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HENRIQUE RAFAEL PONTES FERREIRA

AVALIAÇÃO DE PADRÕES COMPORTAMENTAIS EM MODELOS EXPERIMENTAIS UTILIZANDO DIFERENTES ESTÁGIOS DA VIDA DE MOSCAS (DIPTERA: SARCOPHAGIDAE) E IMPLICAÇÕES PARA A ENTOMOLOGIA FORENSE

Recife
2024

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Tese apresentada ao Programa de Pós-Graduação em Biologia Animal, Centro de Biociências, da Universidade Federal de Pernambuco, como Requisito para obtenção do título de Doutor em Biologia Animal.

Linha de pesquisa: Biologia e Comportamento Animal

Orientador: Prof. Dr. Simão Dias Vasconcelos
Coorientador: Prof. Dr. Taciano de Moura Barbosa

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Aprovada em: 18/09/2024.

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A incapacidade mora no medo, ela consegue nos contaminar, mas a resiliência cura e mostra que só o tempo transforma a dor em força e luta.

Henrique Pontes (Autor, 2024)

RESUMO

O objetivo desta tese foi investigar aspectos comportamentais de espécies de relevância forense da família Sarcophagidae com a utilização das diferentes fases da vida de moscas, *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) e *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830). O capítulo I teve como objetivo explorar os aspectos bionômicos e reprodutivos da *P*. (*P*.) *chrysostoma*. Foi investigado dados quantitativos sobre o ciclo de vida, medições bionômicas (comprimento e peso) de larvas e pupas e descrição do desenvolvimento intrauterino de ovos e larvas. As fêmeas apresentaram ovários com ovos desenvolvidos e larvas entre 8 e 10 dias ($\overline{x}$ = 23,3 ovos/fêmea) e realizavam postura de ovos inférteis. O tempo médio de desenvolvimento para os estágios imaturos foi de 22,24 h e 21,36 h para o 1º e 2º estágios, respectivamente, o 3º estágio apresentou um tempo médio de desenvolvimento de 80,47 h, o estágio de pupa apresentou maior duração ($\overline{x}$ = 295,69 h) e longevidade de adultos foi de aproximadamente 30 dias. O objetivo do Capítulo II foi investigar a influência do fotoperíodo em aspectos reprodutivos de *P*. (*P*.) *chrysostoma* e *P*. (*S*.) *lambens*. Foi realizado: I. Teste de larviposição e II. Teste de desenvolvimento intrauterino. Os experimentos foram realizados nos regimes de fotófases (L:D): de 0:24, 6:18; 12:12; 18:6 e 24:0 por 96 h. A larviposição ocorreu na escuridão total para as espécies em todos os tratamentos ($P > 0,05$). A duração da luz afetou o percentual de fêmeas com desenvolvimento intrauterino avançado de ambas as espécies ($P < 0,05$), com maior percentual de fêmeas com estágios avançados nos tratamentos de maior duração de luz. O objetivo do Capítulo III foi avaliar modelos experimentais para investigar comportamentos de larvas de terceiro estágio (72 horas de idade) e pupas (até 24 horas de idade) de *P*. (*P*.) *chrysostoma* e *P*. (*S*.) *lambens*. Três experimentos foram conduzidos: mobilidade larval horizontal, enterramento vertical em diferentes solos e ascendência de adultos em diferentes solos e profundidades. *P*. (*S*.) *lambens* levou mais tempo para localizar a vermiculita na Arena de Mobilidade Larval (AML) e que *P*. (*P*.) *chrysostoma* demonstrou movimento direcional organizado (25% e 22% das larvas se movendo para a mesma região da AML). Maior profundidade de enterramento para *P*. (*P*.) *chrysostoma* ($\overline{x}$ = 8.96 ± 2.29). O tipo de solo ($P < 0,05$) e a profundidade de enterramento ($P < 0,05$) influenciaram as taxas de sobrevivência das espécies. Os resultados apresentados ao longo dos capítulos contribuem para o avanço do conhecimento em EF, utilizando espécies da região Neotropical, oferecendo novas linhas de investigação sobre o comportamento, reprodução e bionomia.

Palavras-chave: Desenvolvimento larval. Fotófase. Insetos Sarcossaprófagos. IPM min. Mobilidade Larval.

ABSTRACT

The objective of this thesis was to investigate behavioral aspects of forensically relevant species within the Sarcophagidae family, focusing on *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830). Chapter I aimed to explore the bionomic and reproductive aspects of *P.* (*P.*) *chrysostoma*, providing quantitative data on the life cycle, bionomic measurements (length and weight) of larvae and pupae, descriptions of intrauterine development of eggs and larvae, and analyses of egg/larrviposition behavior by gravid females. Females displayed developed ovaries with eggs and larvae between 8 and 10 days old ($\overline{x}$ = 23.3 eggs/female) and laid infertile eggs. The mean development times for immature stages were 22.24 h and 21.36 h for the 1st and 2nd stages, respectively, with the 3rd stage showing a mean development time of 80.47 h, while the pupal stage lasted the longest ($\overline{x}$ = 295.69 h), and adult longevity was approximately 30 days. Chapter II examined the influence of photoperiod on reproductive aspects of *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens*. Experiments: I. Larviposition test: females were paired with males aged over 8 days, and II. Intrauterine development test: females over 8 days old and mated were isolated individually. Experiments were conducted in photophase regimes (L:D) of 0:24, 6:18, 12:12, 18:6, and 24:0 for 96 h. Larviposition occurred only in complete darkness for both species across all treatments ($P > 0.05$). Light duration affected the percentage of females with advanced intrauterine development in both species ($P < 0.05$), with higher percentages of females showing advanced stages in treatments with longer light durations. Chapter III aimed to assess experimental models to investigate immature dipteran behaviors within the FE context. Third instar larvae (72 hours old) and pupae (up to 24 hours old) of *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* were used. Three experiments were conducted: horizontal larval mobility, vertical burial in different soils, and adult emergence in various soils and depths. *P.* (*S.*) *lambens* took longer to locate vermiculite in the Larval Mobility Arena (LMA), while *P.* (*P.*) *chrysostoma* showed organized directional movement (25% and 22% of larvae moving towards the same LMA region). Greater burial depth was observed in humus-rich soil ($P < 0.001$) for both species, with *P.* (*P.*) *chrysostoma* burying more deeply ($\overline{x}$ = 8.96 ± 2.29). Soil type ($P < 0.05$) and burial depth ($P < 0.05$) significantly influenced survival rates of both species. The findings across these chapters contribute to advancing FE knowledge, utilizing Neotropical species as models to study sarcophagids and providing new insights into their behavior, reproduction, and bionomics.

Keywords: Larval Development. Photophase. Sarcosaprophagous Insects. min PMI. Larval Mobility.

LISTA DE ILUSTRAÇÕES

CAPÍTULO 1: Bionomics, reproductive traits and assessment of forensic relevance of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae)

CAPÍTULO 2: Differential effects of photophase on the reproductive behaviour of two species of medico-legal relevance, *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) (Diptera: Sarcophagidae)

LISTA DE TABELAS

CAPÍTULO 1: Bionomics, reproductive traits and assessment of forensic relevance of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae).

CAPÍTULO 2: Differential effects of photophase on the reproductive behaviour of two species of medico-legal relevance, *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) (Diptera: Sarcophagidae).

CAPÍTULO 3: Post-feeding mobility, burial behaviour and survivorship of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) (Diptera: Sarcophagidae) under laboratory conditions

SUMÁRIO

1 INTRODUÇÃO

Os dípteros muscóides das famílias Calliphoridae, Sarcophagidae e Muscidae possuem ampla distribuição na região Neotropical. Devido ao hábito necrófago de muitas espécies dessas famílias, elas desempenham um papel vital na ciclagem de nutrientes no solo e são amplamente utilizadas como modelos para estimar o intervalo pós-morte mínimo (IPM min) (Amendt, 2017).

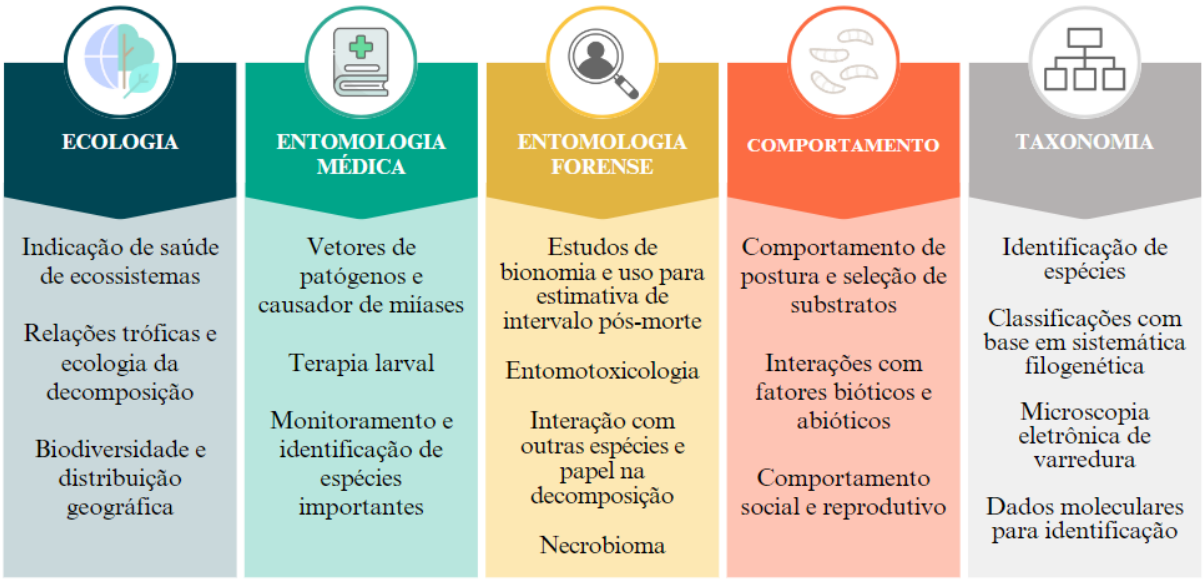
Os estudos sobre a família Sarcophagidae abrangem diversas áreas do conhecimento. Na ecologia, pesquisas exploram o papel dessas moscas na decomposição de matéria orgânica, investigando como seu comportamento necrófago contribui ecologicamente na dinâmica do processo de decomposição e como interagem com outros decompositores no necrobioma (Carvalho et al., 2004; Fremdt; Amendt, 2014; Almeida Silva; Carvalho Filho; Brasil, 2024). Na entomologia médica, os estudos focam na investigação de espécies que impactam a saúde humana e animal, seja como vetores de patógenos ou como causadoras de miíases (Pasquale et al., 2019; Martins; Barbosa; Gama, 2021; Barratt et al., 2001). Além disso, algumas espécies de Sarcophagidae têm demonstrado importância no tratamento de feridas e são utilizadas na terapia larval (Yoon et al., 2022).

Com o seu papel definido dentro do processo de decomposição, sarcofagídeos foram encontradas colonizando carcaças modelos (porcos e camundongos) em estudos forense (Barbosa et al., 2009; Jales et al., 2020; Szpila et al., 2015; Sousa; Carvalho-Filho; Esposito, 2015) e cadáveres humanos (Meira *et al.*, 2020; Oliveira; Vasconcelos, 2010).

O hábito alimentar necrófago de Sarcophagidae fornece informações pertinentes e podem ser evidências processuais, com aplicações dentro da Entomologia Forense (EF) a partir dos padrões de sucessão cadavérica e principalmente pelo tempo de desenvolvimento de larvas que são utilizados como recurso para estimar o IPM min (Anderson, 2019; Catts; Goff., 1992).

O estudo desses dípteros tem avançado significativamente em aspectos comportamentais, incluindo a descrição de padrões de movimentação e reprodução e na taxonomia com aplicações para desvendar entraves taxonômicos sobre a identificação de fêmeas e estágios imaturos (Silva Xavier et al., 2015; Silva-Xavier; Carvalho Queiroz, 2016; Barbosa et al., 2019; Dias et al., 2024) (Figura 1).

Figura 1: Eixos investigativos e principais aplicações práticas utilizando espécies de Sarcophagidae em diferentes áreas de estudo e técnicas associadas.



Fonte: Autor (2024).

Na região neotropical, aproximadamente 800 espécies são conhecidas, e no Brasil, mais de 380 espécies foram reportadas (Pape, 1996; Buenaventura et al., 2020; Mello-Patiu et al., 2024). *Peckia* Robineau-Desvoidy (1830), está distribuído no Novo Mundo, com 67 espécies descritas (Pape, 1996; Pape; Andersson, 2000; Buenaventura; Pape, 2013) e significativa relevância na EF no Brasil, ao ser encontrada colonizando cadáveres (Guimarães et al., 2022; Vasconcelos; Soares; Costa, 2014; Gaedke; Silva Mouga, 2017).

Durante a fase imatura da família Sarcophagidae, as larvas passam por três estágios, e, no terceiro, entram no período pós-alimentar, caracterizado pela busca de locais seguros para pupação. Esse comportamento ajuda a evitar predadores e condições adversas, favorecendo sua transformação em adultos e a continuidade do ciclo de vida (Denlinger, 2022). Ao atingir a fase adulta, os Sarcofagídeos exibem estratégia de busca ativa por recursos energéticos e proteicos para garantir a reprodução eficaz (Rathman; Lanza; Wilson, 1990).

O estudo dessa família é baseado nas associações ecológicas e a colonização de cadáveres pela identificação dos machos (Carvalho; Mello-Patiu, 2008; Barbosa; Mello-Patiu; Vasconcelos, 2021). A busca pela identificação de fêmeas se faz de grande valia, pois as fêmeas são mais abundantes em coletas de campo (Barbosa et al., 2021; Szpila et al., 2015). Para isso, novas aplicações têm sido propostas, por exemplo as que integram taxonomia e genética (Guo

et a., 2012; Pimsler et al., 2014), a morfometria geométrica e a miscroscopia eletrônica de varredura também (Ling et al., 2023; Bhattacherjee; Ghosh; Banerjee, 2021).

Estudos que usam fêmeas podem elucidar alguns aspectos da história de vida do grupo, como a larviposição, comportamento de voo, utilização de recursos efêmeros, competição e aspectos reprodutivos e influencia na dinâmica dos processos de decomposição. Por muitos anos, a investigação focada no tempo de desenvolvimento dos estágios imaturos em condições laboratoriais tem sido a principal abordagem para Sarcophagidae.

No processo de colonização, o estudo do necrobioma merece destaque por tentar explicar como os fatores abióticos como temperatura, umidade e fotoperíodo, quando analisados de forma integrada, podem influenciar no tempo de desenvolvimento de insetos associados, resultando em variações e consequentemente no IPM min (Amendt, 2017; Byrd; Sutton, 2020). Ainda, ao se deparar com condições favoráveis de desenvolvimento que o necrobioma fornece, os sarcofagídeos dividem esse recurso com outros insetos, de mesma guilda trófica ou que desempenham papéis como predadores e parasitoides (Voss, Spafford, Dadour, 2009; Sereno, Salvo, Battán-Horenstein, 2016; Cruz et al., 2021).

Comportamentos típicos de larvas diz respeito a mobilidade, alimentação voraz, dispersão e enterramento no solo. O momento pós-alimentar das larvas fornece informações valiosas para a história de vida do inseto, por exemplo, o tempo e a distância da dispersão horizontal e a distância vertical de enterramento (Greenberg, 1990; Jales et al., 2023).

A busca de pupas por peritos criminais é uma prática comum e deve ser incentivada, pois, pode fornecer dados mais robustos para a estimativa de IPM min. Por outro lado, há um cenário quase inexplorado na pesquisa forense quando se fala do comportamento larval, pois a depender das condições abióticas (chuva, temperatura, tipo de solo, compactação), condições bióticas (fuga de predadores e parasitoides) e uso de substâncias químicas, pode acontecer alterações consideráveis na mobilidade e dispersão de larvas (Arnott; Turner, 2008; Jales et al., 2023).

Para os adultos, o comportamento de voo e localização de recursos é mediada principalmente pela pluma odorífera liberada pelos recursos efêmeros como carcaças e cadáveres (Sukontason et al., 2004). São inatas ao inseto ações como identificação do composto, direcionamento, voo, pouso e postura no recurso (Harvey, 2024). O olfato é o principal sentido utilizado por moscas para essa interação com o meio, porém, o visual também pode mediar o direcionamento (Mcfadden; Hans, 2019).

Homicídios geralmente ocorrem em locais fechados, com pouca ou nenhuma luz e de difícil acesso ou durante a noite a demonstração experimental da localização de recursos por moscas, a deposição noturna de ovos e larvas em cadáveres nessas condições é de extrema importância. A escotofase (período sem luz) pode retardar a localização de um cadáver em ambientes ocultos (Amendt, Zehner, Reckel, 2008) e pode afetar funções fisiológicas como o desenvolvimento e deposição de larvas (Silva Mello, Borja, Carvalho-Queiroz, 2012; Carneiro et al., 2021; Amendt, Zehner, Reckel, 2008; Soares, Vasconcelos, 2016). Como resultado, a estimativa do PMI min pode ser significativamente afetada se o cadáver estiver nessas condições, atrasando assim a colonização (Amendt, Zehner, Reckel, 2008).

A complexidade comportamental dos sarcofagídeos oferece múltiplas linhas de investigação que ressaltam sua relevância para avanços na pesquisa forense. Nesse contexto, é crucial testar metodologias para a amostragem de moscas em locais de crime, visando não apenas uma coleta mais eficaz, mas também a melhoria da qualidade das evidências entomológicas. O desenvolvimento de técnicas abrangentes pode fornecer insights sobre o comportamento desses insetos, aprimorando a interpretação de dados forenses e contribuindo significativamente para a resolução de investigações criminais.

Para o desenvolvimento dessa tese, foi escolhido a família Sarcophagidae por ser um táxon pouco explorado em pesquisas científicas, principalmente no que diz respeito ao comportamento, essas lacunas são evidentes. São encontrados frequentemente colonizando cadáveres em todo o globo, novos achados fornecem perspectivas promissoras para as discussões sobre o grupo.

O objetivo desta tese foi investigar aspectos comportamentais e bionômicos de duas espécies do gênero *Peckia* que apresentam importância na EF, a partir de três eixos principais: i) comportamento: análise da larviposição de insetos adultos, bem como a mobilidade horizontal e enterramento das larvas; ii) reprodução: estudo das atividades reprodutivas de insetos adultos sob diferentes condições de fotófase; e iii) bionômicos: caracterização do tempo de desenvolvimento dos estágios imaturo, desenvolvimento intrauterino e longevidade de adultos.

2 ESTRUTURA DA TESE

O estudo foi desenvolvido em diferentes eixos temáticos, voltado para utilização de diferentes fases da vida de moscas do gênero *Peckia*, especificamente, *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) e *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830). Foi elaborado novas estratégias de investigação direcionados a elucidar aspectos pertinentes para a EF.

O capítulo I, publicado em abril de 2024 no periódico *International Journal of Legal Medicine* está estruturado dentro dos eixos de bionomia e reprodução. Teve como objetivos: (a) descrever o ciclo de vida de *P.* (*P.*) *chrysostoma*, em termos de duração dos estágios imaturos e longevidade dos adultos; (b) mensurar parâmetros bionômicos das larvas e pupas (comprimento e peso); e (c) fornecer uma descrição preliminar do desenvolvimento de ovos e larvas intrauterinos. Utilizando a espécie *P.* (*P.*) *chrysostoma*, foi observado comportamentos atípicos que são importantes para entender a sua história de vida, como a deposição de ovos, sugerindo novas descobertas na investigação sobre o comportamento tipicamente larvíparo.

O capítulo II, submetido na revista *Medical and Veterinary Entomology*, foi estruturado para eixos de comportamento e reprodução em diferentes regimes de fotófase com o objetivo de investigar a influência do fotoperíodo em aspectos reprodutivos de *P.* (*P.*) *chrysostoma* e *P.* (*S.*) *lambens*. Especificamente, abordamos as seguintes questões: i) Essas espécies larvipositam no escuro? ii) Diferenças no fotoperíodo afetam a quantidade de larvas depositadas por essas espécies? e iii) A maturação ovariana é influenciada pelo fotoperíodo? Estabelecemos linhas argumentativas pertinentes para mudanças na vida das espécies *P.* (*P.*) *chrysostoma* e *P.* (*S.*) *lambens* influenciadas pelo fotoperíodo e implicações para a EF.

O capítulo III foi desenvolvido a partir do eixo de comportamento utilizamos larvas e pupas de *P.* (*P.*) *chrysostoma* e *P.* (*S.*) *lambens* com o objetivo de desenvolver modelos comportamentais para investigação de aspectos comportamentais desses estágios, sendo mais específico: a) caracterizar o comportamento de mobilidade horizontal de larvas; b) mensurar a profundidade do enterramento vertical em diferentes tipos de solo; e c) avaliar a ascendência de adultos emergidos de pupas enterradas em diferentes profundidades e tipos de solo. Compreendemos a possibilidade da ampliação de novas discussões sobre o potencial ilimitado de comportamentos inatos dos estágios imaturos e como podem influenciar em investigações de EF e história de vida.

3 CAPÍTULO 1 - Bionomics, reproductive traits and assessment of forensic relevance of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae)

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Abstract

Peckia (*Peckia*) *chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae) is a colonizer of cadavers in the Neotropical Region. Nevertheless, data on development for the *P.* (*P.*) *chrysostoma* (e.g., instar duration) and behavioral strategies used by the species for locating and colonizing a corpse are scant. We aimed to explore bionomic and reproductive aspects of the flesh fly *P.* (*P.*) *chrysostoma*, and in this article we: (a) provide quantitative data on the life cycle of *P.* (*P.*) *chrysostoma*; (b) present bionomic measurements (length and weight) of larvae and pupae; (c) describe intrauterine egg and larvae development; and (d) analyze the ovo/larviposition behavior by gravid females. Females showed ovaries with discernible eggs and larvae between 8 and 10 days ($\overline{x}$ = 23.3 eggs/female). This study reports the first observation of egg deposition, an atypical behavior for the species. The average development time for immature stages was 22.24 h and 21.36 h for 1st and 2nd respectively, and 3rd showed an average development time of 80.47 h. Pupa had the longest duration ($\overline{x}$ = 295.69 h). A direct increase was observed in weight ($P < 0.05$) and length ($P < 0.05$) throughout time. The average survival time of males and females is approximately 30 days. This study expands the knowledge on *P.* (*P.*) *chrysostoma*, such as facultative ovoviviparity under laboratory conditions and the life cycle, which may benefit future studies for accuracy in entomology-based estimation of minimum post-mortem interval (min PMI).

Keywords: Development. Forensic entomology. Flesh fly. Min PMI. Sarcosaprophagous insects.

Introduction

Accuracy in entomology-based estimation of minimum post-mortem interval (min PMI) results from reliable data on insect development of necrophagous insect species. Mathematical formulae solely based on temperature at the crime scene associated with data on the insect's life cycle, such as the duration of immature stages, may produce many-fold variability on min PMI estimate if sources of variation are overlooked [1,2]. Confounding factors include (but are not limited to) weather conditions, time of the day, access to cadaver, chemical cues emitted by the decomposing body [3] and – importantly – physiological features related to insect egg laying and development [4].

According to Villet [1], min PMI estimates are affected not only by the time when gravid female insects reach a corpse, but also on whether oviposition is stimulated and successfully performed. In this temporal window, the time elapsed for egg development and fertilization is of critical importance. Necrophagous flies (Diptera) exhibit a variety of reproductive traits: oviparity (females oviposit eggs that develop and hatch in the external environment), ovoviviparity (females lay eggs at advanced stage of embryological development, and the larva exits the egg shell during or immediately following oviposition), viviparity (females deposit live larvae, i.e., larvae that have already hatched within the female) and pupiparity (the puparium is already formed inside the female, and the laid offspring is immobile) [5].

Precocious egg development refers to the embryonic development in the oviduct of a gravid female fly when a fertilized female has sufficient time to convert a protein meal into mature eggs but lacks suitable oviposition sites (e.g., cadaver) [1,6,7]. During this period, an egg will become fertilized as it passes the opening of the spermathecal duct, and will develop until the point where it may hatch shortly after oviposition [8]. Because criminal investigators prioritize sampling the largest (and probably oldest) larvae on a cadaver, precocious development can lead to overestimates in min PMI [6]. Lutz and Amendt [4] propose that the most appropriate way to deal with this problem could be to subtract the embryonation time from an age estimation for species where there is evidence of precocious eggs.

Curiously, plasticity in reproductive strategies has been registered for species of the most relevant families in forensic entomology, that is, Calliphoridae, Sarcophagidae, Fanniidae, Muscidae and Phoridae. Ephemeral substrates, such as dung, carcasses and cadavers, quickly undergo changes in their physical and chemical properties, and are rapidly depleted [9]. The

deposition of more advanced offspring will confer an advantage for viviparous species over an oviparous species, whose larvae may take a few days to after oviposition [5].

The influence of differential developmental times for egg- or larviposition on the colonization of cadavers has been poorly addressed in legal medicine studies. This is particularly relevant for Sarcophagidae, a family of Diptera that comprises over 3,000 species distributed across 100 genera worldwide, especially in the Neotropical Region [10,11]. Species of Sarcophagidae have evolved reproductive strategies that can facilitate prompt colonization of a cadaver, such as rapid response to chemical cues released from decomposing substrate [12] and viviparity [5]. The prevailing viviparity has been questioned by recent studies which argue that it is not an obligatory trait for all species. For example, laboratory-reared *Blaesoxipha stallengi* (Lahille, 1907) were reported to lay viable eggs under controlled laboratory conditions [10]. Nevertheless, ovoviviparity is still poorly documented among most flesh fly species [13].

Species of the genus *Peckia* (Robineau-Desvoidy, 1830) (Sarcophagidae) are frequently recorded in field inventories in the Neotropical region [14–16]. In recent years, they have been reported colonizing carcasses, animal baits, and cadavers, and have been associated with human myiasis [17–20]. *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830), in particular, has been documented in cadavers found indoors [21] which stimulates studies on bionomics and reproductive strategies under a forensic perspective.

The scarcity of quantitative data on the development time of *P.* (*P.*) *chrysostoma* limits its applicability in forensic entomology. A recent survey performed among forensic experts from Brazil shows that the lack of data on insect rearing and development is a major obstacle for the application of entomological evidence in criminal investigations [22]. Briefly, estimations of min PMI incorporate the accumulated degree-days (ADD), a value that represents the amount of continuous heat necessary for an insect to reach the developmental stage observed at the time of collection [23]. Nevertheless, before ADD tables are built, a thorough description of each stage must be based on realistic temperature conditions, which, for *P.* (*P.*) *chrysostoma* seems to be around 18 ºC and 27 ºC [24]. Under that perspective, methodical measurement of size, weight and duration of immature stages of *P.* (*P.*) *chrysostoma* can assist forensic experts in producing realistic estimates of min PMI.

As part of a scientific cooperation between entomological research centers and the forensic police in Northeastern Brazil, we were motivated to describe the developmental stages of *P.* (*P.*) *chrysostoma*, including reproductive and bionomic traits in the laboratory.

Specifically, we aimed to (a) describe the life cycle of *P.* (*P.*) *chrysostoma*, in terms of duration of immature stages and longevity of adults; (b) measure bionomic parameters of larvae and pupae (length and weight); and (c) provide a preliminary description of intrauterine egg and larva development. Because recent studies have questioned the obligate viviparity as a reproductive strategy among Sarcophagidae, exemplified in *Blaesoxipha* spp [10,13,25]. We examined the reproductive behavior through controlled experiments and investigated the possibility of facultative ovoviviparity in *P.* (*P.*) *chrysostoma*. Our underlying assumption is that the forensic potential of *P.* (*P.*) *chrysostoma* can be strengthened by providing quantitative data and practical criteria to assess its validity as entomological evidence. This species is particularly important in forensic studies, making it a valuable indicator for criminal investigations

Methods

Peckia (*P.*) *chrysostoma* used in this study were collected in an urban area in Recife (8°22'54" S, 34°56'53" W), Brazil, using suspended traps baited with a mixture of minced pork and beef based on the methodology described by Oliveira et al. [26]. The larvae were reared until adult emergence and identification, based on the observation of the terminalia of males that were temporarily anesthetized by being placed in a freezer (-20°C) for one minute. The adults were maintained under controlled conditions of temperature (25 ± 2 °C), relative humidity (60% ± 10%), and a 12:12 (Light: Dark) photoperiod. Sixty adults (30 pairs) were then reared in plastic cages (40 cm X 60 cm X 40 cm) fed on 10% sucrose solution and decomposing minced beef as a substrate for laying, under the same laboratory conditions.

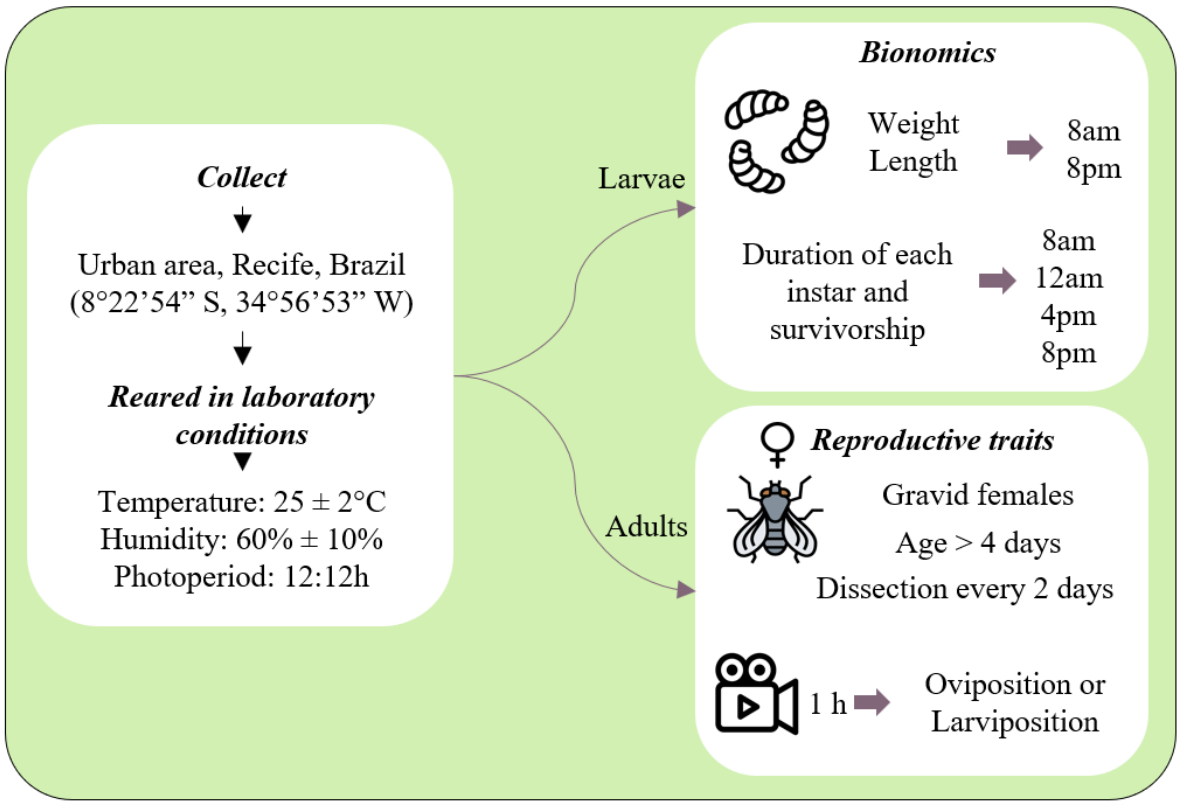
For this study, two sets of experimental procedures were designed according to the main objectives: (i) description of bionomics of the species, focusing on the duration, weight and length of immature stages, and (ii) characterization of reproductive strategies and intrauterine egg development (Fig. 1).

Bionomics: instar duration, body length and weight and survivorship

For the study of bionomics, all observations took place in the laboratory under controlled temperature (25 ± 2 °C), relative humidity (60% ± 10%) and photoperiod 12:12 (Light: Dark). A set of 10 cages containing adults and decomposing minced beef were observed

at every 1 h-interval to detect larviposition. Freshly laid larvae were transferred in groups of ten individuals to thirty plastic containers of 150 mL with minced beef (2 g per larva) as food and vermiculite as substrate for pupation. Three hundred individuals (10 larvae X 30 plastic containers) were observed daily at 08:00 h, 12:00 h, 16:00 h, and 20:00 h. The following variables related to the immature stages (2nd and 3rd instar, prepupa, and pupa) were measured: duration of each developmental stage (in hours), body length (in mm), body weight (in mg), and survivorship at each stage (%).

Figure 1: A summarized description of the variables addressed in this study, focusing on the bionomics and the observation of reproductive traits of *Peckia* (*Peckia*) *chrysostoma* reared under laboratory conditions.



We used destructive sampling, that is, at each observation time we selected a subsample of larvae for one-time measurements. Prior to each measurement, larvae were individually washed to remove any adhering food and killed by immersion in warm water (3–5 s) at 50 ºC. Immediately, body length was measured using a digital caliper rule with 0.1 mm precision, and the measurements were analyzed using ImageJ® software. Larvae were weighed using an analytical balance (Tecnal® 0.1 mg precision) and the instar was determined by examination

of the morphology of the posterior spiracles under microscope (20X). To build survivorship tables, we recorded the mortality at first, second and third instar, at the pre-pupa and the pupa stage. Insects were reared until death, and we recorded the longevity and the sex ratio of the adults.

Reproduction traits

Reproductive traits were examined in two experiments. Firstly, we characterized the intrauterine development of eggs and larvae. For this assessment, we reared groups of 20 pairs under controlled conditions and selected a subsample of 10 females at every two days. Each female at each timepoint was dissected using a stereoscopic microscope (40X magnification), for the visualization and counting of the number of eggs and intrauterin larvae. The stage of development of eggs and larvae of *P.* (*P.*) *chrysostoma* followed the protocol with adaptations proposed by Chaiwong et al. [28] who classified it into three stages: (i) initial stage, characterized by the visualization of spherical follicles; (ii) intermediary stage, characterized by the visualization of oocysts expanding to fill one-third to half of the total follicle length and (iii) advanced stage, characterized by the visualization of eggs and fully developed larvae.

In the second experiment, we recorded and quantified the occurrence of egg deposition or larval deposition, ensuring that the adults were at the appropriate age of sexual maturation and had undergone proper development for oviposition. We also evaluated the viability of the offspring for each reproductive strategy. For this purpose, we used five batches of 20 pairs of adult flies kept in plastic cages (60 x 40 x 30 cm) with decomposing minced beef ad libitum to stimulate mating and provide additional protein source. After five days, sufficient time for copulation and egg maturation, we removed the food source and maintained the adults for 72 h for complete egg maturation [28]. We then selected ten females and proceeded with the filming register of egg or larviposition, using a Sony HDR-CX130 digital camera. Each female was filmed for 60 min, a minimal time for offspring deposition, based on pilot tests. The following variables related to reproductive aspects were measured: (a) number of eggs deposited; (b) number of larvae; and (c) viability of eggs.

Data analysis

For the experiment on bionomics, we used 30 replicates, each replicate consisting of an observation unit of 10 larvae. For the experiments on reproduction, we used five replicates: each replicate consisting of an observation unit of 20 pairs. We estimated the mean values and

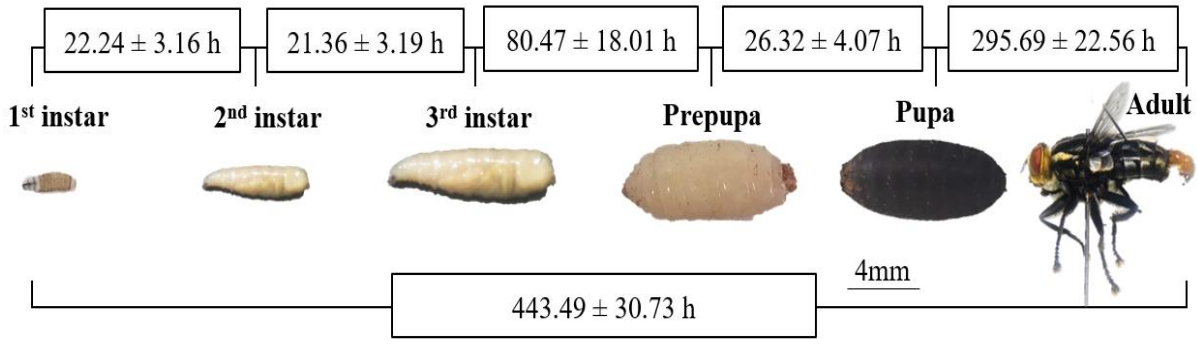
the standard deviation of the following variables: (i) larval weight, (ii) length and (iii) development time. We also calculated the survivorship for each stage and performed regression analysis to establish the relationship between larval weight and length at different stages of development and derive the corresponding model equation. The sex ratio (number of females/total number of females and males) of emerged adults and the longevity of adults were estimated, by calculating the mean ($\overline{x}$) and median (Md), in days. We analyzed reproductive traits by counting the number and the viability of eggs and larvae. Lastly, in the other experiment we calculated the mean number of eggs at each developmental stage within the gravid female. Statistical analysis was conducted using t-tests and regression, with the R software (version 4.1.2), and a significance level of $P < 5\%$ was considered.

Results

Instar duration, body size and weight, and survivorship

The total developmental time from first instar to adult, under the conditions tested, averaged 443.5 ± 30.73 h (Fig. 2), varying from a minimum of 384 h and maximum of 488 h (Table 1). The duration of the first instar (22.24 ± 3.16 h) and second (21.36 ± 3.19 h) instar did not differ significantly ($P > 0.05$), lasting less than a day. The third instar was significantly longer ($P < 0.05$), reaching 106.8 ± 19.9 h, but after approximately 80.0 h the larvae stop feeding and abandoned the food source and buried in the vermiculite, entering a phase characterized as the post-feeding stage (prepupa), which lasted, on average, 26.3 h. The pupal stage lasted 295.69 ± 22.56 h, being the longest phase of the *P.* (*P.*) *chrysostoma* cycle. The minimum and maximum duration of each stage varied, and this interval was longer in the 3rd instar (from 56 to 121 h) and in the pupa (from 264 to 336 h).

Figure 2: Developmental time (h) of immature stages of *Peckia* (*Peckia*) *chrysostoma* reared under laboratory conditions.



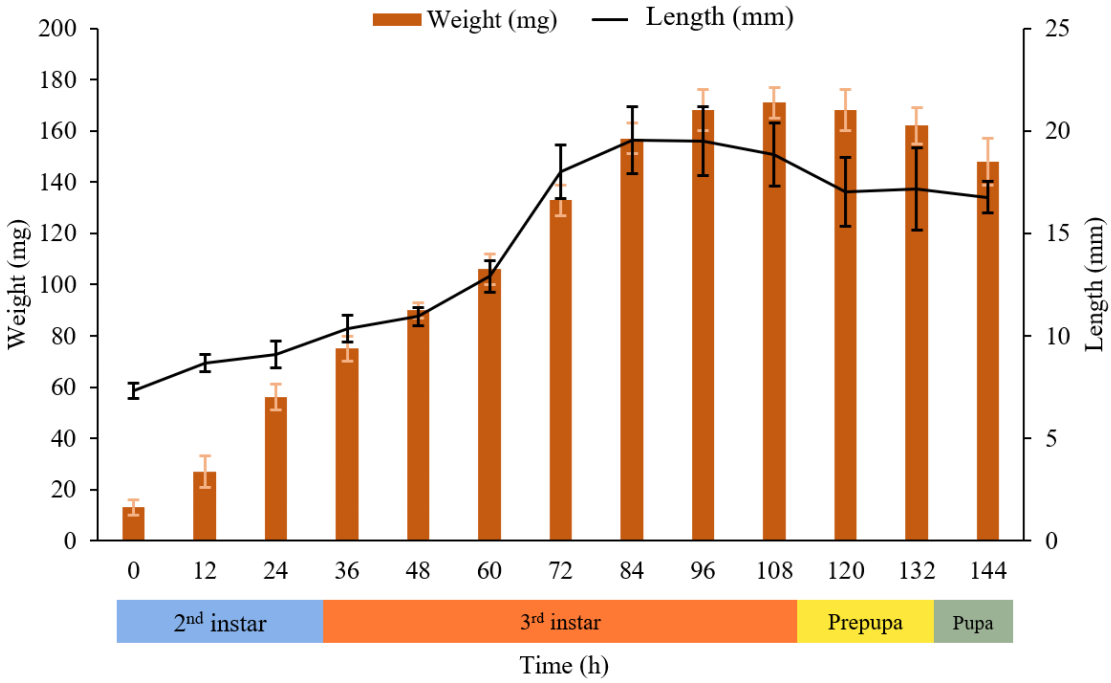
Survivorship exceeded 80% in all stages, and no mortality was observed during the transition from the post-feeding to the pupal stage. When considering the entire development process from the 1st stage to adult emergence, the overall survivorship of the species was 61.0%. The pupal stage exhibited the highest survivorship, with 90.4% of the pupae successfully reaching the adult stage, resulting in a sex ratio of 0.55, which did not favor either sex ($P > 0.05$) (Table 1).

Table 1: Estimation of the minimum and maximum development time and survivorship observed for the immature stages of *Peckia* (*Peckia*) *chrysostoma* maintained under laboratory conditions (25 ± 2 °C, 60 ± 10% RH and 12 h of photoperiod). TDT – Total development time.

Stages	Range (N) Min. – max. (h)	Range (N) Min. – max. (days)	Survivorship (%)
Egg	-	-	-
1st instar	18 – 28	0.75 – 1.17	82.67 (n= 248)
2nd instar	16 – 27	0.67 – 1.13	86.29 (n= 214)
3rd instar	56 – 121	2.3 – 5.04	94.85 (n= 203)
Prepupa	24 – 42	1 – 1.75	100 (n= 203)
Pupa	264 – 334	11 – 13.91	90.41 (n= 183)
TDT (1st instar – adult)	386 – 518	16.08 – 21.58	61.00 (n= 183)

The weight and length of the larvae increased significantly as they aged (Fig. 3) (weight: P = 2e-16, R^2 = 0.88, y = 1.195x + 31.053; length: P = 2.2e-16, R^2 = 0.80, y = 0.093x + 8.0624). Weight increased approximately 10 times from early 2nd instar (ca. 18 g) to late 3rd instar (ca. 180 g). Third instar larvae increased up to three times in weight and twice in size as they aged, with these changes being most noticeable between 36 h and 84 h. There was also a slight decrease in weight and size for the prepupa and pupa stages when compared to 3rd, that is, after 84 h.

Figure 3: Weight (mg) and length (mm) of immature stages of *Peckia* (*Peckia*) *chrysostoma* reared under laboratory conditions (25 ± 2 °C, 60 ± 10% RH and 12:12 h (L:D)).

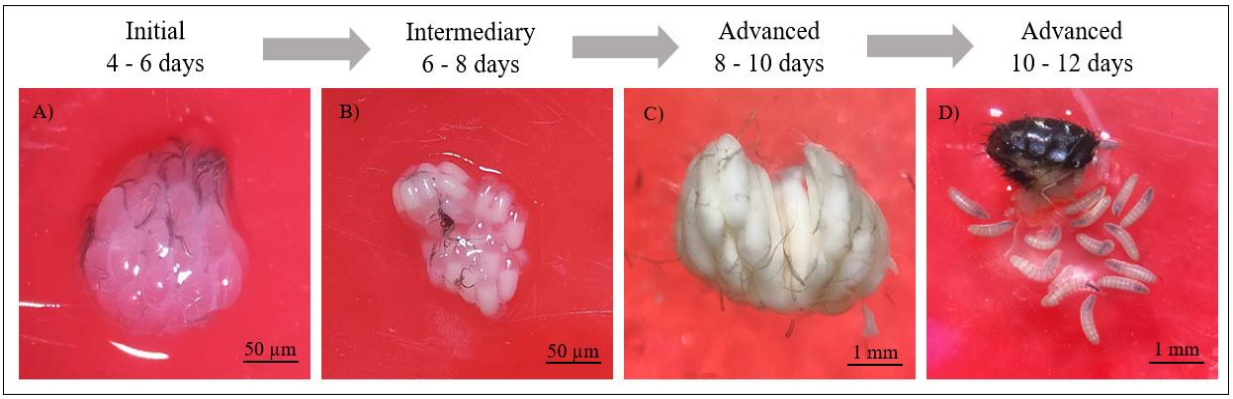


Reproductive traits

The longevity of males ($\bar{x}$ = 30.29 ± 18.27, Md: 26 days) and females ($\bar{x}$ = 31.41 ± 16.69, Md: 24) did not differ significantly (P = 0.28). Females dissected on days 4 and 6 after exposure to males had eggs at the initial or intermediate stages. Only from the 8th day, fully developed eggs were visible, categorized as advanced stage, and on the 10th day, developing larvae inside the female were evident. We observed an average of 23.9 ± 5.02 eggs in the advanced stage per female, while we observed an average of 15.3 ± 4.18 larvae per female.

All three stages of offspring development were visualized in the analysis. In the initial stages of development, it was possible to visualize spherical follicles (Fig. 4A). The intermediary stage is characterized by the first indication of visibly developing oocyte within the follicles (Fig. 4B), progressing to great elongation of the follicles as the oocyte expands. For the advanced stage, the developed oocyte appeared to fill the entire follicle, with the visualization of bright white and elongated eggs with an appearance similar to small grains of rice (Fig. 4C). The developed larvae were visualized inside the follicles and had a yellowish color with sclerotized bands in the thoracic and abdominal segments, corresponding to cuticular spines that surround the entire body (Fig. 4D).

Figure 4: Egg and larval intrauterine development of *Peckia* (*Peckia*) *chrysostoma*: A) Initial stage; B) Intermediate stage; C) Advanced stage with visualization of developed eggs; D) Visualization of developing larvae (40X).



As a complementary observation on the reproductive behaviour, approximately 80% of the adults of *P.* (*P.*) *chrysostoma* engaged in copulation within the first 6 to 8 h after emergence. Also, for ca. 75% of the observed females, the first oviposition (eggs) occurred on average, between the 8th and 10th day of life. Larval deposition was frequently observed in females averaging over 12 days old. In the recorded experiments, the group of females observed simultaneously deposited both eggs and larvae, and it was not possible to determine if a single female was oviparous, ovoviviparous, viviparous, or both (Supplementary Material #1). However, none of the laid eggs was viable, given that there was no larval hatching for the whole period of observation (15 days after oviposition). Conversely, 100% of the deposited larvae were alive. Egg deposition was more frequent in the trials using young females, accounting for

61.1% and an average of 37.2 eggs per deposition. The remaining depositions consisted of larvae, comprising 38.9% and an average of 17.2 larvae.

Discussion

We describe here the development of *P.* (*P.*) *chrysostoma*, which can be a starting point for the incorporation of entomological evidence based on the species in criminal investigations. Overall, the length and weight curves for *P.* (*P.*) *chrysostoma* follow the pattern observed in other Sarcophagidae species [29], with a significant increase in these variables in the third instar. We propose here a tentative model for fitting the bionomic variables of weight and length related to time of development ($R > 0.8$), and the appropriate curves can be fitted to predict the elapsed time until the larva reaches a specific stage, as these variables support the estimation of larval age on the day of body discovery [3].

We document the facultative oviposition strategy of *P.* (*P.*) *chrysostoma*, a behavior that has been increasingly recorded in among Sarcophagidae species, such as *Blaesoxipha* (*Gigantotheca*) *plinthopyga* Wiedemann, 1830, *B.* (*G.*) *stallengi* Lahille 1907, and *Sarcophaga* (*Liosarcophaga*) *aegyptica* Salem, 1935 [10,13,25,30]. The evidence that young *P.* (*P.*) *chrysostoma* females can deposit a large quantity of infertile eggs should be taken into account to prevent overestimation of the min PMI [1]. Facultative egg deposition can be a strategy to retain a quantity of eggs for the subsequent maturation of the larvae, ensuring that the gravid female will deposit (i) when the larvae are fully developed, or (ii) when the females encounter ideal conditions (substrates) for larval development. Delayed resource seeking behavior results in the deposition of larger, more robust larvae. This retention period can be equivalent to the embryonic development time of eggs in calliphorid flies, for example, ranging up to 24 hours [31]. We reinforce the suggestion by Lutz and Amendt [4] that the estimation of embryonation time should be subtracted from the age estimate for the species when early egg evidence is present and variations can affect the PMI min.

The deposition of infertile eggs by typically viviparous species, although not commonly reported, has been recorded for *Calliphora augur* Fabricius, 1775 [32], *C. hilli* Patton, 1925 [33] and *C. dubia* (Macquart, 1855) [34], viviparous species that can alternatively lay soft and infertile eggs for a few days prior to regular larviposition, mirroring the observations made here for *P.* (*P.*) *chrysostoma*. This behavior was not observed it in natural conditions for

Sarcophagidae, even with repeated sampling [35]. We believe that the deposition of infertile eggs during the female's sexual maturation may be a strategy to reduce the offspring and prioritize the more viable progeny, thus avoiding increased competition among the immatures. Larviparity is an efficient characteristic to avoid interspecific competition for resources, as seen in predatory species of *Chrysomya* (Diptera: Calliphoridae) [36]. This is similar to what is observed in *Lucilia cuprina* (Wiedemann, 1830) (Calliphoridae), where the reabsorption of some oocytes occurs to ensure the survival of a smaller brood in response to low food availability [37].

According to Villet et al. [1], the accuracy and precision of the information used in predictive models of min PMI are the main challenges faced by forensic entomologists. Based on the data obtained here, we propose qualitative criteria adapted from Vasconcelos et al. [38] to validate the relevance of *P.* (*P.*) *chrysostoma* in legal medicine investigations, as presented in Table 2.

Table 2. A qualitative assessment for the validation of *Peckia* (*Peckia*) *chrysostoma* (Diptera: Sarcophagidae) as a species of potential forensic importance (adapted from Vasconcelos et al. 2023). Legend: - = not important; + = little importance; ++ = moderate importance; +++ = strong importance; ? = unknown.

Criteria	Category	Ref
Likelihood of cadaver colonization	++	[21]
Carrion colonization	+++	[16,39,40]
High abundance colonizing carrion	+	[39,41]
Occurrence in specific countries or geographic regions	-	[18,38,40,42]
Association to specific types of habitats, such as urban or forested areas	-	[16,39,42,43]
Seasonal occurrence throughout the year (Potential use as indicators of season of death, e. g., rainy or dry seasons)	-	[16,44]
Experimental data on reproductive strategies	+	this study
Protocol for rearing under laboratory conditions	+++	this study, [24,45]
Availability of identification keys	++	[27]
Comprehensive morphological description of all stages	++	[46]

Estimation of min PMI (data on bionomics, development, instar duration)	++	this study, [24]
Potential use in forensic entomotoxicology	+	[39]
Available data in genetic databases (molecular identification)	++	[47]

Necrophagy is the main feeding habit of *P.* (*P.*) *chrysostoma*, so its frequent association with cadavers, associated with its ability to rapidly locate animal carcasses as quickly as Calliphoridae species typically regarded as early colonizers, reinforce its forensic relevance [21,39].

The species occurs in many environments in the Neotropical Region – rainforests, agroecosystems, dry forests, urban and forested areas – with overlap of habitats – both indoors and outdoors; thus, its value as an indicator of site of death is insubstantial [13,18,31,36,38,42]. It also occurs throughout the year, in dry and rainy periods in Brazil [16,43] so that it is an unpredictable indicator of season of death.

The species is relatively easy to rear in the laboratory [24], which makes it amenable to bionomical studies using available protocols. It appears to show a direct response to laboratory-tested variables such as temperature, photoperiod, type of substrate, among others, so it can be used as model for further field variables. Documented association of *P.* (*P.*) *chrysostoma* with drug-intoxicated carcasses [39] justifies its use as a tool for the detection of chemical compounds (forensic entomotoxicology). The presence of genetic data in databases [47] also provides valuable information for investigations involving molecular biology-based identification.

The scarcity of data on the colonization of cadaver's victims of different conditions of death hinders an objective assessment of *P.* (*P.*) *chrysostoma* as an additional tool in elucidating the type of death, but a congeneric *Peckia* sp. has been sampled from a human corpse found hanging in Northeastern Brazil [48. For the estimation of the min PMI, bionomic data regarding the duration of larval instars are limited, but this study provides a starting point for development time and complements it with variables such as weight and length for the validity of the min PMI. We hope that our findings stimulate further studies to validate the species' suitability for legal medicine investigations in areas of similar landscape and climatic conditions. Such detailed information contributes to a better understanding of the species and enhances its potential as a forensic tool.

Conclusions

We provide an integrative description of the bionomics and reproductive traits of a flesh fly species, *P.* (*P.*) *chrysostoma*, which holds significance in the fields of medico-veterinary and forensic sciences. The results shed light on the development time and survivorship of immature stages, providing insights into the ovarian development and highlighting the ability to deposit eggs as a reproductive strategy that was previously poorly understood within the Sarcophagidae family.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1007/s00414-024-03242-y.

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Author contributions

All authors contributed to the study conception, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, roles/writing - original draft, writing - review & editing.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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4 CAPÍTULO 2 - Differential effects of photophase on the reproductive behaviour of two species of medico-legal relevance, *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) (Diptera: Sarcophagidae)

Manuscrito submetido à edição especial de Entomologia Forense do periódico Medical and Veterinary Entomology

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Abstract: The aim of this study was to assess the light regimes under which females of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) would larviposit and understand how these variables influence intrauterine development in these flies. The experiments were conducted in an experimental room (lux = 100) in two independent experiments: I. Larviposition test: Females mated with males of over 8 days old. II. Intrauterine development test: females over 8 days old and mated, were individually isolated. Both experiments were performed under photophases (L:D) of 0:24, 6:18; 12:12; 18:6 and 24:0 for a period of 96 h. Larviposition occurred in total darkness for *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* in all treatments, without significant differences among photophase treatments ($P > 0.05$). Photophase influenced the timing of larviposition, occurring as early as 24 hours in 12:12 L:D conditions, while no larviposition was observed in the first 24 hours in the 0:24 treatment for either species. The light duration significantly affected the percentage of gravid females of both species ($P < 0.05$), with higher percentages of gravid females in treatments of longer light duration. Females at the initial and intermediate stages of egg development were more strongly associated with treatments of continuous darkness (0:24) or short photophase (6:18), whereas females with an advanced stage of egg development were more prevalent in the 12:12 treatment. The implications of these findings for forensic entomology are profound, challenging conventional knowledge by revealing that necrophagous insects are not limited to diurnal activity patterns.

Keywords: Forensic entomology; larviparity; flesh flies; circadian rhythm; photoperiodism.

Resumo: O objetivo deste estudo foi avaliar a influência de regimes de luz para fêmeas de *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) e *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) na deposição de larvas e entender como essa variável influencia no desenvolvimento intrauterino dessas moscas. Dois experimentos independentes foram conduzidos em uma sala de experimentação (lux = 100): I. Teste de larviposição: Fêmeas acasaladas com machos com

mais de 8 dias de idade. II. Teste de desenvolvimento intrauterino: fêmeas com mais de 8 dias de idade e acasaladas foram isoladas. Ambos os experimentos foram realizados sob os regimes de luminosidade (Light:Dark) de 0:24, 6:18; 12:12; 18:6 e 24:0 h por um período de 96 horas. A larviposição ocorreu em total escuridão (0:24) para *P.* (*P.*) *chrysostoma* e *P.* (*S.*) *lambens*, sem diferenças significativas na quantidade de larvas depositadas entre os tratamentos ($P > 0,05$). A duração da luz influenciou o momento da larviposição, ocorrendo nas primeiras 24 horas na condição de 12:12 L:D, enquanto nenhuma larviposição foi observada nas primeiras 24 horas no tratamento de 0:24 para ambas as espécies. A duração da luz afetou significativamente a porcentagem de fêmeas grávidas de ambas as espécies ($P < 0,05$), com percentuais mais altos de fêmeas grávidas em tratamentos de maior duração de luz. Fêmeas nos estágios inicial e intermediário de desenvolvimento intrauterino foram mais fortemente associadas a tratamentos de escuridão contínua (0:24) ou pouca luz (6:18), enquanto fêmeas com um estágio avançado de desenvolvimento de ovos foram mais prevalentes no tratamento de 12:12. As implicações dessas descobertas para a entomologia forense são profundas, desafiando o conhecimento convencional ao revelar que insetos necrófagos não estão limitados a padrões de atividade diurna.

Palavras-chave: Entomologia forense; Larviparidade; Moscas da carne; Ritmo circadiano; Fotoperiodismo.

INTRODUCTION

Internal circadian clocks regulate physiological processes in insects, with consequences for their development, reproduction and behaviour. Circadian-based models proposed nearly one hundred years ago (Bünning, 1936) suggest that separate ‘dawn’ and ‘dusk’ oscillators are involved, with behavioural and developmental events accelerated or delayed according to the length of the photophase (Saunders, 2012). For most insects, simple one-day information on day length is not sufficient to change photoperiodic phenotypes, and a certain number of short or long days are required (Hamanaka et al., 2023). This suggests that the presence of a counter mechanism that accumulates unknown signal titres up to an internal threshold to trigger photoperiodic effects for a certain period, so that above an internal threshold, the neurosecretory system switches on or shuts off the endocrine system (Hamanaka et al., 2023).

Information on diel activity of insects can help elucidate key aspects of applied entomology. For example, hourly variations in plant-pollinator activity during the day can shape entire plant communities (Nagano, 2023) whereas the mating, oviposition and emergence of parasitoids used in biological control of insect pests are highly dependent on the timing of the the photophase (Teng et al., 2023). In medical entomology, the efficiency of chemical

control of mosquito vectors depends on knowledge of the adult activity at different hours of the night (Wilke et al., 2023).

Understanding of the influence of photophase (exposure to light) and scotophase (darkness) on the behaviour of species of medico-legal importance is poorly supported by quantitative data. Blowflies (Diptera: Calliphoridae) and flesh flies (Diptera: Sarcophagidae) are essentially diurnal in their foraging and mating activity (Smith et al., 2016; Soares and Vasconcelos 2016). In a rainforest fragment, *Chrysomya albiceps* (Wiedmann, 1819), *C. megacephala* (Fabricius, 1794), *Hemilucilia segmentaria* (Fabricius, 1805) and *H. semidiaphana* (Rondani, 1850) (Calliphoridae) were only registered in diurnal samplings (Soares and Vasconcelos, 2016). Similarly, *C. albiceps* and *Cochliomyia macellaria* (Fabricius, 1775) were not collected in nocturnal samplings in a dry forest (Oliveira and Vasconcelos, 2020).

Darkness seems to hinder flight and oviposition in *Calliphora vicina* Robineau-Desvoidy, 1830 (Saunders; Cymborowski et al., 2008; Bonacci et al., 2016), and to delay the larval development of *C. albiceps* (Silva Mello et al., 2012). Records of nocturnal oviposition of Calliphoridae species vary from none (Baldridge et al., 2006; George et al., 2012) to low (Amendt et al., 2007). However, studies performed worldwide have challenged the assumptions of exclusive diurnal activity for forensically-important dipteran species. Greenberg (1990) reported low frequency of nocturnal flight and oviposition of *C. vicina*, *Lucilia sericata* (Meigen, 1826), and *Phormia regina* (Meigen, 1826) in the United States, whereas *C. vicina*, *C. megacephala*, and *Chrysomya rufifacies* (Macquart, 1843) were reported to fly and lay eggs at night (Singh and Bharti 2001).

Because homicides tend to occur mostly at night, that is, in the absence of natural light or under conditions of confinement with limited access to luminosity, the experimental demonstration of nocturnal egg and larval deposition on cadavers is of utmost importance. The scotophase may also delay the location of a cadaver in concealed environments (Amendt et al., 2008). As a result, the estimation of minimum PMI can be significantly affected if the cadaver is located in these conditions, thus delaying colonization (Amendt et al. 2008). Similarly, for sarcophagid species the effect of light:dark cycles on intrauterine larval development, copulation, fertility, and laying behaviour are all poorly understood.

To our knowledge, Sarcophagidae species have not been recorded in nocturnal sampling in the Neotropical region. Reproduction during the scotophase has not been

documented either. Females of Sarcophagidae have an average lifespan of approximately 30 days and can deposit larvae in successive events throughout their life, but tend to deposit a higher number of larvae in the initial days after reaching the adult stage (Nascimento et al., 2020; Silva Xavier et al., 2015). Recognition of a substrate for larval development is therefore critically dependent on short-term clues, either visual or olfactive (Gomes et al., 2007). The extent to which visible light determines the likelihood of larviposition remains unknown.

Sarcophagid species predominantly exhibit larviparous behavior, although a few cases of oviparity have been reported under laboratory conditions (Charabidze et al., 2015; Barbosa et al., 2019). Oocyte development is characterized by an initial period of mitotic division and tissue differentiation, which later leads to the proliferation of follicular cells and oocyte growth, along with the formation of vitelline membranes and chorion (Kokaro 1983). Follicular cell development and vitellogenesis are influenced by body size and dietary protein (Kokaro 1983; Kenny et al., 1992). The stages of ovarian growth and vitellogenesis have been described for *Sarcophaga argyrostoma* (Robineau-Desvoidy, 1830), and the rate of ovarian maturation was unaffected by the photoperiod but influenced by body size and protein meal (Kenny et al., 1992).

Here, we investigated the influence of photoperiod on reproductive aspects of two forensically relevant flesh fly species, *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830). We use these species because they have been reported as colonizers of human cadavers, and the females deposit first instar larvae on recently deceased bodies (Vasconcelos et al., 2014; Gaedke and Silva Moura, 2017). Specifically, we addressed the following questions: **i)** Do *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* larviposit in the dark? **ii)** Do differences in photoperiod affect the number of larvae laid by these species? and **iii)** Is ovarian maturation affected by photoperiod? The findings of the study are discussed in the context of forensic entomology.

METHODS

Insect collection and maintenance

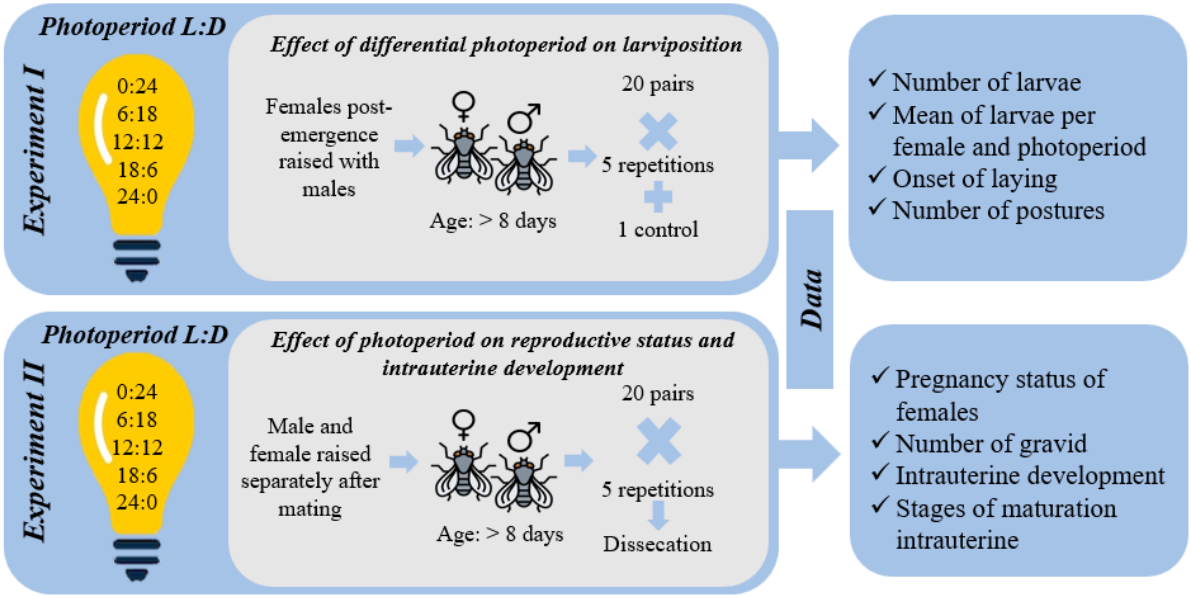
Specimens used in the experiments were obtained from laboratory colonies reared for at least three generations under controlled conditions of temperature (25 ± 2 °C), relative humidity (70% ± 5%), and photoperiod of 12:12 h light:dark. Adults were reared in 5-litre cages, provided with a 50% sugar solution in water *ad libitum*, and induced to oviposit with decomposing ground beef. Twenty-four hours before the experiments, recently emerged adults were transferred to transparent cages (40 cm x 60 cm x 40 cm) and decomposing ground beef was provided to stimulate ovarian development.

The trials took place in an experimental room with controlled humidity and temperature, sealed to prevent any external light except for the photoperiod treatments. Light intensity was measured using a lux meter (TLUX100, Incoterm®) (Figure 1). Two experiments were performed, constituting separate and independent observations with distinct objectives (Figure 1).

Experiment I: Effect of differential photophase on larviposition

A group of 200 recently-emerged adults (100 males and 100 females) were maintained together in a transparent cage (40 cm x 60 cm x 40 cm) and allowed to copulate for eight days. During this period, they were exposed to a 12:12 L:D cycle and in the absence of a substrate for larviposition. Preliminary tests indicated that this period was sufficient for mating but not for larviposition. After this period, we confirmed pregnancies by randomly selecting ten females from additional cages maintained under identical conditions, and by dissecting those females as described previously (Chaiwong et al., 2012). After confirming that a minimum of 80% of females were pregnant, we proceeded with the experimental trials.

Figure 1: Experimental design and variables addressed in the assessment of larviposition and intrauterine maturation in *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens* reared in different light:dark replicates.



Prior to the experiments, we transferred 20 pairs of flies of each species to an experimental cage (20 cm x 30 cm x 40 cm), with decomposing ground beef (50 g) to incite larviposition. Five cages were used, each assigned to one of the following photophases (L:D): 0:24, 6:18; 12:12; 18:6 and 24:0. At every 24 h interval, we observed larviposition and replaced the substrate. The procedure lasted 96 hours, during which time we registered the following variables: **i)** the occurrence of larviposition; and **ii)** the number of larvae deposited.

Experiment II: Effect of photophase on reproductive status and intrauterine development

For this experiment, we selected recently emerged adults from pupae previously maintained individually in plastic vials. The adults were sexed, and 20 pairs of each species were transferred to an experimental cage (20 x 30 x 40 cm) containing 50 g of decomposing ground beef to stimulate larviposition, under a 12:12 L:D photoperiod. They remained for eight days to allow mating and were then separated to ensure that all females were at the same mating stage and had the same ovarian maturation.

After this period, each cage was assigned to one of the following photophases 0:24, 6:18; 12:12; 18:6, and 24:0 (L:D), maintained for 96 hours, with decomposing ground beef.

We then proceeded to assess successful fertilization by examining the ovarian development of females in each treatment. To accomplish this, females were dissected in distilled water under a stereoscope (40x magnification) to ascertain their reproductive status, which we categorized as either pregnant or non-pregnant. For pregnant females, the stage of ovarian development was categorized according to the classification proposed by Chaiwong et al. (2012), with adaptations in: **I** – *initial* stage (observation of spherical follicles); **II** – *intermediate* stage (observation of oocysts within the follicles), and **III** – *advanced* stage (observation of elongated eggs and/or fully developed larvae).

Data analysis

In Experiment I, the number of larvae deposited was recorded daily for each light regime. The mean number of larvae per female for each cage (20 females), the mean number of larvae per light regime (number of larvae divided by the number of positive observations of larvae), and the range (minimum and maximum number of larvae deposited). To evaluate larviposition in different light regimes, reference indices were utilized:

I) Positivity Rate (PR): the number of positive events, i.e., number of cages in which larviposition was observed at 24 h intervals in each light regime:

$$\frac{Total\ number\ of\ positive\ observations}{Total\ number\ of\ observations}$$

II) Daily Reproductive Efficiency (DRE): that assesses the temporal distribution of larviposition, calculated daily, with a total cumulative value for each photophase also considered.

$$\frac{Total\ number\ of\ larvae}{Total\ number\ of\ females}\ X\ Number\ of\ days$$

III) Fecundity, expressed as the mean Larviposition Rate per Female (LRF)

$$\frac{Total\ number\ of\ larvae}{Total\ number\ of\ females}$$

After confirming the normality of the data using the Shapiro-Wilk test, we performed an analysis of variance (ANOVA) with Tukey's *post hoc* test to assess the influence of photophase on the quantity of deposited larvae and DRE for both species. In case of non-normality, the non-parametric Kruskal-Wallis test and the Bonferroni *post hoc* test (Pohlert,

2014) were performed. The positivity of larval occurrence in each treatment was compared using Fisher's test, based on a contingency table and event counting (positive (1) = presence of larvae, negative (0) = observation without larvae).

For the comparison of qualitative variables (pregnancy status of females and ovarian developmental stage), the Chi-squared test was employed, and the developmental ovarian status was assessed using Multiple Correspondence Analysis (MCA). All tests were conducted with a significance level of $P< 0.05$, using R® software version 4.3.1.

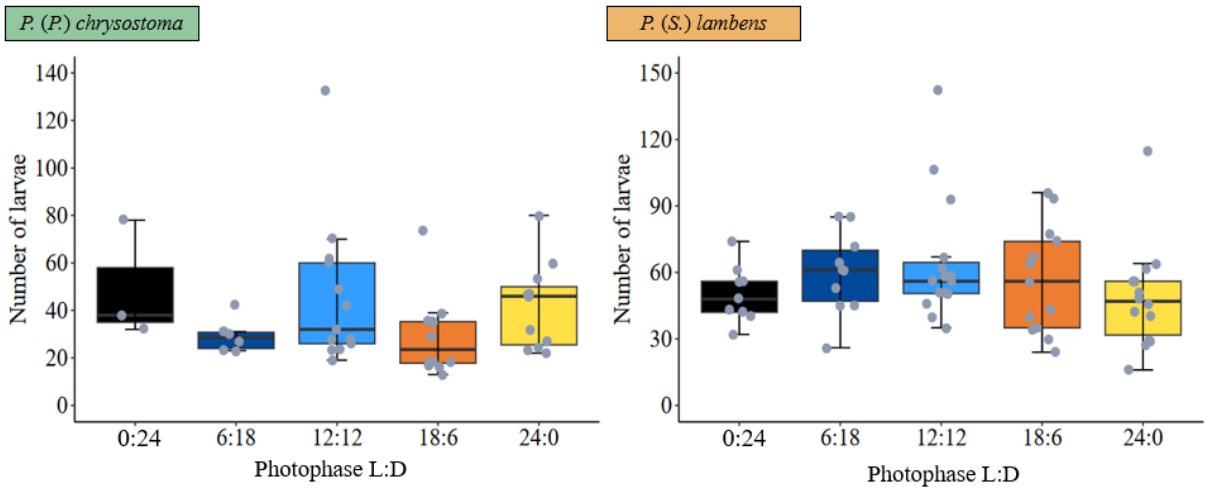
RESULTS

Experiment I: Effect of photophase on larviposition

Larviposition occurred in total darkness for both *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens*. Indeed, the event of larviposition was recorded under all photophases, without significant differences between them ($P > 0.05$). Photophase influenced the temporal pattern of reproduction. Larviposition was observed as early as 24 hours after the start of the experiment in cages exposed to light, while no larviposition occurred in the first 24 hours in experimental cages in continuous darkness (0:24 L:D) for both species.

Peckia (*S.*) *lambens* deposited a higher number of larvae during the 96 hours experimental period (N = 3,434) compared to *P.* (*P.*) *chrysostoma* (N = 1,729) ($\chi^2 = 20.38$, df = 1, $P < 0.001$). No variation was observed in the number of larvae deposited across the different photophases for *P.* (*P.*) *chrysostoma* ($\chi^2 = 6.92$, df = 4, $P = 0.14$), or for *P.* (*S.*) *lambens* ($\chi^2 = 4.65$, df = 4, $P = 0.32$). Additionally, throughout the analyzed period, the median values of larvae deposited and the distribution of collected data did not differ across different photophases (Figure 2).

Figure 2: Number of larvae laid by (A) *Peckia* (*Peckia*) *chrysostoma*, and (B) *Peckia* (*Sarcodexia*) *lambens* under different photophases in the laboratory. Values represent each observational unit, which comprises the sum of 20 females.



Overall, the number of offspring by both species was low in the 96-h period, independently of the light regime. The mean number of larvae deposited per *P.* (*P.*) *chrysostoma* varied from 2.47 ± 1.25 (under 0:24 L:D), ranging from 32 to 38 larvae per observation unit (20 females) per day to 2.23 ± 1.60 (12:12 L:D), with a range of 19 to 133 larvae per observation. Mean number of larvae laid by *P.* (*S.*) *lambens* varied from 2.51 ± 0.63 (under 0:24 L:D), ranging from 32 to 74 larvae per observation to 3.23 ± 1.41 (12:2 L:D), ranging from 35 to 142.

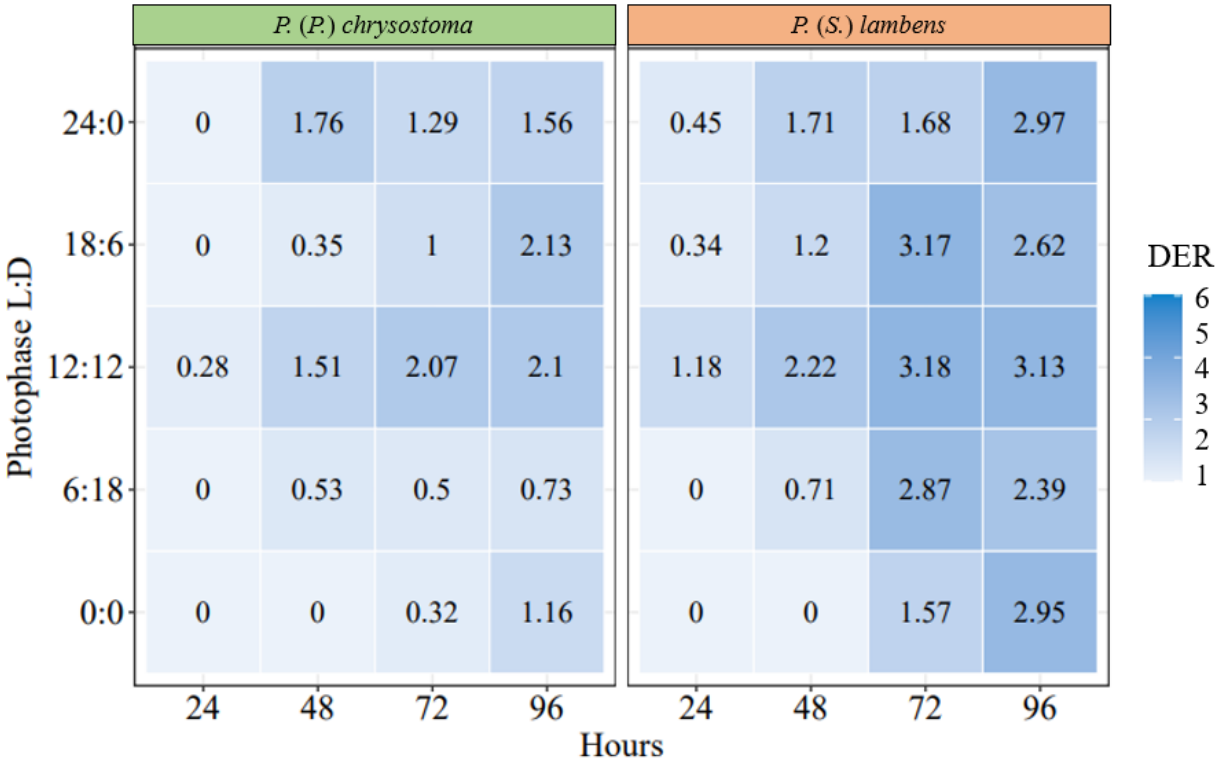
In the DRE per photophase, the lowest values were concentrated in the 0:0 and 6:18 regimes, and tripled in the 12:12 condition for *P.* (*P.*) *chrysostoma* and approximately doubled for *P.* (*S.*) *lambens*. The positivity rate also showed a significant increase for *P.* (*P.*) *chrysostoma* ($P < 0.001$), with lower values for the 0:24 and 6:18 photophases and a variation from 0.15 in 0:24 to 0.65 in 12:12. There was no significant difference in PR for *P.* (*S.*) *lambens* ($P = 0.25$), with a variation from 0.45 in 0:24 L:D to 0.75 in 12:12 (Table 1).

Table 1: Mean number of deposited larvae, range, larvae per female ratio, and cage positivity for each light regime for *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens*. $\bar{x}$: mean; SE: Standard error; DRE: Daily Reproductive Efficiency; PR: Positivity Rate.

Species	Photophase (L:D)	Larvae per female ($\bar{x}$+ SD)	Range of larvae deposited (N)	Larvae per cage ($\bar{x}$ + SD)	DRE	PR
Peckia (*P.*) *chrysostoma*	0:24	2.47 ± 1.25	32 – 38	49.33 ± 25.0	4.2	0.15
	6:18	1.47 ± 0.35	23 – 42	29.33 ± 7.06	5.08	0.3
	12:12	2.23 ± 1.6	19 – 133	45.84 ± 31.1	17.32	0.65
	18:6	1.47 ± 0.81	13 – 74	29 ± 16.9	10.32	0.6
	24:0	2.09 ± 0.91	22 – 80	41.9 ± 18.34	13.16	0.55
Peckia (*S.*) *lambens*	0:24	2.51 ± 0.63	32 – 74	45.11 ± 10.97	18.08	0.45
	6:18	2.98 ± 0.9	26 – 85	49.4 ± 17.03	23.88	0.5
	12:12	3.23 ± 1.41	35 – 142	50.66 ± 26.95	38.84	0.75
	18:6	2.81 ± 1.2	24 – 96	50 ± 21.27	29.32	0.65
	24:0	2.43 ± 1.18	16 – 115	42.85 ± 21.24	27.24	0.7

The number of larvae increased with time of observation in all treatments. The highest DRE was observed at the 96-h period for both *P.* (*P.*) *chrysostoma* ($\chi^2 = 12.021$, df = 3, $P < 0.005$) and *P.* (*S.*) *lambens* ($\chi^2 = 16.8$, df = 3, $P < 0.001$). Overall, DRE did not differ between the photophases for either *P.* (*P.*) *chrysostoma* ($\chi^2 = 4.71$, df = 4, $P = 0.31$) or *P.* (*S.*) *lambens* ($\chi^2 = 4$, F = 0.44, P = 0.7). However, DRE was higher for *P.* (*P.*) *chrysostoma* when compared to *P.* (*S.*) *lambens* ($\chi^2 = 8.5238$, df = 1, $P < 0.005$) (Figure 3).

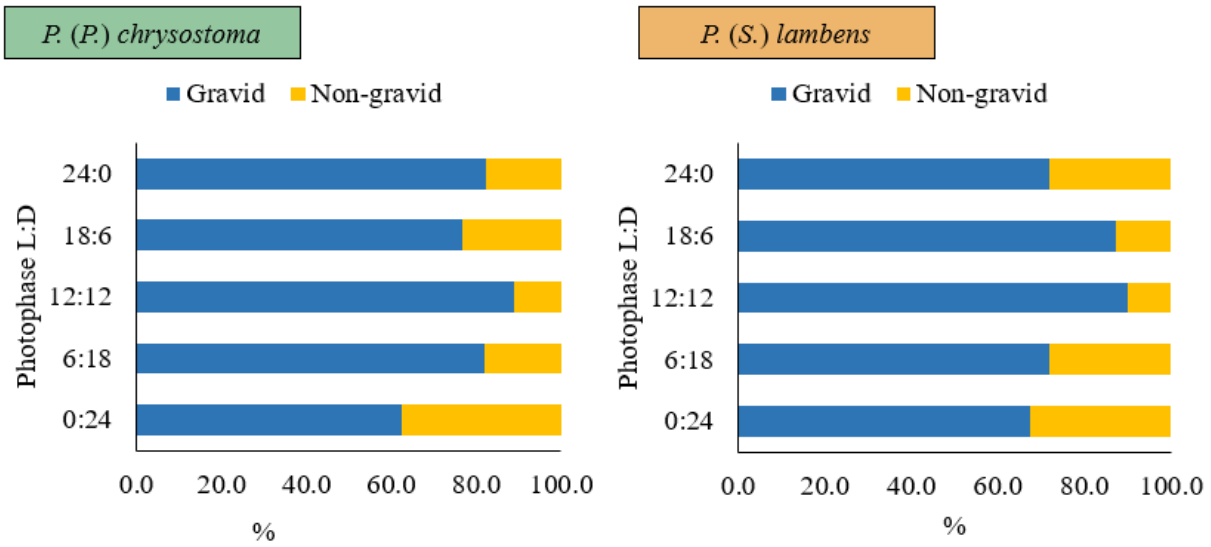
Figure 3: Daily Reproductive Efficiency (DRE) of *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens* under different photophases in the laboratory.



Experiment II: Effect of photophase on reproductive status and ovarian development

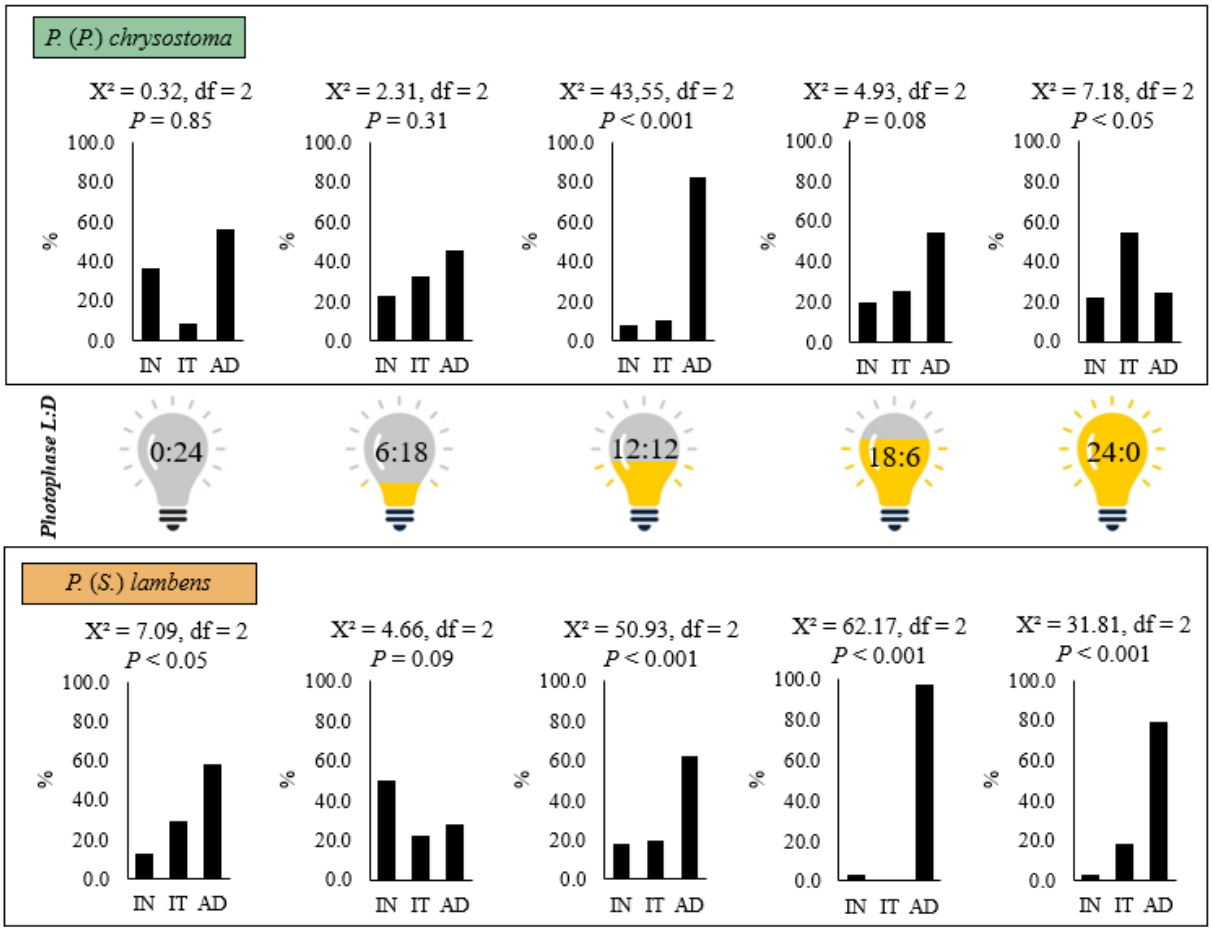
Photophase duration affected the percentage of gravid females of both species ($P < 0.05$), with higher percentages of gravid females in cages exposed to longer light duration. The effect was similar for both species. In total darkness, 37.5% of *P.* (*P.*) *chrysostoma*, and 32.7% of 37.5% of *P.* (*S.*) *lambens* were not gravid. A significantly higher ($P < 0.05$) percentage of gravid females occurred under 12:12 L:D, in similar proportions for both species, 88.9%, for *P.* (*P.*) *chrysostoma* and 90.0% for *P.* (*S.*) *lambens* (Figure 4).

Figure 4: Percentage of gravid and non-gravid females of *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens* under different photophases in the laboratory.



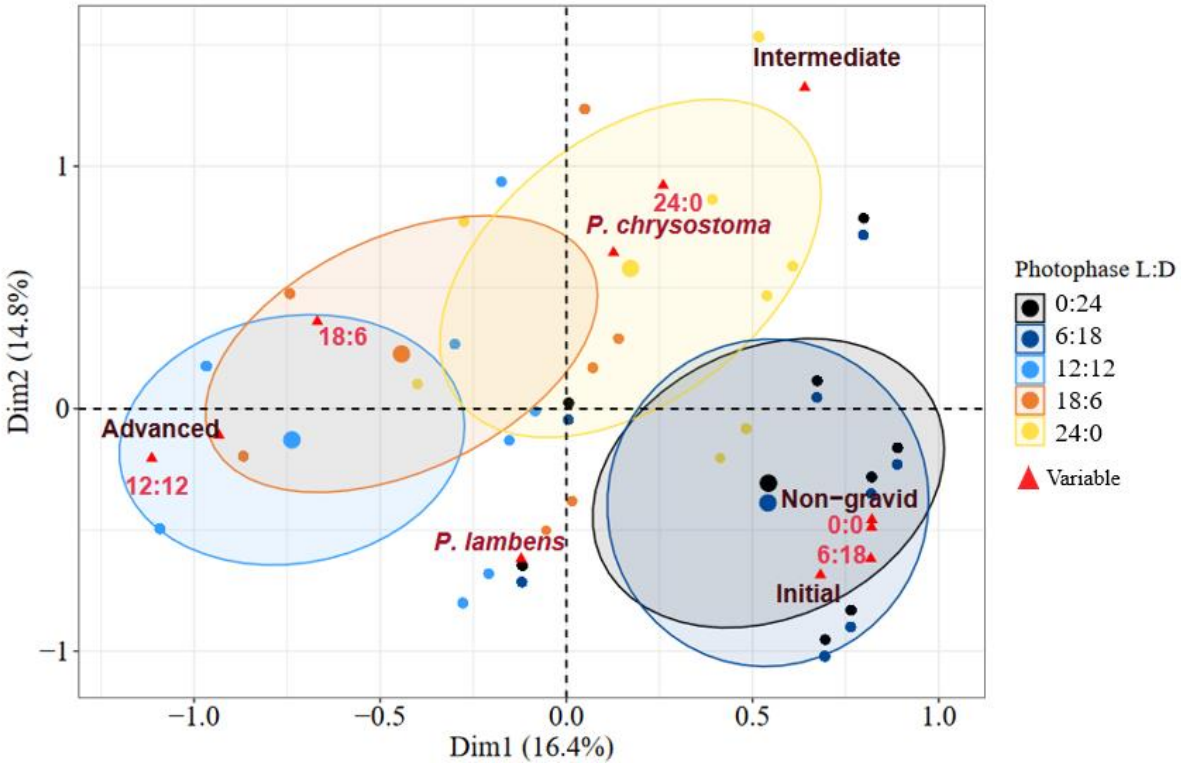
The photophase influenced the stage of intrauterine maturation in both species. Scotophase (0:24) and short light duration (6:18) caused a delay in egg development. For *P.* (*P.*) *chrysostoma*, the highest percentage of eggs in the initial stage of development (36.0%) was observed under total darkness. In the 6:18 regime, there was a predominance of the initial stage for *P.* (*S.*) *lambens* (50.0%, $P = 0.09$ compared to other stages). Notably, the intermediate stage was more prevalent in the 24:0 regime for *P.* (*P.*) *chrysostoma* (54.1%, $P < 0.05$), while for *P.* (*S.*) *lambens*, eggs at advanced development were more abundant (78.8%, $P< 0.001$). In the 12:12 and 18:6 treatments, advanced development was predominant for both species, reaching 82.5% for *P.* (*P.*) *chrysostoma* in 12:12 ($P < 0.001$) and 97% for *P.* (*S.*) *lambens* in 18:6 ($P < 0.001$) (Figure 5).

Figure 5: Percentage of individuals at different stages of ovarian development for A) *Peckia* (*Peckia*) *chrysostoma* and B) *Peckia* (*Sarcodexia*) *lambens* exposed to different photoperiods. IN: initial stage; IT: intermediate stage; AD: advanced.



The MCA evidenced clear associations between the photophase and the reproductive stage. Females at early stage of egg development were more strongly associated with treatments of scotophase (0:24) or short light duration (6:18), while females with advanced stage of egg development were more strongly associated with 12:12 L:D (Figure 6).

Figure 6: Multiple Correspondence Analysis of qualitative variables on the reproductive status of *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens* females exposed to different light regimes.



DISCUSSION

Homicides occur mostly in poor-lit environments in Brazil (Ceccato et al., 2021; Mendes and Silva, 2020). Sarcophagidae females must be able to rapidly locate the ephemeral resource to guarantee the deposition of viable offspring (Barbosa et al., 2023; Battán-Horenstein et al., 2021). Flight activity of Sarcophagidae species has been described as essentially diurnal (Soares and Vasconcelos, 2016). We empirically demonstrate that the absence of light does not impede gravid *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* females to deposit viable larvae. Contrarily to what has been observed for several necrophagous species, the photophase duration was not associated with altered frequency of larviposition and the number of deposited larvae.

To our knowledge, this is the first documented report of nocturnal (i.e., darkness) larviposition by flesh fly species in the Neotropical Region. These findings can enhance our understanding of the reproductive behavior of species in the *Peckia* genus, there by expanding the theoretical framework of forensic entomology for Sarcophagidae. Nocturnal larviposition

offers an evolutionary advantage by extending the time available for actively seeking food resources and reducing competition, as fewer predators and competitors are active at night, promoting reproductive success and survival.

Viviparity among Diptera is often associated to the need for exploiting ephemeral resources exposed to high competition and also to pathogens, such as dung and carrion (Meier et al., 1999). Because Calliphoridae species are the main competitors of flesh flies, as both are early colonizers of cadavers, larvae of *Peckia* face predation by the highly competitive species *Chrysomya albiceps* (Calliphoridae) (Barbosa et al., 2021). From that perspective, two advantages stand out, the viviparity and the anticipation of the timing of offspring deposition allow that Sarcophagidae can successfully outcome competition and/or predation. We demonstrate a third advantage here, which is the ability to larviposit in the dark, a behaviour rarely observed for Calliphoridae species.

According to Wooldridge et al. (2007), the probability of oriented flight and oviposition among Calliphoridae species at night is low, so that the colonization of cadavers in the scotophase is virtually null. However, data from literature are contradictory. *Calliphora vicina* (Calliphoridae) can lay eggs in the absence of light, although in that experiment the females were reared under 12:12 L:D (Bonaci et al., 2016). *Chrysomya putoria* laid eggs at night when females were kept under total darkness and under artificial light (Carneiro et al., 2022).

We postulate that the ability to larviposit in the dark is not accidental for flesh flies. Firstly, the frequency of occurrence of larviposition did not differ throughout the photophases, from complete darkness to full-time illumination. Fecundity did not differ between extremes of light and darkness. No minimal threshold light limit was detected, which suggests that mated females can successfully lay larvae on cadavers in the dark, in quantities similar to diurnal conditions. The median of larva deposited, a parameter that perhaps describes more accurately the fertility (by reducing the weight of outliers) did not differ among treatments for either species.

When cumulative larviposition is measured, however, the mean number of larvae was approximately three times higher in the 12:12 condition compared to total scotophase for *P.* (*P.*) *chrysostoma* and more than twice as high in *P.* (*S.*) *lambens*. The offspring tended to increase with time, as the highest DER for both species was registered at 96 h after the

beginning of the experiments. Therefore, we postulate that short durations of light (or even total absence) cause delay in the process of larviposition.

It is interesting to notice that larviposition took at least 48 h to occur under total darkness compared to as early as 24 h in the 12:12 L:D, for both species. Delayed larviposition may result from longer time needed for egg development in the female ovaries. It is reasonable to argue that the longer *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* are kept in the scotophase, the longer they will start larviposition – with consequences for the estimation of the min PMI.

Comparison with other families of medical and forensic relevance offers interesting counterparts. Although the frequency of oviposition was similar under day and night conditions, the mean number of eggs was significantly lower for *M. scalaris* kept in the dark (Bostock et al., 2017). Contrarily, no difference occurred in the number of eggs laid by *C. putoria* in the day and the night (Carneiro et al., 2022), which suggests that more complex variables (e.g., temperature, time post-mating) have synergistic effect in effective ovi/larviposition.

Nevertheless, there seems to be a mechanism for conditioning the females to adapt to dark conditions without interrupting the reproductive sequence. That is, after some days of exposure, the DER tend to be similar among different photophases. Arguably, chemical cues are as important as visual stimuli in substrate recognition by females (George et al., 2012). From a forensic standpoint, the ability to lay larvae in the dark, in numbers similar to diurnal conditions, may offer advantages for survival (Saunders 2009; Soares and Vasconcelos 2016).

Singh and Barthi (2008) offered preliminary observations on nocturnal larviposition of two *Sarcophaga* species in India, although that experiment was not replicated. We provide here empirical, quantitative and replicated evidence that *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* not only larviposit at night, but can also lay an offspring comparable to females reared under 12:12 L:D.

We also provide a classification of egg development stages, based on the framework proposed by Chaiwong et al. (2012). However, due to the lack of detailed descriptions of intrauterine egg development in Sarcophagidae, we focused on the three main stages. These stages are sufficiently informative to indicate the time required for larvae to fully develop within the uterus and to detect differences in developmental timing.

Clearly, gonotrophic cycles are mediated by light. Oocyte production is regulated by internal (e.g., hormones) and external factors, such as light intensity, duration and temperature (Schiesari et al., 2011; Cardinal-Aucoin et al., 2013) and, in the case of sarcophagids, a protein

meal that enhances egg maturation despite autogeny occurring (Chadha and Denlinger, 1976; Wentworth and Roberts, 1984; Hahn et al., 2008). Surprisingly, total darkness delayed – but not prevented – successful larviposition.

These results support those of Kenny et al. (1992), who evaluated the effect of photoperiod on the development of *S. argyrostoma*, indicating that photoperiod is important during the later stages of ovarian growth. These authors reported that photoperiod induces earlier larviposition in periods of prolonged light (18:6 L:D), which may explain the high number of females in advanced maturation stages during periods with more light.

Endogenous circadian control of behavioural functions enables organisms to synchronize important events by interpreting enthralling signals from their environment (Prohaska et al., 2018). So far, the best insect model to understand the molecular and neurogenetic bases of circadian behaviour rhythms is *Drosophila melanogaster* Linnaeus, 1758. In that species, peptidergic neuronal circuits are assumed to link circadian-clock neurons in the brain to motor circuits in the ventral nerve cord (King et al., 2017). In the only available study on circadian rhythm of flesh flies, Prohaska et al. (2018) demonstrated that *Sarcophaga crassipalpis* Macquart, 1839 exhibit gender-dependent responses to light:dark cycles with females displaying an extraordinary departure from diurnally, with extended dark activity, which can link physiological events associated with egg provisioning and locomotor activity in that anautogenous flesh fly.

The integration of internal physiological variables—such as metabolic state, circadian time, reproductive drive —with external sensory cues, such as food, mate availability, light, temperature, and suitability for egg deposition (King et al., 2017) is still obscure for Sarcophagidae species. Nevertheless, we suggest that some degree of flexibility in the strict day-night circuit permits that typically diurnal species experience egg development and maturation in the scotophase.

Anthropogenic factors associated with artificial light at night (ALAN) can directly influence the behavior and foraging activities of flies, especially synanthropic species. Insects' activity during the night is influenced by lunar phases and artificial light from urban areas (Kirkpatrick and Olson 2007; Silva, 2014; Soares and Vasconcelos 2016; Williams et al., 2017; Carneiro et al., 2021).

From a forensics perspective, sites that are typically used for discard of cadavers, such as car trunks, concealed sites, etc. can be accessed by Sarcophagidae, providing there are no

mechanical barriers. Secondly, the minimum PMI does not need to account for the egg development period, as the eggs deposited by *Peckia* (*P*.) *chrysostoma* are infertile, with frequent observations confirming this (Ferreira et al., 2024). Additionally, no egg deposition by *Peckia* (*S*.) *lambens* was observed in this study. As research into the nocturnal behavior of flies progresses in the Neotropical Region, which harbors the highest diversity of Sarcophagidae species in the world, we hope the further studies consolidate the importance of flesh flies as entomological evidences in court.

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CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AVAILABILITY OF DATA AND MATERIAL

The data that support the findings of this study are openly available in Dryad at https://doi.org/10.5061/dryad.34tmpg4tz.

AUTHORS CONTRIBUTIONS

All authors contributed to the study conception, data curation, formal analysis, investigation, methodology, project administration, resources, software, supervision, validation, visualization, roles/writing - original draft, writing - review & editing.

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5 CAPÍTULO 3 - Post-feeding mobility, burial behaviour and survivorship of *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830) (Diptera: Sarcophagidae) under laboratory conditions

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Abstract: In forensic entomology (FE), understanding larval dispersal is crucial for determining key data such as the time, distance, trajectory, speed, and burial depth for pupation. This study contributes to this knowledge by examining forensically relevant species and evaluating various techniques. We aimed to: a) characterize the mobility and horizontal directionality of larvae in a Laval Mobility Arena (LMA); b) measure vertical burial depth across different soil types; and c) assess the emergence of adults from pupae buried at varying depths and soil types. Third-instar larvae (72 hours old) and pupae (up to 24 hours old) from different experimental pairs and days were used in three separate experiments. The species were active in the LMA, with *P.* (*S.*) *lambens* taking longer to locate the vermiculite compared *P.* (*P.*) *chrysostoma* to which demonstrated more organized movement, with 25% and 22% of larvae moving to the same LMA region. Greater burial depth was observed in humic and vermiculite soils for both species, with *P.* (*P.*) *chrysostoma* burying deeper than *P.* (*S.*) *lambens*. The latter was only recorded in shallow and moderate layers. Pupal mortality was low, with over 65% adult emergence for *P.* (*P.*) *chrysostoma* and more than 70% for *P.* (*S.*) *lambens*. Soil type and burial depth influenced survival rates. These findings enhance our understanding of larval behaviour in FE, which is crucial for accurate post-mortem interval (PMI min) estimations.

Keywords: Behavioural Patterns. Flesh fly. Larval Mobility Arena. Post-feeding behaviour. Necrophagous insects.

INTRODUCTION

Female flies of the Sarcophagidae are commonly attracted to odours released from carcasses or ephemeral resources with high nutritional value, where they deposit their larvae, which remain until the final stages of development (George, Archer, and Toop, 2012; Gomes, Godoy, and Von Zuben, 2006). This behaviour gives many species high forensic potential, particularly for estimating the minimum post-mortem interval (minPMI), which is the time from death until the body is discovered (Vasconcelos, Soares, and Costa, 2014).

Sarcophagids undergo distinct phases during which the larvae feed as much as possible until resources are exhausted or until enough energy to complete their life cycle is accumulated. After the feeding period, the larvae at late 3rd instar begin searching for an ideal location for pupation. This process is known as post-feeding larval dispersal (Gomes, Godoy, and Von Zuben, 2006; Greenberg, 1990).

Feeding and growth in size cease at pupation, which facilitates the exploitation of ephemeral resources and enhances the probability of successful metamorphic transition after achieving developmental size thresholds (Levot, Brown and Shipp, 1979; Ribeiro and Von Zuben, 2010). The profound tissue and organ transformation involved in the passage from immature to adult requires that an insect locates and exploits its surroundings in search of a suitable place. For necrophagous Diptera, the larvae typically move away from the resource and avoid predators and desiccation by burying themselves.

In the context of forensic entomology (FE), studies on larval dispersal have contributed to the understanding of key data, particularly in comprehending the dynamics of colonization within the necrobiome, as well as factors that influence the time required for larvae to begin dispersing from the feeding site, the distance traveled, trajectory, speed, and depth of burial for pupation (Gomes, Godoy, and Von Zuben, 2006; Gomes and Von Zuben, 2005; Jales et al., 2023). This body of knowledge provides valuable insights for developing techniques aimed at improving minimum PMI estimates.

The horizontal movement behaviour of immature stages has been evaluated in some studies, which have identified biotic factors that can influence the distance travelled. These factors include fly species, the search for an ideal pupation site, chemical influences, competition levels, and predation (Arnott and Tuner, 2008; Greenberg, 1990; Reigada and Godoy, 2005; Robinson et al., 2018; Singh and Bala, 2010). Post-feeding dispersal behaviour

is also mediated by abiotic factors, such as soil type, soil compaction, and temperature, rainfall, humidity, and photoperiod. In seasonal environments, like tropical dry forests (Caatinga), high temperature and low relative humidity accelerate decomposition rates and larval development, shortening the time window for investigators to collect samples (Oliveira and Vasconcelos, 2018).

In addition to post-feeding movement behavior, burial is another crucial aspect to consider. During evidence collection at death scenes, forensic investigators may find pupae and larvae buried in the soil, depending on the species, which can vary in burial depth and distance from the resource (Gomes, Godoy, and Von Zuben, 2006; Gomes and Von Zuben, 2005).

Understanding these factors can aid in the forensic application of species, particularly *Peckia* (*Peckia*) *chrysostoma* (Wiedemann, 1830) and *Peckia* (*Sarcodexia*) *lambens* (Wiedemann, 1830), which have been documented as colonizers of human cadavers in Brazil (Costa, Mello-Patiu, and Lopes, 2001; Vasconcelos, Soares, and Costa, 2014).

This study was motivated by the advancements made with the proposed results for the Neotropical region regarding mobility behaviour across horizontal surfaces, burial, and vertical survival of immatures within the Sarcophagidae family. It aims to contribute data on the behaviour of forensically important species and to test tools and techniques in this area. Specifically, the study seeks to: a) characterize the horizontal mobility of larvae at post-feeding stage; b) measure the depth of vertical burial in different soil types; and c) evaluate the emergence of adults from pupae buried at different depths and in different soil types.

METHODS

Insect rearing

The insects used in the experiments were obtained from laboratory colonies reared for at least five generations under controlled conditions at a temperature of 25 ± 2°C, relative humidity of 60% ± 10%, and a photoperiod of 12:12 (Light: Dark). Adults were reared in 40 cm x 60 cm x 40 cm cages, provided with a 50% sugar solution in water *ad libitum* and decomposing minced beef for larviposition. Female oviposition was stimulated from the 5th day of age, using 24-hour decomposed ground beef. As females laid eggs, the larvae were

transferred and kept in 250 mL plastic containers with ground beef as food (2 mg/larva) and vermiculite as a substrate for pupation (Silva Xavier et al., 2015; Barbosa et al., 2019).

Soil Grain Analysis

For the characterization of clayey soil and the determination of the average grain size of the soils used in the tests, techniques were employed following Suguio's (1973) protocol. Clayey soil was defined as having at least 30% silt-clay content, with the sample containing 34.6%. The weight of the soil used to fill the arena in Experiment II was measured using a precision scale (0.1 g). The soils used in Experiments II and III were sieved, and only soils with a maximum grain size of 2 mm were used (Table 1).

Table 1: Overall soil characterization for experiments based on 30-gram samples

Soils	Predominant grain size (mm)	Proportion for predominant size (%)
Sandy	0.5	36.53
Clayey	0.5	33.93
Humic	2	24.83
Vermiculite	1	38.47

Experiment I: Mobility of larvae at the post-feeding stage

To assess the horizontal mobility of larvae at the post-feeding stage, we selected third-instar larvae of uniform age (approximately 72 hours post-moulting) and size (about 10 mm for *P.* (*P.*) *chrysostoma*, 6 mm for *P.* (*S.*) *lambens*), which exhibit increased activity in searching for a burial substrate.

We designed an experimental arena referred to as the Larval Mobility Arena (LMA), which consisted of a plastic square (50 cm per side) lined with a 1 x 1 mm grid. Each side of the square was surrounded by a 10 cm wide and 5 cm deep cavity containing vermiculite, at the same level as the floor. An additional area (10 cm) wide surrounding the vermiculite-filled fissure was laid to evaluate whether the larva would keep moving after finding a place for burial.

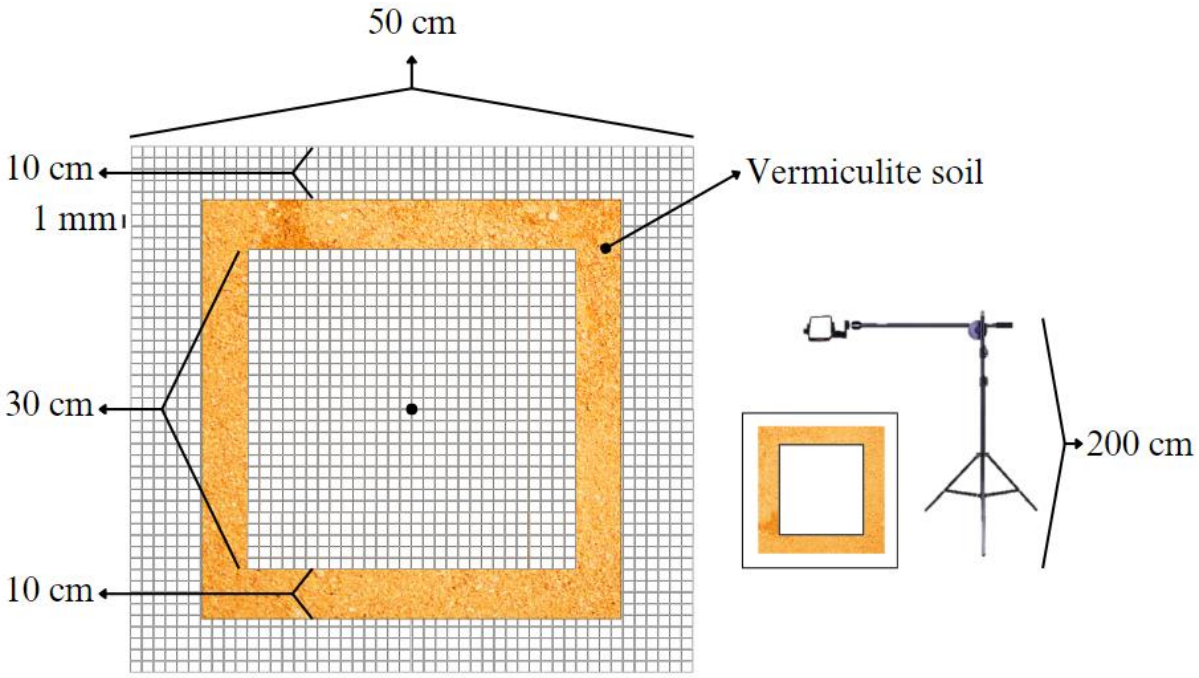
A batch of 10 larvae was placed in the centre of the arena using soft forceps and the behaviour was observed for 5 minutes. Ten repetitions per batch were conducted for each species. A digital camera (4K quality, 30 fps, MP4 format) was positioned 2 meters above the LMA to record larval movement, ensuring the entire arena fit within the frame without

compromising image quality, and providing a clear, high-resolution view of the millimeter paper (Figure 1).

To minimize external factors such as vibrations, odours, and sounds, the experiment was conducted in a room under controlled conditions of temperature (25 ± 2°C) and relative humidity (60% ± 10%) and no person was allowed in the room after the beginning of the experiment.

The video recordings were analysed using the software Tracker (version 5.1.4). Each larva was analysed using a point on the cephalic region (anterior region) of the larva as a reference point (Berrigan & Pepin, 1995), evaluating the trajectory from the initial point in the centre of the LMA.

Figure 1: Methodological scheme for the experimental Larval Mobility Arena (LMA), to evaluate and film the horizontal movement of 3rd instar larvae in the post-feeding phase, focusing on locating ideal sites for pupation.



We characterized larval mobility as follows: (a) the total distance travelled by each actively moving larva (mm); (b) the time (s) each larva remains active until reaching the substrate; (c) speed (cm/s); (d) movement direction to assess gregarious behaviour. The variables were partially based on Berrigan & Pepin (1995) and Jales et al. (2023).

The total distance travelled was obtained using the equation:

$$d = \int_{i=1}^{n} \sqrt{(x_i - x_{i-1})^2 + (y_i - y_{i-1})^2}$$

Where d is the total distance travelled by the larva (in mm, subsequently converted to cm), x and y represent the Cartesian coordinates at a given point (i). The velocity obtained for each sample considers the larva's displacement as uniform rectilinear motion, measured at each larval movement, using the measurement/position at each point (i) in the Cartesian plane from time (t) and position variations (y) of the subsequent (ti+1 and yi+1) and previous (ti-1 and yi-1) points, using the formula:

$$v_{y,i} = \frac{y_{i+1} - y_{i-1}}{t_{i+1} - t_{i-1}}$$

The time spent by each larva in the LMA was measured by subtracting the initial time from the final time when the larva exited (in milliseconds, later converted to seconds). The same procedure was used to measure the time of burial upon encountering the vermiculite. To assess directionality, the entire trajectory was monitored using markers on each larva, and the location where the larva encountered the vermiculite was recorded by dividing the LMA into five quadrants, each 10 cm on each side.

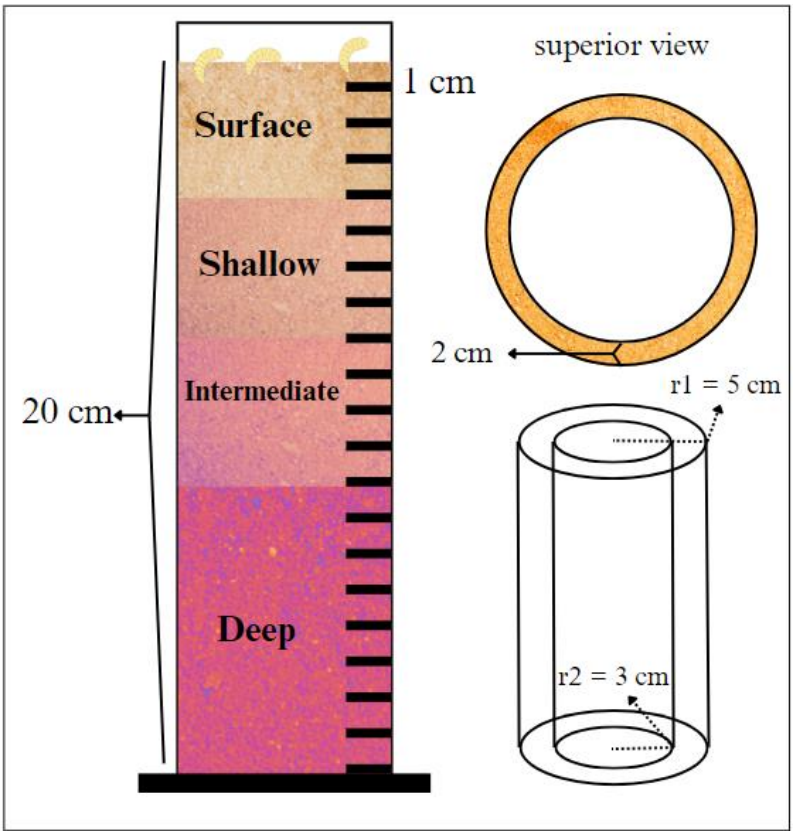
Experiment II: Depth of burial by larvae at the post-feeding stage

For this study, we designed an arena for burial observation (ABO), which consisted of two transparent acetate cylinders (0.3mm thick, 25 cm long, 5 cm radius) and one inside (3 cm radius). The 2-cm gap was filled with one of the following treatments: sandy soil, humic soil, clayey soil, or vermiculite. The outer cylinder had a 1 cm grading on its external side. A batch of 10 larvae at the post-feeding stage (3rd instar, over 72 h post moulting) was placed on the surface using soft forceps.

The arena with soil/vermiculite and larvae was covered with nylon mesh and kept under laboratory conditions. After 72 hours, we measured the depth of burial of insects by observing the position (cm) of the pupae through the external scale. Additionally, the arenas were inspected, and the soils were gradually removed using a small shovel to measure the depth at which each pupa were buried, designing four levels: surface (1-4 cm), shallow (5-8 cm), intermediate (9-12 cm), and deep (> 12 cm).

We defined as variables: soil type, depth (independent) and the number of pupae (dependent). The pupae were categorized as either fully developed or malformed, when incomplete tissue change was observed. Only fully developed pupae were accounted for.

Figure 2: Methodology for assessing the depth of burial of *Peckia* spp. larvae.



Experiment III: Survivorship following burial

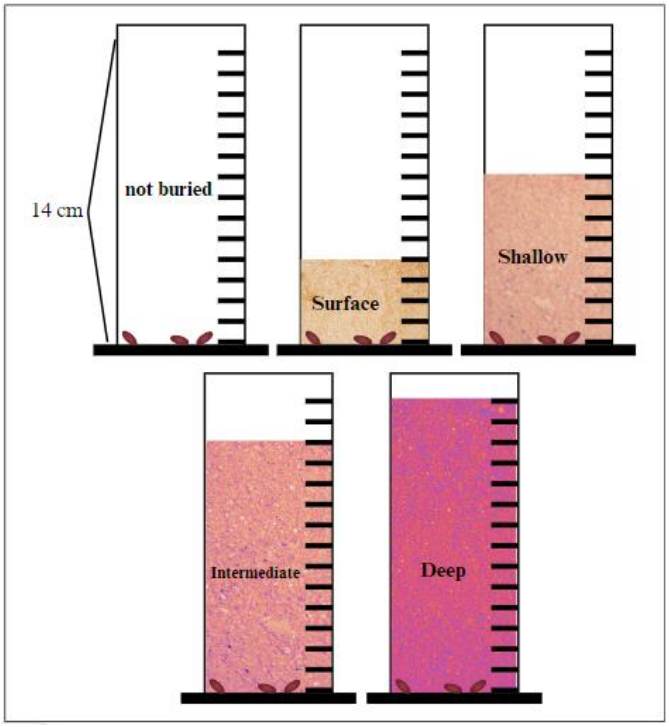
To examine survivorship of specimens under burial, we used an experimental cylinder-shaped arena made of acetate plastic (0.3 mm), with a diameter of 10 cm. The experimental unit consisted of a batch of 10 pupae (24 hours old), with 10 repetitions for each species, buried in one of the following types of soil: sandy, humic, clayey, and vermiculite (Figure 3).

Soil depth layers were considered to evaluate how deeply adults could emerge from the buried substrate, with each layer being 4 cm in depth: surface (4 cm), shallow (8 cm), intermediate (12 cm), and deep (14 cm). Preliminary assessments showed larval depths of up to 12 cm, and a control test was also conducted to assess the viability of pupae in conditions where they were not buried. All substrates were sieved through a 2 mm mesh to remove plant

debris and stones. The experiment was evaluated by counting the adults that emerged on the surface.

Survival (dependent variable) was measured by counting the adults that emerged in the experiment, which was useful for calculating the survival rate (= number of survivors / total number) for the independent variables: soil type and depth. The adults were categorized as either fully developed or malformed, when incomplete tissue change was observed. Only fully developed adults were accounted for.

Figure 3: Methodology for estimating the survivorship of pupae of *Peckia* spp. following burial at different depths



Data analysis

For Experiment I, the video recordings of each larva were analysed using Tracker® software (version 5.1.4). Mass point markings were made every 100 frames, as the videos had approximately 10,000 frames, resulting in the best quality for viewing the experiments.

The time spent in the LMA was measured by subtracting the initial time from the final time (s) when the larva exited. To assess directionality, the entire trajectory was monitored using markers on each larva, and the location where the larva encountered the vermiculite was recorded by dividing the LMA into five quadrants, each 10 cm on each side.

Quantitative data related to different species and variables were tested for normality using the Shapiro-Wilk test. Normally-distributed data were analysed using ANOVA and Tukey's post hoc test. For non-normally distributed data, the Kruskal-Wallis and Bonferroni's post hoc test were used. All tests were conducted using R® software (version 4.4.0), at 5% significance level.

RESULTS

Experiment I: Horizontal Movement of Larvae

The species were active in the LMA, 100% of the larvae leaving the centre of the LMA to reach in the vermiculite. The larvae of *P.* (*P.*) *chrysostoma* had a greater length (χ^2 = 109.95, df = 1, $P < 0.001$) compared to *P.* (*S.*) *lambens*. The time spent in the LMA until they found the vermiculite varied for both species, with *P.* (*S.*) *lambens* staying longer in the arena (χ^2 = 54.71, df = 1, $P < 0.001$). Other variables also showed differences, with the average speed (χ^2 = 9.2, df = 1, $P = 0.002$) and the mean total distance travelled (χ^2 = 3.89, df = 1, $P = 0.04$) being greater for *P.* (*P.*) *chrysostoma* than for *P.* (*S.*) *lambens* (Table 2).

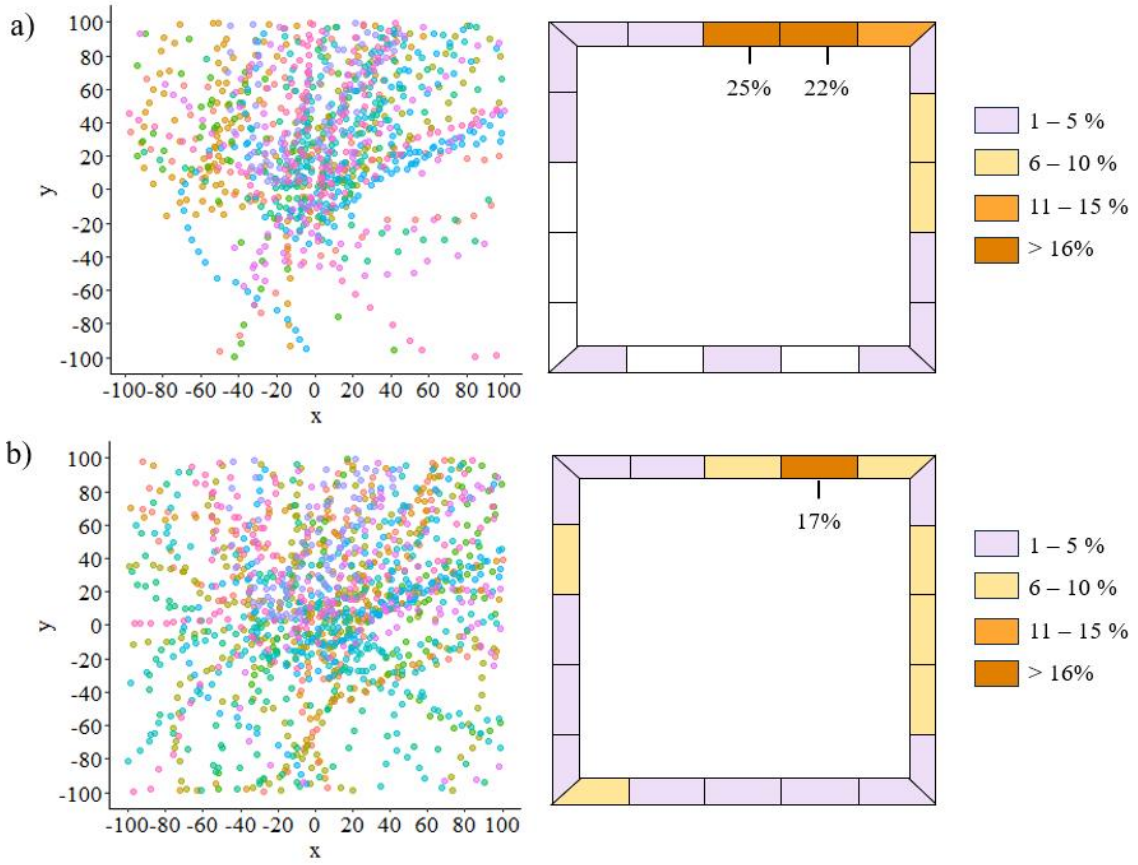
Table 2: Estimated time of larvae in the Larval Mobility Arena (LMA), average speed, total distance traveled, time to burrow, along with weight and length data for *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens*.

P.* (*P.*) *chrysostoma	**Mean**	**Median**	**Range (N)**
Weight (mg)	167.5 ± 6.8	168.2	159.63 – 176.91
Length (mm)	19.55 ± 1.6	19.83	18.23 – 21.82
Distance travelled by larvae (cm)	61.58 ± 27.82	58.46	25.43 – 149
Time until reaching substrate (s)	52.58 ± 25.62	45	26.66 – 136.66
Speed (cm/s)	1.58 ± 0.94	1.41	0.2 – 4.8
P.* (*S.*) *lambens			
Weight (mg)	98.6 ± 4.2	98.62	95.74 – 102.01
Length (mm)	9.18 ± 0.6	9.23	8.56 – 9.54

Distance travelled by larvae (cm)	53.21 ± 20.51	47.82	25.13 – 125.41
Time until reaching substrate (s)	84.12 ± 36.05	80	41.66 – 263.33
Speed (cm/s)	1.31 ± 0.82	1.26	0.11 – 3.56

When considering the direction of larval movement, *P*. (*P*.) *chrysostoma* exhibited less tortuous/sinuous movement toward one side of the ALM. This species showed a predominant direction in two areas, with 25% and 22% of the tested larvae moving towards these regions. In contrast, *P*. (*S*.) *lambens* did not display a directional pattern, with the larvae moving across all sections of the LMA, but 17% of the larvae moved to the same location (Figure 4).

Figure 4: Larval movement in the experimental arena and preferred burial area, highlighting the percentage of *Peckia* (*Peckia*) *chrysostoma* larvae (a) and *Peckia* (*Sarcodexia*) *lambens* larvae (b) distributed in each area.



Experiment II: Depth of larval burial

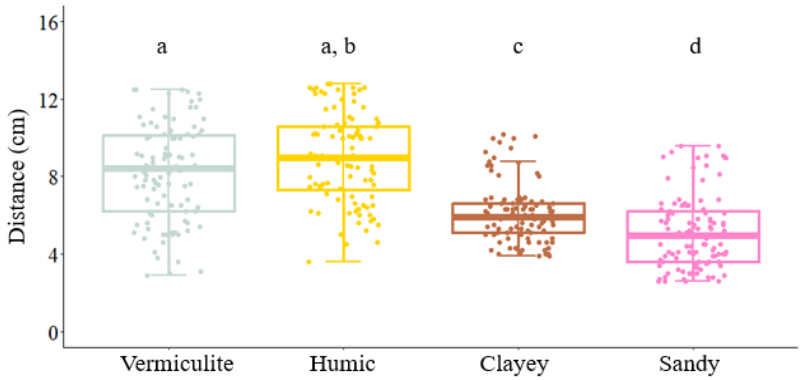
All larvae of both species buried themselves, and all pupae were fully developed, that is, no deformed pupae were registered. The maximum depth of burial was 12.8 cm for *P.* (*P.*) *chrysostoma* and 8.5 cm for *P.* (*S.*) *lambens*. It was possible to record greater depth of burial in humic and vermiculite soils for both species. When comparing the species, there was a difference in vertical burial distance ($\chi^2 = 296.0$, df = 1, $P < 0.001$), with *P.* (*P.*) *chrysostoma* burying at greater depths, regardless of the soil type tested (Table 3).

Table 3: Burial Depth (in cm) of *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens* larvae in experimental arenas exposed to different soil types. SD: standard deviation

P.* (*P.*) *chrysostoma	**Mean ± SD**	**Median**	**Range (N)**
Sandy	5.12 ± 1.89	5.0	2.6 – 9.8
Clayey	6.08 ± 1.56	5.9	3.9 – 10.6
Humic	8.96 ± 2.29	9.0	3.6 – 12.8
Vermiculite	8.2 ± 2.5	8.4	2.9 – 12.5
P.* (*S.*) *lambens			
Sandy	2.44 ± 0.6	2.4	1.2 – 3.5
Clayey	2.58 ± 0.78	2.6	1.1 – 4.0
Humic	5.37 ± 1.27	5.1	2.0 – 7.8
Vermiculite	4.89 ± 1.5	4.8	1.3 - 8.5

The greatest burial depths for *P.* (*P.*) *chrysostoma* were recorded in humic soil and vermiculite. However, statistical comparisons showed variations between soil types ($\chi^2 = 143.07$, df = 3, $P < 0.001$), with no difference observed only between vermiculite and humic soil ($P > 0.05$). The highest number of larvae (71%) reached a moderate depth in clay soil. Few larvae reached the extreme depth, with 7% in vermiculite and 14% in humic soil. No buried pupae were retrieved at extreme depth for sandy and clay soils (Figure 5).

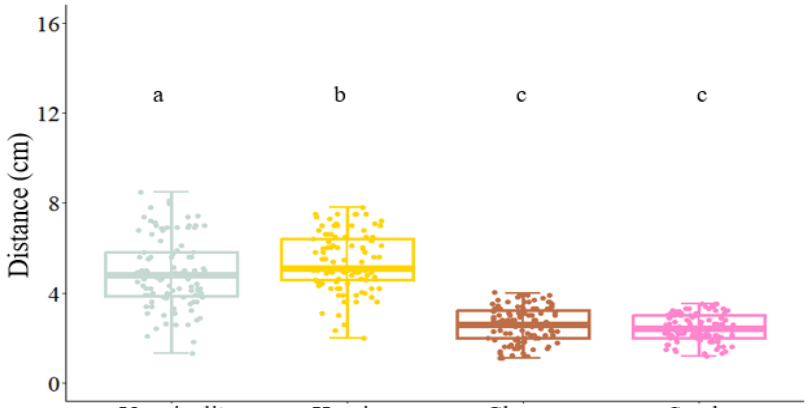
Figure 5: Burial depth in different soil types for post-feeding larvae of *Peckia* (*Peckia*) *chrysostoma*. Different letters indicate statistical differences ($P < 0.001$).



	Vermiculite	Humic	Clayey	Sandy
Shallow	8%	3%	21%	50%
Moderate	48%	43%	71%	41%
Deep	37%	40%	8%	9%
Extreme	7%	14%	-	-

The species *P.* (*S.*) *lambens* also showed differences in burial depth across soil types ($\chi^2 = 250.36$, df = 3, $P < 0.001$), with no differences observed between sandy and clay soils. However, larvae of this species were only recorded in the shallow and moderate layers. In clay and sandy soils, 100% of the larvae burrowed into the shallow layer of the arena. Conversely, 61% and 40% of *P.* (*S.*) *lambens* larvae were buried at moderate depths in humic and vermiculite soils, respectively (Figure 6).

Figure 6: Vertical burial depth in different soil types for post-feeding larvae of *Peckia* (*Sarcodexia*) *lambens*. Different letters indicate statistical differences ($P < 0.001$).



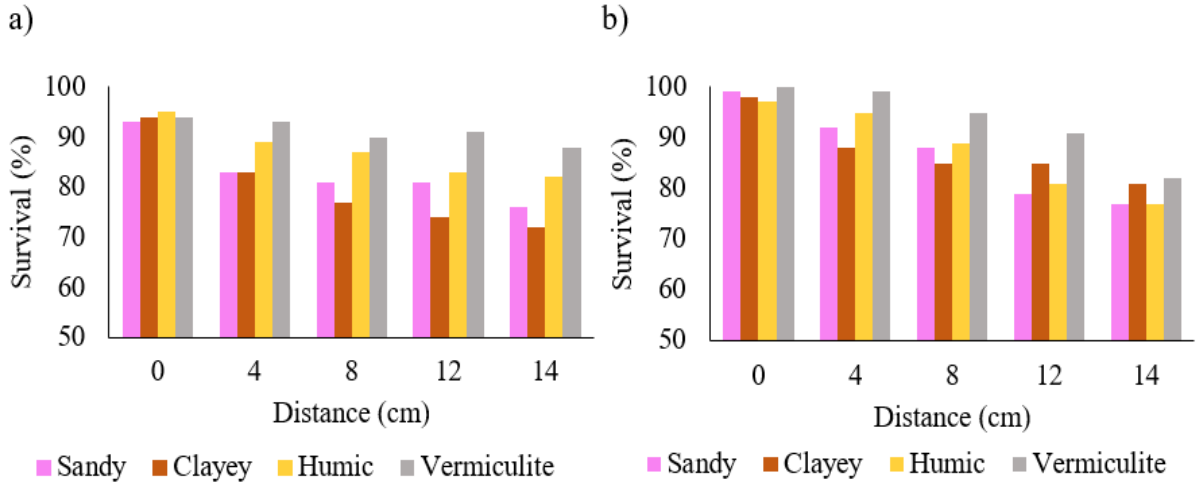
	Vermiculite	Humic	Clayey	Sandy
Shallow	60%	39%	100%	100%
Moderate	40%	61%	-	-
Deep	-	-	-	-
Extreme	-	-	-	-

Experiment III: Survival of pupae based on burial depth conditions

When evaluating the survivorship of pupae under different layers of burial, high rates of survival were recorded, as both *P.* (*P.*) *chrysostoma* and *P.* (*S.*) *lambens* could survive the maximum depth tested in the study (14 cm). Overall, more than 65% of buried pupae emerged as adults for *P.* (*P.*) *chrysostoma* and over 70% for *P.* (*S.*) *lambens*.

Different soil types influenced the survival of *P.* (*P.*) *chrysostoma* ($\chi^2 = 15.424$, df = 3, $P = 0.001$) as well as different depths ($\chi^2 = 14.939$, df = 4, $P = 0.004$). For *P.* (*S.*) *lambens*, soil type ($\chi^2 = 12.739$, df = 3, $P = 0.005$) and depth ($\chi^2 = 37.994$, df = 4, $P < 0.001$) also affected survival (Figure 7).

Figure 7: Survival rates of pupae and adult emergence of *Peckia* (*Peckia*) *chrysostoma* (a) and *Peckia* (*Sarcodexia*) *lambens* (b) based on burial in different types of soil and depths.



DISCUSSION

We present experimental setups to investigate the behavioral aspects of two forensically relevant species, *Peckia* (*Peckia*) *chrysostoma* and *Peckia* (*Sarcodexia*) *lambens*. This study empirically demonstrates not only their similarities to other necrophagous species but also highlights distinct parameters unique to these *Peckia* species.

These parameters can be used by forensic professionals for predictions and to enhance the rigor of forensic analyses and these techniques will be applied in further studies, allowing the data to be compared and refined, ultimately contributing to the continuous improvement of forensic entomology practices. Through the assessment of burial and mortality in experimental

arenas with different soil types, we explored a nearly unexplored behaviour in the Sarcophagidae family, aligning new approaches and obtaining relevant and novel data for the family.

The experimental LMA model, designed according to the evaluative criteria established by Oliveira et al. (2019), was created at a low cost, facilitating its replication and experimentation in various locations and conditions. To ensure that the design of the LMA accurately represents the natural conditions in which larvae search for burial sites, factors such as a single light source, controlled humidity and temperature, and burial conditions with vermiculite were carefully monitored to simulate the natural environment, this accessible approach contributes to a broader understanding of ecological and forensic processes.

The ability of flies (e.g., Calliphoridae and Sarcophagidae) to colonize cadavers has been extensively studied and documented for many years, highlighting their significant role in forensic entomology. Since the emergence of behavioural foundations that can be used for various applications in FE, such as PMI min estimation, chemical compound detection, cadaver displacement, and DNA identification, insects have been crucial as significant traces. In this study, we provided new perspectives on the post-feeding movement behaviour of Sarcophagidae, a family that is under-researched behaviourally but includes several taxa with forensic potential (Carvalho; Mello-Patiu, 2008).

In this study, we empirically demonstrated that horizontal mobility, burial, and survival in various simulated field conditions can modulate the spatial dynamics of post-colonization ecology of *Peckia* species. The differences in the results among the species in this study may have been mediated by variations in weight and length. The data found can be useful for the development of mathematical models that predict the maximum dispersal of larvae from a cadaver, considering factors such as total distance travelled, weight, length, and the speed of each species, there by strengthening the use of immature stages to estimate the min PMI.

In a thorough death scene investigation, specimen collection is typically directed towards older larvae (larger or further from the corpse). Post-feeding larvae disperse over a period, leaving the food substrate to seek an appropriate location for pupation (Andrade et al., 2002). This behaviour, known as post-feeding dispersal, is an intrinsic moment for the insect, seeking an ideal location for stage change due to exposure to predators and parasitoids. Larvae bury themselves at considerable vertical distances for escape.

Besides the dispersal itself, larval movement is manifested through body contractions, resting time, and factors that may make them more conspicuous to predators and chemicals (Jales et al., 2023). The phase change mainly occurs due to the ecological concept of ideal energetic feeding for stage change, where larvae feed voraciously to accumulate energy needed for the transition.

Roux et al. (2006), in a field study, observed that *Protophormia terranovae* (Calliphoridae) (Robineau-Desvoidy, 1830) exhibited irregular dispersal, similar to what was observed in this study for *P.* (*S.*) *lambens*. Each sample showed differential movement patterns, with only 11% of the larvae showing a directional pattern similar to *P.* (*P.*) *chrysostoma*, which had a higher proportion of larvae migrating to the same quadrant of the arena. In real situations, larval movement is influenced by micro-variations in factors such as soil density, solar exposure, light direction, and humidity (Greenberg, 1990; Roux et al., 2006).

Other explanations for larval migratory behaviour include horizontal movement based on food resource availability, which can also involve larval density. Depending on these variables, larvae may pupate very close to the food source, but longer migration may be influenced by the availability of this resource, with larvae tending to avoid predators and parasitoids (Gomes; Von Zuben, 2005). For example, larvae found on cadavers in indoor environments may need to travel longer distances from the corpse until ideal pupation sites are readily available. Thus, factors such as speed, presence or absence of obstacles, energy reserves, and body length can influence the horizontal distance larvae can travel.

The practical intent of forensic experts to search for larvae at long distances from the corpse may arise from the understanding that older larvae, having reached a post-feeding physiological condition, might be found far from the cadaver. However, the evidence found in this study suggests that once larvae find an ideal location for pupation, they immediately bury themselves. For FE, finding pupae can also provide information consistent with a longer period of cadaver colonization, and shallow excavation may be sufficient for sampling these species at the death scene.

It is known that larvae migrate from food resources upon reaching a stage suitable for pupation, driven by the energy accumulated during feeding and their sensitivity to soil contact in finding a suitable pupation site. During burial, larvae face pressure and low oxygen density, which impacts the survival of pupae and their emergence as adults. Immature fly stages have a remarkable ability to survive burial, with about 25% survival rate when buried 33 cm deep, and

adults of both species have the potential to reach the surface when buried as immatures at a depth of 50 cm (Balme et al., 2012).

Feeding is a significant variable influencing burial and phase transition. Even with limited resources, flies can develop to the third stage and achieve over 80% survival in burial conditions. Although the authors did not find significant differences in adult emergence between burial conditions of 5 and 25 cm, very few flies emerged from extreme burial conditions (50 cm) (Balme et al., 2012).

Our study illustrates that adults of *P*. (*P*.) *chrysostoma* and *P*. (*S*.) *lambens* reach the surface in different types of soil and at varying depths. However, we could not determine the limiting factor for how far larvae can ascend through the soil column. Future research should focus on assessing the adult emergence capacity under extreme burial conditions. The mechanisms explaining the vertical ascent of adult flies through soil remain unclear, as reaching the surface requires significant energy expenditure influenced by gravity and pressure from soil compaction, with flies using unknown mechanisms to surface (Balme et al., 2012).

To expand the discussion on how environmental factors such as humidity, temperature, and soil composition affect larval behavior in the pupation and burial process, it is important to emphasize that humidity and temperature are crucial for larval survival. Very dry environments can increase larval mortality due to dehydration (Byrd; Tomberlin, 2019; George et al., 2013), and these conditions can also directly influence larval development and pupation rates. Soil composition, particularly compaction and grain size, can affect the larvae's ability to move and the effort required for burial. More compact soils may present a physical barrier, limiting burial depth, while looser soils facilitate digging and the pupation process (Gomes et al., 2005; Balme et al., 2012).

The difference in maximum burial depth between *P*. (*P*.) *chrysostoma* and *P*. (*S*.) *lambens* can be explained by a combination of factors, including: (I) species-specific characteristics (larval weight and length), (II) soil properties (compaction and grain size), with some conditions favoring deeper burial for larger larvae, and (III) the edges of the arena, which may have contributed to the deeper burial of the larvae. In many cases, the pupation depth for other species varies between 4-5 cm, as in *Cochliomyia macellaria* (Fabricius, 1775), and reaches up to 11 cm in *Lucilia sericata* (Meigen, 1826) (Greenberg, 1990; Godoy et al., 1995).

In Calliphoridae, post-feeding larvae are more likely to pupate close to the food source (Godoy et al., 1996). Our findings were similar to those observed for Calliphoridae, suggesting

that larvae, upon finding an ideal location, immediately burrow. Furthermore, *Chrysomya albiceps* (Wiedemann, 1819) is commonly associated with the predation of larvae from other fly species, significantly impacting prey species and particularly reducing native species (Faria & Godoy, 2001), with a high predation potential on interspecific species such as *P.* (*P.*) *chrysostoma* (Barbosa et al., 2021).

The experimental arenas evaluated represent examples of variability in observing larval behaviour. Several factors observed in the development of these arenas might influence larval movement and burial, primarily concerning: I) The arena size, which could have influenced the greater distance of larval burial; II) The absence of food resources in the centre of the arena; III) The evaluation of survival rates at greater depths in experiments involving buried pupae.

Our findings provide insights into behavioral patterns relevant for understanding the life history of species, inferring decomposition dynamics in the necrobiome, and offering valuable information for forensic entomology (FE). Understanding the mechanisms by which necrophagous flies colonize carcasses, move, and bury themselves is crucial. In forensic investigations, searching for pupae or larvae within a certain radius from the death scene is expected, as older larvae migrate to suitable sites and bury themselves. Critically examining the death scene can reveal that smooth domestic floors without suitable burrowing sites may lead to larval migration until energy is depleted, possibly resulting in pupation in feeding sites or gaps in furniture and walls.

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6 CONSIDERAÇÕES FINAIS

- O estudo proporciona uma descrição detalhada do ciclo de vida, incluindo a duração dos estágios imaturos e a longevidade dos adultos de *Peckia* (*Peckia*) *chrysostoma*, além da mensuração de parâmetros bionômicos importantes em condições laboratoriais controladas e aplicadas para a Entomologia Forense (EF);
- Registra-se, de forma inédita, a ocorrência de comportamentos atípicos em *P.* (*P.*) *chrysostoma*, como a deposição de ovos, apesar de serem inférteis, estimula novas discussões sobre a estratégia reprodutiva dominante, a ovoviviparidade;
- Nas condições de laboratório, o fotoperíodo afeta significativamente a postura e a quantidade de larvas depositadas pelas espécies *P.* (*P.*) *chrysostoma* e *Peckia* (*Sarcodexia*) *lambens*;
- A maturação ovariana é afetada pelas variações nos regimes de fotoperíodo, destacando a importância de considerar influencias no IPM min e investigações na EF;
- São propostos modelos experimentais replicáveis que podem ser usados para caracterizar a mobilidade, comportamento de enterramento das larvas e ascendência de adultos emergidos de pupas em diferentes tipos de solo;
- A partir da obtenção de dados de velocidade, deslocamento horizontal e distância de enterramento, reforçamos as práticas e métodos de procura de evidências e escavação superficial estabelecidos na EF, confirmando a eficácia de metodologias tradicionais.

7 PERSPECTIVAS

- Os resultados apresentados ao longo dos capítulos contribuem para a Entomologia Forense na região Neotropical, utilizando espécies como modelo para futuros estudos, com métodos replicáveis, condições de criação em laboratório, oferecendo novas linhas de investigação sobre o comportamento, história de vida, reprodução e bionomia;
- Os achados podem ser aplicados em investigações criminais, auxiliando na determinação do IPM min utilizando *Peckia* (*Peckia*) *chrysostoma* e na interpretação de evidências entomológicas em locais de crime, como a influência da fotófase e do comportamento larval em estágio pós-alimentar;
- Os padrões comportamentais e biológicos observados podem ser comparáveis com outros grupos, sugerindo uma possível ressignificação no uso dessas espécies no contexto forense a partir de aplicações mais precisas e abrangentes;
- A tese abre possibilidades para novas conquistas sobre a influência de fatores ambientais no comportamento e história de vida de moscas de importância forense;
- A continuidade das investigações sobre o comportamento reprodutivo e o desenvolvimento de moscas pode revelar condições de campo ainda não observadas para Sarcophagidae, contribuindo para o aprimoramento das práticas em Entomologia Forense e ecologia;
- As técnicas testadas para o comportamento de larvas indicam que os métodos tradicionais de coleta, que envolvem a escavação superficial do solo em torno de corpos ou áreas de interesse forense, continuam sendo adequados, com a recomendação de manter o rigor na observação dos diferentes estratos de solo e condições abióticas.

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APÊNDICE A: artigo publicado no periódico International Journal of Legal Medicine

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ORIGINAL ARTICLE

Bionomics, reproductive traits and assessment of forensic relevance of *Peckia (Peckia) chrysostoma* (Wiedemann, 1830) (Diptera: Sarcophagidae)

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Abstract

Peckia (Peckia) chrysostoma (Wiedemann, 1830) (Diptera: Sarcophagidae) is a colonizer of cadavers in the Neotropical Region. Nevertheless, data on development for the *P. (P.) chrysostoma* (e.g., instar duration) and behavioral strategies used by the species for locating and colonizing a corpse are scant. We aimed to explore bionomic and reproductive aspects of the flesh fly *P.* (*P.*) *chrysostoma*, and in this article we: (a) provide quantitative data on the life cycle of *P.* (*P.*) *chrysostoma*; (b) present bionomic measurements (length and weight) of larvae and pupae; (c) describe intrauterine egg and larvae development; and (d) analyze the ovo/larviposition behavior by gravid females. Females showed ovaries with discernible eggs and larvae between 8 and 10 days ($\overline{x} = 23.3$ eggs/female). This study reports the first observation of egg deposition, an atypical behavior for the species. The average development time for immature stages was 22.24 h and 21.36 h for 1st and 2nd respectively, and 3rd showed an average development time of 80.47 h. Pupa had the longest duration ($\overline{x} =$ 295.69 h). A direct increase was observed in weight ($P < 0.05$) and length ($P < 0.05$) throughout time. The average survival time of males and females is approximately 30 days. This study expands the knowledge on *P.* (*P.*) *chrysostoma*, such as facultative ovoviviparity under laboratory conditions and the life cycle, which may benefit future studies for accuracy in entomology-based estimation of minimum post-mortem interval (min PMI).

Keywords Development · Forensic entomology · Flesh fly · Min PMI · Sarcosaprophagous insects

Introduction

Accuracy in entomology-based estimation of minimum post-mortem interval (min PMI) results from reliable data on insect development of necrophagous insect species. Mathematical formulae solely based on temperature at the crime scene associated with data on the insect's life cycle, such as the duration of immature stages, may produce many-fold variability on min PMI estimate if sources of variation are overlooked [1,2]. Confounding factors include (but are not limited to) weather conditions, time of the day, access to cadaver, chemical cues emitted by the decomposing body [3] and – importantly – physiological features related to insect egg laying and development [4].

According to Villet [1], min PMI estimates are affected not only by the time when gravid female insects reach a corpse, but also on whether oviposition is stimulated and successfully performed. In this temporal window, the time elapsed for egg development and fertilization is of critical

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importance. Necrophagous flies (Diptera) exhibit a variety of reproductive traits: oviparity (females oviposit eggs that develop and hatch in the external environment), ovoviviparity (females lay eggs at advanced stage of embryological development, and the larva exits the egg shell during or immediately following oviposition), viviparity (females deposit live larvae, i.e., larvae that have already hatched within the female) and pupiparity (the puparium is already formed inside the female, and the laid offspring is immobile) [5].

Precocious egg development refers to the embryonic development in the oviduct of a gravid female fly when a fertilized female has sufficient time to convert a protein meal into mature eggs but lacks suitable oviposition sites (e.g., cadaver) [1,6,7]. During this period, an egg will become fertilized as it passes the opening of the spermathecal duct, and will develop until the point where it may hatch shortly after oviposition [8]. Because criminal investigators prioritize sampling the largest (and probably oldest) larvae e on a cadaver, precocious development can lead to overestimates in min PMI [6]. Lutz and Amendt [4] propose that the most appropriate way to deal with this problem could be to subtract the embryonation time from an age estimation for species where there is evidence of precocious eggs.

Curiously, plasticity in reproductive strategies has been registered for species of the most relevant families in forensic entomology, that is, Calliphoridae, Sarcophagidae, Fanniidae, Muscidae and Phoridae. Ephemeral substrates, such as dung, carcasses and cadavers, quickly undergo changes in their physical and chemical properties, and are rapidly depleted [9]. The deposition of more advanced offspring will confer an advantage for viviparous species over an oviparous species, whose larvae may take a few days to after oviposition [5].

The influence of differential developmental times for egg- or larviposition on the colonization of cadavers has been poorly addressed in legal medicine studies. This is particularly relevant for Sarcophagidae, a family of Diptera that comprises over 3,000 species distributed across 100 genera worldwide, especially in the Neotropical Region [10,11]. Species of Sarcophagidae have evolved reproductive strategies that can facilitate prompt colonization of a cadaver, such as rapid response to chemical cues released from decomposing substrate [12] and viviparity [5]. The prevailing viviparity has been questioned by recent studies which argue that it is not an obligatory trait for all species. For example, laboratory-reared *Blaesoxipha stallengi* (Lahille, 1907) were reported to lay viable eggs under controlled laboratory conditions [10]. Nevertheless, ovoviviparity is still poorly documented among most flesh fly species [13].

Species of the genus *Peckia* (Robineau-Desvoidy, 1830) (Sarcophagidae) are frequently recorded in field inventories in the Neotropical region [14–16]. In recent years, they have been reported colonizing carcasses, animal baits, and cadavers, and have been associated with human myiasis [17–20]. *Peckia (Peckia) chrysostoma* (Wiedemann, 1830), in particular, has been documented in cadavers found indoors [21] which stimulates studies on bionomics and reproductive strategies under a forensic perspective.

The scarcity of quantitative data on the development time of *P.* (*P.*) *chrysostoma* limits its applicability in forensic entomology. A recent survey performed among forensic experts from Brazil shows that the lack of data on insect rearing and development is a major obstacle for the application of entomological evidence in criminal investigations [22]. Briefly, estimations of min PMI incorporate the accumulated degree-days (ADD), a value that represents the amount of continuous heat necessary for an insect to reach the developmental stage observed at the time of collection [23]. Nevertheless, before ADD tables are built, a thorough description of each stage must be based on realistic temperature conditions, which, for *P. (P.) chrysostoma* seems to be around 18 °C and 27 °C [24]. Under that perspective, methodical measurement of size, weight and duration of immature stages of *P.* (*P.*) *chrysostoma* can assist forensic experts in producing realistic estimates of min PMI.

As part of a scientific cooperation between entomological research centers and the forensic police in Northeastern Brazil, we were motivated to describe the developmental stages of *P.* (*P.*) *chrysostoma*, including reproductive and bionomic traits in the laboratory. Specifically, we aimed to (a) describe the life cycle of *P.* (*P.*) *chrysostoma*, in terms of duration of immature stages and longevity of adults; (b) measure bionomic parameters of larvae and pupae (length and weight); and (c) provide a preliminary description of intrauterine egg and larva development. Because recent studies have questioned the obligate viviparity as a reproductive strategy among Sarcophagidae, exemplified in *Blaesoxipha* spp [10,13,25]. We examined the reproductive behavior through controlled experiments and investigated the possibility of facultative ovoviviparity in *P. (P.) chrysostoma*. Our underlying assumption is that the forensic potential of *P. (P.) chrysostoma* can be strengthened by providing quantitative data and practical criteria to assess its validity as entomological evidence.

Methods

Peckia (P.) chrysostoma used in this study were collected in an urban area in Recife (8°22'54" S, 34°56'53" W), Brazil, using suspended traps baited with a mixture of minced pork and beef based on the methodology described by Oliveira et al. [26]. The larvae were reared until the emergence and

identification [27] of adults under controlled temperature conditions (25 ± 2 °C), relative humidity (60% ± 10%) and photoperiod 12:12 (Light: Dark). Sixty adults (30 pairs) were then reared in plastic cages (40 cm X 60 cm X 40 cm) fed on 10% sucrose solution and decomposing minced beef as a substrate for laying, under the same laboratory conditions.

For this study, two sets of experimental procedures were designed according to the main objectives: (i) description of bionomics of the species, focusing on the duration, weight and length of immature stages, and (ii) characterization of reproductive strategies and intrauterine egg development (Fig. 1).

Bionomics: instar duration, body length and weight and survivorship

For the study of bionomics, all observations took place in the laboratory under controlled temperature (25 ± 2 °C), relative humidity (60% ± 10%) and photoperiod 12:12 (Light: Dark). A set of 10 cages containing adults and decomposing minced beef were observed at every 1 h-interval to detect larviposition. Freshly laid larvae were transferred in groups of ten individuals to thirty plastic containers of 150 mL with minced beef (2 g per larva) as food and vermiculite as substrate for pupation. Three hundred individuals (10 larvae X 30 plastic containers) were observed daily at 08:00 h, 12:00 h, 16:00 h, and 20:00 h. The following variables related to the immature stages (2nd and 3rd instar, prepupa, and pupa) were measured: duration of each developmental stage (in hours), body length (in mm), body weight (in mg), and survivorship at each stage (%).

We used destructive sampling, that is, at each observation time we selected a subsample of larvae for one-time measurements. Prior to each measurement, larvae were individually washed to remove any adhering food and killed by immersion in warm water (3–5 s) at 50 °C. Immediately, body length was measured using a digital caliper rule with 0.1 mm precision, and the measurements were analyzed using ImageJ® software. Larvae were weighed using an analytical balance (Tecnal® 0.1 mg precision) and the instar was determined by examination of the morphology of the posterior spiracles under microscope (20X). To build survivorship tables, we recorded the mortality at first, second

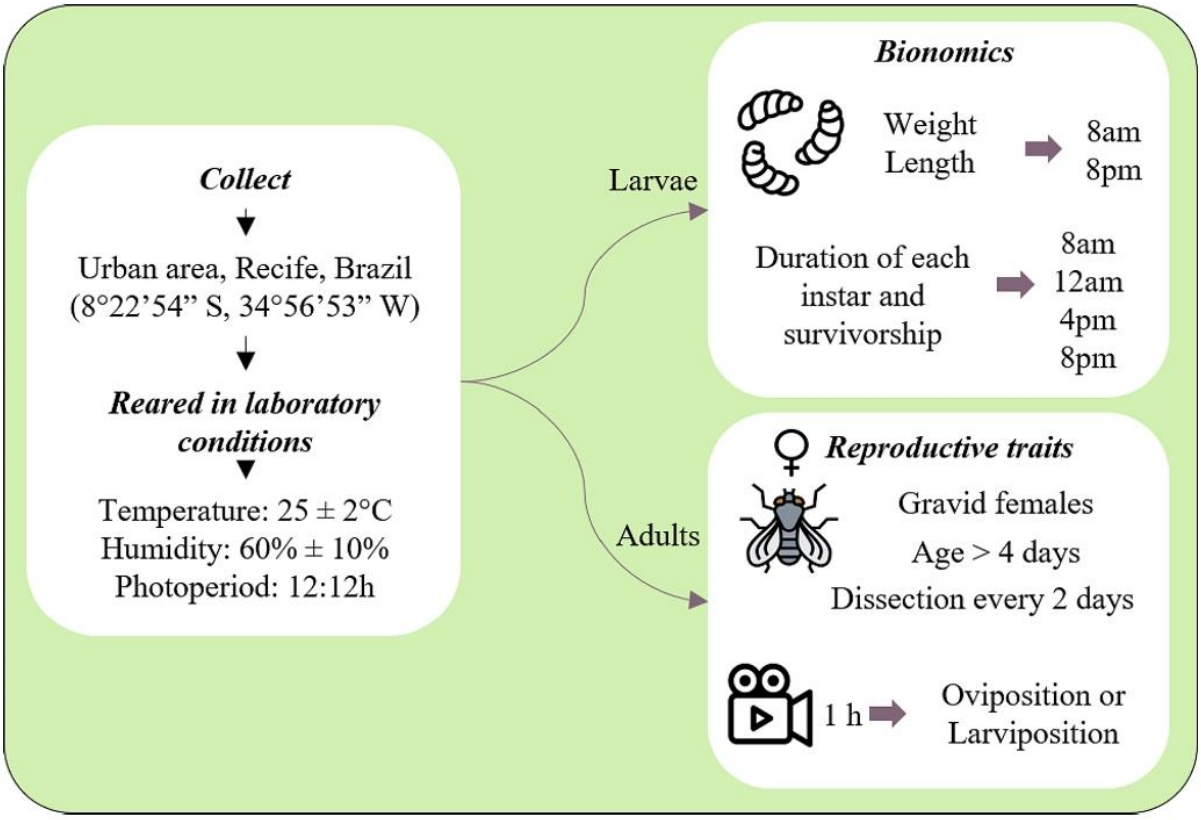

Fig. 1 A summarized description of the variables addressed in this study, focusing on the bionomics and the observation of reproductive traits of *Peckia (Peckia) chrysostoma* reared under laboratory conditions

and third instar, at the pre-pupa and the pupa stage. Insects were reared until death, and we recorded the longevity and the sex ratio of the adults.

Reproduction traits

Reproductive traits were examined in two experiments. Firstly, we characterized the intrauterine development of eggs and larvae. For this assessment, we reared groups of 20 pairs under controlled conditions and selected a subsample of 10 females at every two days. Each female at each time-point was dissected using a stereoscopic microscope (40X magnification), for the visualization and counting of the number of eggs and intrauterin larvae. The stage of development of eggs and larvae of *P.* (*P.*) *chrysostoma* followed the protocol with adaptations proposed by Chaiwong et al. [28] who classified it into three stages: (i) initial stage, characterized by the visualization of spherical follicles; (ii) intermediary stage, characterized by the visualization of oocysts expanding to fill one-third to half of the total follicle length and ii) advanced stage, characterized by the visualization of eggs and fully developed larvae.

In the second experiment, we recorded and quantified the occurrence of egg deposition or larval deposition and evaluated the viability of the offspring for each reproductive strategy. For this purpose, we used five batches of 20 pairs of adult flies kept in plastic cages (60×40×30 cm) with decomposing minced beef ad libitum to stimulate mating and provide additional protein source. After five days, sufficient time for copulation and egg maturation, we removed the food source and maintained the adults for 72 h for complete egg maturation [28]. We then selected ten females and proceeded with the filming register of egg or larviposition, using a Sony HDR-CX130 digital camera. Each female was filmed for 60 min, a minimal time for offspring deposition, based on pilot tests. The following variables related to reproductive aspects were measured: (a) number of eggs deposited; (b) number of larvae; and (c) viability of eggs.

Data analysis

For the experiment on bionomics, we used 30 replicates, each replicate consisting of an observation unit of 10 larvae. For the experiments on reproduction, we used five replicates: each replicate consisting of an observation unit of 20 pairs. We estimated the mean values and the standard deviation of the following variables: (i) larval weight, (ii) length and (iii) development time. We also calculated the survivorship for each stage and performed regression analysis to establish the relationship between larval weight and length at different stages of development and derive the corresponding model equation. The sex ratio (number of females/total number of females and males) of emerged adults and the longevity of adults were estimated, by calculating the mean ($\bar{x}$) and median (Md), in days. We analyzed reproductive traits by counting the number and the viability of eggs and larvae. Lastly, in the other experiment we calculated the mean number of eggs at each developmental stage within the gravid female. Statistical analysis was conducted using t-tests and regression, with the R software (version 4.1.2), and a significance level of $P < 5\%$ was considered.

Results

Instar duration, body size and weight, and survivorship

The total developmental time from first instar to adult, under the conditions tested, averaged 443.5 ± 30.73 h (Fig. 2), varying from a minimum of 384 h and maximum of 488 h (Table 1). The duration of the first instar (22.24 ± 3.16 h) and second (21.36 ± 3.19 h) instar did not differ significantly ($P > 0.05$), lasting less than a day. The third instar was significantly longer ($P < 0.05$), reaching 106.8 ± 19.9 h, but after approximately 80.0 h the larvae stop feeding and abandoned the food source and buried in the vermiculite, entering a phase characterized as the post-feeding stage (prepupa), which lasted, on average, 26.3 h. The pupal stage

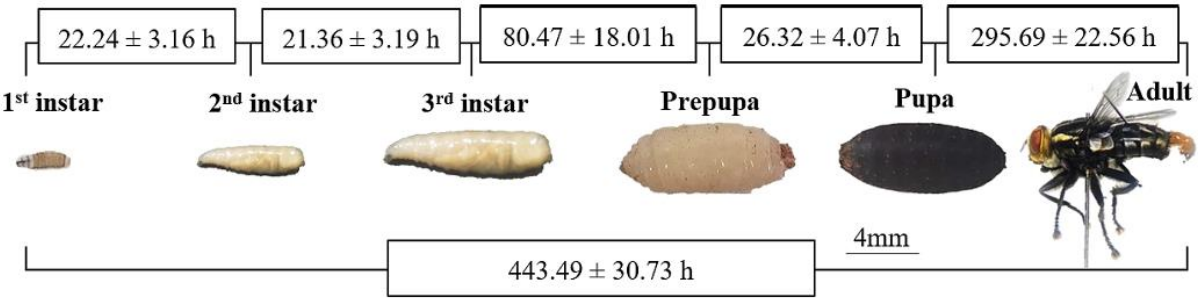

Fig. 2 Developmental time (h) of immature stages of *Peckia (Peckia) chrysostoma* reared under laboratory conditions

Table 1 Estimation of the minimum and maximum development time and survivorship observed for the immature stages of *Peckia (Peckia) chrysostoma* maintained under laboratory conditions (25 ± 2 °C, 60 ± 10% RH and 12 h of photoperiod). TDT – Total development time

Stages	Range Min. – max. (h)	Range Min. – max. (days)	Survivorship (%)
Egg	-	-	-
1st instar	18–28	0.75–1.17	82.67 (n = 248)
2nd instar	16–27	0.67–1.13	86.29 (n = 214)
3rd instar	56–121	2.30–5.04	94.85 (n = 203)
Prepupa	24–42	1.00–1.75	100 (n = 203)
Pupa	264–334	11.00–13.91	90.41 (n = 183)
TDT (1st instar – adult)	386–518	16.08–21.58	61.00 (n = 183)

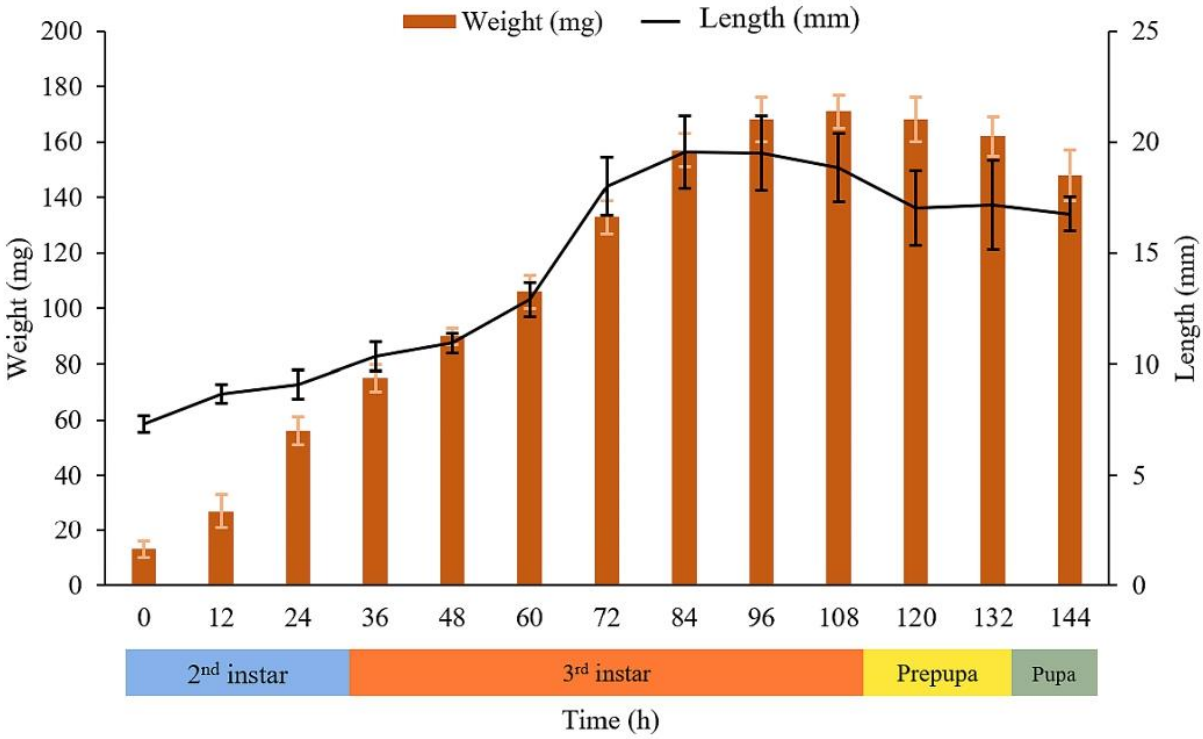

Fig. 3 Weight (mg) and length (mm) of immature stages of *Peckia* (*Peckia*) *chrysostoma* reared under laboratory conditions (25 ± 2 °C, 60 ± 10% RH and 12:12 h (L: D))

lasted 295.69 ± 22.56 h, being the longest phase of the *P.* (*P.*) *chrysostoma* cycle. The minimum and maximum duration of each stage varied, and this interval was longer in the 3rd instar (from 56 to 121 h) and in the pupa (from 264 to 336 h).

Survivorship exceeded 80% in all stages, and no mortality was observed during the transition from the post-feeding to the pupal stage. When considering the entire development process from the 1st stage to adult emergence, the overall survivorship of the species was 61.0%. The pupal stage exhibited the highest survivorship, with 90.4% of the pupae successfully reaching the adult stage, resulting in a sex ratio of 0.55, which did not favor either sex ($P > 0.05$) (Table 1).

The weight and length of the larvae increased significantly as they aged (Fig. 3) (weight: P = 2e-16, $R^2 = 0.88$, $y = 1.195x + 31.053$; length: P = 2.2e-16, $R^2 = 0.80$, $y = 0.093x + 8.0624$). Weight increased approximately 10 times from early 2nd instar (ca. 18 g) to late 3rd instar (ca. 180 g). Third instar larvae increased up to three times in weight and twice in size as they aged, with these changes being most noticeable between 36 h and 84 h. There was also a slight decrease in weight and size for the prepupa and pupa stages when compared to 3rd, that is, after 84.

Reproductive traits

The longevity of males ($\bar{x} = 30.29 \pm 18.27$, Md: 26 days) and females ($\bar{x} = 31.41 \pm 16.69$, Md: 24) did not differ significantly ($P = 0.28$). Females dissected on days 4 and 6 after exposure to males had eggs at the initial or intermediate stages. Only from the 8th day, fully developed eggs were visible, categorized as advanced stage, and on the 10th day, developing larvae inside the female were evident. We observed an average of 23.9 ± 5.02 eggs in the advanced stage per female, while we observed an average of 15.3 ± 4.18 larvae per female.

All three stages of offspring development were visualized in the analysis. In the initial stages of development, it was possible to visualize spherical follicles (Fig. 4A). The intermediary stage is characterized by the first indication of visibly developing oocyte within the follicles (Fig. 4B), progressing to great elongation of the follicles as the oocyte expands. For the advanced stage, the developed oocyte appeared to fill the entire follicle, with the visualization of bright white and elongated eggs with an appearance similar to small grains of rice (Fig. 4C). The developed larvae were visualized inside the follicles and had a yellowish color with sclerotized bands in the thoracic and abdominal segments, corresponding to cuticular spines that surround the entire body (Fig. 4D).

As a complementary observation on the reproductive behaviour, approximately 80% of the adults of *P. (P.) chrysostoma* engaged in copulation within the first 6 to 8 h after emergence. Also, for ca. 75% of the observed females, the first oviposition (eggs) occurred on average, between the 8th and 10th day of life. Larval deposition was frequently observed in females averaging over 12 days old. In the recorded experiments, the group of females observed simultaneously deposited both eggs and larvae, and it was not possible to determine if a single female was oviparous, ovoviviparous, viviparous, or both (Supplementary Material #1). However, none of the laid eggs was viable, given that there was no larval hatching for the whole period of observation (15 days after oviposition). Conversely, 100% of the deposited larvae were alive. Egg deposition was more frequent in the trials using young females, accounting for 61.1% and an average of 37.2 eggs per deposition. The remaining depositions consisted of larvae, comprising 38.9% and an average of 17.2 larvae.

Discussion

We describe here the development of *P.* (*P.*) *chrysostoma*, which can be a starting point for the incorporation of entomological evidence based on the species in criminal investigations. Overall, the length and weight curves for *P.* (*P.*) *chrysostoma* follow the pattern observed in other Sarcophagidae species [29], with a significant increase in these variables in the third instar. We propose here a tentative model for fitting the bionomic variables of weight and length related to time of development ($R > 0.8$), and the appropriate curves can be fitted to predict the elapsed time until the larva reaches a specific stage, as these variables support the estimation of larval age on the day of body discovery [3].

We document the facultative oviposition strategy of *P. (P.) chrysostoma*, a behavior that has been increasingly recorded in among Sarcophagidae species, such as *Blaesoxipha (Gigantotheca) plinthopyga* Wiedemann, 1830, *B.* (*G.*) *stallengi* Lahille 1907, and *Sarcophaga (Liosarcophaga) aegyptica* Salem, 1935 [10,13,25,30]. The evidence that young *P.* (*P.*) *chrysostoma* females can deposit a large quantity of infertile eggs should be taken into account to prevent overestimation of the min PMI [1]. Facultative egg deposition can be a strategy to retain a quantity of eggs for the subsequent maturation of the larvae, ensuring that the gravid female will deposit (i) when the larvae are fully developed, or (ii) when the females encounter ideal conditions (substrates) for larval development. Delayed resource-seeking behavior results in the deposition of larger, more

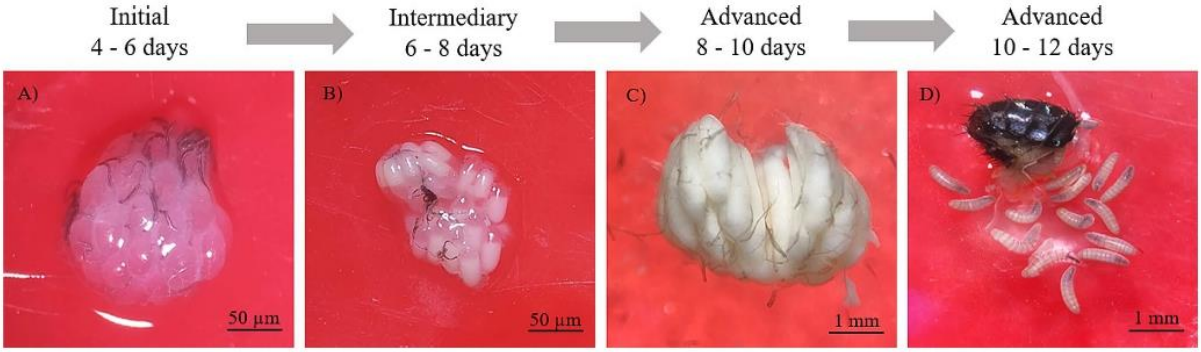

Fig. 4 Egg and larval intrauterine development of *Peckia* (*Peckia*) *chrysostoma*: (**A**) Initial stage; (**B**) Intermediate stage; (**C**) Advanced stage with visualization of developed eggs; (**D**) Visualization of developing larvae (40X)

robust larvae. This period of retention can be equivalent to the embryonic development time of eggs in calliphorid flies, for example, ranging from up to 24 h [31]. We reinforce the suggestion by Lutz and Amendt [4] that estimating the time of embryonation from an age estimate for species should be subtracted where evidence of early eggs is present.

The deposition of infertile eggs by typically viviparous species, although not commonly reported, has been recorded for *Calliphora augur* Fabricius, 1775 [32], *C. hilli* Patton, 1925 [33] and *C. dubia (Macquart, 1855)* [34], viviparous species that can alternatively lay soft and infertile eggs for a few days prior to regular larviposition, mirroring the observations made here for *P. (P.) chrysostoma*. This behavior was not observed it in natural conditions for Sarcophagidae, even with repeated sampling [35]. We believe that the deposition of infertile eggs during the female's sexual maturation may be a strategy to reduce the offspring and prioritize the more viable progeny, thus avoiding increased competition among the immatures. Larviparity is an efficient characteristic to avoid interspecific competition for resources, as seen in predatory species of *Chrysomya* (Diptera: Calliphoridae) [36]. This is similar to what is observed in *Lucilia cuprina* (Wiedemann, 1830) (Calliphoridae), where the reabsorption of some oocytes occurs to ensure the survival of a smaller brood in response to low food availability [37].

According to Villet et al. [1], the accuracy and precision of the information used in predictive models of min PMI are the main challenges faced by forensic entomologists. Based on the data obtained here, we propose qualitative criteria adapted from Vasconcelos et al. [38] to validate the relevance of *P. (P.) chrysostoma* in legal medicine investigations, as presented in Table 2.

Necrophagy is the main feeding habit of *Peckia (P.) chrysostoma*, so its frequent association with cadavers, associated with its ability to rapidly locate animal carcasses – as quickly as Calliphoridae species typically regarded as early colonizers, reinforce its forensic relevance [21,39]. The species occurs in many environments in the Neotropical Region – rainforests, agroecosystems, dry forests, urban and forested areas – with overlap of habitats – both indoors and outdoors; thus, its value as an indicator of site of death is insubstantial [13,18,31,36,38,42]. It also occurs throughout the year, in dry and rainy periods in Brazil [16,43] so that it is an unpredictable indicator of season of death.

The species is relatively easy to rear in the laboratory [24], which makes it amenable to bionomical studies using available protocols. It appears to show a direct response to laboratory-tested variables such as temperature, photoperiod, type of substrate, among others, so it can be used as model for further field variables. Documented association of *P. (P.) chrysostoma* with drug-intoxicated carcasses [39] justifies its use as a tool for the detection of chemical compounds (forensic entomotoxicology). The presence of genetic data in databases [47] also provides valuable information for investigations involving molecular biology-based identification.

The scarcity of data on the colonization of cadaver's victims of different conditions of death hinders an objective assessment of *P.* (*P.*) *chrysostoma* as an additional tool in elucidating the type of death, but a congeneric *Peckia* sp. has been sampled from a human corpse found hanging in Northeastern Braz[il] [48]. For the estimation of the min PMI, bionomic data regarding the duration of larval instars are limited, but this study provides a starting point for development time and complements it with variables such as weight and length for the validity of the min PMI. We hope that our findings stimulate further studies to validate the species' suitability for legal medicine investigations in areas of similar landscape and climatic conditions. Such detailed

Table 2 A Qualitative assessment for the validation of *Peckia* (*Peckia*) *chrysostoma* (Diptera: Sarcophagidae) as a species of potential forensic importance. (adapted from Vasconcelos et al. 2023). Legend: - = not important; + = little importance; ++ = moderate importance; +++ = strong importance; ? = unknown

Criteria	Category	Ref
Likelihood of cadaver colonization	++	[21]
Carrion colonization	+++	[16,39,40]
High abundance colonizing carrion	+	[39,41]
Occurrence in specific countries or geographic regions	-	[18,38,40,42]
Association to specific types of habitats, such as urban or forested areas	-	[16,39,42,43]
Seasonal occurrence throughout the year (Potential use as indicators of season of death, e.g., rainy or dry seasons)	-	[16,44]
Experimental data on reproductive strategies	+	this study
Protocol for rearing under laboratory conditions	+++	this study [24,45],
Availability of identification keys	++	[27]
Comprehensive morphological description of all stages	++	[46]
Estimation of min PMI (data on bionomics, development, instar duration)	++	this study [24],
Potential use in forensic entomotoxicology	+	[39]
Available data in genetic databases (molecular identification)	++	[47]

information contributes to a better understanding of the species and enhances its potential as a forensic tool.

Conclusions

We provide an integrative description of the bionomics and reproductive traits of a flesh fly species, *P. (P.) chrysostoma*, which holds significance in the fields of medico-veterinary and forensic sciences. The results shed light on the development time and survivorship of immature stages, providing insights into the ovarian development and highlighting the ability to deposit eggs as a reproductive strategy that was previously poorly understood within the Sarcophagidae family.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00414-024-03242-y.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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