetles did not differ in their SBC between localities (Včelná pod Boubínem and Ploužnice) (t value = -1.455 , df: 72.57, p value = 0.15).

Effect of Photoperiod

The two tested photoperiod regimes (long- and short-day) did not indicate any statistical variability (t value = -1.388 , df: 529.75, p value = 0.1660).

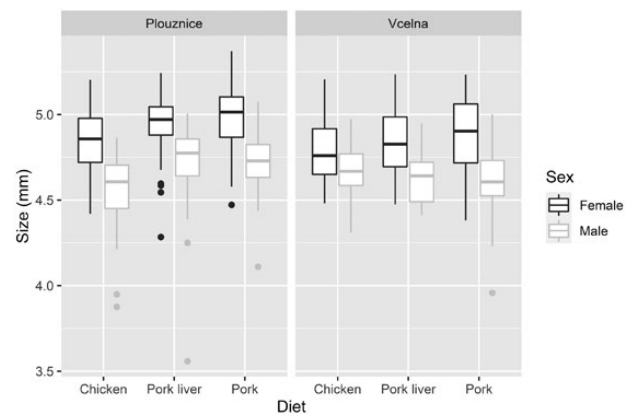


Fig. 2. Boxplot charts of the size for reared *T. sinuatus* beetles based on their sex, diet, and parental locality (Plouznice: Ploužnice, Vcelna: Včelná pod Boubínem). Horizontal lines within the boxes indicate median values; upper and lower boxes indicate the 75th and 25th percentiles, respectively; whiskers indicate the values with the 1.5 interquartile ranges; small, black dots are outliers.

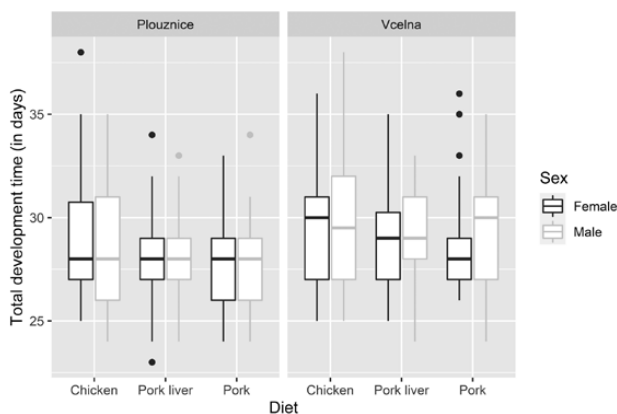


Fig. 1. Boxplot charts of the total development time for reared *T. sinuatus* beetles based on their sex, diet, and parental locality (Plouznice: Ploužnice, Vcelna: Včelná pod Boubínem). Horizontal lines within the boxes indicate median values; upper and lower boxes indicate the 75th and 25th percentiles, respectively; whiskers indicate the values with the 1.5 interquartile ranges; small, black dots are outliers.

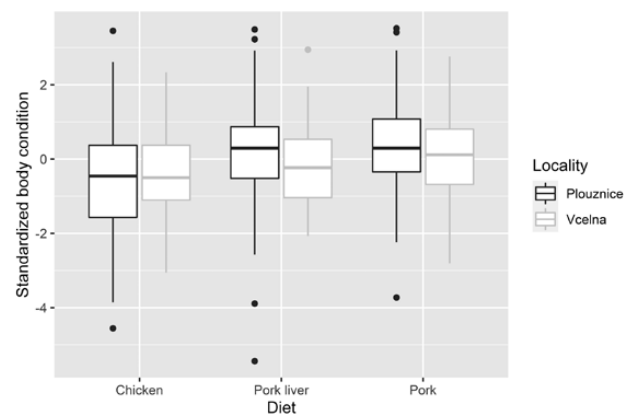


Fig. 3. Boxplot charts of standardized body condition for reared *T. sinuatus* beetles based on their diet, and parental locality (Plouznice: Ploužnice, Vcelna: Včelná pod Boubínem). Horizontal lines within the boxes indicate median values; upper and lower boxes indicate the 75th and 25th percentiles, respectively; whiskers indicate the values with the 1.5 interquartile ranges; small, black dots are outliers.

Discussion

The immature stages of *T. sinuatus* were assessed for potential developmental changes caused by three distinct diets (pork liver, pork muscle, and chicken muscle) from two animal species, in addition to the effects of two cyclic photoperiod regimes (16 h L: 8 h D, and 12 h L: 12 h D) under the constant temperature of 20°C.

The diets introduced indeed affected the development time of the *T. sinuatus* immature stages, as it was shortened with the pork muscle, followed closely by pork liver, and it was prolonged with chicken muscle. These results were almost comparable to our previous experiment involving the sister species *Thanatophilus rugosus* (Linnaeus, 1758) (Qubaiová et al. 2021). However, the fastest development in the latter was seen on pork liver, closely followed by pork muscle, and chicken muscle extended the development time as well.

The final body size and body condition (weight/size) of the developed *T. sinuatus* beetles differed among the tested diets. Individuals fed with pork muscle and liver were larger in size and in better physiological condition than those fed with chicken muscle, which could offer them a competitive advantage. This contrasted our findings on both *T. rugosus* and *Oiceoptoma thoracicum* (Linnaeus, 1758) (Qubaiová et al. 2021, 2022) where size was not significantly affected by the proposed diets. Such variable results further imply that diet can affect the phenotypic characters in different ways in closely related species. Additionally, a disparity in size between the sexes was observed in *T. sinuatus*, which matched with the results of *T. rugosus* but not *O. thoracicum* (Qubaiová et al. 2021, 2022).

Survival rates of *T. sinuatus* did not seem to be impacted by any of the tested diets. In comparison, both *T. rugosus* and *O. thoracicum* (Qubaiová et al. 2021, 2022) exhibited survival variability, and the worst rates were observed in individuals fed with chicken muscle. Perhaps *T. sinuatus* is less selective and more adapted to survive on a lower-quality diet even when it prolongs its development time, as long as the external conditions are optimal, such as, the temperature and humidity, and the absence of pathogens or parasites.

The findings of the current study and the two previous ones (Qubaiová et al. 2021, 2022) indicated that one optimal (high-quality) diet for rearing necrophagous beetles could be pork muscle and pork liver, as the shortest development and/or high survival rates are achieved. Both diets in accord can provide developmental data that are as close as possible to the point of passing and eliminate or at least minimize the PMI overestimation. Over more, these results can be interesting for the forensic community, regarding carrion beetle rearing or research, as pigs are the most popular analogues for human bodies (Matuszewski et al. 2020), thus their tissue could serve as a good, proposed feeding diet as well, instead of using other animal species. Our experiments also showed the constraints of utilizing a lower-quality feeding diet, in our case, the chicken muscle. For this reason, even when chicken meat is commonly used for rearing beetle larvae (Gennard 2013, Byrd and Tomberlin 2019b), we do not recommend it as a rearing diet, mainly because it prolongs the development time and increases larval mortality. However, since we tested only the breast muscle, other chicken parts [e.g., the liver which is considered highly nutritious (USDA 2019)] may indicate different results. Nevertheless, there may be other factors that make chicken muscle less desirable by the developing larvae. For instance, it liquidizes quickly, and if not changed regularly during rearing, small larvae (L1) often tend to drown in it. In contrast, pork muscle and liver hold their consistency for a longer period (personal observation), and their nutritional values appear to be more favorable (USDA 2019), also both diets always result in excellent development and/or survival (see Qubaiová et al. 2021, 2022).

Variation among *T. sinuatus* populations from different localities, where also observed. Reared beetles from parents collected at Včelná pod Boubínem developed slower and were smaller in size compared to those from the Ploužnice locality on all diets tested. Further analysis was conducted, where *T. sinuatus* population from the Lysolaje locality (from the *Photoperiod experiment*) was compared to both previously mentioned populations. We discovered that the Ploužnice population had the quickest development time, followed by Lysolaje, while Včelná pod Boubínem lagged behind. The Lysolaje locality displayed an average temperature, during the peak activity months of the species from May-August, of 22.5°C (temperature data obtained from [Meteoblue weather 2022](#)), at which it could be assumed that the development would be faster than in the Ploužnice locality with an average of 20.5°C, or Včelná pod Boubínem (18.25°C). Yet, this was not the case and the Ploužnice locality showed the fastest development. The reason behind this could possibly be that it had the nearest temperature to the one deployed in our experiment and the developing beetles were more adapted to it. Nevertheless, it is necessary to note that these findings were performed under one temperature in the laboratory, and the localities of the populations utilized were not on a broad latitudinal gradient. Thus, considering the previous factors, further specific experiments on the topic would be appropriate.

In our preceding work, [Jakubec et al. \(2019\)](#), *T. sinuatus* larvae were reared on fish meat (*Scomber scombrus* Linnaeus, 1758), and the development time was, on average, 38.99 days (SD = 3.199). This number is almost 10 d higher than our current findings on the three diets tested at the same temperature in both studies. These results are not very similar, which could be attributed to other aspects, not only the different diet used. Compared to the current study with 543 individuals, [Jakubec et al. \(2019\)](#) had a much lower number of individuals (20) that reached adulthood due to high mortality (probably caused by the disturbance of the post-feeding stage), which might have also impacted the results. However, the main factor could be the different rearing methodologies applied; in [Jakubec et al. \(2019\)](#), larvae were separated individually from L1 to minimize competition, which may have prolonged the development and caused the high mortality. This was similarly indicated in [Gruszka and Matuszewski \(2021\)](#) work on *Necrodes littoralis* (Linnaeus, 1758). The possible effects of heat production and cooperative feeding are beneficial when aggregating in a group, as noticed by [Gruszka et al. \(2020\)](#) and [Matuszewski and Mądra-Bielewicz \(2021\)](#), yet this can perhaps be true only for L1 and L2 *Thanatophilus* larvae, as the L3 larvae are more independent.

In our other study [Montoya-Molina et al. \(2021\)](#), the development of *T. sinuatus* took 38.94 days (SD = 3.126 d) at 20°C on pork muscle. These results are not comparable to our current findings either, as we presume the prolonged development time was due to the mixed origin of the parental beetles (five different locations within the Czech Republic). Hence, the possible effects of a broad spectrum of thermal sensitivities and climatic gradient variations ([Gilchrist 2000](#); [Tsai et al. 2020](#)) may be at play here. Moreover, even though in [Montoya-Molina et al. \(2021\)](#), larvae were separated at L3 (as in the current study), high mortality was still present (most likely due to the high occurrence of pathogens and parasites) and a low number of specimens (21 individuals) reached adulthood, which could have also influenced the duration of development.

There was no significant effect of the photoperiod regimes tested on the developing larvae of *T. sinuatus*. The species may be adapted for both day lengths, which is perhaps sensible as it starts activity in mid-April, where days start short (cc. 13.7 h L) (daylight data acquired from [Eglitis 2022](#)), and continues through the summer, where

the days are longer (around 16 h L), until September where days are shorter again (cc. 12.6 h L). Similar results were, to our knowledge, obtained in only one other study regarding a necrophagous insect, the blow fly *Chrysomya rufifacies* (Bauer et al. 2020). Three photoperiod regimes (two were comparable to ours) had no influence on larval development time, growth rate, or body size. It is highly plausible that if geographically distinct populations of *T. sinuatus* (broader latitudinal cline), with different seasonal adaptations, were assessed, there could be a visible disparity in the development under different photoperiod regimes.

In conclusion, the diet used during rearing influences various life history traits of each forensically relevant species, or even closely related species, in a distinct manner. This is not only dependent on the type and/or source of the diet but also, as our findings indicated, on the origin locality of the population, including those with a narrower geographical distribution. Therefore, it is advised to consider these aspects, and not merely the common temperature influence, when conducting experiments or obtaining data sets necessary for forensic investigations and research.

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6. Chapter 6: Age and sex drives the chill recovery times of a forensically important beetle, *Thanatophilus sinuatus* (Coleoptera: Silphidae)

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The last chapter of the thesis deals with natural history factors influencing the Chill Coma Recovery times (CCRT) in the forensically important species *Thanatophilus sinuatus*. CCRT is the time it takes for an organism to recover after entering into chill coma caused by cold stress. Chill coma is the state induced by low temperatures and is associated with the loss of locomotion. We demonstrate that age and sex of the beetles are the two major factors influencing the recovery of the species after stressful low temperatures. Understanding the thermal sensitivity of the species will help understanding insect occurrence in crime scene are temperature-dependent (e.g., PAI or pre-appearance interval). Hence, improving the accuracy of the model estimates.

Authors contributions: **SMM**: Concept, field work, lab work, data analysis and writing. **PJ**: Concept, data analysis and writing. **JQ**: field work, lab work and writing. **D.N.A**: Concept and writing.

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10 **Age and sex drives the chill coma recovery times of a forensically important beetle,**

11 ***Thanatophilus sinuatus* (Coleoptera: Silphidae)**

12

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17

18 **Abstract**

19 Temperature is one of the main extrinsic factors influencing the physiological responses of
20 ectotherms. As all animals, beetles must endure variation in climatic conditions during their
21 life cycle. Unpredictable short periods of cold temperatures are likely to occur throughout the
22 year, and species vary in their response depending on their life history traits such as body size
23 and physiological age. In temperate regions, organisms likely survive stressful temperatures
24 with behavioural and physiological responses that allow them to avoid cold shock or adjust
25 their baseline levels of cold tolerance. Here, to quantify the response to cold stress of a

26 forensically important beetle *Thanatophilus sinuatus* Fabricius, we calculated the Chill Coma
27 Recovery times (CCRt) in adult beetles of three different age groups (10, 20 and 30 days-old)
28 at 0°C. We experimentally proved that newly emerged beetles (10 days-old) had shorter CCRt
29 than older beetles. For all ages, females took longer to recover than males. Our results show
30 that the relationship between age and sex significantly influences CCRt, and these together
31 (CCRt + age + sex) may explain the distributional and occurrence patterns of *T. sinuatus* in
32 carcasses throughout its geographic range. We did not find significant influence of exposure
33 time to cold or beetle weight on CCRt. Other factors such as exposure time and beetle weight
34 did not significantly influence CCRt.

35

36 **Keywords:** *Silphinae, cold resistance, temperature tolerance, carrion beetle, necrophagous*

37

38 **Introduction**

39 The activity, development, and survival of ectotherms is influenced by abiotic conditions,
40 especially temperature (Castañeda et al. 2005; Angilletta 2009), which in turn impacts species
41 dynamics, abundance, and geographical distributions (Addo-Bediako et al. 2000; Angilletta
42 2009; Bennet et al. 2021). Specifically, cold tolerance and cold tolerance plasticity are crucial
43 for the survival and distribution of ectotherms (Bale 2002; Denlinger and Lee 2010; Angilletta
44 2009; David et al. 1998; Gilbert and Huey 2001) since both are necessary to withstand
45 environmental changes and extreme temperature conditions (Chevin and Hoffmann 2017).
46 Therefore, understanding the factors influencing species-specific cold tolerance and cold
47 tolerance plasticity are important for predicting thermal tolerance traits necessary for accurate
48 assessments of species occurrence and distribution patterns.

49

50 Many insects are incapable of withstanding extended periods of low temperature because they
51 lack necessary mechanisms to survive or mitigate physiological damage caused by sub-zero
52 exposure (Sinclair 1999; Overgaard and MacMillan 2016). As a result, the degree of
53 physiological injury caused by cold temperatures is linked to the duration and the intensity of
54 cold exposure (MacMillan and Sinclair, 2011; Overgaard and MacMillan 2016). These injuries
55 can affect an individual's ability to move after a cold stress or even the ability to restore
56 essential physiological functions. Some of these physiological injuries are linked to a disruption
57 of ion and water homeostasis (Kostal et al. 2004; MacMillan et al. 2015; Findsen et al. 2014),
58 resulting in cell depolarization (Bayley et al. 2018).

59
60 Cold tolerance can be measured in several different ways, e.g., critical thermal minima (CT_{min}),
61 chill mortality, and chill coma recovery time (CCRT; Sinclair et al. 2015). CCRT is measured
62 as the time it takes for an organism to recover after succumbing to chill coma caused by cold
63 stress. Chill coma is the comatose state induced by low temperatures and is associated with the
64 loss of neuromuscular function caused by the loss of muscle membrane potential (Hosler et al.
65 2000; Overgaard and MacMillan 2015). Depending on the magnitude and the duration of cold
66 exposure, chill coma can be lethal or reversible (Angilletta 2009; Delinger and Lee 2010;
67 Kostal et al. 2004; Kostal et al. 2006). CCRT is used as a proxy for a natural situation in which
68 an animal might experience a cold stress (e.g., rapid decrease in temperature at night) and
69 eventually recover as temperatures rise again (e.g., with the morning sun; David et al. 1998).
70 The inability to recover quickly from cold exposure may leave an individual vulnerable to other
71 risks (e.g., predation).

72
73 The geographic distributions of ectotherms such as beetles, are largely influenced by the
74 temperature range in which they can perform and survive (Bale 2002; Angilletta 2009; Chown

75 and Terblanche 2006; Bennett et al. 2021; Gotcha et al. 2021). This range of temperatures is
76 linked to an increased probability of experiencing extreme low temperatures varying in both
77 intensity and length (Easterling et al. 2000). The species *Thanatophilus sinuatus* (Fabricius,
78 1775) (Coleoptera: Silphidae) is a trans-Palearctic distributed species found throughout Central
79 Europe and is of recent forensic importance due to its rapid colonization and prevailing
80 occurrence on large carrion (Dekeirsschieter et al. 2011; Montoya-Molina et al. 2021;
81 Qubaiová et al. 2023). *Thanatophilus sinuatus* activity peak is during the warmer months (June
82 - Augusts) (Frątczak–Łagiewska and Matuszewski 2018; Esh and Oxbrough 2021;
83 Matuszewski 2021), having two generations during the active period (Frątczak–Łagiewska and
84 Matuszewski 2018). Thus, the species wide distribution along the Palearctic region may be
85 linked to the cold response mechanism used by the beetles to endure short periods of low
86 temperatures.

87

88 As mentioned above, the magnitude and time of exposure to stressful cold conditions are some
89 of the major factors influencing insect cold tolerance. For that reason, these two variables have
90 drawn a large proportion of cold tolerance research in insects. Biological factors, such as age
91 and size, may also have a sizable impact on thermal sensitivity (Angilletta et al. 2004; Bowler
92 and Terblanche 2008), but have received less attention across taxa. Therefore, this work aims
93 to describe the impact of biological factors on the thermal tolerance of a forensically important
94 species in Europe, *Thanatophilus sinuatus*. Since newly emerged adults likely allocate more
95 resources to physiological maintenance compared to old beetles that are investing in
96 reproduction, we hypothesized that recovery from cold stress (CCRT) will be positively
97 correlated with age and negatively correlated with weight. Results presented here will help
98 improve estimates used in the forensic sciences (pre-appearance (PAI); Matuszewski and
99 Szafałowicz 2013) and understanding the patterns of colonization in carcasses.

100

101 **Material and Methods**

102 Laboratory experiments were performed using the carrion beetle *Thanatophilus sinuatus*. The
103 *T. sinuatus* beetles were sampled in a transitional area between agricultural and urban land of
104 Prague-Lysolaje, Czech Republic (50°07'33.0"N, 14°21'44.0"E). Beetles were collected
105 during spring and summer of 2022, as the species is active throughout this period of the year
106 (Jakubec and Ružička, 2015; Frątczak–Łagiewska and Matuszewski 2018; Esh and Oxbrough
107 2021). Collected beetles were transferred to the laboratory at the Czech University of Life
108 Sciences in Prague and kept under constant conditions (described below).

109

110 All collected adult beetles were left to oviposit in breeding boxes (Hagen Exo Terra Faunarium
111 37 x 22 x 24.5 cm). The breeding boxes contained approximately 13 cm of gardening soil.
112 Inside, a plastic tube filled with water and tapped with cotton was placed above the soil surface
113 as a water resource. Beetles were fed *ad libitum* with pieces of pork muscle (*Sus scrofa*
114 Linnaeus, 1758). More detailed information regarding beetle breeding can be found in our
115 previous articles (Montoya-Molina et al. 2021a; Qubaiová et al. 2023). Laid eggs were
116 transferred into separate Petri dishes to follow offspring development. All beetles used in the
117 experiment were bred and developed under constant temperature 20°C and 16 L: 8 D
118 photoperiod regime. Newly emerged beetles were placed in separate breeding boxes with the
119 same setup and thermal conditions as the parental generation. Twenty-four hours prior to the
120 experiment, beetles were isolated from the breeding boxes into a smaller box. Sixteen beetles
121 were placed in each box to reduce beetle density and competition for food, assuring beetles had
122 the best body condition.

123

124 Chill Coma Recovery time (CCRt) was measured in 383 beetles of three different physiological
125 ages (10, 20 and 30 days-old). Beetles of each age were randomly selected and exposed to one
126 of two different chill times, 2 hours (n = 191) and 16 hours (n = 192), at zero degrees Celsius.
127 More than 48 beetles were used per treatment combination (chill time and age). It is important
128 to mention that 10 day-old beetles were unmated and 20 and 30 day-old beetles were mated.

129

130 To measure CCRt, we followed the methodology described by David et al. (1998). The
131 experiment consisted of placing sixteen beetles inside an Eppendorf tubes (1.5 ml; one beetle
132 per tube) organized into a foam rack and exposing them to 0 °C inside a thermal bath. Zero
133 degrees Celsius is an ecologically relevant temperature which all Holarctic insects must endure
134 for some period annually (Bale 1989). We used a piece of cotton to push the beetles into the
135 bottom of the Eppendorf tubes and the foam rack was used to help maintain the Eppendorf
136 tubes floating in a thermal bath (ECO-Lauda) filled with ethylene glycol. The temperature
137 inside the thermal bath was confirmed with the temperature sensor of the bath and two
138 independent thermocouples placed near the foam rack. Once the thermal bath was at the desired
139 temperature, the foam rack with the beetles was placed at the surface of the bath and the start
140 time was recorded.

141

142 At the end of each chill period, beetles were removed from the bath and placed on a white tray
143 at room temperature (20°C - controlled by air conditioning), where their recovery was video
144 monitored. Beetles were initially placed on their elytra and the time interval between removal
145 from the thermal bath and the point when they were able to get from the elytra back on their
146 legs (recovery) was recorded (following David et al 1998; 2003). Recovered beetles were
147 removed from the tray immediately to prevent interference with other recovering beetles.

148

149 We confirmed that the chill exposure treatments used were non-lethal by maintaining the tested
150 beetles after the assay and observing successful reproduction of a new generation.

151

152 *Statistical analysis*

153 To investigate the effects of beetle age, sex, and weight on CCRt we used a Generalized Linear
154 Regression Model (GLM) with a Gamma distribution to account for the non-normal
155 distribution of the CCRt values. To find the best fitting model, we created ten different models
156 that contained both core variables (age and chill time) and one or two optional variables (sex
157 and weight) (see Table 1). The models differed in their complexity (simple = additive effect,
158 complex = interaction), and were compared using Akaike information criterion with a
159 correction for small sample size (Taddy 2017).

160

161 Data management and all analyses were carried out using the statistical program R (R Core
162 Team 2020). Graphical outputs were created using ggplot2 and sjPlot packages (Wickham
163 2009, Lüdecke 2020). Critical levels of p values and AICc were 0.05 and 2 respectively. In
164 case of tie between AICc values, we used Occam's razor principle and decided the winner
165 model by comparing number of used degrees of freedom (df).

166

167 **Results**

168 The model with the lowest AIC and degrees of freedom (model 8) included the additive effect
169 of sex, age, and chill time, and the interaction effect of age and chill time (bolded value in
170 Table 1: \sim age * chill_time+sex; AIC value: 4928.076). Overall, age had a significant positive
171 effect on CCRt since older beetles had the longest CCRt (t value = 3.551, p value < 0.001) (see
172 Fig. 1; Table 2). Male beetles had significantly shorter CCRt compared to females (t value = -
173 3.704, p value < 0001). However, beetle weight did not have a significant effect on CCRt

174 despite females being significantly larger than males. Interestingly, chill time did not have a
175 significant effect on CCRt either (t value = 1.548, p value = 0.122). Though, the p value for the
176 interaction between chill time and age was < 0.1 (t value = -1.698, p value = 0.09), suggesting
177 some pattern (Table 2). This pattern was similar for males and females; young beetles (10-days
178 old) had long recovery times with long chill exposure, while old beetles (30-days old) had short
179 recovery times with long chill exposure (see Fig. 1; Table 2).

180

181 **Discussion**

182

183 In this work, we provided a description of the thermal sensitivity of the forensically important
184 carrion beetle, *Thanatophilus sinuatus*, on a fine time scale. Our results indicated that the cold
185 recovery response (CCRt) of *T. sinuatus* is mediated by the age and sex. In contrast, we did not
186 find an association between body weight and CCRt. As an ectotherm, *T. sinuatus* performance
187 is directly linked to environmental temperature and to physiological traits such as age and size
188 (Angilletta et al. 2004; Kingsolver and Huey 2008). Based on the results here, the age and sex
189 of beetles, more so than past cold exposure time, may be predictive of whether individuals of
190 this species are present or absent in a specific area or region (e.g., crime scenes in specific
191 counties).

192

193 Our results show that ontogeny is a primary factor affecting the chill coma recovery of *T.*
194 *sinuatus* beetles. Age has a major effect on the capacity of ectotherms to recover from cold
195 stress (Angilletta 2009; Bowler and Terblanche 2008). Our results show that newly emerged
196 beetles (10 day-old) recovered faster than older beetles. The CCRt values increased as beetles
197 aged. An insects' thermal tolerance is subject to constant change during its life cycle, resulting
198 in age-specific susceptibility or resistance to cold stress during particular stages of life (Bowler

199 and Terblanche 2008; Marais et al. 2009; Mitchell et al. 2013). In newly emerged beetles,
200 energy resources are primarily assigned to locomotive and maintenance functions, and basic
201 survival requirements as they likely to experience low environmental temperatures (Huey and
202 Berrigan 2001; Kozłowski et al. 2004; Nespolo et al. 2008). As beetles mature, energy
203 resources are invested into reproduction; resulting in a reproduction-survival trade-off (Bubliy
204 et al. 2002; Ellers and vanAlpen 1997; Roff et al. 2002). In ectotherms, reproduction is a costly
205 investment (Hoglund and Sheldon 1998; Lehtonen 2012). In our experiment, mated beetles
206 (twenty and thirty days-old), which are allocating energetic resources towards reproduction
207 (e.g., sexual maturation, clutch size in females), had the longest recovery times from cold
208 stress. Furthermore, female cold tolerance was weaker than that of males, which might result
209 from comparatively higher reproductive investment (egg formation) than in males (Kindsvater
210 and Alonzo 2014). For this reason, our results suggest that female reproduction comes at the
211 expense of energetic resources that could be allocated to important responses such as the cold
212 tolerance response (CCRT).

213

214 Body weight did not significantly affect the CCRT in *Thanatophilus sinuatus*. One explanation
215 is that CCRT measurements are not sensitive enough to show differences as weight may depend
216 on other physiological factors. Body weight and potentially body shape/size still impact cold
217 tolerance performance if the sampled individuals were to represent better variation likely found
218 in the field. Like most insects, beetles depend on environmental temperature to thermoregulate,
219 and large-bodied organisms take longer to absorb heat from the environment than small-bodied
220 ones affecting their physiological processes (Ashton and Feldman, 2003; Blanckenhorn and
221 Demont 2004; Mousseau 1997).

222

223 Our results show that there was no significant effect of chill time on CCRt. This is in stark
224 contrast to previous literature that clearly demonstrates an inverse relationship between chill
225 exposure time and recovery time (David et al. 1998; 2003; MacDonald et al. 2004; Ramadan
226 et al. 2020). However, we did find an interaction effect between chill time and beetle age.
227 Young beetles recovered fastest, but only when the exposure time was short. Older beetles
228 recovered slightly faster with a long cold exposure time compared to young beetles (see Fig.
229 1). This suggests that cold sensitivity, specifically beetle sensitivity to short non-lethal cold
230 exposure, changes with time. Much of the research on the impact of age (Anderson et al. 2005;
231 David et al. 1998) and the time of exposure (Bublyi et al. 2002; David et al. 1998) on the cold
232 recovery responses have been studied previously in *Drosophila* flies Meigen 1830. Those
233 results demonstrated that age and time exposure impact negatively the CCRt. Nevertheless, in
234 *T. sinuatus* this pattern was reversed, suggesting a species-specific interaction between chill
235 time and age.

236

237 We found that chill exposure and subsequent recovery did not negatively affect beetle survival
238 or fecundity, as all beetles recovered successfully and were able to reproduce. This is most
239 likely because the experimental conditions (time of exposure and magnitude) were within the
240 thermal limits of the species (S. Montoya-Molina personal observations). If beetles were
241 exposed to more extreme conditions, then survival and reproduction would likely have been
242 negatively affected since exposures to 0 °C for over 24 hours are associated with the
243 accumulation of chill injuries in insects (MacMillan and Sinclair 2011).

244

245 Thermal sensitivity is an important factor in the biology of economically important species,
246 native or alien (Athanasios et al. 2018a; 2018b; Nyamukondiwa et al. 2010). The silphid
247 beetle *T. sinuatus* is considered a promising bioindicator for forensic sciences due to its close

248 association with the necrobiome and its fast colonization of it (Matuszewski and Szafałowicz
249 2013), and the established quantity of information regarding its developmental biology
250 (Montoya-Molina et al. 2021; Qubaiová et al. 2023). Despite of the species proven importance,
251 information regarding its thermal sensitivity is still scarce. Our study showed that newly
252 emerged adult beetles are tolerant to cold exposures for at least 16 hours. However, old-mated
253 adults show signs of increased thermal sensitivity to low temperatures with prolonged recovery
254 times compared to younger individuals, possibly because of an energetic reproductive-survival
255 trade-off. Many of the models used in the forensic sciences for understanding insect occurrence
256 are temperature-dependent (e.g., PAI or the pre-appearance interval). Hence, understanding the
257 response of this species to low temperature exposure provides necessary information of a basic
258 life history trait, improving the accuracy of model estimates and improving our understanding
259 of functional limits of the species biology. Further efforts should attempt to describe the
260 thermal biology of other forensically important beetle species to accurately account for species-
261 specific cold recovery responses. These descriptions may help improve the use of insects for
262 solving legal cases.

263

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268

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444

445

446 **Tables and figures**

447

448 **Table 1:** Comparison of all models for the GLM analysis of the chill coma recovery time, with their
 449 delta AICc values and degrees of freedom. The best model is in bold.

450

ID.	Model	AICc	df
1	~ age * chill_time*sex+weight	4931.287	10
2	~ age * chill_time+sex+weight	4928.048	7
3	~ age + chill_time*sex+weight	4931.628	7
4	~ age + chill_time+sex+weight	4929.830	6
5	~ age + chill_time+weight	4951.232	5
6	~ age + chill_time+sex	4930.771	5
7	~ age * chill_time+weight	4949.330	6
8	~ age * chill_time+sex	4928.076	6
9	~ age * chill_time	4949.478	5
10	~ age + chill_time	4950.486	4

451

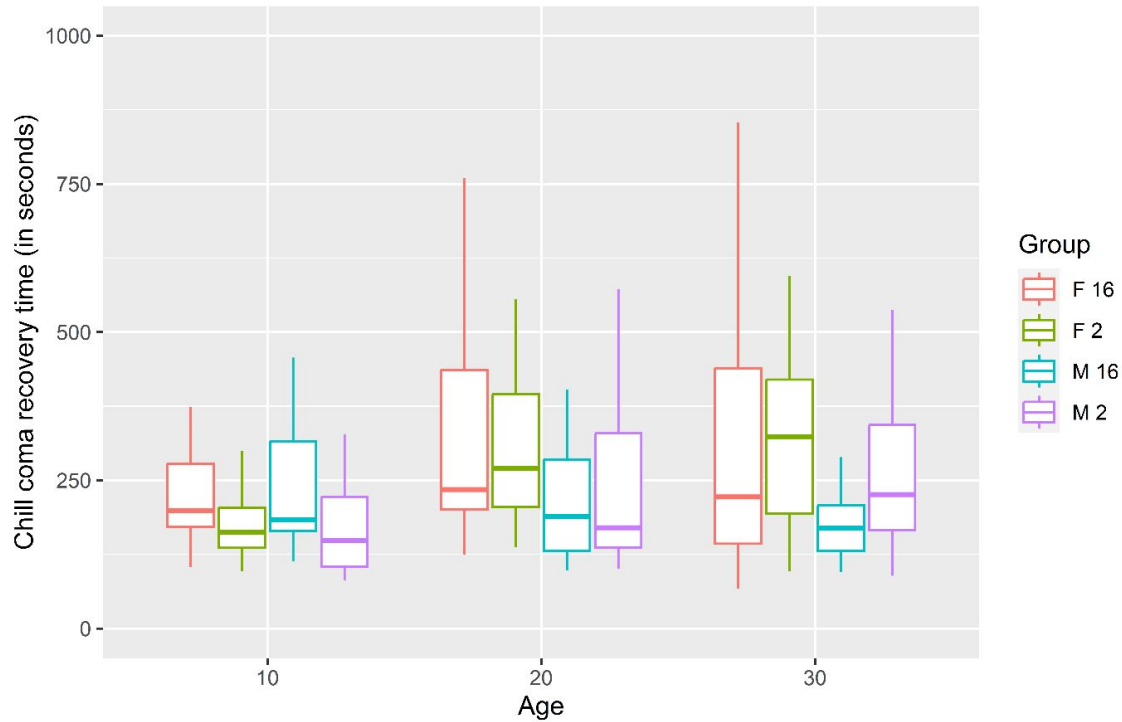
452

453 **Table 2:** Model statistics of the best GLM model. Variables that showed a statistically significant effect
 454 in bold.

Coefficients	Estimate	Std. Error	t value	p value
Intercept	193.099	39.848	4.846	> 0.001

Age	6.287	1.770	3.551	> 0.001
Chill time (16)	86.203	55.670	1.548	0.122
Sex (male)	-83.773	22.617	-3.704	>0.001
Age: Chill time	-4.307	2.536	-1.698	0.09

455



456

457

458 **Figure 1:** Boxplot of differences in Chill Coma Recovery times (CCRT) for sex, age groups and chill
 459 times for *Thanatophilus sinuatus*. F and M abbreviations indicate beetles' sex and the number that
 460 follows the exposure time. Horizontal lines inside the boxes indicate the median values; upper and
 461 lower boxes indicate the 75th and 25th percentiles respectively. Whiskers indicates the values within the
 462 1.5 quartile ranges.

463

7. Chapter 7: General Discussion

This chapter will analyse the thermal biology (Thermal Summation Model constants, Developmental times, and Chill Coma Recovery time) of *Thanatophilus sinuatus* and *T. rugosus*. It will attempt to demonstrate the role of these two beetles in the forensic sciences.

The use of carrion beetles developmental information is still a novelty, however, is quickly being studied by many researchers worldwide (e.g., Charabidze et al. 2014; Dekeirsschieter et al. 2011; and Matuszewski 2016; 2018; Gruszka and Matuszewski 2021; 2023; Jakubec, 2016; Hu et al. 2019; 2020; Midgley et al. 2009; Midgley et al. 2010; Ridgeway et al. 2014; Martin-Vega et al., 2017; Montoya-Molina et al. 2021a; 2021b; Qubaiová et al. 2021; 2022; 2023; Wang et al., 2016; 2022; etc.).

As previously stated, historically, beetles have been neglected in the forensic sciences as they were considered late colonizers, meaning that they arrive later than flies after death, therefore, a lousy bioindicator for estimating the PMI and the Time of Colonization (Dekeirsschieter et al. 2011; Midgley et al. 2010). Indisputably, beetles may not be able to arrive as fast as the flies, (e.g., *Calliphora spp.*) and compete with them in relevance during the first minutes after death colonizing the carcasses (Jarmusz et al. 2020; Matuszewski et al. 2010; 2011). Still, they are dominant and abundant taxon during the carcass decomposition and even more abundant over the later stages of the decomposition providing evidence for the PMI estimation when Diptera are no longer found on the corpse (Midgley and Villet 2009; Midgley et al. 2010). Moreover, nowadays methods using late arriving insects to calculate the post-mortem interval (pre-appearance also known as PAI) are being used and improved (Matuszewski 2011; Matuszewski and Szafałowicz 2013; Matuszewski and Mądra-Bielewicz 2016). The above mentioned, categorize Coleoptera as of high priority for research in the legal and criminal sciences.

Unequivocally, Coleoptera is the most diverse group of animals in nature, fulfilling many ecological functions previously mentioned in the introduction (Bánki et al. 2023). During carcasses decomposition process, it is observed that there is the same amount of beetle species attracted to the carcass as Diptera (Midgley et al. 2010). Hence, the information that could be extracted from Coleopterans and Dipterans together may contribute robust evidence in legal

investigations. Additionally, novel studies in selected necrophagous beetles have proven the historical belief wrong. Some beetle species may arrive to the corpse soon after death (Matuszewski and Szafałowicz 2013; Midgley et al. 2009; Midgley et al. 2010; Ridgeway et al. 2014).

Some species can be classified of higher value in the forensic sciences than others, the former depending on its biology and the amount of information that may provide for forensic investigations (Amendt et al. 2011; Matuszewski 2021; Matuszewski et al. 2010). Not long ago, Matuszewski and collaborators (2010) provided a list of insects of forensic importance in Central Europe. In the study, the evaluation of different carrion taxa was based on their patterns of residency in three different seasons and land covers. When analysing the utility of some Coleoptera species frequenting the carrion, surprisingly, many recognized species of forensic use fell into categories of no usefulness, including *T. rugosus*. On the other hand, *T. sinuatus* was categorized as species of moderate usefulness.

In disagreement with Matuszewski et al. (2010), *Thanatophilus rugosus* and *T. sinuatus* do bear high usefulness as other listed beetle species, such as *Necrodes litorallis* and *Creophilus maxillosus* listed in the same category. *T. sinuatus* and *T. rugosus* adults, feeds, breeds and oviposit in the carrion, being considered strictly necrophagous (Ikeda et al. 2008). The species are often collected in large carcasses (Esh and Oxbrough 2020; Fratzczak-Lagiewska and Matuszewski 2018) being rapid colonizers (*T. sinuatus* = 1.4 days) and widely distributed (Matuszewski and Szafałowicz 2013). They prior fit the requirements for a species to be used in forensic entomology. Besides Matuszewski and collaborators (2010) sampling was carried out in different types of forest in Western Poland. Habitat preferences of the species were left overlooked as both species are common to occur in habitats with no land cover (e.g., grasslands, meadows) or little land cover (Esh and Oxbrough 2021; Fratzczak-Lagiewska and Matuszewski 2018; Kočárek 2001; Růžička 1994; *Personal observation*). In their process to reach this conclusion, the usefulness of the taxa was not appropriately evaluated, leaving important ecological factors such as the land cover preference disregarded.

The importance of *T. sinuatus* and *T. rugosus* in forensic investigations in Europe is highlighted in many of the previous studies published by Bonacci et al. (2010) in Italy, Dekeirsschieter et al. (2011), Dekeirsschieter (2012) in Belgium and Fratzczak-Lagiewska and Matuszewski (2018) in Poland. In the Czech Republic, both species are sampled in criminal

cases and used as entomological evidence (*T. sinuatus* = 443 cases between 2003 and 2022) and (*T. rugosus* = 343 cases between 2003 and 2020). The occurrence of the species in criminal cases during the active months of the year supports the utility of these two taxa in forensic investigations. In Southern Italy, during the winter season, Bonacci et al. (2011) noticed that larvae and adults of *T. sinuatus* and *T. rugosus* may replace blowflies during decay due to low temperatures and adverse climatic conditions.

The importance of the taxa used in the legal sciences lies in the quantity and quality of useful information produced after meticulous research following straightforward methodologies. In this sense, *T. sinuatus* and *T. rugosus* comprise a series of studies published by Dekeirsschieter (2012) in the Developmental times, Jakubec et al. (2019) in the description of the immature stages and the morphology of *T. sinuatus* and, studies by Novák et al. (2018) providing the morphological description of the immature stages for *T. rugosus*. Fratzak-Lagiewska and Matuszewski (2018) analysed both species' seasonality and resource partitioning. They demonstrate the importance of these two species in Central Europe on the late stages of decomposition of large carcasses (Matuszewski et al. 2014).

To date, from the five *Thanatophilus* species occurring in Europe: *T. dispar* (Herbst, 1793), *T. lapponicus* (Herbst, 1793), *T. ruficornis* (Kuster, 1851), *T. rugosus* (Linnaeus, 1758) and *T. sinuatus* (Fabricius, 1775), only the two latter (*studied in this thesis*), developmental information is known. In 2012, Dekeirsschieter provided an approximation of the developmental times of *T. sinuatus* at two ecologically relevant temperatures, 18°C and 23°C. Results of total development vary significantly between studies. The total development at 18°C was 33.7 days, compared to our results 43.4 days at the same temperature. Many differences in the breeding methodology used by Dekeirsschieter (2012) compared to our study (e.g., diet, locality of the parentals, breeding method may have contributed to contrasting developmental times.

Other development results available for *Thanatophilus* species were published by Ridgeway et al. (2014) for both Afrotropical species *T. micans* (Fabricius, 1794) and *T. capensis* (Wiedemann, 1821). Our results at 20°C shows that, *T. sinuatus* and *T. rugosus* takes 42.04 and 30.45 days correspondingly to complete the total development. Compared to obtained by Ridgeway et al (2014), where *T. capensis* and *T. micans* takes 39.6 days and 22.64 days to complete total development (Ridgeway et al. 2014). The differences in the developmental times and TSM constants obtained for *T. capensis* at 20°C compared to *T. micans* and the similarity with the Palearctic sister's species might be predicted (Table 2). As

results of TSM and total development could be explained by the geographical distributions of the species. Similar results might be expected for *T. capensis*, *T. sinuatus*, and *T. rugosus* as they all inhabit temperate regions compared to *T. micans*, widely distributed in Africa. This relationship between cold and warm-adapted species distribution is broadened later when analysing the thermal summation parameters results for *T. sinuatus* and *T. rugosus*.

In chapters 2-3, we provided the thermal summation model (TSM) constants of Lower Developmental Threshold (LDT) and Accumulative Degree Days (ADD) for *Thanatophilus sinuatus* and *T. rugosus*. Measurements of the TSM constants constitute an essential tool in forensic sciences as they are used to calculate the PMI. Further, these measurements may help elucidate much of the biology of these two Palearctic distributed species. Results of LDT values (LDT.sinuatus = 9.8 ± 0.2 ; LDT.rugosus = 8.5 ± 0.08) were comparable to the others forensically important beetles in Europe such as *Creophilus maxillosus* and *Necrodes littoralis* (Table 2). Our results of LDT are close in similarity to the ones of *Necrodes littoralis* (LDTlittoralis = 9.0 ± 0.5) and *Creophilus maxillosus* (LDTmaxillosus = 11.6 ± 0.2). Values of LDT may explain possible adaptations of the species (Honěk 1996; Trudgill et al. 2005), suggesting that in populations distributed in high and low latitudes, lower LDT and fast development are favoured by selection due to climatic conditions (Buckley et al. 2017). Thence, explaining the colonization patterns of these species during extensive periods along the year, withstanding low temperatures close to 0°C during the spring and fall (Matuszewski et al. 2010).

Values of ADD (ADDsinuatus = 360.4 ± 10.7 ; ADDrugosus = 362.7 ± 4.9) were visibly lower than *N. littoralis* (ADDlittoralis = 434.7 ± 28.8) and *Creophilus maxillosus* (ADDmaxillosus = 405.1 ± 14.6). *T. sinuatus* and *T. rugosus* ADD constants significantly contrast with other forensically important species. Values of ADD are associated with the size of ectotherms. High ADD values are often linked with the adult insect's size increase (Honěk 1999; Trudgill et al. 2005). Interestingly, the body lengths/size of *N. littoralis* and *C. maxillosus* are apparently larger than the ones of the *Thanatophilus* species. Palearctic *Thanatophilus* species are smaller in size than *N. littoralis* and *C. maxillosus*. However, when compared to the African species of the genus, ADD values do not reflect this ADD hypothesis, as males and females may reach the size of *N. littoralis*.

ADD and LDT constants are associated with the organism's phenology (Trudgill et al. 2005 and Buckley et al. 2017). The species aforementioned, exhibit partially similar ecological, seasonal, and habitat preferences, colonizing large-sized carcasses during the active and advanced decay stages of decomposition inhabiting open grasslands habitats (Charabidze et al. 2014; Gruszka and Matuszewski 2021; Matuszewski et al. 2008). The slight differences in the constants of LDT and ADD among these four species could explain possible mechanisms to regulate competition between them. This could be linked to differences in the species environmental adaptations and distributional and seasonal patterns (Rebaudo and Rabhi 2018).

Table 2. List of coleoptera species of forensic importance that with values of LDT and ADD published and publicly available.

Species	Family	LDT (°C)	ADD (°C)	References
<i>Creophilus maxillosus</i>	Staphylinidae	9.6±0.5 11.6±0.2	492.0±23.6 405.1±14.6	Wang et al. 2016; Frączak-Łagiewska et al. 2020
<i>Dermestes tessellatocollis</i>	Dermestidae	12.0±0.5	664.3±55.8	Wang et al. 2021
<i>Eupilosus azureus</i>	Histeridae	9.31	37.58	Caneparo et al. 2017
<i>Necrobia rufipes</i>	Cleridae	16.6±0.6	591.0±39.5	Hu et al. 2020
<i>Necrobia ruficollis</i>	Cleridae	14.5±0.5	684.1±33.8	Wang et al. 2022
<i>Necrodes littoralis</i>	Silphidae	9.0±0.5	434.7±28.8	Gruszka and Matuszewski 2022
<i>Necrophila brunnicollis</i>	Silphidae	15.3±0.3	78.6±6.2	Jakubec et al. 2021
<i>Omosita colon</i>	Nitidulidae	10.6±0.1	514.1±8.7	Wang et al. 2020
<i>Sciodrepoides watsoni</i>	Leiodidae	12.5±1.6	1207.4±489.2	Jakubec 2016
<i>Thanatophilus micans</i>	Silphidae	13.2±0.5	197.9±19.7	Ridgeway et al 2014
<i>Thanatophilus mutilatus/capensis</i>	Silphidae	9.0±0.3	384.1±16.9	Ridgeway et al 2014
<i>Thanatophilus rugosus</i>	Silphidae	8.5±0.08	362.7±4.9	Montoya-Molina et al. 2021
<i>Thanatophilus sinuatus</i>	Silphidae	9.8±0.2	360.4±10.7	Montoya-Molina et al. 2021

Aside from temperature, arthropod development is directly influenced by other factors such as the photoperiod and diet quality (Ikeda-Kikue and Numata 1992; Saunders 2014). In chapters 3-4 we highlight the effect of diet in the developmental times of both *T. sinuatus* and *T. rugosus*. In all of the cases, diet type had significant effect on the species *T. sinuatus* and *T. rugosus* life history. Results on diet influence were almost comparable in both species.

Although, they were not the same. Studies have shown similarities in food preference among phylogenetically related species as they tend to share similar traits (e.g., size, feeding, and habitat preferences) than distant ones (Eklöf et al. 2012). Interestingly, our results showed that *T. sinuatus*, shortest developmental times were found when larvae were bred with Pork muscle (31.9 days), followed by pork liver (32.9 days), and lastly chicken muscle (32.5 days). Unlike *T. sinuatus*, *T. rugosus* faster development was obtained when feeding the immature stages with pork liver (36.4 days) followed by pork muscle (38.5 days), and lastly chicken muscle (40.1 days). A possible resource competition could explain the little changes observed between these two closely related species. Thus, resources competition is what might determine the type of food from where they feed and not the phylogenetic relatedness between species (Elias et al. 2013). In this sense, in nature during the months when both species occur, these two *Thanatophilus* species might be found consuming the same alimentary resource. Specialization for certain types of tissues based on the lipidic or protein contents necessary for developing might be plausible (Chaudhury et al. 2015). Moreover, this possible specialization might serve as strategy (carcass partitioning) to avoid overlapping/competing when both species are found active and occupying same resource (Denno and Cothran 1975).

Diet quality influences the survival of the immature stages of *T. rugosus*. These results are consistent with those obtained by Qubaiová et al. 2022 with *Oiceoptoma thoracicum* (Linnaeus, 1758) where mortality rates were negatively influenced when fed with chicken muscle. Intriguingly, in our study survival of *T. sinuatus* was not affected by any of the tested diets. It might be possible that *T. sinuatus* is adapted to survive on a lower-quality diet when conditions are not optimal. Possible physiological mechanisms (e.g., nutrient regulation = *feeding plasticity*?) are triggered in response to the variation of food resources (Smykal and Raikhel 2015). Overall, high mortality is associated with the least nutritious dietary resource in most species (Chaudhury et al. 2015; Qubaiová et al. 2021; 2022; Scriber and Slansky 1981). Still, less specialized species, like *T. sinuatus*, might be capable of activating compensatory physiological mechanisms (e.g., *increased digestive efficiency*) in response to the absence of a nutritious diet allowing them to continue development without dying (Chown and Nicolson 2004).

Photoperiod, on the other hand, had no statistical differences. Similar results were obtained by Bauer et al. (2020) when testing the photoperiod using the flesh fly *Chrysomya rufifacies*. *T. sinuatus* seasonal activity starts mid-April (cc. 13 h L) and throughout the summer (cc. 16 h L)

until September (cc. 12.6 h L). Over this period of activity, light regimes vary by approximately 6 hours of Light difference (Eglitis 2022). The changes in the tested light regimes (16h L: 8 h D and 12h L: 12 h D) studied were insignificant to be perceived by *T. sinuatus* adapted to significant changes in the light regime. Photoperiodism strongly influences Palearctic species, triggering photoperiodic responses such as diapause in adults (Ikeda-Kikue and Numata, 1992). In insect larvae, photoperiod cues might disrupt normal daily rhythms (e.g., metabolism, feeding), affecting the immatures growth and survival (Scriber and Slansky 1981). Contrary to expected, our results demonstrate that larval development in *T. sinuatus* might be insensitive to photoperiod stimulus. Similar results were obtained by Kutcherov and Lopatina (2022) when analysing the developmental times in four phytophagous leaf beetles (Coleoptera; Chrysomelidae) in the Palearctic. Moreover, the effects of photoperiod in *T. sinuatus* developmental times might not be the same in other geographical regions, as photoperiodism might be an adaptation to local specific conditions (Owings et al. 2014). Future research on this topic is necessary to confirm the local responses of *Thanatophilus* species to photoperiod.

Like all ectotherm physiology and biology, Silphids are linked to temperature (Castañeda et al. 2005; Angilletta 2009). Geographical variation of insect physiology results from specific local adaptations to temperatures experienced by the populations (Bennet et al. 2021; Kipyatkov and Lopatina 2010, Lopatina et al. 2012). We found important differences in developmental times between Chapter 2 (Montoya-Molina et al. 2021) and Chapter 4 (Qubaiová et al. 2023) using the same laboratory conditions and methodologies. At same breeding temperature 20°C, in Chapter 2 the development of *T. sinuatus* took 38.94 (SD = 3.126) days, compared to 27.98 (SD = 2.28) days in Chapter 4. Interpretation of the possible variation in the developmental times result from changes in thermal constants directly associated to the ectotherm's local thermal conditions in the natural habitats (Honek 1996 and Trudgill 1995). In this sense, the prolonged development might be the effects of a mixed origin of the parentals (five different locations inside the Czech Republic – Chapter 2). Therefore, developmental times presented in Chapter 2, might be the possible effects of a broad spectrum of thermal sensitivities and climatic gradient variations (Gilchrist 2000; Tsai et al. 2020) of the parental's beetles when compared to Chapter 4 where only two different parental populations were used.

Changes in developmental times in forensically important beetle was also discussed by Frątczak–Łagiewska et al. (2020) when comparing the development of *Creophilus maxillosus* from Central Europe with the ones from Asia and United States. They observed that the total days needed for the Central Europe population to complete development (15 °C = 122.21 days)

doubled the days needed by the North American population (16 °C = 65.47 days). They advocate these findings to multiple factors (e.g., beetles diets and rearing conditions); but none of them connected to possible geographical adaptations.

Beetles thermal variability among populations is the way species response to local selective pressures to increase fitness in their local environment (Kingsolver and Huey 2008; Sinclair et al. 2012). These measurements are important as they are the way how ectotherms will act towards the changes in thermal conditions (Denlinger and Lee 2010). We have studied these variations in *Thanatophilus sinuatus* and, results show significant differences between the studied populations (Prague, Czech Republic; Frankfurt am Main, Germany and Venezia, Italy). Moreover, geographical variation not only influenced developmental times and values of LDT and ADD, but other ecological relevant measurements such as the Critical Thermal Minima (CT_{min}). The aforementioned, might be of great value for the forensic investigations as values of LDT, ADD and CT_{min} can be directly associated to the insects locality of origin. Under a climate change and global warming scenario, where decline on forensically relevant insects is evident (Amendt 2021), information of thermal responses and thermal sensibilities are to be considered as priority to understand, species specific responses, changes on necrophagous communities and, the impact on carrion decomposition.

In the last chapter of the thesis, Chapter 5, we attempt to start a new line of investigation in the thermal biology of forensically important insects. This study explored thermal sensitivity using the Chill Coma Recovery time (CCRt) measurement. This measurement are been used in economically important species (e.g., native or alien) to understand their thermal sensitivity for management and control (Athanasios et al. 2018a; 2018b). Our results showed that younger *T. sinuatus* beetles could recover faster from a Chill coma than older ones. Interestingly, old beetles tend to increase their thermal sensitivity to low temperatures when the periods of exposure last longer due to possible reproductive-survival trade-offs. Cold tolerance measurements are important for the forensic sciences as the appearance of certain species in a legal case might be subject to the species' capacity to withstand natural drops in environmental temperature. Besides, measurements used in forensic entomology are dependent (e.g., pre-appearance interval) on cold response measurements.

The previous chapters of the thesis (chapters 1-6) and the cited articles provide a broad picture of scientific literature on forensically important beetles growth and development. Many

important manuscripts were cited, still, other extent of articles that might be relevant were left uncited. This literature exhibits the range of tools and crucial information that forensic entomologists can provide and use in the legal investigations. The most frequent use of the insects in the forensic sciences is for estimating the PMI (Matuszewski 2021). Almost thirty percent (N = 282 out of 1087) of the articles published between 1998 and 2017 in forensic entomology were associated to “Estimation of postmortem interval” (Lei et al. 2019b). Intriguingly, just the ten percent of the studies were associated to “Insect development” being this one the key stone of PMI estimations. Future studies in forensic sciences ought to be done in understanding the variation of the insects development, due to the external factors variation. More research is needed to understand the thermal and developmental biology of carrion species. Thus, significantly improving the PMI estimates, hence consolidating the forensic entomology as key scientific evidence for solving legal cases.

8. Conclusions

The present thesis provides novel information regarding the developmental biology of *Thanatophilus sinautus* and *T. rugosus* to be used in the carrion ecology and, for the forensic entomologist as evidence in legal trials. Overall, this thesis fill some of the remaining gaps in the knowledge related to the thermal and developmental biology (Thermal Summation Models, CCRt) of two Central European forensically important species.

To our knowledge, The Thermal Summation Model (TSM) parameters of Lower Developmental Threshold (LDT) and Accumulative Degree Days (ADD) here calculated, are presented for the first time in both species. *T. sinuatus*, developmental times were calculated before by Dekeirsschieter (2012) at two different constant temperatures. In our work, we complement the work by the latter on the developmental times of *T. sinuatus* by breeding the species at seven different constant temperatures. Further, the times of development calculated for *T. rugosus* under six constant temperatures are provided for the first time. The TSM parameters calculated, are relevant as they can be used to provide an approximation of the age of the developmental stage found on carcasses.

At intermediate environmental temperatures, larval development was successfully completed resulting in viable adults. On the other hand, extreme temperatures, higher and lower resulted on developmental failures; high larval and pupal mortality and deformed adults. Interestingly, at the same time, in all the cases, higher temperatures were linked to the fastest development. High temperatures might be favourable in a way to have more generations during the year (Benrey and Denno, 1997) but at the expenses of an increased developmental failures.

Results of the effects of diet type on the development of *T. sinuatus* and *T. rugosus* under constant temperature provide us with an important finding. We found that *T. sinuatus* fast development was obtained when the species was fed with pork muscle followed by the pork liver and lastly the chicken muscle. Differently, *T. rugosus*, shorter development was obtained when the beetles were fed under pork liver, followed by pork muscle and lastly chicken muscle. This slight impacts on the development due to dissimilar diets suggest the species might be separated in the corpse due to preferences on the type of tissue avoid competition during the time of the year that both species might be occurring at the same time.

Parameters of TSM varied significantly for *Thanatophilus sinuatus*. This was obtained in this thesis (Montoya-Molina et al. (2020a) vs Qubaiová et al. (2023)) and, by other researchers when comparing their results with the ones from other research groups using the same species (e.g., Fratzak-Lagiewska and Matuszewski (2020) vs Wang et al. (2017) in *Creophilus maxillosus*) for their studies. The prior doesn't mean that developmental information and TSM parameters locally calculated are not useful in other geographical regions. They can be used as an approximation on the species thermal requirements and the species development performance under different environmental conditions. It is crucial to highlight the importance of these kind of studies on a narrower geographical level (population). The latter will help us understand the specific response mechanisms (e.g., thermal adaptation, thermal plasticity) used by *Thanatophilus* species to different environment thermal conditions, will give us an approximation to comprehend the species ecological function, distribution and seasonality.

Many of the models used in the forensic sciences are dependent on temperature. Hence, exploring other thermal biology measurements (e.g., CCRT) on forensically important species might help us improve the models dependant on insects occurrence (Pre appearance interval) and, improving our understanding on the functional limits of the species.

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ACTIVE RESEARCH GROUPS

1. Systematic and Forensic Entomology – **Czech University of Life Sciences Prague, Prague, Czech Republic.**
2. Detritivores and micetocoles beetles- **Federal University of Viçosa, Viçosa, Brazil.**
3. Group on Ecology, Agro-ecosystems and Natural Habitats (**GEAHNA**)- **University of Valle, Cali, Colombia.**

BASIC AND ACADEMIC FORMATION

Under-graduation studies/University

UNIVERSIDAD DEL VALLE, Biology-Entomology August 2007 – June 2014 Cali- Colombia. **Research:** Dung beetles (Coleoptera: Scarabaeinae) diversity in three land uses in the Cesar River Valley. **Advisor:** Dr. Carolina Giraldo-Echeverry Ph.D. **Co-advisor:** Dr. James Montoya-Lerma Ph.D.

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Pos-graduation/University

UNIVERSIDAD FEDERAL DE MATO GROSSO. Master in Ecology and Biodiversity Conservation (2016-2018) Cuiabá-MT, Brasil. **Research:** Taxonomic review of the *Dichotomius* (*Luederwaldtinia*) *agenor* species group (Coleoptera: Scarabaeidae: Scarabaeinae) **Advisor:** Dr. Fernando Z. Vaz-de-Mello Ph.D.

This work was supported by National Council for Scientific and Technological Development (CNPq) Grant: Protax/CNPq 440604/2015s0.

Pos-graduation/University (Ongoing)

CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE. Ph.D Student at the Ecology department, Faculty of Environmental Sciences. **Research:** An overview of the thermal biology of the Central European *Thanatophilus* (Coleoptera: Silphidae) species: carrion beetles as an important tool for the forensic sciences. **Supervisor:** doc. Ing. Jan Růžička, Ph.D. - Czech University of Life Sciences Prague; **Advisor:** Ing. Pavel Jakubec, Ph.D - Czech University of Life Sciences Prague.

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COMPLEMENTARY STUDIES

1. Workshop in Biosecurity and Genetically Modified Organisms- Universidad del Valle: March 2012
2. Course Analysis of the Diversity - Ecology Institute, A.C. INECOL. April 2014 – Mayo 2014
3. Practical identification and phylogenetic topics of American Scarabaeinae - Federal University of Mato Grosso, Brazil. October 2015.
4. Practical identification of American Scarabaeinae - Federal University of Mato Grosso, Brazil. July 2016
5. Course Determination of Latin American Staphylinidae. Lecturer: Angélico F. Asenjo (Ph.D)- UFMT, Brasil. Universidad del Cauca, 14-18 May 2018.
6. Summer course in “Systematics of Beetles 2018” University of Prague. Prague, Czech Republic. 8-21 de July 2018
7. Course of Biology and Ecology and Taxonomy of Colombian Scarabs (Coleoptera: Scarabaeoidea). 12-13 November 2020

OTHER STUDIES

1. Scientific visiting to Scarabaeiology Laboratory- Federal University of Mato Grosso, Brazil. May-July, 2014. Direct person in charge: Dr. Fernando Vaz-de-Mello Ph.D.
2. Scientific visiting at the Coleoptera area, curation of the *Dichotomius* genus, Natural History Museum London-NHML. London, United Kingdom. August - October 2017. Direct person in charge: Dr. Max Barclay Ph.D. (Coleoptera curator).
3. Doctoral Traineeship – ERASMUS. Monitoring and modelling the growth of forensic relevant flies (Diptera). Johann Wolfgang Goethe-Universität Frankfurt am Main, Institute of Legal Medicine. February – April 2022. Direct person in charge: Dr. Jens Amendt Ph.D.

PROJECTS PARTICIPATION

1. Administrative Department of Science, Technology and Innovation-Colciencias and Federación Colombiana de Ganaderos- FEDEGAN (Grant: 527-2011). Under- graduation Grant.

2. National Council for Scientific and Technological Development (CNPq) Grant: Protax/CNPq 440604/2015s0. Pos-graduation Grant.
3. Internal Grant Agency (IGA)- CZU (Prague): Larval morphology and developmental biology of Central European species of *Thanatophilus* (Coleoptera: Silphidae). IGA - Faculty of Environmental Sciences - CZU: 20194208. Research grant: 42110/1312/3159
4. Internal Grant Agency (IGA) - CZU (Prague): Intraspecific variability of thermal biology traits in *Thanatophilus sinuatus* (Coleoptera: Silphinae). IGA - Faculty of Environmental Sciences - CZU: 20194208. Research grant: 42110/1312/3145
5. University Grant Competition (UGC) - CZU (Prague): Unexplored aspects of insect mating behaviour: parasitic manipulations and necrophilous inclination in *Harmonia axyridis*. 71/2021. (Co-Investigator).

PROFESSIONAL EXPERIENCE

1. University of Valle- Assistant at the museum, Entomology.
2. GAIA FOUNDATION (Cali, Colombia)-Propuesta de declaratoria y formulación de plan de manejo para un área de bosque seco y subxerofítico de la cuenca del río grande, municipios de Restrepo, La Cumbre y Dagua, Valle del Cauca, Colombia-Inventario de coprófagos.
3. CIPAV FOUNDATION (Cali Colombia) – Project Ganadería Colombiana Sostenible (Colombian Sustainable Livestock)
4. Wildlife Conservation Society (WCS) Cali, Colombia- Inventario de escarabajos coprófagos. Monitoreo de escarabajos. Proyecto de Compensaciones por Pérdida de Biodiversidad, San José del Nus, Antioquia.
5. CALIDRIS FOUNDATION- Monitoreo biológico de los escarabajos coprófagos presentes en las unidades de muestreo existentes en las siete cuencas seleccionadas en el marco del Convenio Especial de Cooperación para la Transferencia de Tecnología No. 084 de 2013 suscrito entre CVC y RARE, INC
6. CZECH UNIVERSITY OF LIFE SCIENCES – DOHODA O PROVEDENÍ PRÁCE. Laboratorní analýzy. Odborný vědecký pracovník projektu – 221360. (1.5.2021 – 31.12.2021)
7. CZECH UNIVERSITY OF LIFE SCIENCES – DOHODA O PROVEDENÍ PRÁCE. Odborný vědecký pracovník projektu 71/2021 (May 2021 – May 2022)

RESEARCH AREAS OF INTEREST

Natural Sciences - Biological Sciences -Entomology- Ecology- Forensic Sciences- Silphidae and Scarabaeinae Taxonomy- Carrion and Dung beetles diversity.

LANGUAGES: Spanish (native speaker), English, Portuguese.

REFEREE IN CIENTIFIC JOURNALS

1. Revista Colombiana de Entomología (Colombian Journal of Entomology) – July 2018
2. European Journal of Taxonomy – June 2019; February 2021
3. Zootaxa – November 2020
4. Journal of Medical Entomology – July 2022
5. Biodiversity Data Journal – December 2022

PARTICIPATION CIENTIFIC EVENTS

1. **Name of the event:** IX Coloquio de insectos sociales –IUSSE 2013 Sección Bolivariana. **Cali, 21 al 24 de agosto de 2013**
2. **Name of the event:** II congreso Nacional de estudiantes de biología, Bogotá 28 de septiembre al 2 de Octubre de 2009
3. **Name of the event:** XX congreso Federación Latinoamericana de parasitología y XV congreso Asociación Colombiana de parasitología y medicina tropical. Bogotá 27 de septiembre al 1 de octubre de 2011
4. **Name of the event:** IUSSE Sección Bolivariana Bogotá 19, 20 y 21 de Agosto de 2009
5. **Name of the event:** Digital world conference of Forensic Entomology. September 15-18 of 2020

PRESENTATIONS IN CIENTIFIC EVENTS

1. **Name of the event:** XXIV congreso Brasileiro de Entomología. Curitiba, Brazil 16-20 September 2012. **Running title:** Diversidad de escarabajos coprófagos (Scarabaeidae: Scarabaeinae) en la cuenca del río Dagua, Valle del Cauca, Colombia. Authors: **Santiago Montoya-Molina**; Lina Marcela Isaza-López; Guillermo Reina- Rodríguez; Carlos A. Cultid-Medina
2. **Name of the event:** III Congreso Colombiano de Zoología, Medellín, Colombia. **Running title:** División morfológica y variación del forrajeo de obreras de *Atta cephalotes* (Hymenoptera: Myrmicinae) Authors: Jonathan Rodríguez, Lina M. Isaza, **Montoya-Molina, Santiago**.
3. **Name of the event:** Congreso Sociedad Colombiana de Entomología (SOCOLEN 2014). Cali, Colombia 15 -18 August 2014 **Running title:** *Digitonthophagus gazella*: Especie introducida naturalizada en los sistemas ganaderos del valle del río cesar. 41 Congreso de la Sociedad Entomológica Colombiana. Authors: Giraldo, C. **Montoya-Molina, S.**, Escobar, F. Montoya-Lerma, J. & Chará, J.
4. **Name of the event:** Congress of the Entomological Society of America ESA 62ND Annual Meeting. November 2016 Portland, Oregon, EE.UU. **Running title:** Behavior of *Digitonthophagus gazella* and native dung beetles (Coleoptera: Scarabaeinae) in silvopastoral systems in the Caribbean region of Colombia. Authors: Giraldo, C., **Montoya-Molina, S.**, Montoya-Lerma, J., Chará, J. & Escobar, F.

5. **Name of the event:** Congreso Colombiano de Zoología, Simposio de escarabajos coprófagos. Cartagena, Colombia 1-5 December 2014. **Running title:** Escarabajos (Coleoptera: Scarabaeinae) en tres fincas ganaderas, valle del río Cesar, Colombia. Authors: **Montoya Molina, Santiago;** Giraldo Echeverri, Carolina; Montoya-Lerma, James.
6. **Name of the event:** Congreso Brasileiro de Zoología, Universidade Federal de Mato Grosso, Brasil: 03/2016 **Running title:** Interacciones del escarabajo introducido *Digitonthophagus gazella* (Coleoptera: Scarabaeinae) con los escarabajos nativos en el valle del río Cesar- Colombia. Authors: Giraldo-Echeverri Carolina, **S. Montoya-Molina,** Montoya-Lerma James, Chará J.
7. **Name of the event:** XI Reunión Latinoamericana de Scarabaeoidología. **Running title:** Distribución de las especies de los géneros neotropicales *Deltorhinum* Harold, 1869 y *Lobidion* Génier 2010. Authors: **S. Montoya-Molina,** F.A. González, F Z Vaz-de-Mello Universidad de Lavras, Brasil: 10/2016
8. **Name of the event:** Congresso Brasileiro de Entomologia, Gramado, Brazil, September 2018 **Running title:** Funciones ecológicas de los escarabajos coprófagos en paisajes Ganaderos del Valle del río Cesar, Colombia Authors: **Montoya-Molina, S.,** Giraldo-Echeverri, C., Montoya-Lerma, J.
9. **Name of the event:** Congresso Brasileiro de Entomologia, Gramado, Brazil, September 2018 **Running title:** Revisão taxonômica do grupo de espécies *Dichotomius (Luederwaldtinia) crinicollis* (Coleoptera: Scarabaeidae: Scarabaeinae). Authors: **Montoya-Molina, S. & Vaz-de-Mello, F.Z.**
10. **Name of the event:** European Association of Forensic Entomology – EAFE 2019 Meeting. June 2019. **Running title:** Developmental times of the forensically important carrion beetle *Thanatophilus sinuatus* at three constant temperatures. Authors: **Montoya-Molina, S.** Jakubec, P. & Ruzicka, J.
11. **Name of the event:** XXI International Congress of Entomology (ICE 2022). **Running title:** Times of development of the forensically important carrion beetle *Thanatophilus sinuatus* (Silphidae: Silphinae) at constant temperatures. Helsinki, Finland. Authors: **Montoya-Molina, S.** Jakubec, P. & Ruzicka, J.
12. **Name of the event:** European Association of Forensic Entomology – EAFE 2022 Meeting. **Running title:** Intraspecific variability of thermal biology in *Thanatophilus sinuatus* (Coleoptera: Silphidae). University of Alcalá, Madrid, Spain. Authors: **Montoya-Molina, S.,** Jakubec, P., Ipatová, I., Qubaiová, J., Novák.
13. **Name of the event:** European Association of Forensic Entomology – EAFE 2022 Meeting. **Running title:** Finding the best way to preserve larva. University of Alcalá, Madrid, Spain. Authors: Novák, M., Jakubec, P., Mahlerová K., **Montoya-Molina, S.,** Qubaiová, J. M. Ruzicka, J.
14. **Name of the event:** Ecology and Environmental Protection, XXII International Multidisciplinary Scientific GeoConference. Surveying, Geology and Mining, Ecology and Management - SGEM 2022. **Running title:** Biotope preferences of *Oiceoptoma thoracicum* (Coleoptera: Silphidae). Varna, Bulgaria. Authors: Jakubec, P., **Montoya-Molina, S.,** Qubaiová, J., Novák, M., Větrovská, M.

I. SCIENTIFIC PRODUCTION

1. Isaza-López, L.M., **Montoya-Molina, S.**, Giraldo-Echeverri, C., León- González, J., González-Alvarado, F.A. and Montoya-Lerma, J. 2015. Nuevos registros de distribución de *Digitonthophagus gazella* (Fabricius, 1787) (Coleoptera: Scarabaeinae) en Colombia. *Biota Colombiana*.
2. C. Giraldo-Echeverri; **S. Montoya-Molina**; K. Castaño; J. Montoya-Lerma; F. Escobar, J. Chará & E. Murgueitio. 2015. Sistemas silvopastoriles intensivos: Elementos claves para la rehabilitación de la función ecológica de los escarabajos del estiércol en fincas ganaderas del valle del río Cesar, Colombia. In: 3° Congreso Nacional de Sistemas Silvopastoriles - VIII Congreso Internacional de Sistemas Agroforestales, Argentina.
3. **Montoya-Molina, S**; Giraldo-Echeverri, C; Montoya-Lerma, J; Escobar, F; Chará, J. & Murgueitio, E. 2015. Diversidad de escarabajos coprófagos en sistemas silvopastoriles del Valle del río Cesar, Colombia. In: 3° Congreso Nacional de Sistemas Silvopastoriles - VIII Congreso Internacional de Sistemas Agroforestales, Argentina.
4. **Montoya-Molina, S.**, C. Giraldo-Echeverri, J. Montoya-Lerma, J. Chará, F. Escobar, Z. Calle. 2016. *Land sharing vs. land sparing in the dry Caribbean lowlands: A dung beetles' perspective*. *Applied Soil Ecology* 11/2015; 98. DOI:10.1016/j.apsoil. 2015.10.017
5. **Montoya-Molina, S.**, Isaza-López, L.M., León-González, J. 2016. Dung beetles (Scarabaeidae: Scarabaeinae) of the Dagua river basin, Valle del Cauca, Colombia. *Dugesiana*.
6. **Montoya-Molina, S.** & Vaz-de-Mello, F.Z. 2019. Notes on taxonomy and geographic distribution of *Deltorhinum* Harold 1869 (Coleoptera: Scarabaeidae: Scarabaeinae) with description of a new species, a new identification key, and a generic transfer. *Journal of Natural History*. DOI: 10.1080/00222933.2019.1606359
7. **Montoya-Molina, S.** & Vaz-de-Mello, F.Z. 2019. Rediscovering *Dichotomius foveicollis* (Kirsch, 1871) (Coleoptera: Scarabaeinae), new combination, revalidation and other taxonomic notes. *Journal of Natural History*. DOI: 10.1080/00222933.2020.1749322
8. Franco-Galindo, L.S., Ochoa-Sanz, D.M., Isaza-López, L.M. & **Montoya-Molina, S.** 2019. Preliminary list of coprophagous beetles (Coleoptera: Scarabaeinae) from the natural reserve Cañon del Río Claro, Antioquia, Colombia. *Boletín del Museo de Entomología de la Universidad del Valle* 19(1): 21-27.
9. **Montoya-Molina S.** & Vaz-De-Mello F.Z. 2021. Taxonomic review of the *Dichotomius* (*Luederwaldtinia*) *agenor* species group (Coleoptera: Scarabaeidae: Scarabaeinae). *European Journal of Taxonomy* 734: 1–64. <https://doi.org/10.5852/ejt.2021>.
10. **Montoya-Molina, S.** P. Jakubec, J. Qubaiová, M. Novák, H. Šuláková, J. Růžička. 2021. Developmental models of the forensically important carrion beetle *Thanatophilus sinuatus* (Fabricius, 1775) (Coleoptera: Silphidae). *Journal of Medical Entomology* 58(3): 1041-1047. DOI: 10.1093/jme/tjaa255

11. **Montoya-Molina, S.** Arturo González-Alvarado, Carolina Giraldo-Echeverri, Fernando Z. Vaz-de-Mello. 2021. *Ateuchus fedescobari* – a new dung beetle (Coleoptera: Scarabaeinae) species from Colombia, and redescription of the rare *A. punctatissimum* (Génier, 2010) from Brazil. *Journal of Natural History* 55 (1-2): 115-124. DOI: 10.1080/00222933.2021.1895348
12. Qubaiová, J., Jakubec, P., **Montoya-Molina, S.**, Novák, M. Šuláková, H. 2021. Influence of Diet on Development and Survival of *Thanatophilus rugosus* (Coleoptera: Silphidae). *Journal of Medical Entomology* 58(6): 2124-2129. DOI: 10.1093/jme/tjab141
13. **Montoya-Molina, S.** P. Jakubec, J. Qubaiová, M. Novák, H. Šuláková, J. Růžička. 2021. Developmental models of the carrion beetle *Thanatophilus rugosus* (Linnaeus, 1758) (Coleoptera: Silphidae). *Scientific Reports* 11(1): 19377. DOI: 10.1038/s41598-021-98833-9
14. Qubaiová, J., Jakubec, P., **Montoya-Molina, S.**, Novák, M., Suláková, H. 2022. Diet Impact on the Development and Survival of *Oiceoptoma thoracicum* (Coleoptera: Silphidae). *Journal of Medical Entomology*, 1-6.
15. Qubaiová, J., P. Jakubec, **S. Montoya-Molina**, M. Novák, H. Šuláková. 2023. The impact of diet and photoperiodism on the life history of *Thanatophilus sinuatus* (Coleoptera: Silphidae). *Journal of Medical Entomology*, 1-7.
16. Vacek Z, Cukor J, Vacek S, Václavík T, Kybicová K, Bartoška J, Mahlerová K, **Montoya-Molina S.** 2023. Effect of forest structures and tree species composition on common tick (*Ixodes ricinus*) abundance—Case study from Czechia. *Forest Ecology and Management*. 529: 120676. DOI: <https://doi.org/10.1016/j.foreco.2022.120676>
17. Giraldo–Echeverri, C., **Montoya–Molina, S.**, Montoya–Lerma, J., Chará, J.D., Escobar, F. Is *Digitonthophagus gazella* (Fabricius, 1787), undergoing a process of naturalization?: A study case in a tropical dry forest of northern Colombia. Has been successfully submitted online to *Caldasia Journal*.
18. Clavijo-Bustos, J., Cárdenas-Bautista, J.S. Torres, E.D., Torres, M.A., Neita-Moreno, J.C., **Montoya-Molina, S.** A new species of the *Dichotomius agenor* species-group (Coleoptera: Scarabaeidae: Scarabaeinae) from Colombia. Has been successfully submitted online to *Caldasia Journal*.
19. **Montoya-Molina, S.**, Jakubec, P., Awde D.N., Qubaiová, J. Age and sex drives the chill recovery times of a forensically important beetle, *Thanatophilus sinuatus* (Coleoptera: Silphidae). Has been successfully submitted online to *Journal of Medical Entomology*.

BOOKS

1. Giraldo–Echeverri, C.; **Montoya–Molina, S.** & Escobar, F. 2018. *Escarabajos del estiércol en paisajes ganaderos de Colombia*. Fundación CIPAV.