UNIVERSIDADE FEDERAL DO PARANÁ

SABRINA MACHADO DA SILVA

INTRAPUPARIAL DEVELOPMENT OF *Hemilucilia semidiaphana* (RONDANI, 1850) (DIPTERA, CALLIPHORIDAE) AND ITS USE IN FORENSIC ENTOMOLOGY

CURITIBA

2018

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Dissertação apresentada ao curso de Pós-Graduação em Ciências Biológicas, Área de Concentração em Entomologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Mestre em Entomologia.

Orientador: Prof. Dr. Mauricio Osvaldo Moura

CURITIBA

2018

Universidade Federal do Paraná. Sistema de Bibliotecas.
Biblioteca de Ciências Biológicas
(Telma Terezinha Stresser de Assis –CRB/9-944)

Silva, Sabrina Machado da
Intrapuparial development of *Hemilucilia semidiaphana* (Rondani, 1850) (Diptera, Calliphoridae) and its use in forensic entomology. / Sabrina Machado da Silva. – Curitiba, 2018.
48 p. : il. ; 30cm.

Orientador: Mauricio Osvaldo Moura
Dissertação (Mestrado) - Universidade Federal do Paraná, Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências Biológicas (Entomologia).

1. Entomologia forense. 2. Mosca. 3. Microscopia. 4. Diptero. I. Título II. Moura, Mauricio Osvaldo. III. Universidade Federal do Paraná. Setor de Ciências Biológicas. Programa de Pós-Graduação em Ciências Biológicas (Entomologia).

CDD (20. ed.) 595.77

MINISTÉRIO DA EDUCAÇÃO
SETOR CIÊNCIAS BIOLÓGICAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS BIOLÓGICAS
(ENTOMOLOGIA)

TERMO DE APROVAÇÃO

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Curitiba, 30 de Maio de 2018.

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AGRADECIMENTOS

Ao Prof. Dr. Mauricio O. Moura, por toda a ajuda na elaboração desta dissertação, mas principalmente pelas conversas infindáveis e reconfortantes durante o café.

Aos meus pais Rozane e Claudemir e ao meu irmão Cleyton, que ao longo de toda a minha vida tem me apoiado fielmente em todas as minhas escolhas, tornando essa jornada mais acolhedora e feliz. Agradeço também pelos insetos que todos me ajudaram a coletar.

Ao meu namorado Rodrigo de Jesus Quintiliano, por me aguentar falando sobre insetos, pela companhia em minhas vindas e noites que passei no laboratório, cataias e cervejas e principalmente por ser meu parceiro nessa jornada.

A todos os meus colegas do Laboratório de Dinâmicas Ecológicas pela companhia e conversas, não tão extensas porem muito significativas. Em especial a Julia por ser minha parceira de coleta e experimentos.

Aos meus amigos, em especial Sze Mei Lo e Karin Cristina Camargo, pelas conversas e saídas para cafés, almoços e comidinhas todos maravilhosos.

A todas as moscas que contribuíram para a elaboração desta descrição.

A CAPES pela concessão da bolsa de Mestrado.

Ao Projeto Táxon-Line - Rede Paranaense de Coleções Biológicas, pelas fotos.

A todos que contribuíram para elaboração deste trabalho.

PREFÁCIO

> "Cada um de nós tem a sua própria morte, transporta-a consigo num lugar secreto desde que nasceu, ela pertence-te, tu pertence-lhe."
>
> José Saramago

Este texto está sendo escrito (ou, no caso, foi escrito) pela autora desta dissertação (ou seja, a culpa é toda minha) que concluiu ser necessário a apresentação das motivações e objetivos gerais deste trabalho, sem deixar de incluir ao menos nesta seção um quê informal e poético que ela acredita ser de bom tom, porém raro no meio cientifico. A epígrafe, por exemplo, foi retirada de um livro chamado "As intermitências da Morte", do ilustríssimo escritor português José Saramago, cuja leitura se deu durante algumas das minhas muitas viagens de volta para casa nesses dois anos de mestrado. Ao escrever esta dissertação, a frase me veio à lembrança, e com ela algumas indagações do por que estudar a área forense médico-legal e como ela se relaciona com a morte.

No âmbito cientifico, o estudo na área de entomologia forense se traduz nas perguntas: Como? Quando? Onde? Elas podem ser respondidas em parte quando empregamos as informações biológicas dos insetos, o que torna o nosso estudo significativo. A morte em si é um evento natural e ainda assim gera comoção, mas aquela em que as circunstâncias são um enigma acabam por ser a base da entomologia forense. Dito isso e colocando o estudo forense como algo importante para resolver as inquietações do ser humano, cheguei a outro ponto que considero significativo: o estudo detalhado de cada uma das espécies de interesse forense.

Os insetos compreendem a classe mais diversa do planeta e, no entanto, este estudo foca em uma etapa do ciclo de vida de uma das espécies da região Neotropical. Mas existe um contraponto: a riqueza de espécies e sua especificidade faz com que o estudo dessa etapa do ciclo de vida seja útil para a resolução de várias questões, notadamente o como e o onde, já que a espécie de interesse é restrita a ambientes florestais, ocorre com mais frequência na primavera e no estágio de fermentação (dentre as etapas de decomposição).

Ainda que não houvessem n motivos para o estudo desta espécie e etapa de desenvolvimento, o ato de querer compreender a vida e seus pequenos detalhes é algo que nós, cientistas e não-cientistas (por profissão), fazemos. Apesar da já conhecida infinitude do universo (e aqui, o que eu sei que estavam esperando, o clichê do clímax), nós somos poeira estelar e não seria muito legal da nossa parte deixar essa imensidão de lado e uma última pergunta sem resposta: O por quê?

Little fly,
Thy summer's play
My thoughtless hand
Has brushed away.

Am not I
A fly like thee?
Or art not thou
A man like me?

For I dance
And drink and sing,
Till some blind hand
Shall brush my wing.

If thought is life
And strength and breath,
And the want
Of thought is death,

Then am I
A happy fly,
If I live,
Or if I die.

William Blake (1757-1827) P. 1793

RESUMO

Em entomologia forense, a estimativa do intervalo post mortem mínimo (IPM*min*) geralmente é baseada no imaturo mais velho de Calliphoridae recuperado de um local de morte. O tempo que as moscas da família Callliphoridae passam no período intrapuparial compreende mais de 50% do seu ciclo de vida. Isso demonstra a importância de estimativas precisas do tempo de desenvolvimento nesse período, o que deve aumentar a acurácia das estimativas de IPM mínimo. A mosca varejeira *Hemilucilia semidiaphana* (Rondani,1850) foi registrada em seis cenas de morte na cidade de Curitiba e Região Metropolitana. Na literatura já existem dados para os instares larvais e para a taxa de desenvolvimento de *H. semidiaphana*, no entanto não há dados para o período intrapuparial. Para preencher essa lacuna nosso objetivo é fornecer descrições detalhadas para as mudanças morfológicas durante o desenvolvimento intrapuparial de *H. semidiaphana,* possibilitando seu uso em estimativas de IPM min. Amostras do período intrapuparial de *H. semidiaphana* foram obtidas a partir de imaturos criados com dieta artificial em incubadoras ajustadas em temperaturas constantes a 20ºC e 25ºC. As pupas foram fixadas em intervalos de três e seis horas até a emergência do adulto e analisadas utilizando microscopia de luz para a determinação de características morfológicas externas e estágios de desenvolvimento que permitem determinar a idade do imaturo. As análises do período intrapuparial de *H. semidiaphana* forneceram vinte e uma características, das quais identificamos nove características chaves relacionadas à idade que dividem o tempo total de desenvolvimento (144 horas à 25ºC e 192 horas à 20ºC) em intervalos menores. Nas primeiras horas de desenvolvimento as características mais informativas são o cefaloesqueleto e a eversão dos apêndices torácicos e da cabeça. A medida que o desenvolvimento progride as principais características para determinação da idade estão relacionadas à tagmatização completa entre cabeça, tórax e abdômen, ao desenvolvimento das antenas e das peças bucais, à coloração dos olhos e, finalmente, à pigmentação das cerdas são as características mais importantes para determinar a idade intrapuparial. Os dados de desenvolvimento fornecidos, juntamente com a linha do tempo, permitem um modo prático de realizar comparações interespecíficas, bem como para estimativa de idade de *H. semidiaphana* baseada no desenvolvimento intrapuparial.

Palavras-chaves: IPM*min*, microscopia de luz; morfologia; mosca varejeira; pupa.

ABSTRACT

In forensic entomology, the minimum postmortem interval (*min*PMI) estimative is usually based on the oldest immature recovered from a local of death. The time spent by fly immatures in the intrapuparial period comprises more than 50% of their complete life cycles. An accurate estimate of the duration of this period will improve *min*PMI estimates. The blowfly *Hemilucilia semidiaphana* (Rondani,1850) was found in six criminal cases in the city of Curitiba. Even though there is data on the morphology of the larval instars and developmental rate of *H. semidiaphana*, the intrapuparial period has not been investigated. Here we provide a detailed description of the intrapuparial morphological changes of *H. semidiaphana*, which might be useful to estimate minimum PMI. Samples of *H. semidiaphana* in the intrapuparial period were obtained from immatures reared on an artificial diet in incubators adjusted to 25°C or 20ºC temperature regimes. Pupae of *H. semidiaphana* were fixed at intervals of three and six hours until emergence of the adult. The external morphological traits of sampled pupae were analyzed using light microscopy, which enabled the determination of their age. Our analysis of the intrapuparial period of *H. semidiaphana* provided 21 traits from which nine were age informative. These nine characteristics divide the developmental time (144 hours at 25ºC and 192 hours at 20ºC) into smaller sections. In the first hours of development the most informative characters are in the cephaloskeleton: the eversion of the thoracic appendages and the eversion of the head. As the development proceeds, the boundaries between head, thorax and abdomen, the development of the antenna and mouthpieces, the coloration of eyes, and finally the pigmentation of bristles, were the most informative traits to estimate intrapuparial age. The developmental data provided, together with the time line allows a practical way to make interspecific comparisons as well as to estimate the age of *H. semidiaphana* based on the intrapuparial development.

Keywords: blowfly; minPMI; morphology, light microscopy; pupa;

FIGURES

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SUMÁRIO

1. RESUMO EXTENDIDO

A Entomologia Forense é o estudo que utiliza informações biológicas de insetos para aplicação no contexto criminal com o intuito de levantar informações úteis para investigações (Catts e Haskell, 1990, Hall, 2001). Este estudo costuma ser dividido em subáreas que se relacionam com problemas legais: a área de produtos estocados, a área urbana, a área médico-criminal e a área ambiental (Lord e Stevenson, 1986; Oliveira-Costa, 2011). Em sua área médico-criminal a principal análise se baseia na presença ou tempo de desenvolvimento dos insetos encontrados no cadáver e o tempo decorrido desde a colonização (Benecke, 2001) que leva a estimativa de um intervalo de tempo que o inseto está associado com o corpo. Após a morte, o corpo passa por várias etapas de decomposição, entre elas: o *rigor* e o *livor mortis*, cujas características são utilizadas para a estimativa de intervalo pós morte pela de patologia forense (Campobasso et al., 2001). No entanto, essas características têm maior grau de confiabilidade se utilizadas nas primeiras 72 após a morte. Com o decorrer do tempo, o corpo em decomposição se torna um recurso valioso para diversos insetos e artrópodes que passam a utilizar a carcaça de acordo com suas preferências biológicas, incluindo a utilização como recurso alimentar (Mégnin, 1894; Deonier, 1940), o que torna esses colonizadores importantes para a entomologia forense e o cálculo de IPM*min* que passa a ser a forma mais acurada para a estimativa de intervalo pós-morte.

Na entomologia médico-criminal, uma das principais questões a ser respondida é a quanto tempo a morte ocorreu (Benecke, 2004). Esse tempo corresponde ao intervalo decorrido desde a morte até a descoberta do corpo, denominado intervalo pós-morte mínimo (IPM), que por sua vez é baseado no tempo de colonização dos insetos nos corpos (PIA, Tomberlin et al., 2011). Isso ocorre porque após a morte, o cadáver libera odores que atraem uma gama de insetos para o corpo (Anderson, 2001). Parte desses insetos possuem hábito necrófago, colonizam o corpo e possuem papel importante no processo de decomposição (Catts e Goff, 1992). Com isso, os insetos necrófagos ficam associados ao corpo por um período de tempo, sendo por isso utilizados para o cálculo de intervalo pós-morte.

Duas maneiras são mais usualmente empregadas para a estimativa de IPM utilizando o tempo de colonização de insetos nos corpos: baseando-se na comunidade ou na população de insetos recuperados de um local de morte (Smith, 1986; Catts e Haskell, 1990; Byrd e Castner, 2001; Villet et al., 2010). A estimativa de intervalo pós-morte baseada na comunidade de

insetos depende da existência de base de dados do padrão de sucessão de insetos no cadáver de acordo com as etapas de decomposição em determinada região geográfica e climática (Anderson, 1996). Esses dados de ocupação, quando comparados com a amostra coletada em um local de morte, fornecem uma estimativa máxima de intervalo pós-morte (Schoenly et al., 1996). Por outro lado, a estimativa baseada na população de insetos coletados depende da determinação da idade dos imaturos mais velhos recuperados em um local de morte (Amendt et al., 2004). Essa estimativa é obtida através de cálculos matemáticos que utilizam medidas de peso e tamanho dos imaturos, ou dos tempos de desenvolvimento e fornecem um IPM*min* (Catts e Haskell, 1990; Byrd e Castner, 2001, Grassberger e Reiter, 2001).

Dentre os colonizadores de um cadáver os mais abundantes pertencem às ordens Diptera e Coleoptera, grupos que possuem papel importante na decomposição do corpo e são, portanto, de importância forense (Benecke, 2001; Amendt et al., 2010). Em Diptera, as principais famílias associadas à decomposição de cadáveres são Calliphoridae, Sarcophagidae, Muscidae, Phoridae e Fannidae (Gennard, 2007). As moscas varejeiras, Calliphoridae, são as primeiras a chegar ao cadáver, que se torna um sítio para encontro de parceiros para cópula, para a postura de ovos e recurso alimentar para as larvas (Anderson e Van Laerhoven, 1996; Amendt et al., 2010). As moscas da família Calliphoridae são insetos holometábolos que passam grande parte do seu ciclo de vida no corpo, e por isso são comumente utilizados para cálculo de IPM mínimo.

Estudos da morfologia e biologia de Calliphoridae têm fornecido uma grande quantidade de informações utilizadas para o cálculo de IPM (Introna et al., 1998; Starkeby, 2001; Oliveira-Costa e Mello-Patiu, 2004; Arnaldos et al., 2005; Sukontason et al., 2005; Sukontason et al., 2007; Pujol-Luz et al., 2012; Kosmann et al., 2011; Ying et al., 2013; Vairo et al., 2015; Ramos-Pastrana e Wolff, 2017). No entanto, o tempo que as moscas varejeiras gastam com os estágios de ovo e larva corresponde a menos de 50 % do ciclo de vida dos imaturos, o restante do tempo de desenvolvimento corresponde ao estágio de desenvolvimento intrapuparial (Brown et al., 2015). Neste período o imaturo não se alimenta e não se movimenta, e permanece por mais tempo associado ao corpo após a morte (Ma et al., 2015). Apesar da importância em determinar de forma mais acurada a idade do imaturo durante esta etapa de desenvolvimento, os estudos que tem como objetivo a descrição detalhada do período intrapuparial em Calliphoridae para estimativa de idade, e que consequentemente podem ser utilizados para a estimava de IPM*min* ainda são poucos (Brown et al., 2012, Karabey e Sert, 2014; Proença et al., 2014; Ma et al., 2015; Brown et al., 2015; Barros-Cordeiro et al., 2016; Hall et al., 2017; Ramos-Pastrana et al., 2017; Flissak e Moura 2018; Zhang et al., 2018). A

descrição do período intrapuparial é baseada em estágios de desenvolvimento que correspondem a um conjunto de eventos e processos que ocorrem dentro do pupário (Fraenkel e Bhaskaran, 1973; Martín-Vega et al., 2016). Esses estágios foram estabelecidos a partir de diversos estudos da metamorfose em Diptera (Hinton, 1946; Fraenkel e Bhaskaran, 1973; Denlinger e Ždárek, 1994; Martín-Vega et al., 2016), sendo que a terminologia mais comumente utilizada corresponde a seis estágios de desenvolvimento: pupariação, (pré-pupa) apólise larva-pupa, pupa criptocefálica, apólise pupa-adulto e adulto farado (Fraenkel e Bhaskaran, 1973; Martín-Vega et al., 2016).

A descrição do período intrapuparial tem sido feita a partir da determinação de característica morfológicas externas, após a remoção do pupário, associadas à idade do imaturo (Karabey e Sert, 2014; Proença et al., 2014; Ma et al., 2015; Brown et al., 2015; Barros-Cordeiro et al., 2016; Hall et al. 2017; Ramos-Pastrana et al. 2017; Flissak e Moura, 2018; Zhang et al., 2018). Tal descrição é realizada com técnicas simples, em que amostras em intervalos regulares de tempo, após fixadas, são analisadas em microscopia de luz e registradas com imagens (Ma et al., 2015; Zhang et al., 2018; Flissak e Moura, 2018). Uma segunda forma de descrição do período intrapuparial é baseada em técnicas mais complexas como a histologia ou microtomografia computadorizada 3D, que permitem a observação de mudanças durante a metamorfose, como por exemplo, as alterações da morfologia cerebral e do intestino, ou ainda de formação de cutículas (Davies e Harvey, 2012; Hall et al., 2017). A técnica de descrição morfológica de características externas, embora seja a mais empregada em estudos da área possui vieses metodológicos que foram criticados (Martín-Vega et al., 2016). Essa crítica se fundamenta na determinação da idade do imaturo durante o período intrapuparial baseada apenas em características externas. Isso porque, alguns estágios de desenvolvimento, como a apólise larva-pupa e a apólise pupa-adulto, são difíceis de observar em microscopia de luz, principalmente o processo de separação de cutículas entre as fases de pupa e adulto farado, fundamental para a delimitação da apólise. Assim, se a determinação de idade do imaturo for feita utilizando apenas os estágios de desenvolvimento intrapuparial, essa poderia conter erros, decorrentes das imprecisões de delimitação morfológica, na estimativa do IPM mínimo (Martín-Vega et al., 2016), diminuindo sua acurácia. No entanto, quando a determinação da idade é baseada nas principais características morfológicas externas relacionadas à idade, o erro associado se torna menor (Flissak e Moura, 2018). Além disso, a utilização de técnicas histológicas e tomográficas são complexas e custosas (Brown et al., 2015), o que as torna de difícil aplicação na rotina pericial em laboratórios forenses.

Hemilucilia semidiaphana (Rondani,1850) (Diptera: Calliphoridae) tem distribuição Neotropical com registro para a América Central, Trinidade, Bolívia, Paraguai e Brasil (James, 1970; Linhares, 1981; Kosmann et al., 2013). Em Curitiba e região metropolitana *H. semidiaphana* foi registrada em cinco locais de morte em ambiente aberto e em um caso criminal em ambiente fechado, todos próximo a áreas florestadas (Vairo et al., 2015; R. Correia comunicação pessoal). Além dos registros em locais de morte, *H. semidiaphana* já foi utilizada para estimar o intervalo pós-morte na cidade de Batatais, no Estado de São Paulo (Thyssen et al., 2018).

Os dados biológicos disponíveis para *H. semidiaphana* incluem a descrição morfológica dos ovos e instares larvais e, ainda, o tempo de desenvolvimento em diversas temperaturas (Thyssen, 2005). No entanto, não existem estudos para o estágio intrapuparial. Para preencher essa lacuna, este trabalho teve como objetivo a descrição morfológica do período intrapuparial de *H. semidiaphana* em duas temperaturas, permitindo a sua utilização para estimar o intervalo pós-morte mínimo.

Para isso, adultos de *H. semidiaphana* foram coletados na cidade de Curitiba (25°25'S e 49°14'W) utilizando armadilhas do tipo shannon com iscas à base de frango em decomposição. Os casais coletados em campo foram criados em laboratório sob condições controladas e alimentados com uma dieta composta de açúcar, leite em pó, mel e água *ad libitum*. Para a obtenção das posturas uma placa de Petri contendo uma mistura de fígado, carne bovina moída, frango e peixe foi deixada à disposição das fêmeas e observada de hora em hora. Após obtenção de postura, com aproximadamente 300 ovos, os imaturos foram transferidos para recipientes contendo dieta artificial à base de rúmen bovino (Estrada, 2009). Esses recipientes foram colocados em recipientes maiores contendo vermiculita como substrato para pupariação, vedados com tecido scaline e alocados em incubadoras (B.O.D.) mantidas a 25ºC e 20ºC de temperatura, + -70% de umidade e fotoperíodo (12:12 horas). O desenvolvimento dos imaturos foi acompanhado diariamente até ser observado o comportamento de saída da dieta. Após o início do comportamento errante das larvas de terceiro instar, os imaturos foram observados de hora em hora até o início do processo de formação do pupário. Amostragens de 10 pupas e 6 pupas, respectivamente para as temperaturas de 25ºC e 20ºC, foram realizadas em intervalos de 3 horas até as primeiras 24 horas de desenvolvimento e em intervalos de 6 horas até a emergência do adulto. No total 280 (à 25ºC) e 230 exemplares (à 20ºC) foram sacrificados em água quente e fixados em solução AFA (70% Etanol, 40% formaldeído e ácido acético). Após o término do experimento as amostras foram dissecadas para remoção do pupário e

observadas em microscopia de luz para a determinação das principais características associadas à idade.

Os dados obtidos para cada exemplar foram utilizados na construção de tabelas contendo a idade mínima e máxima em que cada conjunto de características consideradas uteis para a determinação da idade de *H. semidiaphana* foi identificado. Também, é apresentada a média ponderada e o tempo mínimo e máximo de duração para cada estágio de desenvolvimento intrapuparial. Além disso, para operacionalizar a utilização dos caracteres, uma linha do tempo para o desenvolvimento intrapuparial de *H. semidiaphana* contendo as principais características para determinação da idade, foi construída para cada uma das temperaturas.

No geral, 21 características morfológicas externas foram consideradas relevantes para a determinação da idade em *H. semidiaphana* em ambas as temperaturas. Porém nove (9) características foram consideradas chaves para a determinação da idade em *H. semidiaphana.* Nas primeiras horas de desenvolvimento intrapuparial as características chaves para determinação da idade são relacionadas ao posicionamento do cefaloesqueleto, a eversão dos apêndices torácicos e da cabeça. Nas últimas horas de desenvolvimento as principais características para determinação da idade estão relacionadas com a tagmatização completa entre cabeça, tórax e abdômen, o desenvolvimento das antenas e das peças bucais, a coloração dos olhos e, finalmente, com a pigmentação das cerdas. As características descritas ocorrem na mesma sequência para exemplares que se desenvolveram tanto em 20ºC quanto 25ºC embora, o tempo de aparecimento dessas características é maior para a temperatura de 20ºC, o que indica que as mudanças morfológicas são invariáveis à temperatura, que afeta apenas a idade que as características morfológicas são identificadas. Sete estágios de desenvolvimento foram determinados, sendo que a média ponderada de tempo (idade) para cada estágio é maior para as amostras que se desenvolveram em 20ºC.

As características morfológicas externas identificadas como úteis para determinação da idade em *H. semidiaphana* são capazes de dividir o desenvolvimento intrapuparial, que durou um total de 144 horas (=6 dias) à 25ºC e 192 horas (=8 dias) à 20ºC, em intervalos menores que facilitam a utilização dos dados para a estimativa de intervalo pós porte mínimo. Além disso, todas as características são facilmente observadas com o auxílio de microscopia de luz, o que faz com que essa abordagem seja facilmente aplicada em laboratórios forenses na América do Sul.

2. INTRODUCTION

In Forensic Sciences, one of the main questions asked when a body is found is: "when did death occur?". The answer to this question is important to the investigation efforts surrounding the circumstances of the death. In forensic entomology "when did death occur?" is answered by calculating a minimum *post mortem* interval (*min*PMI), which is the time since death until the discovery of a body (Benecke, 2004). After death, the body releases odors that attract several insects that colonize and consume the body (Anderson, 2001). Such an association between insects and the decay process (Catts and Goff, 1992) occurs because necrophagous species spend their entire developmental period in corpses. As a result, the period of insect activity (PIA) (Tomberlin et al., 2011; Davies and Harvey et al., 2012) provides valuable information about the *min*PMI (Greenberg, 1991).

There are two ways to estimate the *post mortem* interval: one is based on the carrion insect communities and the other on the population of immature insects sampled from a corpse (Catts and Haskel, 1990; Smith, 1986; Byrd and Castner, 2001; Villet et al., 2010). When the PMI is based on the carrion insect communities, the insect samples collected from a corpse are compared against a reference database of insect succession patterns in each phase of a body's decay, considering the geographic region and temperature (Anderson, 1996). The estimated results represent the maximum PMI, which application is useful in criminal cases that involve a corpse discovered weeks or months after death (Schoenley et al., 1996) When the PMI is based on the immature insects recovered from the body, it is necessary to determine the age of the immature that best represents the minimum time it has colonized the body (Amendt et al., 2004), that is, the most probable immatures that began to develop very shortly after death. Usually, this age estimate is based on the developmental time of the immatures or on measurements of the weight and the length of the oldest larva recovered (Grassberger and Reiter, 2001; Amendt et al., 2004).

The time of colonization, or period of insect activity (PIA), can be greater, less than or equal to the PMI (Amendt et al., 2007). Overall, in cases when there is negligence followed by death, live humans can become infested by fly larvae, a condition known as myases (Zumpt, 1965; Erzinclioglu, 1996), which increases the time of colonization, and make the period of insect activity greater than the *min*PMI (Amendt, 2004). The PMI in cases of negligence followed by death can be determined, for example, using the age of the maggots that are primarily attracted by feces and urine and colonize the body before death, compared with the

age of maggots that only colonize the body after death (Benecke, 2004). In another scenario, when insects have restricted access to a body, a decrease in the time of colonization compared to the time elapsed since death (Benecke, 2004; Tomberlin et al., 2011) should be expected. When the body is burned, wrapped or hid in enclosed places, insects and other arthropods may not have full access to it, thereby delaying or even preventing the colonization and therefore the estimation of the *min*PMI (Tomberlin et al., 2011; Anderson, 2005; Goff, 1992).

Among the fauna associated with a dead body, the most abundant colonizers are in the orders Diptera and Coleoptera, both of which have an important function in the process of decomposition and are thus forensically important (Benecke, 2001; Amendt et al., 2010). In Diptera the most common families associated with corpses are Calliphoridae, Sarcophagidae, Muscidae, Phoridae and Fannidae (Gennard, 2007). Usually, Calliphoridae (blowflies) are the first to arrive at a cadaver. They arrive shortly after death, attracted by the odors emanating from the body (Anderson and Van Laerhoven, 1996; Amendt et al., 2010). For necrophagous Calliphoridae, the corpse becomes a place to find mates, and also a site for laying eggs and a food resource for maggot development (Mégnin, 1894; Deonier, 1940).

Morphological and developmental studies of Calliphoridae eggs and larvae has provided a significant amount of data that can be used to estimate the *min*PMI (Greenberg and Szyska, 1984; Queiroz, 1996; Amendt et al., 2007; Richards et al., 2009; Florez and Wolff, 2009; Szpila, 2010; Thyssen, 2014; Lecheta et al., 2015; Zhang et al., 2018). For example, third instar larvae of *Chrysomya albiceps* (Wiedemann, 1819) and pupae of *Sarconesia chlorogaster* (Wiedmann, 1830) recovered from a local of death in the city of Curitiba (Southern Brazil) provided a *min*PMI of 7-8 days based on the developmental rate of *C. albiceps* and on the coloration of the puparium of *S. chlorogaster* (Vairo et al., 2015). This was only possible because there was previous data (Souza, 1999; Lecheta et al. 2015) that enabled the estimation of the age of the flies found in the local of death.

The time flies (Diptera) spend as egg, first, second and third instar larvae is less than 50% of their total life cycle time, while the sedentary intrapuparial period lasts for the remaining of the development time until emergence (Brown et al., 2015). Therefore, the intrapuparial period can be the oldest recovered stage found in association with a cadaver (Vairo et al. 2015; Bala and Sharma, 2016; Ramos-Pastrana and Wolff, 2017; Thyssen et al. 2018), and in consequence, the most appropriate stage for an accurate estimate of the *min*PMI. Despite the importance of the intrapuparial development to forensic entomology, morphological descriptions of this stage are still scarce and are usually biased toward cosmopolitan species

(Zhang et al., 2018). For instance, the intrapuparial period of blowflies with a large geographic distribution such as *Lucilia cuprina* (Wiedemann, 1830) (Barros-Cordeiro et al. 2016), *Calliphora vicina* Robineau – Desvoidy, 1830 (Brown et al., 2015), *Lucilia sericata* (Meigen, 1826) (Karabey and Sert, 2014), *C. albiceps*, and *Chrysomya megacephala* (Fabricius, 1794) (Zhang et al., 2018) has been described in detail under different temperature regimens.

The timing of the morphological changes during metamorphosis in the intrapuparial period are species-specific and temperature-dependent (Costa et al., 2006). Since insects are ectothermic, their development time is highly affected by temperature (Wells and La Mootte, 2010). Usually, their development is accelerated when the temperature is warm and delayed when it is cold, becoming longer (Greenberg, 1991). For example, in *S. chlorogaster* (Flissak and Moura 2018), an endemic species from South America, the eversion of the head, a key characteristic to determine the age in Calliphoridae, occurs 40 hours after pupariation in controlled temperature (20ºC), whereas the same event occurs 32 hours after pupariation when the temperature is 25ºC.

The intrapuparial period has been separated into stages that correspond to a set of events and process that occur during metamorphosis within the puparium. These stages and the terminology adopted for them have been stablished based on several studies among Diptera (Hinton, 1946; Denlinger and Ždárek, 1994; Fraenkel and Bhaskaran, 1973). The most common terminology includes six stages, which are pupariation, prepupa, larval-pupal apolysis, cryptocephalic pupa, phanerocephalic pupa, pupal-adult apolysis and pharate adult. This framework has been extensively discussed (Hinton, 1946; Denlinger and Ždárek, 1994; Fraenkel and Bhaskaran, 1973; Martín-Vega et al., 2016), becoming the basic framework to describe the intrapuparial period study in Diptera (Pujol-Luz and Barros-Cordeiro, 2012; Defilippo et al., 2013; Karabey and Sert, 2014; Proença et al., 2014; Barros-Cordeiro et al., 2016; Flissak and Moura, 2018).

The external or internal morphological changes that occur in the intrapuparial period can be observed using light microscopy, or more refined techniques, such as tomography (Brown et al., 2015). Although simple, light microscopy requires the removal of the puparium to allow visualization and the generation of images at regular age intervals in order to describe age-related morphological changes (Cepeda-Palacios and Scholl, 2000; Brown et al., 2015; Ma et al., 2015; Barros-Cordeiro et al., 2016; Zhang et al., 2018). Also, this technique is a low-cost option in forensic analyses (Brown et al., 2015). Other techniques, such as histology, visualize

internal chronological changes with histolysis and histogenesis of tissues and organs, observing fat bodies and glycogen storage (Davies and Harvey, 2012). While the micro-computed tomography (micro-CT) method does not require removal of the puparium and provides information about the modifications in the gut, development of the brain, muscles and cuticles (Hall et al., 2017), the costs and time needed to perform this technique render its application in Brazil's forensic laboratories restricted (Brown et al., 2015).

The Neotropical blowfly *H. semidiaphan*a (Diptera: Calliphoridae) occurs in Central American, Trinidad, Bolivia, Paraguay and Brazil (James, 1970; Linhares, 1981; Kosmann et al., 2013). In Brazil, *H. semidiaphana* flies were recovered from crime scenes in five outdoor cases and one indoor case in the city of Curitiba and its vicinities, all close to forested areas (Vairo et al., 2015; personal data). They were also used to provide *min*PMI estimatives in the city of Batatais, state of São Paulo (Thyssen et al., 2018). *Hemilucilia semidiaphana* is usually classified as an asynanthropic species (D'almeida and Lopes, 1983; Parallupi and Castellón, 1994) and is mostly restricted to forested areas (Moura et al., 1997). For this reason, they are considered as indicators of the location of a crime (Otsuka, 2008; Biavatti et al., 2010). The peak occurrence of *H. semidiaphana* is when the temperature is warm (Baumgartner and Greenberg, 1985), usually in spring and summer (Moura et al., 1997; Otsuka, 2008). *H. semidiaphana* flies prefer the fermentation stage (Azevedo, 2016), but can also be found in the bloated stage of decomposition (Moura et al. 1997; Souza et al., 2008; Grisales et al., 2010; Oliveira-Costa et al., 2013). These flies have also been found on a variety of baits like chicken, human feces, fish, rodent carcass and pigs (Otsuka, 2008; August and Wolff, 2009; Moura et al., 1997). Morphological descriptions of the larval instars and the developmental time under different temperatures of *H. semidiaphana* (Thyssen, 2005; Thyssen and Linhares, 2007) are available. However, there is no data on the intrapuparial period. Here we endeavor to provide a detailed description of the intrapuparial morphological changes of *Hemilucilia semidiaphana* under 20ºC and 25ºC, to be used in *min*PMI estimatives.

3. MATERIAL AND METHODS

3.1 Establishment of the colony

The colony of *H. semidiaphana* flies were established from wild adults collected in the city of Curitiba, state of Paraná, Brazil (25°25′S and 49°14′W) on December of 2016 and 2017 with a modified Shannon trap baited with fresh chicken. Adults were identified using the key of Carvalho and Ribeiro (2000).

All adults collected in the field were placed in plastic cages and were fed a diet composed of sucrose, powdered milk, water and honey *ad libidum*. The colony was maintained under constant temperature (25ºC), humidity (60%) and 12:12 hours photoperiod (L: D cycle). A mixture of fish, beef, liver and chicken was placed on a culture dish, into the insect's rearing cage, for the maturation of the ovaries. Flies seemed to show a preference to oviposit below the fish scale, but we also obtained oviposition in the surface of beef and liver. This mixture was checked every hour until oviposition. After oviposition, ca. 300 eggs were transferred to a 500-mL plastic container with a semi-synthetic diet based on bovine stomach (Estrada et al. 2009) provided *ad libidum* for larval development. The containers with the immatures were placed within a larger (1000 mL) container with vermiculite as a substrate for pupariation and placed in incubators adjusted to 25°C (±1), 20ºC (±1) and 30ºC (±1) relative humidity + -70% under constant conditions, and 12:12 hours photoperiod (L: D cycle). Larval development was observed daily until the immatures reached the third instar, stopped feeding or left their diets. The post-feeding larvae were observed every hour until the pupariation process began. However, at 30ºC (±1) eggs hatched, larvae developed and entered the intrapuparial period but did not developed further.

3.2 Sampling of pupae (prepupae or pharate adult) and observation of the morphogenesis

The first sampling began after the irreversible contraction of the anterior segments of the larvae and development of the white puparium at the end of the pupariation process (Fraenkel and Bhaskaran, 1973). The samples were obtained at intervals of three hours until 24

hours after pupariation and then at intervals of six hours until emergence. In each time interval of three or six hours, 10 or 6 pupae, on 25°C and 20°C, respectively were randomly sampled, totaling 144 hours at 25°C and 192 hours at 20ºC. The sampled pupae were euthanized in hot water (approximately 90°C) and fixed in tagged Eppendorfs containing 1.5mL of AFA solution (70% ethyl alcohol, 40% formaldehyde and acetic acid) at a ratio of 8.5:1.0:0.5. All samples were maintained in a freezer at -20°C until dissection. A total of 280 individuals were analyzed at 25°C and 230 individuals were analyzed at 20°C.

All puparia were dissected under a stereomicroscope with the aid of soft forceps and hypodermic needles. This procedure facilitates the complete removal of the puparium, avoiding damage to the sample. The external morphological characters were observed and recorded. The minimum (first appearance) and maximum (last appearance) ages and the weighted mean ($\pm\, standart\ error$) of each characteristic for each stage of development are also provided. Within temperature and period means were weighted based on the numbers of pupae at a given developmental point (expression of a morphological feature) that were assigned to one of the 3h and 6h intervals.

3.3 Images

Morphologically representative specimens of each time interval were photographed with the aid of a LEICA MZ16 stereomicroscopic and LEICA DFC500 camera, compiled and compacted in the LEICA LAS 3D VIEW AND LAS MONTAGE module and edited in the Photoshop C program.

3.4 Terminology

The terminology and descriptions of each developmental stage were based on Fraenkel and Bhaskaran (1973) and Martín-Vega et al. (2016), except for the late cryptocephalic stage, which follow Zhang et al. (2018). The key structures for determining the age of *H. semidiaphana* and its stages of development: post-feeding larvae, pupariation, prepupal stage (larval-pupal apolysis), pupa (early and late cryptocepalic, phanerocephalic and pupal-adult apolysis) and pharate adult was adopted as follow:

Post-feeding Larvae (wandering larvae): third instar larvae cease feeding and leave the carcass.

Pupariation process: initiates when the larvae stop moving and bury themselves into the substrate. At this time the larval segments start shrinking and a **white puparium** begins to form from the cuticle of the third instar larva. The immature in this phase is called **prepupa** and lasts until the complete separation of the cuticles.

Prepupa: the immature is not a larva but cannot yet be called a pupa. In this stage the **larval-pupal apolysis** occurs. When the cuticle of the third instar larva detaches from the puparium, the immature becomes a **pupa.**

Early Cryptocephalic Pupa: In this phase the cuticle of the pupa is fully formed. The anterior region begins to differentiate, showing the **partially everted appendages**. The posterior region of the pupa still resembles a third instar larva.

Late Cryptocephalic Pupa: It begins with the **elongation of the thoracic appendages**, whose length exceeds ¼ of the body, and formation of the **gas bubble**.

Phanerocephalic Pupa: The most conspicuous characteristics of this phase is the **eversion of the head** and the complete eversion of the legs and wings. In this phase the head, thorax and abdomen are fully recognizable.

Pupal–adult apolysis: Occurs when the separation of the adult epidermis from the pupal cuticle begins. This process is visualized by the formation of a **cuticle that covers the entire immature body**. At the end of this process the immature becomes a **pharate adult**.

Pharate adult: At this timepoint pupal-adult apolysis is complete and the pupa becomes a pharate adult because it is **nearly morphologically complete as an adult**.

4. RESULTS

The intrapuparial period of *H. semidiaphana* lasts 144 hours (six days) under 25ºC and 192 hours (eight days) under 20ºC. At 25ºC it corresponds to 51% of the total immature developmental time, while at 20ºC it corresponds to 61 % of it. The color of the puparium provides information on the age of the fly only in the first nine hours of development. After that, the color can no longer be used to determine age because it does not vary. Twenty-one (21) general traits (Table I) and nine (9) key morphological characteristics (Table I; Fig. 6) that can be used to age the intrapuparial period of *H. semidiaphana* are described and then associated with the seven delimited developmental stages.

At 20ºC the morphological changes that define the prepupal stage (including larval-pupal apolysis) and cryptocephalic pupa occur for the first time at almost the same age as they occur at 25ºC. However, the weighted averaged time (age) of occurrence of each stage increased when *H. semidiaphana* was reared at 20ºC (Table II). In general, the key characteristics used to determine age take longer to first appear at 20ºC, but the sequence in which they occur are the same as at 25ºC. In other words, the sequence of morphological changes does not vary with temperature. In view of that, here we describe the intrapuparial development of *H. semidiaphana* only at 25ºC. The time of the first and last appearance (minimum and maximum age) of each group of visible external morphological traits are provided for the intrapuparial development of *H. semidiaphana* reared under 25ºC and 20ºC in Table I. The duration of the seven (7) delimited stages of development at 25ºC and 20ºC, and the weighted average that delimits these seven (7) stages are provide in Table II.

Post-feeding Larvae: After leaving the diet substrate, the post-feeding larvae started wandering on vermiculite for approximately thirty hours (**Fig. 1**).

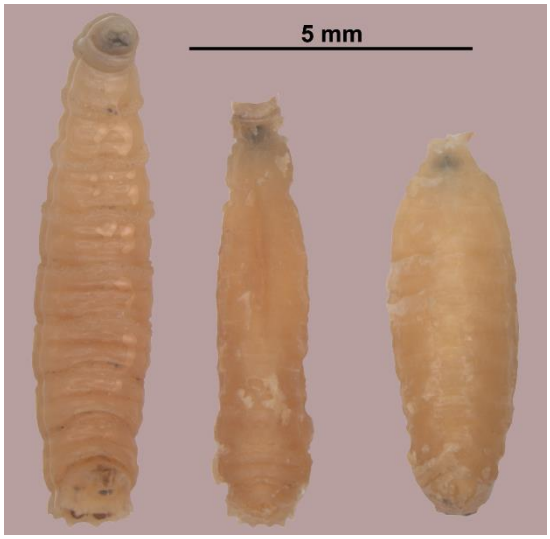

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Figura 2. The process of pupariation of *Hemilucilia semidiaphana* (Rondani, 1850) at 25ºC. From left to right: Post-feeding larvae of third instar in ventral view; Contraction of the anterior segments of the of the third instar larvae; A fully formed white puparium wrapping the prepupa;

Puparium morphology:

The puparia were 6.43mm ± 0.19mm (mean ± se, n=10) in length and 2.62mm ± 0.15mm (mean ± se, n=10) in width. Their general shape is ellipsoidal, with the anterior and posterior portions almost the same length but with the middle portion approximately three times larger than the anterior or posterior portions (Fig 2). The surface of the puparium maintains the larval segmentation and spine band areas with a smooth surface between the bands.

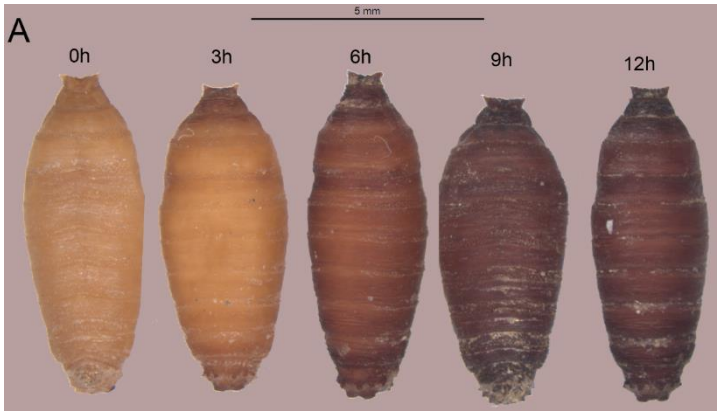

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Figura 3. Tanning of the puparium of *Hemilucilia semidiaphana* (Rondani, 1850) at 25ºC between 0h and 12h.

4.1 Age related morphological changes in 25ºC

0 hr: After wandering, the post-feeding larva initiates the irreversible contraction of its anterior segments (Fig. 1), which ends with the formation of the white puparium (**pupariation**), wrapping the prepupa. At this time, the cuticle of the puparium is soft and translucid, and is difficult to dissect due to the close attachment of the prepupa and puparium, which often causes the prepupa to break (Fig. 1). Morphologically, the prepupa resembles the larva but is shorter and has elliptical shape. The entire cephaloeskeleton is embedded in the anterior body region (Fig. 3A) and is fused with the inner wall of the puparium.

3 to 12 hours: The puparium becomes gradually more tanned, turning light brown in the beginning, between 3 and 6 hours after pupariation, and changing to a dark-brown color after 9 hours (Fig. 2). Also, it becomes gradually easier to remove the puparium from the **prepupa**, which shows that the process of secretion and formation of the pupal cuticle is in progress (**larval-pupal apolysis**). This process of apolysis initiates at the anterior portion of the prepupa and progresses toward the posterior end. When it is complete, at 12 hours after pupariation, it becomes easier to separate the posterior wall of the puparium from the posterior wall of the prepupa. At this time, the apex of the oral hooks of the cephaloeskeleton are exposed (Fig. 3B).

12 hours: The puparium becomes dark brown and is easy to remove. Soon after the larval-pupal apolysis is complete, the thoracic appendages evert (Fig 3.C). Initially, at **15** hours the thoracic appendages reach a quarter of the total length of the pupa (**early cryptocephalic pupal stage).** The abdominal portion of the pupa is still narrow and segmented, like the larvae (Fig. 3C). At **18** hours after pupariation, it is possible to see, internally on the abdomen, the gas bubble, which occupies ca. ¼ of the body length (Fig. 3D). Also, at this time, the thoracic appendages elongate (**late cryptocephalic pupal stage),** reaching almost half of the body (Figs. 3E1, E2). The cephaloeskeleton has now the mouth hooks region exposed (Figs. 3E1, E2).

24 hours: The head is completely everted, the legs and wings elongate, the length of the appendages exceeds more than half of the body (**phanerocephalic pupal stage**). Head, thorax and abdomen begin to differentiate, but the boundaries between them are still incomplete (Figs

3F1, F2). The mouthparts, at first, are fused, without a distinction among the morphological elements (Figs. 3F1, F2). The abdomen shrinks and acquires a U-shape with lines of segmentation that should match the adult segmentation (Figs. 3F1, F2). There is a pair of respiratory horns spreading from the dorsal region of the thorax, next to the base of the wings, which reach the base of the head dorsally (Figs. 3F1, F2). The cephaloeskeleton is completely extruded, but the posterior half (dorsal and ventral cornua, vertical plate and dorsal bridge) continues externally attached to the cuticle (Figs. 3F1, F2). At 30 hours after pupariation, the emergence of the abdominal spiracles (6 in each side) is first observed (Figs. 3F1, F2). At 36 hours a transparent cuticle on the surface covers the abdomen, legs and wings **(pupal-adult apolysis**). In the head, the labellum is double-lobed.

48 hours: The limits between head and thorax and between thorax and abdomen are complete (Figs. 3G1, G2). The legs are slender, and the wings are unfolded. In the head the antenna become visible but still lacks clear margins and arista. Most of the morphological structures of the adult have already developed, the pedicel and the palpus begin to differentiate. Pupal-adult apolysis is complete all over the body, so the pupa can be called a **pharate adult.**

At **54** hours after pupariation, a pair of elongate and narrow palps are clearly distinguished (Fig. 3H1). At 60 hours the clypeus and labrum begins to differentiate. The compound eyes lack a typical delimitation and have no pigmentation. After **78** hours of pupariation the margins of the compound eyes become fully discernible (Fig. 4I1) and the ptilinal invagination is present at the front of the head.

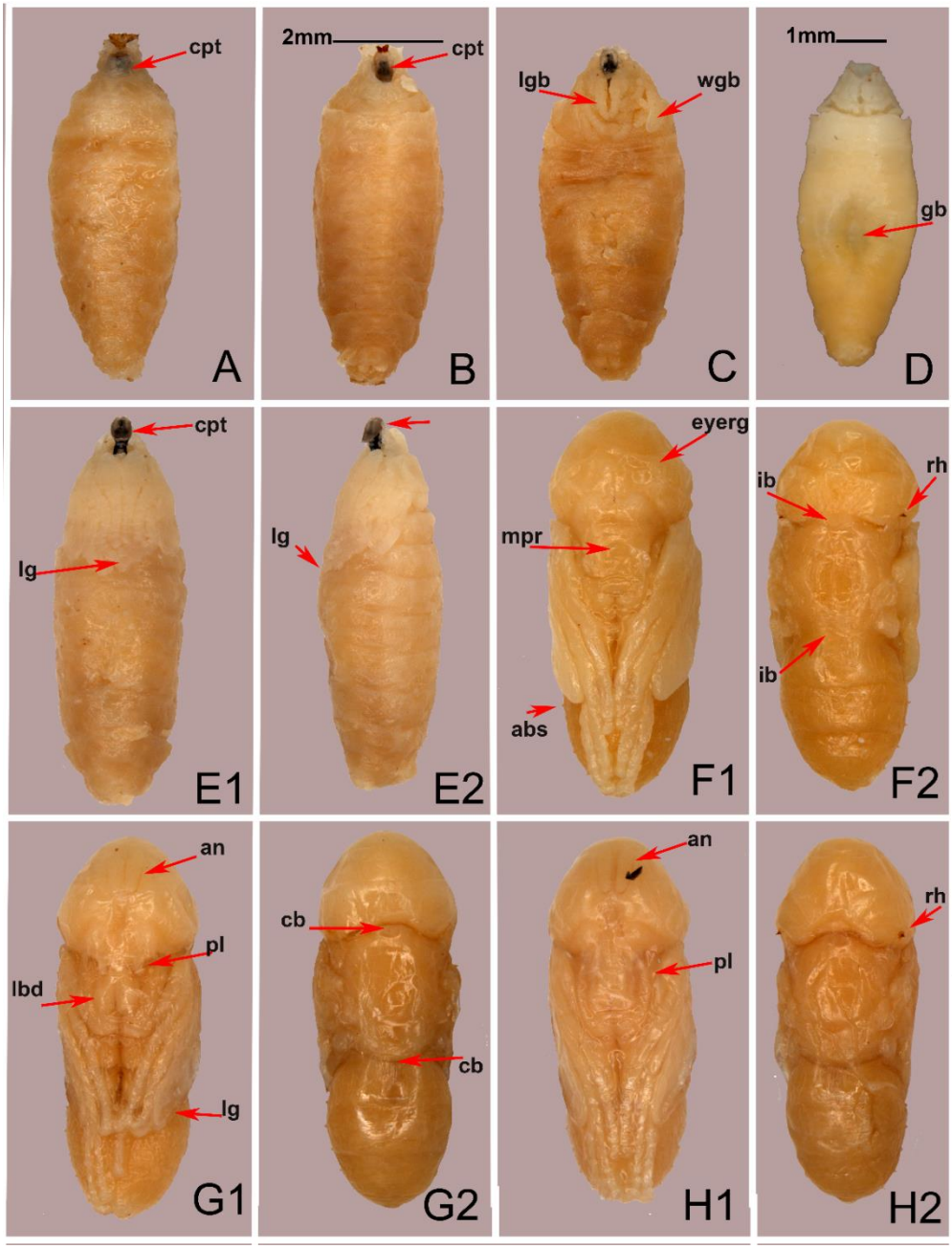

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Figura 4. Intrapuparial development of *Hemilucilia semidiaphana* (Rondani, 1850) at 25ºC. (A) Ventral view of prepupa at 0 h with cephaloeskeleton embedded; (B) prepupa at 9h with partially exposed oral hook in ventral view; (C) pupa at 12h with the apical region of the body differentiated into thoracic appendages;(D) pupa at 15 h showing the gas bubble internally, which occupies about ¼ of the body; (E1;E2) pupa at 18 h showing evident oral hook and elongated thoracic appendages, reaching almost half of the length of the pupal body in ventral view and lateral view respectively; (F1) pupa at 30 h with distinct head, thorax and abdomen, and respiratory spiracles apparent in ventral view; (F2) Dorsal view of pupa at 30 hours, showing no complete boundaries between head and thorax and thorax and abdomen and respiratory horn; (G1) pharate adult at 48h showing the antenna and labellum double-lobed in ventral view;(G2) dorsal view of pharate adult at 48h showing complete boundaries between head and thorax and thorax and abdomen; (H1) pharate adult at 54h showing the outline of antenna, the differentiated maxillary palpus in ventral view and (H2) complete boundaries between head and thorax and thorax and abdomen. Scale 2mm to all the images except "D". Abs, abdominal spiracle; an, antenna; cb, complete boundaries; cpt, cephaloeskeleton; eyer; eye region; ib, incomplete boundaries; gb, gas bubble ; lbd: labellum double lobed; lg, legs; lgb; leg buds; mpr, mouth parts region; pl, palps; rh, respiratory horns; wgb, wing buds.

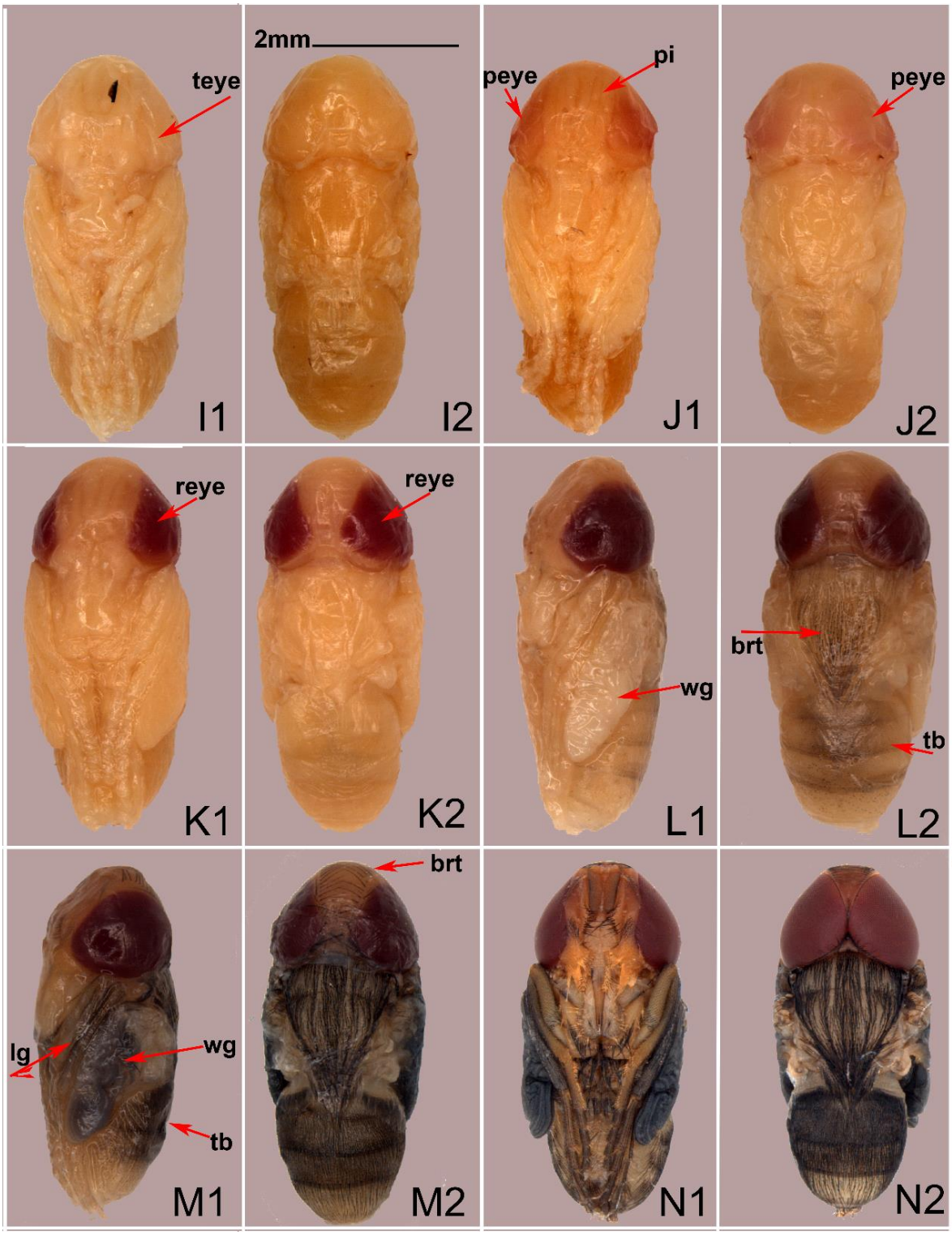

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Figura 5. Intrapuparial development of *Hemilucilia semidiaphana* (Rondani, 1850) at 25ºC. (I1, I2) ventral and dorsal views of pharate adult at 78 h, showing the delimited margins of the transparent eyes; (J1; J2) ventral view and dorsal view of pharate adult at 90h, eye becoming reddish;(K1, K2) ventral and dorsal views of pharate adult at 96 h with red-colored eyes; (L1) lateral view of pharate adult at 108h, showing the folded wings; (L2) dorsal view of the pharate adult stage at 108h, showing bristles on the head and thorax; (M1) lateral view of the pharate adult at 114h, showing the pigmented wings, (M2) dorsal view of the pharate adult stage at 114h, arrows illustrate the presence of bristles all over the body; (N1, N2) ventral and dorsal views of imago at 132h. brt, bristles; lg, legs; pi, ptlinial invagination; peye, pinksh eyes; reye, red eyes; tb, transverse bands; teye, transparent eyes; wg, wings. Scale 2mm to all the images.

90 hours: at this time period, the most striking modification in the external morphology is the compound eyes, which become pink (reddish) (Figs. 4J1, J2). At 96 hours the eyes become entirely red and the head becomes orange (Figs. 4K1, K2; 5 A, B). At 108 hours of development the bristles and hairs on the dorsal surface of the thorax and on the vertex of the head begin to blacken (Figs. 4L1, L2). Soon after, at 114 hours, the bristles and hairs together with the transverse bands of the abdominal tergites are black and the wings and legs start becoming pigmented (Figs. 4M1, M2). At 120 hours after pupariation, the wings and legs are fully pigmentated. In the head the front bristles and the arista are black, and the clypeus becomes brown (Figs. 4N1; 5C, D). Until emergence, at 144 hours after pupariation, all other bristles and hairs have developed and become black.

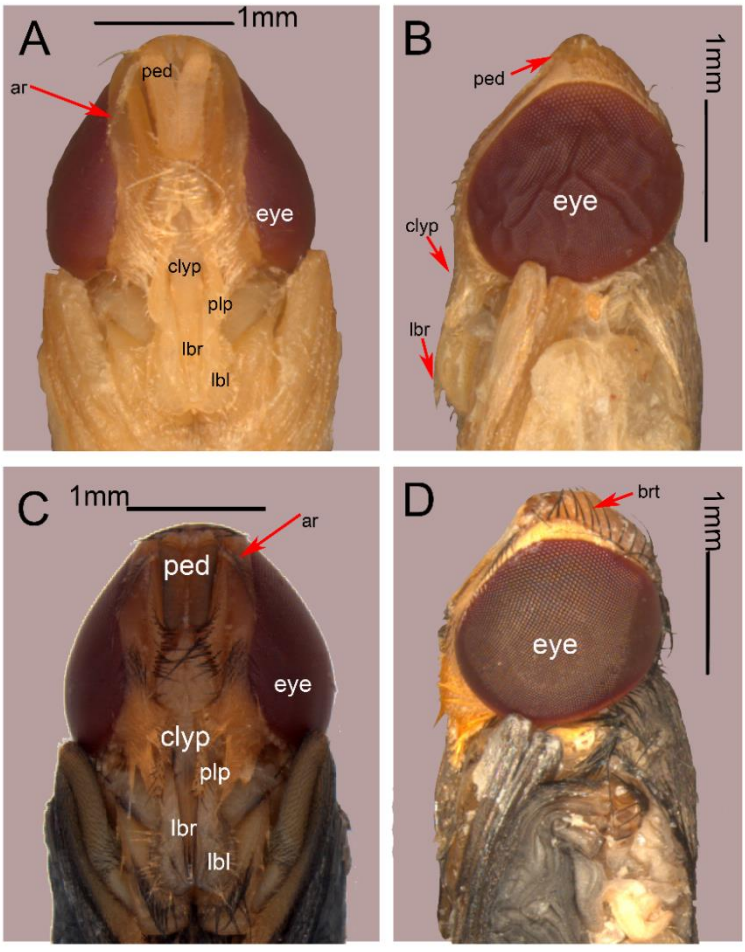

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Figura 6. Development of the head of *Hemilucilia semidiaphana* (Rondani, 1850) at 25ºC. (A, B) Pharate adult at 102h with mouthparts increasingly evident and no pigmentation of head bristles in ventral and lateral view; (C, D) imago at 132h showing mouthparts fully formed. Brt, bristles; clyp, clypeus; ped, pedicel; lbr, labrum; lbl, labellum; plp, palpus; Ar, arista.

4.2 Key characteristics relate to develop of *H. semidiaphana* under 25ºC and 20ºC

During the description of the intrapuparial morphological changes under light microscopy, 21 characteristics were potentially informative (Table 1). This set of characteristics can be applied to determine the age of *H. semidiaphana*. However, nine (9) of those traits are the most informative (key characteristics). Those key traits were chosen because they are easily observed and allow the separation, in both temperatures, of the entire developmental time into shorter periods of time and can be used to estimate the *min*PMI. We provide (Fig. 6) a timeline for the intrapuparial development of *H. semidiaphana* based on these key traits.

Table I. Time of appearance (Min and Max) of the key intrapuparial morphological characters that are potentially informative to estimate the age (in hours) of *Hemilucilia semidiaphana* (Rondani, 1850) reared at constant temperatures (20 and 25ºC).

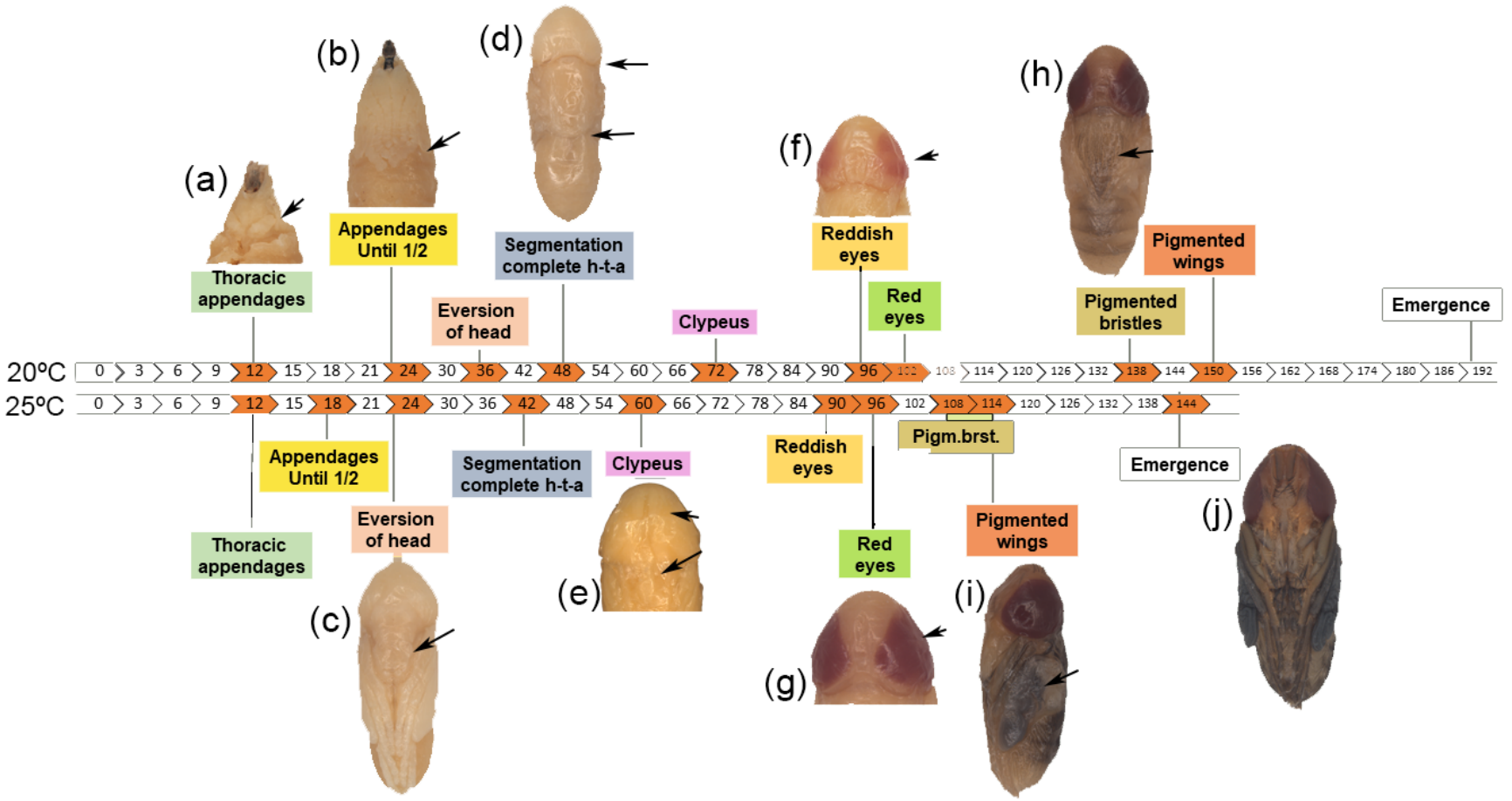
	25ºC		20ºC	
Morphological markers related to age	Min	Max	Min	Max
Whitish Prepupae, cephaloeskeleton embedded,translucid puparium	0		0	
Oral hooks of cephaloeskeleton exposed, Eversion of thoracic appendages	12	30	12	24
Thoracic appendages exceed ¼ of the legth of body, gas bubble	15	30	24	30
Head and thoracic appendages completely evert, U-shape of the abdomen, abdominal spiracles emergence, without cuticle on the appendages	24	30	36	42
Thoracic appendages covered by the cuticle	36	42	42	48
Segmentation between thorax and abdomen are complete, antenna and a labellum doubled lobed; transparent eyes	48	60	48	72
Clypeus	60	90	72	96
Reddish compound eyes	90	102	96	102
Red compound eyes	96	108	102	108
Black bristles on thorax and abdomen	108	120	138	144
Pigmentation of wings and hairs of legs and front bristles of head	114	144	150	192

FONTE: O autor (2018)

Table II. The range and weighted mean (wMean) numbers of hours, with the weighted standard error of the mean (wSEM) of each intrapuparial development stage for *Hemilucilia semidiaphana* (Rondani, 1850) reared at constant temperatures (20 and 25ºC). The number of pupae sampled (n) and the coefficient of variation (CV) per stage is also showed.

	25 degrees					**20 degrees**				
Stage of development	**n**	**Range**	**wMean**	**wSEM**	**cv**	**n**	**Range**	**wMean**	**wSEM**	**cv**
Pupariation	10	0	-	-	-	6	0	-	-	-
Prepupa	35	3-12	7.37	5.07	68	23	3-12	7.3	0.95	52
Early cryptocepalic pupa	25	12-24	15.84	0.75	23	20	12-24	18.15	0.74	20
Late cryptocephalic pupa	22	15-30	22	0.89	20	10	24-30	27.6	2.12	15
Phanerocephalic pupa	12	24-42	33.5	1.56	19	9	36-42	38	2.12	11
Pupal-adult apolysis	22	30-48	40.9	1.6	16	19	42-60	51.79	1.81	14
Transparent eyes	71	48-90	69.88	1.67	19	37	60-96	79.29	1.51	15
Reddish eyes	16	90-102	96.75	1.77	5	7	96-108	102	1.69	5
Red eyes	67	96-138	125.1	1.89	12	84	102-186	146.85	1.69	17
Emergence		144					192			

FONTE: O autor (2018)



FONTE: O autor (2018)

Figure 6. Time line of development in hours of the Hemilucilia semidiaphana (Rondani, 1850) at 20ºC and 25ºC and the key traits related to age**.** The arrows indicate (a) the thoracic appendages in the beginning of the differentiation; (b) Thoracic appendages until ½ of the body of the pupa; (c) pupa divided in head, thorax and abdomen and the fused mouthpieces (arrow);Figura 7 (d) the complete boundaries between head and thorax and thorax and abdomen; (e) differentiate clypeus and antenna; (f) the reddish eyes; (g) the red colored eyes; (h) the pigmented bristles of thorax; (i) the pigmented wings and (j) the imago.

5. DISCUSSION

The estimate of a *min*PMI based on the oldest immature recovered from a local of death has a central place in medico-criminal forensic entomology (Catts and Haskell 1990; Grassberger and Reiter 2001; Villet et al., 2010). For the most part, *min*PMIs have been estimated based on larval development time or rate (Introna et al., 1998; Starkeby, 2001; Oliveira-Costa and Mello-Patiu, 2004; Arnaldos et al., 2005; Sukontason et al., 2005; Sukontason et al., 2007; Kosmann et al., 2011; Ying et al., 2013; Vairo et al., 2015; Ramos Pastrana and Wolff, 2017). Fewer *min*PMIs have been based on pupae (Vairo et al., 2015; Bala and Sharma, 2016; Ramos-Pastrana and Wolff, 2017; Thyssen et al., 2018). Since the intrapuparial period takes at least 50% of the developmental time of immatures of Calliphoridae (Brown et al., 2015), it is the immature stage that could remain associated with a dead body for the longest stretch of time (Ma et al., 2015). Therefore, any accurate estimate of development time in the intrapuparial period should improve the estimate of the *min*PMI (Brown et al., 2015). Despite their value for forensic entomology, there are few descriptions of the intrapuparial development of Calliphoridae, one major group of forensic indicators worldwide (Karabey and Sert, 2014; Proença et al., 2014; Ma et al., 2015; Brown et al., 2015; Barros-Cordeiro et al., 2016; Hall et al., 2017; Ramos-Pastrana et al., 2017; Flissak and Moura, 2018; Zhang et al., 2018). For example, in Southern Brazil, the intrapuparial period of only nine Calliphoridae species have been described (Proença et al., 2014; Karabey and Sert, 2014; Ma et al., 2015; Barros-Cordeiros et al., 2016; Martín-Vega et al., 2016; Salazar-Souza et al., 2018; Flissak and Moura, 2018; Zhang et al., 2018), out of the 20 species considered important to forensic entomology (Carvalho and Ribeiro, 2000). This number is even smaller for Sarcophagidae (fleshflies), another major group of forensically important Diptera whose intrapuparial development of only two species have been described, *Peckia Pattonella intermutans* (Walker, 1861) and *Peckia Sarcodexia lambens* (Wiedemann, 1830) (Souza-Cunha, 2014).

Our analysis of the intrapuparial period of *H. semidiaphana* provides nine age-informative characteristics, which divides the entire development time (144 hours at 25ºC and 192 hours at 20ºC) into small sections (Fig 6), that last no more than 30 hours (at 25ºC) or 42 hours (at 20ºC). We also found that the sequence of appearance of the informative characteristics are independent of temperature, which affects only the onset of their development. However, the estimation of age through the intrapuparial period poses challenges because we cannot estimate development time nor the samples can't be measured by length or

weight and then correlated with the developmental time, usual procedures when it comes to larval instars (Greenberg and Kunich, 2002; Amendt et al., 2004). The most common framework to study the intrapuparial period of cyclorrhaphous Diptera demands the removal of the puparium and the description of external traits as they relate to age (Cepeda-Palacios and Scholl, 2000; Pujol-Luz and Barros-Cordeiro, 2012; Defilippo et al., 2013; Proença et al., 2014; Barros-Cordeiro et al., 2016; Ramos Pastrana and Wolff, 2017; Flissak and Moura 2018). Although usually employed, this framework has been criticized because the timing of the intrapuparial stages are difficult to delimit precisely based only on external changes (Martín-Vega et al., 2016), making it difficult to determine the exact time of the beginning or the end of each stage. For example, the delimitation of the pupal-adult apolysis stage demands histological or tomographical sections of the pupae to be accurately defined (Fraenkel and Bhaskaran, 1973; Martín-Vega et al., 2016), which would explain why this stage is absent from some morphological descriptions of the intrapuparial period (Karabey and Sert, 2014; Salazar-Souza et al., 2018; Barros-Cordeiro et al., 2016; Proença et al., 2014). An error in the delimitation of the time of each stage could lead to an inaccurate estimate of the *min*PMI, if based only on this external morphological information (Martín-Vega et al., 2016). However, the use of key characteristics to estimate the age of pupa (prepupae or pharate adult) together with a detailed description of the pupa should increase the reliability of the *min*PMI because it depends on the time of the appearance of the trait and not on the intrapuparial stages, which are only ancillary (Flissak and Moura, 2018). Moreover, the identification of these traits is easily done under light microscopy. As soon as they are identified, it is straightforward to apply the information to estimate the age of the pupa and consequently the *min*PMI (Flissak and Moura, 2018).

We described seven stages of the intrapuparial developmental of *H. semidiaphana,* which is one more than the common six stages of Calliphoridae (Fraenkel and Bhaskaran, 1973). A similar pattern of stages of intrapuparial development had already been described for *Chrysomya megacephala* (Fabricius, 1794) (Zhang et al., 2018). For both species, *H. semidiaphana* and *C. megacephala*, the cryptocephalic stage was split in two, early and late cryptocephalic pupa, enabling a more detailed aging. The additional stage, late cryptocechalic pupa, was defined by the length of the thoracic appendages that exceeds ¼ of the body length of the pupa. In *H. semidiaphana* the weighted average age the early cryptocephalic stage begins is 18 hours after pupariation, whereas the phanerocephalic pupa stage begins 38 hours after pupariation, an interval that lasts 20 hours. For *C. megacephala* the age for the beginning of the

early cryptocephalic stage is a minimum of eight hours after pupariation whereas the phanerocephalic pupa stage begins 24 hours after pupariation, an interval that last 16 hours (Zhang et al., 2018). Subdividing the cryptocephalic pupa stage in two parts divides the 20 hours in periods of nine hours (early cryptocephalic stage) and 11 hours (late cryptocephalic stage) for *H. semidiaphana*. For *C. megacephala* the period of 16 hours was subdivided in two periods of eight hours (early cryptocephalic stage and late cryptocephalic stage) (Zhang et al., 2018). Consequently, the stages that last 20 and 16 hours now are subdivided into shorter periods of time, leading to a more accurate age estimate.

Overall, the intrapuparial stages that depend on apolysis to be identified, such as larval-pupal apolysis and pupal-adult apolysis, are difficult to determine based on the external morphology. For example, the pupal-adult apolysis of *C. megacephala* was not defined in the work of Zhang et al. (2018) even though they mentioned the cuticle, which means that the process of the pupal-adult apolysis was in progress in the abdomen. We have also found no mention to the pupal-adult apolysis of *Lucilia sericata* (Meigen, 1826) (Karabey and Sert, 2014), *Chrysomya albiceps* (Salazar-Souza et al. 2018); *Cochliomyia macellaria* (Fabricius, 1775); *Lucilia cuprina* (Wiedemann, 1830) (Barros-Cordeiros et al., 2016) *Chrysomya putoria* (Wiedemann, 1830) (Proença et al., 2014). We were able to recognize this stage using the time when the adult cuticle covered the thoracic appendages to delimit it. The same reasoning was applied to *Chrysomya rufifacies* (Macquart, 1842). In this species, the time when the cuticle covers the body of the pupa was used to delimit a developmental stage (Ma et al., 2015). However, both studies (present study and Ma et al., 2015) used only external morphological changes to define the stages and ages of the pupa (Ma et al., 2015).

To delimit the larval-pupal apolysis process it is necessary to examine histological or tomographical sections (Fraenkel and Bhaskaran, 1973; Martín-Vega et al., 2016). However, this stage was described, for example, for *C. albiceps*; *L. cuprina*; *C. macellaria* and *S. chlorogaster* (Barros-Cordeiro et al., 2016; Flissak and Moura, 2018; Salazar-Souza et al., 2018) without using histological or tomographical techniques. But, the larval-pupal apolysis could not be delimited for *C. megacephala* (Zhang et al., 2018), *C. rufifacies* (Ma et al., 2015); *L. sericata* (Karabey and Sert, 2014) and *C. putoria* (Proença et al., 2014). This may indicate that it is difficult to delimit the larval-pupal apolysis only based on external morphology. On the other hand, the prepupae stage has been described for all species mentioned above and also for *H. semidiaphana*. The larval-pupal apolysis occurs during this stage, but due to the difficulties in stablishing the exact time it occurs, we choose to describe the prepupa stage in

details, which in *H. semidiaphana* lasts between 0 - 12 hours. The traits that characterize this stage are: the difficulty in removing the puparium (the puparium becomes gradually easier to remove from the apical portion towards the posterior portion as the development advances); and a cefaloeskeleton fused to the apical puparium. This stage lasts a minimum of 12 hours (12 hours for *H. semidiaphana* and 12.2 to *Drosophila melanogaster*) and 16 hours (S. *chlorogaster*) after pupariation under 25ºC (Flissak and Moura, 2018; Gramates et al., 2017). Lower temperatures, such as 20ºC, in our data, did not alter the minimum duration of prepupal stage of *H. semidiaphana* (Table II). This is not consistent with the results of Flissak & Moura (2018), who found that they raise the mean time of occurrence of the prepupal stage in *S. chlorogaster*.

In a previously study (Thyssen, 2005), the developmental time of the prepupal stage of *H. semidiaphana* lasted 36 hours at 20ºC and 24 hours at 25ºC, which is greater than we found, an average of 7.3 hours in both temperatures (20ºC and 25ºC). This represents a difference of 30 or 17 hours, depending on the temperature regimen (20º or 25ºC). The pupal stage (pupa plus pharate adult) in the results of Thyssen (2005) lasted 244 hours at 20ºC, much longer than in our results (180 hours) using the same temperature. The type of diet used to feed the maggots, larval substrate and the geographic position of the population affects the developmental rate of insects (Kaneshrajah and Turner, 2004; Clark et al., 2006; Richards et al., 2008, Flores et al., 2014; Li et al., 2016). The climate of the sites of origin of the two populations (Curitiba and Campinas) are very similar, which rules out the effect of temperature on development. The composition of the diet, however, varies between experimental designs. Thyssen (2005) reared *H. semidiaphana* on an artificial diet based on embryonated eggs (Leal et al., 1982) while we used an artificial diet based on bovine stomach (Estrada et al., 2009). The quality and quantity of animal protein in diets affects the development time of necrophagous Diptera (Leal et al., 1982; Estrada et al., 2009) in such a way that poorer diets will induce a longer development time to compensate for nutritional deficiencies (Dmitriew, 2011; Teder et al., 2014), which could explain the differences we found.

In the first hours of the cryptocephalic stage the thoracic appendages evert in the apical portion of the body (Fraenkel and Bhaskaran, 1973; Martín-Vega et al., 2016), a key character to determine pupa age (e.g. Karabey and Sert, 2014; Flissak and Moura, 2018; Zhang et al., 2018). It took 12 hours for *H. semidiaphana* and *Lucilia sericata* to enter the intrapuparial development (Karabey and Sert 2014) whereas *Drosophila melanogaster* Meigen, 1830 (Gramates et al., 2017) entered it in six hours and *S. chlorogaster* in 16 hours from pupariation.

In *H. semidiaphana*, besides the eversion of thoracic appendages, a visible gas bubble is also important to determine the age of the pupa. This structure, however, has not been widely described for other calliphorids (e.g. *L. sericata*; *C. putoria*, *S. chlorogaster*, *L. cuprina*, *C. albiceps*, *C. megacephala*). The development of a gas bubble is considered an important event in the intrapuparial development (Hall et al., 2017) because it allows the dramatic changes of the metamorphosis to take place (Hall et al., 2017). The formation of the bubble gas occurs together with the elongation of the thoracic appendages in the development of *H. semidiaphana*. The appearance of the gas bubble has been described in detail only for *Calliphora vicina* (Hall et al., 2017) and for *H. semidiaphana* (this work), which was observed 18 hours after pupariation until the eversion of head under 25ºC and 24 hours after pupariation at 20 ºC.

The eversion of the head is the easiest trait to delimit the phanerocephalic pupa stage (Fraenkel and Bhaskaran, 1973; Martín-Vega et al., 2016). It occurs at a similar age for *H. semidiaphana* (24 hours of intrapuparial development), *C. putoria* (24 hours after pupariation) and *L. sericata* (22 hours after pupariation) but later in *S. chlorogaster* (32 hours after pupariation) and *C. vicina* (30 hours after pupariation) (Proença et al., 2014; Karabey and Sert, 2014; Hall et al., 2017; Flissak and Moura, 2018). At lower temperatures, it took about 50% longer for the head to evert in *H. semidiaphana* (36 hours after pupariation), *C. megacephala* (24 hours after pupariation) and *L. sericata* (41 hours after pupariation), whereas in *S. chlorogaster* it took about 25% longer (40 hours after pupariation) (Karabey and Sert, 2014; Flissak and Moura, 2018; Zhang et al., 2018).

The complete segmentation between head, thorax, and abdomen and the beginning of the development of the antenna are other informative traits to estimate the age of *H. semidiaphana*, and other forensically important flies. In *C. megacephala*; *C. rufifacies*; and *S. chlorogaster* (Ma et al., 2015; Flissak and Moura, 2018; Zhang et al., 2018) both traits were described in the beginning of the pharate adult stage. The complete segmentation has also been described for *C. macellaria* and *L. cuprina* in the pharate adult stage (Barros-Cordeiros et al., 2016). In the pharate stage of *H. semidiaphana* the most conspicuous traits can be found on the head: differentiation of the clypeus and a gradual change in the color of the compound eyes, from reddish to red. The color of the eye is largely used to divide the intrapuparial developmental time (Cepeda-Palacios and Scholl, 2000; Karabey and Sert, 2014; Barros-Cordeiros et al., 2016; Flissak and Moura, 2018; Salazar-Souza et al., 2018; Zhang et al., 2018). Usually, the eye color can be described as transparent, yellowish, pinksh and reddish for *L.cuprina*, *C. macellaria, C. putoria* and *C. megacephala*. In *H. semidiaphana* the eye color

changes from a transparent to pinkish and then reddish eye, the same sequence of changes that happen in *C. rufifacies* (Ma et al., 2015).

The pharate adult stage is the longest among the intrapuparial stages. In consequence, to delimit shorter periods of time during this stage enhances the accuracy of the *min*PMI. In *H. semidiaphana*, the time after the compound eyes have become entirely red until when the adult emerges can be divided according to two traits: the pigmentation of the bristles of the head and thorax, and the pigmentation of the wings and legs. The time between the eyes turning red and emergence of the adult is 48 hours at 25°C in *H. semidiaphana.* Twelve hours after the eye has turned red (or 108 hours after pupariation) the head and thorax bristles become darker. The pigmentation of the legs, wings and abdomen begin six hours after pigmentation of the head and thoracic bristles (or 114 hours after pupariation), remaining only 30 hours until the emergence of adult. These characteristics have been used to estimate age in other Calliphoridae such as *S. chlorogaster*; *L. sericata* and *C. megacephala* (Karabey and Sert, 2014; Flissak and Moura, 2018; Zhang et al., 2018). The bristles turn black first in the head and thorax and later the pigmentation of legs, wings and abdomen begin (Karabey and Sert, 2014; Flissak and Moura, 2018; Zhang et al., 2018), a similar pattern we found for *H. semidiaphana*.

6. **CONCLUSION**

The nine key traits we used to stablish the age of the intrapuparial *H. semidiaphana* at 25º and 20ºC can be applied to estimate a minimum *post mortem* interval. The developmental data provided, together with the time line, allows a practical way to make interspecific comparisons as well as to estimate the age of *H. semidiaphana* based on the intrapuparial development.

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