



ANDRESSA CAPORALE DE CASTRO

MECANISMOS DE INDUÇÃO E QUEBRA DE DIAPAUSA EM *Euryades corethrus*
(LEPIDOPTERA: PAPILIONIDAE, TROIDINI).

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Animal, Instituto de Biociências da Universidade Federal do Rio Grande do Sul, como requisito parcial à obtenção do título de mestre em Biologia Animal.

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RESUMO

A diapausa é um tipo de dormência encontrada em insetos, que é causada por uma alteração na programação genética, em resposta a estímulos ambientais, que pausa ou retarda o desenvolvimento. Os mecanismos ambientais que podem estimular esse processo são o fotoperíodo, a temperatura e a umidade, sendo o primeiro mais comum. Ainda existem dois tipos de básicos de diapausa: a obrigatória e a facultativa. Espécies de insetos que habitam zonas de campo são particularmente expostas a condições extremas de sazonalidade, e para superar essas condições a maioria das borboletas entra em dipausa pupal. *Euryades corethrus* é uma das espécies que se utiliza desse processo para evitar condições climáticas adversas. O presente estudo se propõe a avaliar os efeitos de variáveis abióticas na indução e na quebra da diapausa pupal em *E. corethrus*. Para isso, imaturos dessa espécie foram submetidos a três diferentes experimentos, cada qual controlando uma das possíveis variáveis envolvidas na ativação da diapausa. No experimento de fotoperíodo, os indivíduos foram expostos a quatro tratamentos com diferentes durações de luz e escuro (LD): 10:14, 12:12, 14:10 e 16:08. No experimento de temperatura, foram desenhados cinco tratamentos com médias de temperaturas distintas: 15°C, 20°C, 25°C, 30°C e 35°C. No experimento de umidade, foram estabelecidos três tratamentos com diferentes umidades relativas (RH): <20%, 40-80% e >90%. Para avaliar se a supressão de algum fator abiótico particular poderia desencadear o término da diapausa, metade das pupas em diapausa oriundas de cada um dos três experimentos foi submetida a condições que removessem o fator que desencadeou o processo, expondo as pupas dormentes a condições mais amenas. Os resultados sugerem que a diapausa é principalmente induzida por fotoperíodo, mais precisamente por fotofases curtas. Em relação aos experimentos de temperatura, os tratamentos de 15°C e 35°C causaram 100% de mortalidade, isso provavelmente porque eles ultrapassam o limiar fisiológico da espécie, enquanto 30% dos indivíduos expostos a 30°C entraram em dipausa. Todos os outros tratamentos, dos três fatores, mostraram baixas taxas de indução ((LD): 12:12, 14:10, 16:8; (RH): <20%, 40-80% , >90%; 20°C, 25°C). Considerando o término da diapausa, os resultados demonstram que o fotoperíodo não foi

suficiente para acelerar esse processo. O fotoperíodo teve o efeito mais significativo na indução da diapausa, com fotofases curtas ditando a expressão da diapausa. Esse resultado corrobora o comportamento de muitos insetos de regiões temperadas, que entram em diapausa obrigatória em função das fotofases curtas do outono. Cerca de 30% dos imaturos criados em 30°C entraram em diapausa, sugerindo que as temperaturas de verão podem estimular dormência, esta sendo, provavelmente, uma diapausa facultativa. Em relação à quebra de diapausa, os resultados sugerem que este processo é bastante complexo, provavelmente envolvendo outros processos que não só a supressão da condição que induziu a diapausa.

CAPÍTULO 1

INTRODUÇÃO

1.1. Família Papilionidae

A família Papilionidae possui distribuição cosmopolita e é composta por 551 espécies na Região Neotropical (Haüser *et al.* 2005). As borboletas desta família podem ser reconhecidas pelos prolongamentos das veias M3, que geram estruturas semelhantes a caudas, presentes nas asas posteriores de muitas espécies. Porém, as três características morfológicas consideradas sinapomorfias da família Papilionidae são: (1) o osmetério, uma estrutura eversível em forma de “Y”, localizada atrás da cabeça das larvas, que exala compostos de defesa; (2) a morfologia da veia A2 da asa anterior, que chega até a borda da asa, ao invés de se fundir à A1, como em todas as outras famílias; e (3) a presença de escleritos cervicais fusionados, localizados abaixo da cabeça do imago (Miller 1987). A sistemática atual classifica as espécies do grupo quatro subfamílias: Praepapilioninae (extinta), Baroniinae, Parnassiinae e Papilioninae (Hancock 1983).

De acordo com Teston & Corseuil (1998), no Rio Grande do Sul ocorrem 37 táxons da subfamília Papilioninae (considerando todas as subespécies). Entre essas espécies encontram-se representantes das tribos Leptocircini, Papilionini e Troidini.

1.2. Tribo Troidini

A tribo Troidini Talbot, 1939 é composta por 130 espécies divididas em 12 gêneros (Hauser 2005), sendo representada por três gêneros no Brasil: *Battus* Scopoli, 1777 (cinco espécies), *Euryades* Felder & Felder, 1864 (duas espécies) e *Parides* Hübner, 1819 (22 espécies) (ver Tyler *et al.* 1994). A tribo Troidini é reconhecida como um grupo natural, principalmente devido à morfologia bastante homogênea das suas larvas e pupas (Miller 1987). Quanto à distribuição, os Troidini constituem um grupo predominantemente tropical, com centros de distribuição nas florestas de baixada da América Latina e também na região Indo-Australiana. Os Troidini são um dos poucos grupos de borboletas que se alimentam quase que exclusivamente de Aristolochiaceae

(Weintraub 1995). Independente da origem do sistema de defesa química das Aristolochiaceae, os Troidini evoluíram maneiras para sobrepujar as defesas químicas de suas hospedeiras (Ehrlich e Raven 1964). Mais que isso: tornaram-se capazes de utilizar os compostos produzidos pelas plantas em seu próprio benefício, seja tornando-se impalatáveis (quando adultos) ou interferindo na predação por parasitoides (fase imatura) (Greeney *et al* 2012).

1.3. *Euryades corethrus* (Boisduval, 1836)

Segundo Lamas e col. (2004), o gênero *Euryades* é composto por duas espécies: *Euryades corethrus* (Boisduval, 1836) e *Euryades duponchelii* (Lucas, 1839), sendo ambas encontradas na região sul do Brasil e países limítrofes (Núñez-Bustos 2010). Essas espécies possuem estreita relação com as plantas do gênero *Aristolochia*, utilizando-as como sua fonte exclusiva de alimento durante o período larval. Além disso, possuem marcante dicromatismo sexual: os machos possuem asas pretas e opacas, com manchas vermelhas e amarelas, enquanto que as fêmeas apresentam coloração marrom translúcida, com algumas poucas manchas vermelhas nas asas posteriores (Figura 1). Uma característica típica de *Euryades* é a presença do *sphragis*, uma estrutura transferida pelo macho após a cópula que funciona como um “tampão genital”, impedindo que a fêmea já inseminada copule com outro macho (Burmeister, 1879).

Euryades corethrus (Boisduval, 1836) tem distribuição prevista para os estados do sul do Brasil, sudeste do Paraguai, Argentina e Uruguai, sendo encontrada em áreas abertas de savana e campos de altitude. Geralmente está associada a áreas dos Campos Sulinos, preferindo zonas de campo sujo (Atencio 2014). Ela é comumente avistada em voo nas horas mais quentes do dia à procura de parceiros ou de plantas para oviposição, podendo ser encontrada de fevereiro a maio e de setembro a dezembro (Link *et al.* 1977, Costa 2016). Esta espécie utiliza preferencialmente *Aristolochia sessilifolia* como hospedeira (Klitzke e Brown Jr 2000), com alguns autores como Rotteveel (1996) Biezanko *et al.* (1974), Tyler *et al.* (1994), Núñez-Bustos (2010) sugerindo também *Aristolochia fimbriata*, *Aristolochia angustifolia*, *Aristolochia lingua* e *Aristolochia*

brevifolia como fontes de alimento larval. Por apresentar grande fidelidade de hábitat e por ser oligófaga durante sua fase larval, esta espécie é sensível à fragmentação causada pela conversão de áreas de campos naturais (Tyler *et al*, 1994), principalmente em monoculturas comerciais, como arroz, trigo, milho, soja, eucalipto e pinus, sendo menos impactada pelo uso do solo pela pecuária extensiva que não utiliza forragens de inverno.

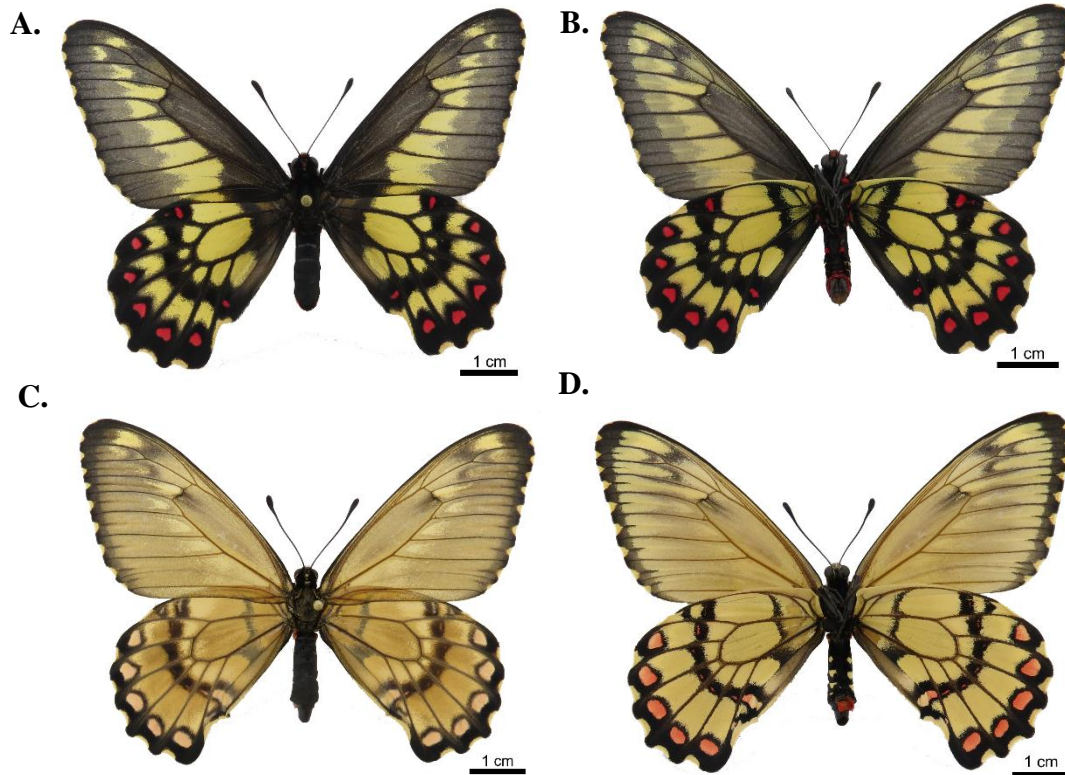


Figura 1. Espécimes de *Euryades corethrus* (Boisduval,1836) da Coleção de Referência do Departamento de Zoologia da UFRGS **A.** macho em vista dorsal; **B.** macho em vista ventral; **C.** fêmea em vista dorsal; **D.** fêmea em vista ventral;

1.4. Diapausa

Insetos, assim como outros invertebrados, podem sofrer um período de dormência ao longo de seu ciclo de desenvolvimento. Dormência é o termo utilizado para definir qualquer estado inativo do desenvolvimento, usualmente acompanhado de supressão metabólica (Košťál 2006, Lee Jr. 2009a). Quando a dormência ocorre no verão é chamada de “estivação” e quando ocorre no inverno, “hibernação”. (Lee Jr. 2009b, Masaki 2009). Dentre esses estados inativos os mais importantes nos insetos são a quiescência e a diapausa. A quiescência é caracterizada por uma

resposta imediata as condições ambientais adversas e pode ocorrer em qualquer estágio do desenvolvimento. Quando as condições se tornam favoráveis novamente o desenvolvimento se restabelece imediatamente (Belozarov 2009, Košťál 2006, Lee Jr. 2009a, Gullan & Cranston 2009).

A diapausa é um processo complexo, sendo desencadeada por uma programação genética que pausa, ou retarda, o desenvolvimento em resposta a estímulos ambientais. Esse processo vem acompanhado de supressão metabólica e ocorre em um único estágio da ontogênese, variando de espécie para espécie. O início da diapausa geralmente precede as condições adversas do ambiente e seu término não necessariamente ocorre com o fim das adversidades. (Gullan & Cranston 2009, Košťál 2006, Belozarov 2009, Lee Jr. 2009a, Andrewartha 1952). Geralmente o inverno é a estação mais evitada através da diapausa em zonas temperadas, mas esse período de dormência também pode servir para evitar calor excessivo, verões secos e períodos de escassez de alimento nos trópicos (Delinger 2009).

Existem dois tipos de diapausa: a facultativa e a obrigatória. Na diapausa obrigatória a espécie apresenta um ciclo de vida univoltino, em que cada indivíduo de cada geração entrará em diapausa, independentemente de qualquer alteração ambiental (apesar de poder ser acelerado por certos fatores). Já a diapausa facultativa surge em resposta a estímulos específicos do ambiente, o que resulta em espécies com ciclos de vida multivoltinos, onde nem todos os indivíduos entraram em diapausa. (Andrewartha 1952, Delinger 2009, Gullan & Cranston 2009). A incidência da diapausa pode ser muito variável, tanto entre espécies como entre populações (Andrewartha 1952).

Os estímulos ambientais que desencadeiam a entrada em diapausa são variáveis, mas geralmente estão ligados a variações de fotoperíodo, temperatura e umidade (Tauber et al 1986). A mudança sazonal na duração dos dias pode ser um bom indicador para prever a mudança de características ambientais que causam a ocorrência de diapausa, mas a diapausa não corresponde necessariamente a uma reação imediata ao fotoperíodo, podendo ocorrer em resposta a sinais recebidos numa fase anterior. A temperatura média diária também pode ser considerada uma boa pista sazonal para a indução da diapausa; com frequência uma resposta a dias curtos é reforçada por

baixas temperaturas (Delinger 2009).

A diapausa pode ser vista como uma adaptação, que preserva as espécies em uma fase resistente aos rigores do inverno. Como consequência, um período de refrigeração pode ser essencial para o término da diapausa. Alguns insetos não necessitam deste período de aclimação para saírem da diapausa, mas outros realizam a quebra da diapausa mais rapidamente se tiverem sido expostos a temperaturas mais baixas durante algum tempo. (Delinger 2009, Andrewartha 1952). Wang e col. (2009) testaram diferentes fotoperíodos e temperaturas para induzir a quebra de diapausa na borboleta *Seriginus montelus* (Papilionidae, Troidini), e encontraram que a diapausa pode ser quebrada sem que haja refrigeração prévia dos indivíduos em estágio pupal.

Em lepidópteros, a diapausa pode ocorrer em todos os estágios do desenvolvimento, sendo mais comum nos estágios imaturos (Delinger 2009). A maioria das espécies da família Papilionidae entra em diapausa pupal. Existem diversos trabalhos a respeito da diapausa nessa família, alguns mostrando os efeitos ambientais na indução ou término da dormência (Deering *et al* 2005, Ishii 1986, Scriber & Sonke 2011, Wang *et al* 2007, Sims 2007, Yamamoto *et al* 2011), variação geográfica da diapausa a estímulos ambientais (Kato 2005, Rockey *et al* 1987, Wang *et al* 2012, Yoshio & Ishii 1998, Yoshio & Ishii 2001), sobrevivência à diapausa (Sims 1983a), herança da indução e intensidade da diapausa (Sims 1983b), e influência da qualidade nutricional das plantas hospedeiras na diapausa (Takagi & Miyashita 2008).

1.5. Justificativa e Hipóteses

Em recente avaliação realizada pela Fundação Zoobotânica do Rio Grande do Sul, durante a atualização da Lista Vermelha de Espécies Ameaçadas do Rio Grande do Sul, *E. corethrus* foi categorizada como vulnerável (Critério VU B2ab(iii)), ver critérios em IUCN 2012) (FZB 2014). Na Lista Vermelha do Estado do Paraná ela aparece como ameaçada desde 2004, e foi categorizada como em perigo (Critério VU B2ab(ii, iii)) (Mikich & Bérnils, 2004). No passado, esta borboleta foi abundante em diversas áreas dos Campos Sulinos, principalmente nos meses do verão e outono. Atualmente ela ocorre em localidades esparsas de campos naturais parcialmente manejados, que

apresentam pouca conectividade com a matriz ambiental natural (Atencio 2014). Pouco se conhece sobre sua história natural, mas observações de campo sugerem a ocorrência de diapausa por hibernação (no inverno) e por estivação (no verão) em populações naturais de *E. corethrus* do Estado do Rio Grande do Sul. Da mesma forma, estudos populacionais preliminares sugerem que a quebra de diapausa pode estar relacionada às condições ambientais encontradas no início da primavera e final do verão. Por se tratar de uma espécie ameaçada, que tem poucos aspectos de sua história natural conhecida, as informações diversas sobre a biologia de *E. corethrus* podem contribuir para a elaboração de futuras ações de manejo e conservação da espécie. A investigação dos mecanismos envolvidos na indução e quebra de diapausa trarão informações inéditas a respeito da espécie, podendo contribuir para o conhecimento da dinâmica de populacional da espécie.

A hipótese inicial de trabalho é que a indução de diapausa em *E. corethrus* seja influenciada pelo fotoperíodo, temperatura e umidade, e que a quebra de diapausa seja causada pela reversão nas condições ambientais que levam à dormência. Como premissas desta hipótese, assume-se que, durante o outono, quando os dias se tornam mais curtos que as noites e as temperaturas médias são inferiores a 15°C no sul do Brasil, as lagartas detectam a chegada das condições adversas do inverno e começam a se preparar para entrar em hibernação pupal. Já durante o verão, quando as temperaturas médias atingem 30°C e a umidade relativa do ar média cai para cerca de 30%, as lagartas seriam capazes de prever a ocorrência de estiagem e se preparariam para entrar em estivação pupal. Com a chegada da primavera os dias e noites se igualam em duração e as temperaturas médias ficam na casa dos 25°C, estimulariam os indivíduos que se encontram em hibernação pupal a continuar seu desenvolvimento até o estágio de imago. Da mesma forma, com a chegada do outono, temperaturas médias amenas (~ 25°C) e a umidade relativa do ar mais alta (~70%) induziriam a retomada do desenvolvimento nos indivíduos em estivação pupal.

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CAPÍTULO 2

Winter is coming: diapause in the subtropical swallowtail butterfly Euryades corethrus is triggered by the shortening of day length.

Manuscrito a ser submetido à revista

Functional Ecology

1 ***Winter is coming: diapause in the subtropical swallowtail butterfly *Euryades****
2 ***corethrus is triggered by the shortening of day length.***

3
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11
12 **Summary**

13 1. Diapause is a type of dormancy found in insects, being modulated by genetically programmed
14 responses to environmental cues that occur in advance of adverse conditions arising. The most
15 common stimulus to trigger this process is the photoperiod, although temperature and humidity
16 can also induce diapause. There are two types of diapause, the obligatory and the facultative.
17 Insect species that inhabit grassland landscapes are particularly overexposed to extreme climate
18 seasonality. To get through the extreme seasonal periods, most of butterfly species enter in
19 pupal diapause. *Euryades corethrus* (Boisduval, 1836) is an open field butterfly, endemic of
20 grasslands from southern South America, which has a known ecological strategy to overcome
21 the adverse conditions of environment using diapause.

22 2. Our goal is to investigate the effects of photoperiod, temperature, and humidity as cues on

23 diapause induction and on diapause termination.

- 24 3. We subject individuals from a stock population to three different experiments. Photoperiod,
25 where the individuals were exposed to four treatments (LD 10:14, LD 12:12, LD 14:10, LD
26 16:08). Temperature, where the individuals were exposed to five treatments (15°C, 20°C, 25°C,
27 30°C, 35°C). Humidity, where the individuals were exposed to three treatments (RH: <20%,
28 40-80%, >90%). To evaluate if the suppression of a particular abiotic factor can trigger
29 diapause termination, 50% of the dormant pupae produced in each of the three experiments
30 were subjected to rearing conditions that removed the adverse factor, exposing the dormant
31 pupae to amenable conditions; the other 50% were kept in the original condition as a control.
- 32 4. The results indicated that diapause is mainly induced by short photophases (LD 10:14). In the
33 temperature experiment, 30% of individuals exposed to 30°C entered in diapause. The 15°C
34 and 35°C treatments caused 100% of mortality of immatures. The other treatments show lower
35 rates of diapause induction ((LD): 12:12, 14:10, 16:8; (RH): <20%, 40-80% , >90%; 20°C,
36 25°C). Regarding diapause termination, photoperiod was not enough to accelerate the process.
- 37 5. Photoperiod had a major significant effect in the induction of diapause in *E. corethrus*, with
38 short-day lengths dictating the expression of diapause. These results corroborate the behavior
39 of many insects that overwinter in diapause in temperate regions. Immatures reared in high
40 temperatures showed some diapause induction, suggesting that summer temperatures can
41 produce some dormancy, which is, probably, a facultative diapause. Our results suggest that
42 diapause termination is a more complex process, probably involving another process than only
43 the suppression of the condition that induced diapause.

44 **Key-words:** dormancy, environmental cues, Lepidoptera, Pampa Biome, overwintering .

45

46

47 **Introduction**

48 Insects, like other invertebrates, may show a dormancy state in some point of their
49 life cycle, which is generally produced by physiological modifications triggered by environmental
50 changes (Košťál 2006, Lee Jr. 2009a). Such response is considered an adaptive process, which
51 allows invertebrates to avoid unfavorable developmental and foraging conditions during their life
52 cycle (Lee Jr 2009a). Diapause is the most abundant and complex dormancy processes found in
53 insects (Kostal 2006), being modulated by genetically programmed responses to environmental cues
54 that occur before of adverse conditions arising (Kostal 2006). Diapause produces a metabolic
55 suppression, occurring only once during the life cycle, and always in the same stage of development
56 on closed related taxa (Delinger 1986, Nechols et al. 2009). There are two types of diapause, the
57 obligatory and the facultative. The obligatory diapause usually occurs in species with a univoltine
58 life cycle, where each individual of each generation enters diapause, regardless of environmental
59 changes (which can be accelerated by some factors). On the other hand, facultative diapause occurs
60 in response to specific environmental stimuli, which occurs in species with multivoltine life cycles,
61 and where not all individuals enter diapause (Delinger 2009).

62 Among the environmental stimuli that can trigger the diapause process, the most common
63 one is photoperiod, although temperature and humidity can also induce diapause (Tauber et al
64 1986). These three factors act as cues for the detection of seasonal changes (Tauber et al 1986),
65 allowing insects to predict the oncoming harsh conditions which can be incompatible with insect
66 normal activities. For instance, the changes in day-length can help insects to predict when the
67 winter is coming. In the same way, daily thermoperiod modifications towards summer solstice also
68 can induce diapause (Leather, Walters & Bale 1995), aiding insects to avoid physiological
69 incompatible temperatures.

70 Diapause induction is not an immediate reaction to environmental changes, and should be
71 considered the final step of very small physiological changes enhanced by abiotic factors stimuli

72 (Delinger 2009), which can likewise be perceived in a different developmental stage where insect
73 dormancy is observed (Danilevskii 1965). The diapause termination can be induced in the same
74 way as diapause induction, but in the opposite direction, using the abiotic stimuli as a trigger to
75 indicate the forthcoming amenable seasonal conditions. However, the suppression of the abiotic
76 factor that triggered diapause does not necessarily cause the reversal of the dormancy state. The
77 dormancy reversal can also be produced when the nutritional reserves of the dormant individual
78 reach the minimum threshold required to complete the life cycle (Andrewartha 1952, Tauber,
79 Tauber & Masaki 1986, Košťál 2006, Belozarov 2009).

80 Insect species that inhabit grassland landscapes are particular overexposed to extreme
81 climate seasonality since the absence of forest refuges may decrease the chances to find a shelter
82 that could provide mild conditions. Thus, in grassland landscapes, extreme climate is expected to
83 induce the diapause process in insects as a physiological adaptation to overcome both rigorous
84 winters and severe summers (Delinger 2009). To get through the extreme seasonal periods, most of
85 butterfly species enter in diapause state as immature, especially during the pupal stage (Delinger
86 2009). Most of Papilionidae species that show diapause as pupae are from temperate and
87 subtropical zones where the severity of seasonal climate are generally associated with winter
88 conditions (Sims 1983, Yoshio & Ishii 1998, Deering et al 2005, Kato 2005, Sim 2007, Wang et al
89 2007, Tagashi & Matsida 2008, Scriber & Sonke 2011, Yamamoto et al 2011, Scalco et al 2015).
90 Subtropical species from grasslands may suffer from two-fold environmental harassments, since
91 they have to overcome winter severity and sometimes also summer extreme conditions, as observed
92 for some other insect species (Tauber et al 1986). For instance, in Argentinean and Uruguayan
93 Pampa, winter temperatures may reach -5°C , and summer temperatures can overpass 45°C . Both
94 situations are incompatible with butterfly normal activities and, to our knowledge, no studies so far
95 have investigated the effects of both winter and summer harsh conditions in the induction of
96 dormancy states in grassland butterflies. We predict that butterfly species from subtropical, zones

97 which live in large grasslands landscapes, among other adaptive strategies, may show adaptive
98 dormancy related to both to winter and summer extreme conditions, if migration does not occur.

99 *Euryades corethrus* (Boisduval, 1836) is an open-field butterfly of the Papilionidae family
100 endemic of grasslands from southern South America (Atencio 2014). Like other species of this
101 family, it has a known ecological strategy to overcome the adverse conditions of the winter using
102 diapause (Tyler et al. 1994). Preliminary population data obtained at the latitude 31°S suggest that
103 dormant states in *E. corethrus* can also happen during summer apex, when temperature can reach
104 over 45°C and relative air humidity can drop below 20% (Link et al. 1977, Costa 2016). The
105 sensibility of species to environmental conditions variation is not known, but life history theory
106 predicts that dormancy in *E. corethrus* should be triggered by specific environmental stimuli in the
107 advance of harsh condition arrival, as observed in closed related species. Considering this gap in
108 dormancy theory, the main objective here is to investigate the dynamics of diapause occurrence in
109 subtropical butterflies from grasslands, which are subject to both winter and summer adverse
110 conditions, using the butterfly *E. corethrus* as a model. We designed controlled experiments under
111 laboratory conditions to investigate the effects of photoperiod, temperature and humidity as cues to
112 diapause induction and to diapause termination. We expected that individuals exposed to lower
113 photoperiod, temperature and humidity would enter in diapause, as well as individuals exposed to
114 higher temperatures. We also expected that dormant individuals would reverse the diapause state
115 when exposed again to amenable conditions.

116 **Materials and Methods**

117 **Model organism and stock population**

118 *Euryades corethrus* occurs in the southern states of Brazil and Paraguay, and also on
119 Argentina and Uruguay, generally in the areas of Pampa and Chaco biomes. It is found in savannas,
120 grasslands and altitude fields, preferring shrublands and meadows (Atencio 2014). The butterflies
121 are sighted in flight in the hottest hours of the day, and show a voltinism concentrated from
122 February to May and from September to December (Link et al. 1977, Costa 2016). This species

123 uses preferably *Aristolochia sessilifolia* as host-plant (Klitzke and Brown Jr 2000), but can also use
124 *Aristolochia fimbriata*, *Aristolochia angustifolia*, *Aristolochia lingua* and *Aristolochia brevifolia*
125 (Biezanko et al. 1974, Rotteveel 1996, Tyler et al. 1994, Núñez-Bustos 2010). *Euryades corethrus*
126 larvae are oligophagous and adult exhibit high habitat fidelity, making the species extremely
127 sensitive to local environmental modifications (Doilibaina et al 2011).

128 To produce the stock population, we collected eggs of *E. corethrus* in branches of *A.*
129 *sessilifolia* in two natural populations near Porto Alegre, RS, Brazil. (30°06'21"S 51°40'33"W,
130 29°53'02"S 51°10'13"W). The eggs collected were individually transferred to cylindrical plastic
131 vials (50 ml), with the bottom covered with paper towel, taken to the laboratory, and then subjected
132 to the experimental conditions. The care of immatures was taken daily; after egg hatching fresh
133 branches of *A. sessilifolia* were supplied *ad libitum* as food source. After switching to fourth instar,
134 the larvae were transferred from 50ml to 200 ml cylindrical plastic vials, covered with fine voile,
135 until adult emergence.

136 All experimental treatments were performed in B.O.D incubators, allowing the maintenance of
137 controlled conditions during trials. The following data on the development of specimens subject to
138 each experimental treatment were registered: data of egg hatching, instar changing, pupation and
139 adult emergence; sex and occurrence of diapause.

140

141 **Experiments for diapause induction**

142 To determine the effects of photoperiod, temperature and humidity on diapause induction,
143 we subjected the individuals from stock population to three different experiments. On each
144 experiment, only one abiotic effect was tested per time, so the other two factors were kept constants.
145 Since the time spent in pupal stage of normal and diapause individuals are not known for *E.*
146 *corethrus*, we stipulate that diapause occurred when the time spent as pupa was superior to 55% of
147 the total time spend from egg to adult emergence.

148 To analyze the effect of photoperiod, groups of eggs were exposed to four treatments; each
149 treatment simulated days (24h) with different durations of light and dark periods (LD): 10:14
150 (N=66), 12:12 (N=54), 14:10 (N=53) and 16:08 (N=53). The first three treatments simulated,
151 respectively, the photophases of winter solstice, spring and autumn equinox, and summer solstice at
152 latitude 31°S; the last treatment was used as a supernormal treatment, simulating the photoperiod
153 found in the southern extreme distribution of *E. corethrus*. The temperature and the relative
154 humidity (RH) of this experiment were kept constant (25°C, 40-80%).

155 To analyze the effect of temperature, groups of eggs were exposed to five treatments; each
156 treatment simulated days (24h) with different mean temperatures: 15°C (N=59), 20°C (N=61), 25°C
157 (N=53), 30°C (N=58), and 35°C (N=109). The temperature range used represented the mean day
158 temperature variation observed from winter to summer days at latitude 31°S. The photoperiod and
159 the RH of this experiment were kept constant (LD 14:10; 40-80%).

160 To analyze the effect of humidity, groups of eggs were exposed to three treatments; each
161 treatment simulated days (24h) with different RH: <20% (N=55), 40-80% (N=53) and >90%
162 (N=65). The humidity range used represented the mean RH variation observed from winter to
163 summer days at latitude 31°S. The photoperiod and the temperature of this experiments were kept
164 constant (LD 14:10; 25°C).

165

166 **Experiments for diapause termination**

167 To evaluate if the suppression of a particular abiotic factor can trigger diapause termination,
168 50% of the dormant pupae produced in each of the three experiments (photoperiod, temperature or
169 humidity) were subjected to rearing conditions that removed the adverse factor, exposing the
170 dormant pupae to amenable conditions, and keeping constant the other two. The other 50% of
171 dormant pupae was maintained in the original condition and used as a control. For photoperiod
172 induced diapause, dormant pupae were removed from the photophase that induced diapause and put

173 under LD 14:10. For temperature induced diapause, dormant pupae were removed from the
174 temperature that induced diapause and put under 25°C. For humidity induced diapause, dormant
175 pupae were removed from the relative humidity that induced diapause and put under RH 40-80%.

176

177 **Results**

178 **Diapause induction**

179 During the diapause induction experiments, a total of 457 individuals were reared. Near 15%
180 of all individuals subjected to the experiments entered in diapause (photoperiod, 24%; temperature,
181 15%, humidity 4%). Diapause was not sex dependent, occurring equally in females and males (Qui-
182 square test, $\chi^2=2.231$, $gl=4$, $p=0.677$).

183 In the photoperiod experiment, the treatments with longer duration of dark phase
184 significantly induced more diapause than treatments with longer duration of light phase (Qui-square
185 test, $\chi^2=116.331$, $gl=2$, $p<0.001$). Under LD 10:14, 88% of pupae entered in diapause, while under
186 LD 14:10 only 8% entered in diapause; under LD 16:08 and LD 12:12 all pupae development
187 without diapause (Fig. 1a). The results also indicated that the duration of life cycle of individual
188 subjected to photoperiod treatments was statistically different from each other (Kruskal-Wallis test,
189 $\chi^2 = 69.350$, $gl=3$, $p<0.001$; Table 1), with the LD 10:14 treatment showing the longest life span.

190 In the temperature experiment, 15°C and 35°C treatments caused 100% of mortality of the
191 exposed individuals and were excluded from the analysis, indicating the existence of a
192 physiological threshold for immature development. All remaining treatments produced a basal rate
193 of diapause induction, despite marginally significant (Qui-square test, $\chi^2=5.619$, $gl=2$, $p=0.060$).
194 Near 13% of individuals from 20°C treatment entered in diapause, while 8% from 25°C and 30%
195 from 30°C had the same fate (Fig. 1b). The results also indicated that the duration of life cycle of
196 individual subjected to the temperature treatments was statistically different from each other
197 (ANOVA test, $F=21.599$, $gl=2$, $p<0.001$; Table 1), with the 20°C treatment showing the longer life

198 spam.

199 In the humidity experiment, none of the treatments significantly increased the diapause
200 incidence (Qui-square test, $\chi^2=3.648$, $gl=2$, $p=0.161$). All individuals from RH <20% treatment
201 development without diapause, while 8% from RH 40-80% and 6% from RH >90% treatment
202 entered in diapause (Fig. 1c). The life cycle duration was statistically different between the three
203 treatments (Kruskal-Wallis test, $\chi^2 =25.471$, $gl=2$, $p<0.001$; Table 1), with RH 40-80% treatment
204 showing the longer life spam.

205

206 **Diapause termination**

207 The diapause induction experiment suggested that dormancy was an effect of experimental
208 treatment only in the photoperiod experiment, so the dormant resultant pupae from temperature and
209 humidity experiments were not considered in the diapause termination experiment. The dormant
210 pupae from the treatment LD 10:14 subjected to LD 14:10 did not differ statically from the control
211 group maintained in the LD 10:14 treatments regarding the number of days needed to adult
212 emergence (t -test = 1.724, $gl=37$, $p=0.093$, Fig. 2). Thus, the reversal of photoperiod condition that
213 induced diapause (*i.e.*: LD 10:14 to LD 14:10) does not seem to be directly related with diapause
214 termination.

215

216 **Discussion**

217 **Diapause induction**

218 The results presented here suggest that photoperiod had a major significant effect in the
219 induction of diapause in *E. corethrus*, with short-day lengths dictating the expression of diapause.
220 The LD 10:14 treatment produced near 90% of individuals on diapause, while in the other
221 treatments there was a low rate of diapause induction. These results corroborate the behavior of
222 many insects that overwinter in diapause in temperate regions (Delinger 2009). One intriguing
223 result observed was the low diapause induction rate on LD 14:10 treatment and the absence of

224 diapause induction observed on the LD 12:12. We expected the opposite result, since LD 12:12 is
225 compatible with autumn equinox, when population density starts to decrease near latitude 31°(Costa
226 2016), and when immature population are starting to prepare itself to overwinter. These findings can
227 be explained by the temperature used at the treatments, which was kept constant at 25°C. According
228 to the literature, temperature may considerably modify, or even abolish, the insect's reaction to
229 photoperiod in some cases (Beck 1980).

230 Photoperiodic cues may be effective in diapause induction only when the temperature is
231 above or below particular thresholds (Xiao et al. 2013). This could explain the result of LD 12:12,
232 where none of individuals entered in diapause. This is so because this treatment was exposed to the
233 optimum temperature of 25°C. If the group was to be exposed to a lower temperature, we would
234 expect individuals to enter diapause, like observed for some other Lepidoptera from Asia (Wang et
235 al. 2009, Yang et al. 2014).

236 Despite not significant, temperature seems to be related to the production of dormancy states
237 in *E. corethrus*, especially considering higher temperatures. Immatures reared in the 30°C treatment
238 had near 30% of diapause induction, suggesting that summer temperatures can produce some
239 dormancy. This rate of induction probably can be considered as facultative diapause, a process that
240 usually results in multivoltine life cycles. In insects with this kind of dormancy only a few
241 individuals from population enter in an obligatory diapause (Andrewartha 1952). This seems to be
242 the case of *E. corethrus*, where preliminary data from an unpublished study shows that the species
243 is multivoltine (M. Costa, per. communication). On the other hand, the 20°C treatment did not
244 induce a significant rate of diapause probably because the photoperiod used in the temperature
245 experiment was LD 14:10.

246 Dry environmental conditions seem not to produce diapause states in *E. corethrus*. The
247 results suggest exactly the opposite, despite not significant: normal (RH 40-80%) and humid
248 (RH >90%) treatments induced more diapause than dry (RH<20%) conditions. In Pampa and Chaco

249 biomes, the natural habitats of *E. corethrus*, landscape may suffer from periodic events of drought
250 during years when the ocean-atmosphere phenomenon La Niña occurs (Poveda & Mesa 1997).
251 Many insect take advantage of the seasonal availability of moisture to forage and reproduce
252 (Leather et al. 1995), thus adaptations to avoid drought are expected to have evolved in species
253 subjected to periods water deficit (Taubet et al 1986). Diapausing insects are able to control water
254 loss, since they are unable to take in water (Nakamura 2011), so pupal diapause during periods of
255 drought could be considered a good strategy to overcome such adverse conditions. In the case of *E.*
256 *corethrus*, if such adaptation exists in nature, it is not triggered by RH.

257 The water and moisture role regarding the diapause induction and termination, are important
258 trigger factors in insects, but in none of the species studies so far drought was involved in the
259 diapause induction (Hodek 2003). In Lepidoptera, high humidity is generally responsible for
260 diapause termination. The main body of work regarding the abiotic cues effects on diapause
261 induction is dedicated to photoperiod and temperature. (Tauber et al. 1986, Leather et al. 1995),
262 different from moisture cues, that are rarely seem in literature on the theme (Masaki 1980, Tauber et
263 al 1998).

264 **Diapause termination**

265 On the LD 10:14 treatment-induced diapause, changing the photoperiod to LD 14:10 was
266 not sufficient to cause diapause termination in most of pupae subjected to the experiment. This
267 result suggests that diapause termination is a more complex process, probably involving another
268 mechanism than only the suppression of the condition that induced diapause. Some studies support
269 that dormant pupae exposed to chilling, followed by high temperatures treatments, may suffer from
270 acceleration on the diapause termination (Xiao et al. 2013, Chen et al. 2014). Diapause may be
271 completed more quickly at higher temperatures (Leather et al. 1995), and some studies have shown
272 that high temperatures and long days often act together to avert diapause whereas low temperature
273 and short days act to induce it (Danilevskii 1965). The interrelationship between temperature and
274 photoperiod can, in many cases, be essential for diapause determination (Beck 1980). It has been

275 shown that chilling is not a prerequisite for the completion of hibernation diapause in many insects,
276 and that in some species the diapause completion also can occur at intermediate or high temperature
277 (Hodek & Hodková 1988). We showed that *E. corethrus* can finish diapause without chilling.
278 Typically, overwintering insects can terminate diapause without an explicit environmental cue
279 during mid to late winter (Chen 2014), but many insects will terminate diapause more quickly if
280 they have first been chilled for a few months (Delinger 2009).

281 *Euryades corethrus* is a subtropical grassland species and, therefore, has to find a way to
282 protect itself from harsh conditions from both summer and winter. The results of the temperature
283 experiment indicate that *E. corethrus* may show a facultative diapause pattern induced by high
284 summer temperatures that average 30°C. On the other hand, the signal that seems to be the most
285 important in warning the butterflies that winter is coming is the shortened of photophase, which also
286 triggers the diapause process.

287 The mechanisms behind diapause termination in *E. corethrus* were not unraveled with the
288 experimental design used were. Individuals induced to diapause by short photophase stimuli did not
289 show diapause termination when exposed to a control photophase, so other cues, or process, may be
290 necessary for the diapause termination in the model studied here. The factors that effectively trigger
291 the end of pupal dormancy states in *E. corethrus* should be addressed in further studies.

292

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301

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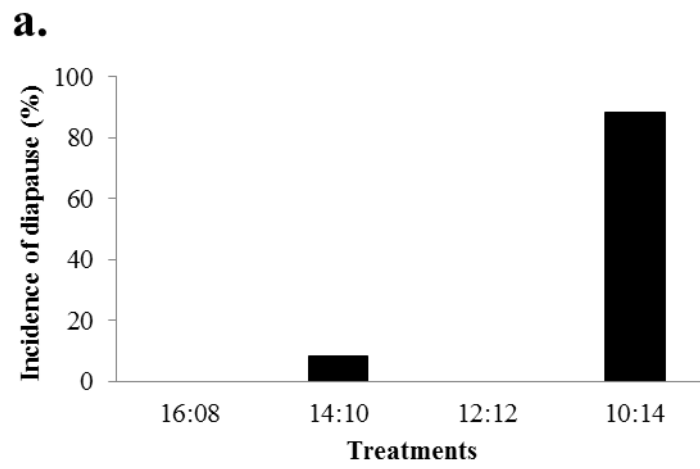
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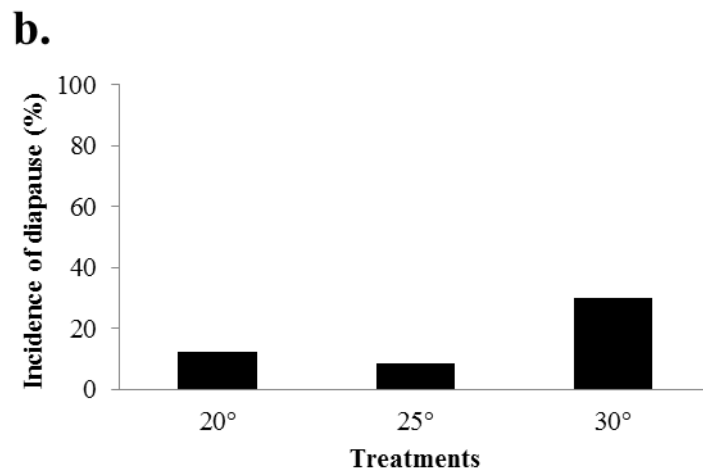
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Figure 1. Incidence of pupal diapause (%) of *Euryades corethrus* under different treatments, Photoperiod (a), Temperature (b) and Humidity (c).



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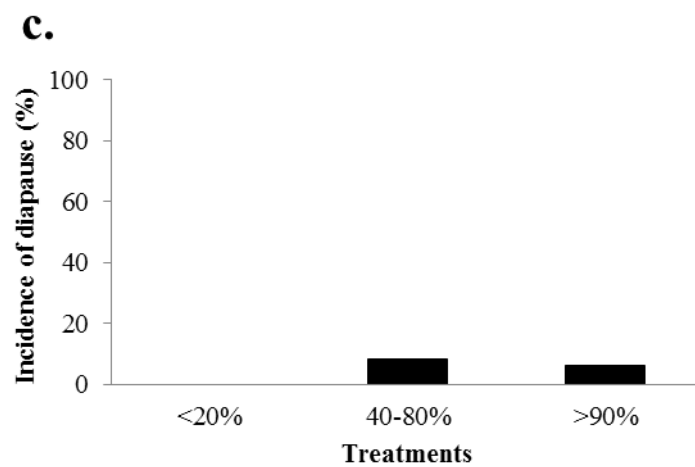


Figure 2. Experiment of diapause termination: Life cycle length of diapausing individuals in two different experiments groups. Control group (LD 10:14) and experimental group (LD 14:10);

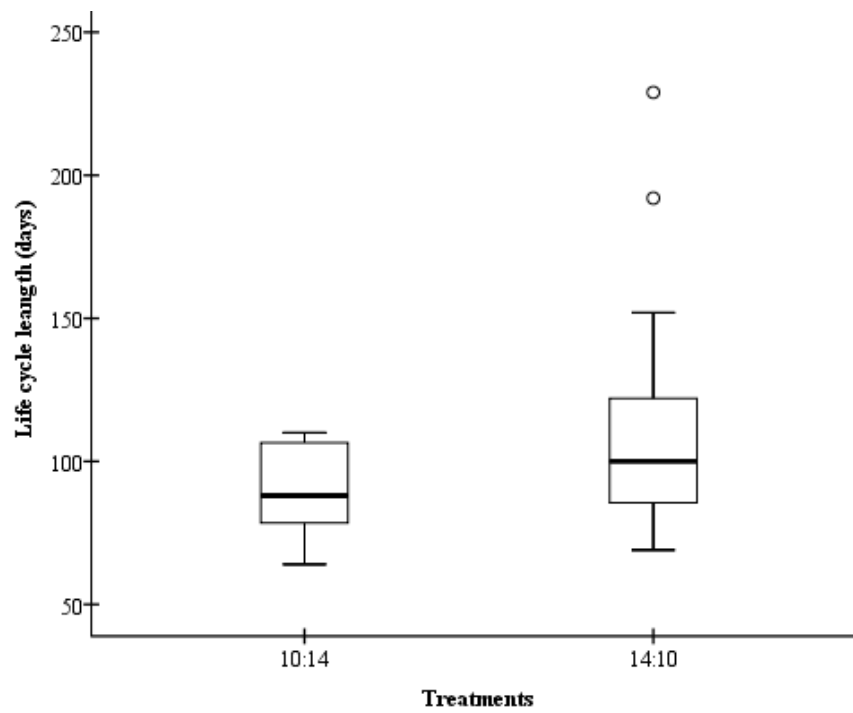


Table 1. The effects of photoperiod, temperature and humidity in the time of development (mean \pm EP) for male and female of *Euryades corethrus* in different treatments. Cells marked with the same letter did not differ significantly (Kruskall-Wallis test, followed by pairwise Mann-Whitney tests after Bonferoni corrections).

Experiment	Treatment	Time of development (days)		
		M	F	M+F
Photoperiod	LD 16:08	31,68 \pm 0.38 (16) ^a	32.00 \pm 0.43 (17) ^a	31.84 \pm 0.28 (33) ^a
	LD 14:10	35.8 \pm 2.22 (21) ^a	39.00 \pm 5.33 (14) ^a	37.02 \pm 2.45 (35) ^b
	LD 12:12	31,04 \pm 0.27 (22) ^b	30,8 \pm 0.59 (10) ^b	30.97 \pm 0.25 (32) ^c
	LD 10:14	102,42 \pm 7.01 (7) ^c	119,62 \pm 10.27 (16) ^c	114.39 \pm 7.55 (23)
Temperature	20°C	52.00 \pm 4.17 (22) ^a	48.7 \pm 0.96 (20) ^a	50.42 \pm 2.22 (42) ^a
	25°C	35.80 \pm 2.22 (21) ^b	39.00 \pm 5.33 (14) ^b	37.02 \pm 2.45 (35) ^b
	30°C	37.69 \pm 4.88 (13) ^b	38.07 \pm 5.14 (14) ^b	37.89 \pm 3.49 (27) ^b
Humidity	RH <20%	36.53 \pm 0.40 (13) ^a	35.91 \pm 0.49 (12) ^a	36.24 \pm 0.31 (25) ^a
	RH 40-80%	35.8 \pm 2.22 (21) ^b	39.00 \pm 5.33 (14) ^b	37.02 \pm 2.45 (35) ^b
	RH >90%	35.72 \pm 0.51 (11) ^a	36.37 \pm 0.56 (16) ^a	36.11 \pm 0.38 (27) ^a

CAPÍTULO 3

Considerações finais

Euryades corethrus é uma espécie de campo de zonas subtropicais e, portanto, adaptou-se para se proteger de condições adversas do verão e do inverno. Para isso ela se utiliza de um estado de dormência, a diapausa.

Neste trabalho ficou evidente que o sinal mais importante para advertir as borboletas que condições adversas do inverno estão chegando é a fotofase curta. Apenas o fotoperíodo foi significativo quanto a indução de diapausa, sendo o tratamento com fotofase curta (LD 10:14) responsável por gerar 88% dos indivíduos que entraram em diapausa.

Os resultados do tratamento de temperatura indicam que *E. corethrus* pode mostrar um padrão de diapausa facultativa induzida pela altas temperaturas do verão, quando as médias variarem em torno dos 30°C. É possível que o valor-*p* muito próximo da significância seja devido ao baixo número de indivíduos utilizados neste tratamento. Talvez, com o aumento no número de réplicas, a significância mude, mostrando que este mecanismo, no caso altas temperaturas, também é importante para a indução da diapausa.

Temperaturas extremas (15°C e 35°C) ultrapassam o limiar fisiológico dessa espécie, causando altas taxas de mortalidade. Temperaturas também afetam diretamente o tamanho do ciclo de vida dessa espécie; quanto menor a temperatura na qual os indivíduos são expostos, maior é o ciclo de desenvolvimento, assim como quanto maior a temperatura, menor o ciclo. Provavelmente estas alterações na duração do ciclo de vida são resultado de diferentes normas de reação da espécie, que são resultantes de alterações metabólicas induzidas pelas condições experimentais. Alguns tratamentos não induziram diapausa em quantidades significativas, sugerindo que a interação entre diferentes tratamentos também possa induzir a diapausa. Autores sugerem que fotoperíodo e temperatura podem funcionar como estímulos reforçadores, causando, assim, a dormência. Isoladamente, o tratamento com baixas temperaturas e o tratamento de fotofase isonômica não

induziram a diapausa. Porém, caso fosse realizado um experimento utilizando os dois mecanismos simultaneamente, seria provável que as taxas de indução de diapausa aumentassem. A literatura apresenta diversos exemplos que dão suporte a essa hipótese.

Foi visto, também, que a diapausa não pode ser interrompida apenas pela mudança de fotoperíodo; outros fatores ambientais, ou bióticos, devem estar envolvidos nesse processo. Apesar de *E. corethrus* conseguir sair da diapausa sem passar por uma fase de resfriamento, é possível que se pupas dormentes fossem expostas a um tratamento com baixas temperaturas durante a diapausa o término da diapausa se daria em menos tempo do que o observado no presente estudo. Isso porque, na natureza, durante o período de dormência, essa espécie enfrenta baixas temperaturas no inverno, antes que haja a quebra da diapausa na primavera, quando as temperaturas se elevam. É possível também que a umidade seja um fator envolvido nesse processo. Existem casos de lepidópteros que quebraram a diapausa quando expostos a altas taxas de umidade, na chegada de estações chuvosas. É provável que o peso crítico do indivíduo também tenha influência neste término. O indivíduo que atingir um peso mínimo deverá completar seu desenvolvimento, saindo do período de dormência, caso esse peso seja menor que o necessário, a pupa não conseguirá completar a metamorfose.

Para que se haja certeza a respeito dos fatores que influenciam o término da diapausa, estes fatores deverão ser investigados em outros estudos. Novos experimentos deverão ser desenhados para que os efeitos possam ser testados isoladamente e também de maneira inter-relacionada.

Anexo 1



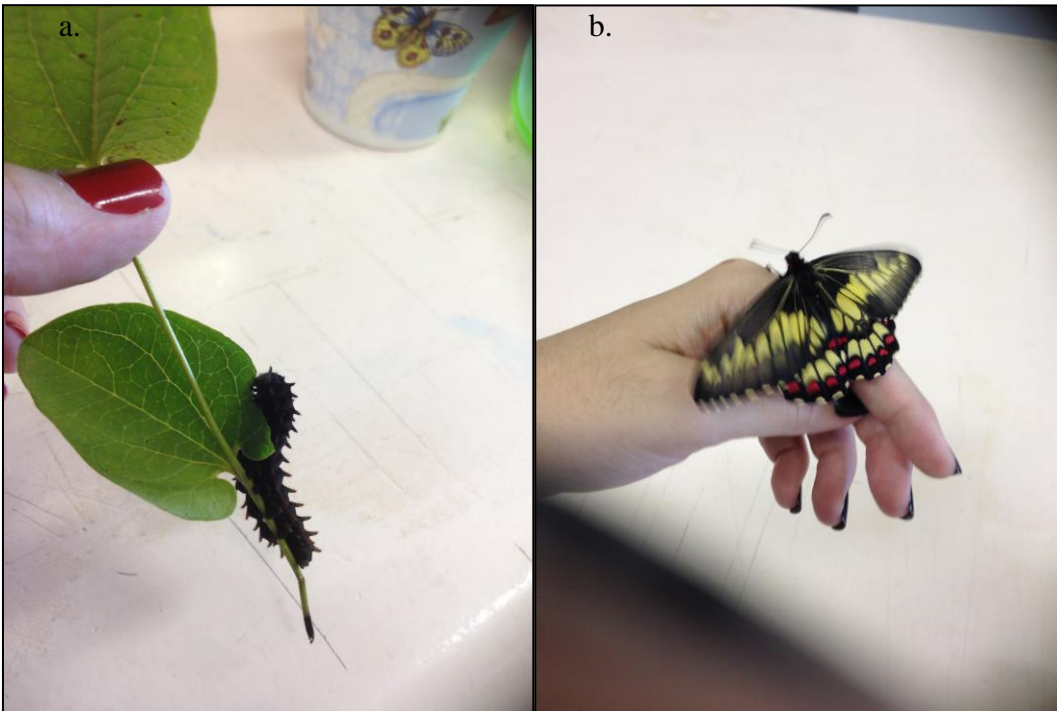
Figura 1. Locais de coleta de ovos. (a) Estação Experimental Agrônômica e (b) Praça São Luis, Canoas



Figura 2. Câmaras de criação (BOD's)



Figura 3. Planta hospedeira utilizada nas criações, *Aristolochia sessilifolia*.



398 Figura 4. Espécimes originados da criação, (a) lagarta e (b) borboleta.

Anexo 2

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