UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
DEPARTAMENTO DE GENÉTICA
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

VARIAÇÃO SAZONAL DE TAMANHO, MORFOMETRIA E COMPORTAMENTO DE ACASALAMENTO EM *DRYAS IULIA ALCIONEA* (LEPIDOPTERA, NYMPHALIDAE, HELICONIINAE) E SUAS IMPLICAÇÕES NA EVOLUÇÃO DO DIMORFISMO SEXUAL

Aluno: Nicolás Oliveira Mega

Tese submetida ao Programa de Pós-Graduação em Genética e Biologia Molecular da UFRGS como requisito parcial para a obtenção do grau de Doutor em Ciências.

Orientador: Aldo Mellender de Araújo

PORTO ALEGRE, AGOSTO DE 2008.

INSTITUIÇÕES E AGÊNCIAS DE FOMENTO

Esta tese foi desenvolvida principalmente no Laboratório de Genética Ecológica, do Departamento de Genética da Universidade Federal do Rio Grande do Sul, com fomento do Conselho Nacional de Desenvolvimento Científico (CNPq) e do Programa de Apoio a Núcleos de Excelência (PRONEX).

Parte da tese foi realizada em colaboração com o Laboratório de Biologia Molecular do Centro Nacional de Pesquisa de Uva e Vinho, Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), com recursos do CNPq, EMBRAPA Uva e Vinho e Fundação de Amparo a Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

FORMATAÇÃO DA TESE

Esta tese foi elaborada seguindo as orientações de formatação e montagem de dissertações, teses e projetos de pesquisa para mestrado ou doutorado a serem submetidos ao PPGBM, disponíveis no site http://www.ppgbm.com.br/.

*"- Pronto! lhe disse o chefe dos esbirros.
Aqui está um companheiro. Imediatamente
baixaram os enormes ferrolhos da porta maciça,
revestida de largas barras. Os dois cativos
ficaram separados do universo inteiro".*

Voltaire, em O Ingênuo.

AGRADECIMENTOS

Nenhum trabalho, por mais simples que seja, consegue ser levado adiante sem a ajuda de outras pessoas. Isto é um fato. Porém, sempre que penso em todas as pessoas que contribuíram de alguma forma para o trabalho, nunca deixo de ficar surpreso. Confesso que não saberia enumerar todas elas sem cometer sérios esquecimentos e espero que os esquecidos me perdoem. Passaram-se mais de quatro anos que entrei no processo de formulação da tese e certamente as memórias do passado já não estão devidamente organizadas onde deveriam estar. Espero não ser colocado na berlinda por isso e ser perdoado se eu eventualmente esquecer alguém.

O primeiro agradecimento vai para o pessoal do laboratório, especialmente para o pessoal do Grupo de Lepidoptera. Ana Carion, Gabriela Pasqualin, Julie Zanin além de outros, de passagem rápida pelo laboratório, ajudaram muito com a manutenção dos estoques de *Dryas*. Agradecimentos especiais a André Klein, Adriano Ferreira e Pedro Rates Vieira que, além de terem ajudado nas criações, foram incansáveis nos trabalhos de campo e nas horas de estrada entre os locais de coleta. Também agradeço a todos colegas pelos momentos de amizade e descontração.

O André mereceria um capítulo de agradecimentos só para ele, pois ajudou praticamente em todas as etapas dos assuntos discutidos aqui, além outras coisas que analisamos e que ficaram de fora da tese em função do espaço. Ele foi o peão de obra, revisor, técnico de laboratório e crítico implacável. Ele é co-autor de um dos trabalhos que apresento aqui, com todos os méritos. *May the force be with you!*

Tenho muito a agradecer pelos momentos de amizade, discussões e trocas de idéias (e também aos desentendimentos!) com os demais doutorandos do laboratório e com o meu orientador. Aprendi muito sobre ciência, e sobre mim mesmo, com o Adriano Ferreira, Luis Ernesto Schmidt, Maurício Almerão e Aldo Mellender de Araújo. Dizem que somos o somatório das experiências que somos capazes de absorver e vocês fazem parte de muitas delas. Admiro muito vocês todos.

Não posso esquecer do Luis Fernando Revers e do pessoal da EMBRAPA, que ofereceram toda a estrutura e apoio necessários para o início das análises moleculares, projeto que ficou de fora da tese, mas que será retomado em breve. Devo muito ao Luis pelo apoio incondicional e pelas conversas de incentivo. Agradeço especialmente a Gisele

Passaia, Alessandra Russi e Fernanda Sbeghen que, com toda a sua simplicidade, proporcionaram suporte técnico e ajuda com as rotinas de laboratório.

Ainda gostaria de agradecer a Aline Quadros, Lisandra Robe, Luis Ernesto Schimidt and Wilson Morandi Jr. por disponibilizar espécimes para a elaboração dos trabalhos de biologia molecular. Outras importantes contribuições foram dadas por Gilson Moreira e Fernando Costa, principalmente em relação à metodologia experimental e aos testes de campo. Também agradeço a Sídia Callegari-Jacques, pelas dicas e críticas nas estatísticas, e ao Luis Ernesto Schmidt e Rodrigo Fornel, pela assistência com a morfometria geométrica, além das críticas nas versões iniciais (e futuras) dos trabalhos de morfometria. Não posso esquecer de agradecer ao Maurício Almerão, pela revisão de alguns dos textos e críticas sinceras sobre os trabalhos, e a Karen Haag, pelo empréstimo das coleções de *Dryas* e pelos puxões de orelha.

Finalmente, mas não por último, tenho que agradecer a Gabi, por tudo (tudo mesmo!). Ela foi para campo, revisou textos, discutiu ciência, emprestou o carro para saídas de campo (e não ficou braba quando eu acertei a cancela do estacionamento do Campus do Vale com o carro dela). Mais importante que tudo, sempre foi uma voz de apoio e motivação para que eu não desistisse de tudo em frente às dificuldades. Tenho certeza que ela sempre acreditou no meu potencial, inclusive quando eu mesmo tinha dúvidas sobre ele.

A todos vocês, e aqueles que eu deixei carinhosamente guardados em algum canto da memória, meu o muito obrigado. Também agradeço a todas as agências de fomento pelo financiamento do projeto e ao CNPq, pela concessão da bolsa.

ÍNDICE

RESUMO

Dryas iulia alcionea é uma espécie de borboleta que apresenta dimorfismo sexual em relação à coloração e ao tamanho das asas. Estudos em populações naturais mostraram que a variação no tamanho das asas dos machos é inferior a das fêmeas, sugerindo a ação de forças seletivas sobre a variabilidade das asas dos machos. Nesta tese, são apresentadas e discutidas evidências comportamentais, de desenvolvimento, genéticas e populacionais que podem explicar parcialmente os padrões morfológicos observados. Os principais resultados da tese são: (1) a forma das asas dos machos parece ser adaptada para vôos de longa duração, com alta eficiência energética, enquanto a forma das asas das fêmeas seria adaptada para vôos curtos, com alta manobrabilidade; (2) o repertório comportamental dos machos é maior do que o das fêmeas e diretamente relacionado à tentativa de efetuar cópula; (3) os machos buscam e cortejam ativamente as fêmeas (estratégia de patrulhamento); (4) as fêmeas parecem ter um papel decisivo no sucesso copulatório dos machos, já que a intensidade do cortejo dos machos parece não influenciar o sucesso copulatório; (5) os machos com padrões comportamentais menos variáveis parecem ter maior sucesso copulatório; (6) machos grandes e pequenos se comportam da mesma maneira frente a fêmeas receptivas, sugerindo que não existam estratégias alternativas para compensar efeitos causados por tamanhos corporais diminutos; (7) o tamanho dos machos não parece influenciar a escolha das fêmeas; (8) não há disputa direta entre machos (comportamento agonístico); (9) o tamanho das asas é uma característica com um moderado fator genético; (10) a correlação genética entre as características das asas dos machos e fêmeas é imperfeita, o que permitiria ou indicaria a ação de seleção sobre o dimorfismo sexual de tamanho das asas; (11) a correlação entre características das asas anteriores e posteriores é baixa, sugerindo uma considerável independência genética entre as características dos dois conjuntos de asas; (12) o tamanho apresenta significativa variação entre populações e estações do ano, sendo fortemente influenciado pelos recursos alimentares e temperatura durante o desenvolvimento larval. Com essas evidências, o modelo do equilíbrio diferencial de evolução poderia ser utilizado para explicar o dimorfismo sexual de tamanho observado, já que afeta as histórias de vidas, ecologia e comportamento. Assim, o dimorfismo sexual em *D. i alcionea* seria um epifenômeno resultante de forças seletivas atuado diferentemente sobre machos e fêmeas.

ABSTRACT

Dryas iulia alcionea is a butterfly species that shows sexual dimorphism in relation to wing color and size. Studies of natural populations showed that the variation in wing size in males is lower than in females, suggesting the action of selective forces on wing size variation in males. In this thesis, I present and discuss behavioral, developmental, genetic and populational evidences that could explain the morphological patterns observed. The main results of the thesis are: (1) wing shape in males seems to be adapted for long lasting flights, with high energetic efficiency, while wing shape of females seems to be adapted for short flights, with improved maneuverability; (2) males have a more numerous mating behavior repertoire than females, which seems to be directly related to attempts of genital coupling; (3) males court and actively seek for mates (patrooling strategy); (4) females seem to have a decisive role in the copulatory success of males, since the intensity of male courtship does not seem to influence male copulatory success; (5) males with less variable behavioral patterns during courtship seem to have greater copulatory success; (6) large and small males court receptive females in the same way, suggesting that they do not use alternative strategies to compensate for effects caused by small body sizes; (7) the male size does not seem to influence female choice during courtship, (8) there is no direct competition between males, with agonistic behavior; (9) wing size has a moderate genetic basis; (10) genetic correlation between males and females, for some wing traits, seems to be imperfect, which could indicate or allow the action of sexual selection on wing size and shape; (11) the genetic correlation between forewings and hindwings is low, suggesting some genetic independence between the two sets of wings; (12) wing size has significant variation among populations and seasons and is strongly influenced by diet and temperature during development. Considering these evidences, the differential equilibrium model of evolution could be used to explain the sexual size dimorphism observed, since it affects life histories, ecology and behavior. Thus, the sexual size dimorphism in *Dryas iulia alcionea* could be an epiphenomenon resulting from selective forces acting differently on males and females.

CAPÍTULO 1

INTRODUÇÃO

INTRODUÇÃO

Considerações Iniciais:

Uma das preocupações centrais da Genética Ecológica, inaugurada por E.B. Ford na década de 30, foi à identificação de adaptações genéticas a contextos ecológicos e seu significado evolutivo. Neste sentido, uma série de estudos tem mostrado que adaptações a distintos ambientes e modos de vida podem levar ao surgimento de novidades evolutivas (para exemplos, ver Freeman e Herron 2001). Estas novidades podem ocorrer em diversos níveis biológicos, como por exemplo, no plano bioquímico, fisiológico, morfológico e comportamental.

Atualmente, um dos principais desafios para os biólogos é explicar os padrões de diversidade em termos dos princípios evolutivos, fornecendo conhecimentos sólidos que possibilitem a preservação das espécies e de seus hábitats. Por sua vez, o estudo da biologia reprodutiva de uma espécie ou grupos de organismos estreitamente relacionados é, no sentido mais profundo, o estudo dos processos que geram a diversidade. Assim, o estudo da biologia reprodutiva, do sistema de acasalamento e das restrições impostas a ambos pode levar-nos a uma maior compreensão do papel das diferenças biológicas e da história evolutiva de uma dada espécie ou grupo.

Biologia reprodutiva e problemas de estudo em *Dryas iulia*

O conhecimento da biologia reprodutiva é importante para o entendimento dos papéis impostos pelas restrições ambientais sobre a reprodução dos organismos na natureza. Esta área de estudo abrange sistemas de acasalamento, dinâmica reprodutiva, dimorfismos sexuais, comportamentos de corte e cópula, entre outros (Paim 1995). Devido a uma série de características, tais como ciclo de vida curto, muitas gerações por ano, coloração das asas muitas vezes distintiva entre machos e fêmeas, facilidades de manuseio e manutenção de estoques e amostragem de populações naturais, os lepidópteros constituem um grupo adequado para abordagens de aspectos relacionados a sistemas de cruzamento e seleção sexual.

Abordagens etológicas constituem um excelente ponto inicial para estudos que envolvem biologia reprodutiva. Este enfoque foi usado em diversos estudos no passado e, hoje em dia, continua sendo usado com bastante sucesso (p. ex., Vaidya 1969, Kingan *et. al.* 1995, Swanson e Monge-Najera 2000, Andersson e Dobson 2003). Apesar dos esforços, pouco se sabe sobre o comportamento reprodutivo de certos grupos de espécies de borboletas, que constituem a segunda maior ordem taxonômica dentro dos insetos. Este é o caso da subfamília Heliconiinae, imensamente estudadas quanto à interação com suas plantas hospedeiras, ecologia e evolução (Ehrlich e Raven 1964, Benson *et. al.* 1975, Dunlap-Pianka *et. al.* 1977, Périco e Araújo 1991, Estrada e Jiggins 2002, entre outros).

Os heliconíneos (Nymphalidae) são borboletas eminentemente tropicais, que usam plantas da família Passifloraceae como hospedeiras (DeVries 1987) (fig.1). Estas plantas são lianas, comuns em áreas de sucessão ecológica e ambientes perturbados (Cervi 1997). Associados a estes habitats, geralmente encontram-se grande quantidade de flores, que são usadas pelas formas adultas (imagos) como fonte de pólen e néctar. Estas borboletas formam populações geralmente grandes, que atingem maior densidade nos meses finais do verão até o meio do outono.

Figura 1. Heliconíneos do Parque Nacional do Corcovado, Costa Rica, e algumas folhas de espécies de Passiflora utilizadas pelas larvas como alimentação. Foto: Missouri Botanical Garden, 2001.

Dryas iulia Fabricius (1775) é um heliconíneo que se distribui por toda a região neotropical, do sul dos Estados Unidos (DeVries 1987) até o norte do Uruguai e Argentina (Emsley 1963) (fig. 2). Doze subespécies são reconhecidas por Emsley (1963), as quais se diferenciam principalmente em relação à coloração das asas e posição das androcônias (escamas glandulares especiais, presentes somente nos machos, com função no acasalamento). A existência dessas diferentes pode ser explicada pela existência de barreiras geográficas e isolamento reprodutivo em sistemas de ilhas genéticas (Davies e Bermingham 2002). Segundo Brown Jr. (1979, 1992) as populações continentais sul-americanas são predominantemente de *Dryas iulia alcionea* Cramer (1779). Esta espécie é encontrada em diversos habitats, estando geralmente associada a locais perturbados (Paim 1995). Nestes locais, a presença de passiflorá~~s~~ceas é bastante comum e abundante. Apesar de usar várias passiflorá~~s~~ceas como hospedeiras, *D. i. alcionea* está fortemente associada ao subgênero *Decaloba* (antigamente denominado Plectostema) (Brown Jr. e Mielke 1972, Benson *et al.* 1975, Périco e Araújo 1991).

Figura 2. Macho de *Dryas iulia alcionea* aquecendo-se ao sol. Foto: Nicolás Oliveira Mega.

Nos últimos anos, vários trabalhos têm sido desenvolvidos pelo Grupo de Genética Ecológica (Departamento de Genética, UFRGS) e Laboratório de Morfologia de Insetos (Departamento de Zoologia, UFRGS), envolvendo diversos aspectos da biologia de *D. i. alcionea*. Entre eles podemos destacar preferências alimentares (Périco e Araujo 1991), escolha de hospedeiras e locais de oviposição (Garcias 1983, Mega 2004), comportamento reprodutivo e territorial (Garcias 1983) estudos sobre os efeitos do endocruzamento e estruturação genética de populações, (Haag *et al.* 1993; Haag e Araújo 1994; Paim 1995), morfometria (Haag *et al.* 1993; Haag e Araújo 1994; Paim 1995; Paim *et al.* 2004), comportamento e sistemas de defesa em larvas (Gröehs 2000; Mega e Araújo 2008) e preferência alimentar (Borges 2005).

O padrão de coloração da superfície dorsal das asas é alaranjado intenso, apresentando manchas negras nos bordos das asas, motivos pelos quais estes heliconíneos são denominados “fogo-no-ar” (Biezanko e Freitas 1938; Biezanko e Link 1972). Geralmente, a fêmea é menos vistosa que o macho, efeito obtido pelo maior tamanho das bordas negras das asas nas fêmeas. A superfície ventral é de coloração ocre, apresentando manchas marrom claras por toda a superfície. Além disso, *D. i. alcionea* apresenta dimorfismo sexual com relação ao tamanho das asas, sendo os machos, em média, maiores dos que as fêmeas (Haag *et. al.* 1993, Paim 1995).

Sabe-se que o tamanho corporal em Lepidoptera pode variar dependendo das condições ambientais e nutricionais as quais são submetidas as larvas durante o desenvolvimento. Paim (1995) sugere que, para *D. i. alcionea*, as diferenças entre os dois sexos são consistentes em todas as estações do ano, mesmo havendo variação na qualidade nutricional das plantas hospedeiras. Informações sobre o componente genético envolvido na variação do tamanho das asas são desconhecidas para esta espécie.

Estudos prévios sugerem que *D. i. alcionea* seja uma espécie poliândrica, com fêmeas copulando com mais de um macho durante seu ciclo de vida (Garcias 1983, Drummon III 1984). Os machos parecem dispersar espacialmente mais do que as fêmeas, já que costumam voar mais alto, mais rápido e de forma mais linear (N.O. Mega, observações pessoais). Dados de campo e de outros estudos sugerem que a vagilidade dos machos seja cerca de cinco vezes maior do que a das fêmeas (Haag *et. al.* 1993), podendo causar efeitos significativos no fluxo gênico entre populações (Burns 1968; Haag *et. al.* 1993).

D. i. alcionea deposita diversos ovos isolados sobre sua planta hospedeira. Seus ovos são comumente encontrados em gavinhas secas, refúgios abandonados (para detalhes do uso de refúgios, ver Mega e Araújo 2008), pedúnculos florais e vegetação associada à hospedeira (DeVries 1987; Garcias 1983; Paim 1995; Mega 2004). Entretanto, quando são analisados os locais de oviposição fora da planta hospedeira, os ovos são geralmente encontrados em estruturas tipo gavinha, refúgio e talo (pedunculares). Tais evidências sugerem que as fêmeas desta espécie são altamente especializadas na escolha dos locais de postura, maximizando a sobrevivência dos seus ovos (Périco e Araújo 1991). Este comportamento evitaria o ataque e a remoção dos ovos e primeiros estádios larvais por parte de predadores, principalmente formigas (Mega 2004).

Dunlap-Pianka e col. (1977) encontraram que fêmeas de *D. iulia* apresentavam um pico inicial de oviposição entre o 5º e 10º dia de vida adulta, diminuindo bruscamente após o 10º dia de postura e atingindo o término da oviposição em torno do 30-40º dia. Garcias (1983) encontrou padrões semelhantes para a curva de oviposição de *D. i. alcionea.* Os dados observados para esta espécie, que se caracteriza por se alimentar somente de néctar, quando comparados com *Heliconius charitonius*, que se alimenta também de pólen, apresentaram grandes diferenças (Dunlap-Pianka *et. al.* 1977). *H. charitonius* não apresenta pico inicial de oviposição, permanecendo estável durante todo o período de oviposição, que dura em torno de 70 dias. Neste caso, é evidente a diferença de investimento reprodutivo por parte das fêmeas de *H. charitonius,* que distribuem ponderadamente seus recursos reprodutivos ao longo do tempo de vida. Este comportamento também é observado em *H. erato phyllis* (N.O. Mega, observações pessoais).

Outras evidências sobre o investimento reprodutivo de *D. iulia* vêm de estudos de longevidade realizados com imagos em ambiente de insetário (Dunlap-Pianka *et. al.* 1977, Garcias 1983). As formas adultas apresentaram uma vida média de 35 dias, sendo que os machos foram, geralmente, mais longevos do que as fêmeas (em torno de 45 dias). Estes dados sugerem que o investimento reprodutivo das fêmeas desta espécie é mais concentrado nos primeiro dias de vida reprodutiva do que quando comparado aos machos. Os picos de produção e postura de ovos são coincidentes com os dias iniciais de vida adulta, exceto pelos primeiros cinco dias, onde as fêmeas são refratárias à corte e cópula. Com o passar dos dias a freqüência de postura de ovos cai, sendo que as fêmeas morrem de 2 a 5 dias após pararem de por ovos. Neste estágio, seus ovários tornam-se praticamente residuais. Os machos parecem ganhar experiência de cortejo sexual nas primeiras semanas de vida, de modo que efetivam cópulas com mais freqüência nas últimas semanas de vida (N.O. Mega, observações pessoais). Entretanto, não existem dados disponíveis na literatura a respeito da aceitação por parte das fêmeas, de machos de idades avançadas. Sabe-se pouco também a respeito das atividades de corte de machos mais velhos

Muitas espécies de borboletas possuem uma fecundidade inversamente proporcional à idade. Os adultos emergem com o número predeterminado de gametas que irão produzirão durante toda sua vida reprodutiva. Além disso, a alimentação e o ritmo de atividade dos machos tem uma relação inversa com a idade, sugerindo que o investimento reprodutivo é maior nas primeiras semanas de vida adulta (Wilkund 2003).

Como já mencionado anteriormente, as fêmeas de *D. i. alcionea* podem copular com mais de um macho durante seu ciclo reprodutivo (Garcias 1983, Paim 1995). Estas cópulas costumam acontecer nos primeiros quatro a cinco dias da fase adulta, entre a emergência e as primeiras oviposições (Garcias 1983). Durante as cópulas, os machos transferem para as fêmeas um ejaculado característico, o espermatóforo. Esta estrutura é constituída de duas ou três camadas de proteínas, carboidratos, diglicerídeos e triglicerídeos, esteróis e fosfolipídios envoltos por uma cutícula. A forma do ejaculado se assemelha a um saco, com uma projeção, o pescoço (*collun espermatoforae*), por onde saem os espermatozóides (Drummond III 1984).

O tamanho e forma dos espermatóforos apresentam grande diversidade dentro dos diferentes grupos de lepidópteros (Drummond III 1984). Muitas vezes, estes formatos são conservados entre as espécies, podendo ser usados do ponto de vista taxonômico. A forma final do ejaculado depende tanto do formato e dimensões da bursa (*corpus bursae*) da fêmea, como também do ducto ejaculatório (*frenum* e *simplex cuticular*) dos machos (Drummond III 1984). Estas informações são úteis para o entendimento do sistema de cruzamento em borboletas, indicando o número de vezes que uma fêmea pode copular, bem como quanto os machos investem em energia (tamanho do espermatóforo) na inseminação das fêmeas.

Em lepidópteros, reprodução e longevidade estão geralmente associados com limitações das reservas de nitrogênio acumuladas durante a alimentação da larva e adulto, que são estocadas nos corpos gordurosos (Dunlap-Pianka *et. al.* 1977). O maior gasto energético das fêmeas parece ser com o esforço reprodutivo, investistido na produção de ovos. Algumas espécies de borboletas, tais como *Danaus plexippus, Heliconius hecale* e *H. erato*, podem retirar nutrientes provenientes dos espermatóforos transferidos pelos machos. Os aminoácidos contidos nestes ejaculados podem ser usados pelas fêmeas na produção de ovos, sendo críticos para a produção máxima dos mesmos. Em *Heliconius*, um único espermatóforo contém nitrogênio necessário para formar 15-30 ovos (Boggs e Gilbert 1979, Boggs 1986). Estes nutrientes são secreções de glândulas sexuais acessórias e são constituídos por um complexo de componentes nutricionais, de alto valor metabólico.

Em *D. i. alcionea*, pouco se sabe da utilização de recursos nutricionais transferidos pelos machos. Estudos de biologia reprodutiva realizados anteriormente (Garcias 1983, Paim, dados não publicados) encontraram que espermatóforos depositados na bursa da fêmea em oviposição são desgastados gradualmente. Estes dados preliminares sugerem, a princípio, que as fêmeas de *D. i. alcionea* podem fazer, eventualmente, uso de espermatóforos como fonte nutricional, a exemplo do que acontece com outras espécies de Heliconiinae.

Outro ponto importante, também decorrente dos hábitos poligâmicos das fêmeas de *D. i. alcionea*, é a possibilidade de precedência de esperma para a fecundação dos ovos. Como pode existir mais de um espermatóforo na bursa das fêmeas, espera-se que os espermatozóides contidos em cada um dos ejaculados disputem a fertilização dos ovos (revisão em Simmons 2001). Numerosos trabalhos publicados (revistos em Drummond III 1984) revelam que muitas espécies de lepidópteros acasalam mais do que uma única vez, o que permite que os ejaculados de dois ou mais machos entrem em competição dentro do trato reprodutivo de uma única fêmea. Em Lepidoptera, geralmente os espermatozóides do último macho a copular com a fêmea são os que acabam fertilizando a maioria dos ovos (Drummond III 1984). Entretanto, alguns machos de certas espécies de Odonata poligâmicas apresentam pênis raspadores, que removem os espermatóforos alojados no interior das fêmeas (Krebs e Davis 1993), posteriormente transferindo o seu próprio ejaculado. Com isso, estes machos maximizariam a porcentagem da prole da qual são pais, impedindo que os espermatozóides dos machos anteriores fertilizem os ovos das fêmeas. Visto que as fêmeas de lepidópteros possuem duas aberturas genitais, a possibilidade de raspagem não se aplica a elas, já que o macho não tem acesso a espermateca. Além disso, não são conhecidas estruturas masculinas especializas para a remoção ou raspagem da bursa. Como Eberhard (1996) sugere, neste caso as fêmeas teriam ganho o conflito de interesses com relação aos machos.

Uma outra possibilidade envolvendo a dinâmica de uso do esperma seria a escolha da fêmea ("Cryptic Female Choice", Eberhard e Cordero 1995, Eberhard 1996) posterior à cópula. Segundo LaMunyon e Eisner (1993), algumas mariposas são capazes de realizar tais escolhas, julgando os ejaculados recebidos e utilizando o esperma dos maiores espermatóforos para fertilizar seus ovos.

Seleção sexual *vs.* seleção natural e as evidências encontradas em *D. iulia*:

O estabelecimento da teoria da seleção sexual, bem como de grande parte da biologia evolutiva, é atribuída a Charles Darwin. No seu livro *On the origin of species* (1859), ele escreveu que a seleção sexual "depende, não somente da luta pela existência, mas também da luta entre os machos pela posse das fêmeas; o resultado não é morte para os competidores sem sucesso, mas pouca ou nenhuma descendência". Desta forma, Darwin tentou explicar a evolução de características, particularmente em machos, que eram prejudiciais para a sobrevivência, mas que promoviam sucesso em competições reprodutivas (este tema foi grandemente expandido na sua obra de 1871, *The descent of man and selection in relation to sex*). Darwin fez distinção entre seleção de características que reforçavam sobrevivência (seleção natural) e aquelas que aumentam o sucesso de um indivíduo em conseguir acasalamentos (seleção sexual). Apesar de atuarem em processos evolutivos independentes, ambas formas de seleção são tipos de seleção natural, já que tanto uma como a outra possuem efeitos no processo de descendência com modificações.

Não há dúvidas de que a evolução do sexo tenha tido grande impacto nas táticas reprodutivas tanto de machos como de fêmeas (Parker 1979). A característica primária dos machos é se empenhar para conseguir acasalamentos, o que ocasiona competição pelo acesso a fêmeas e a evolução de uma gama de características associadas com esta luta. Por outro lado, fêmeas podem ter a possibilidade de escolher entre muitos parceiros em potencial (Thornhill e Alcock 1983). Assim, é esperado que suas preferências aumentem seu sucesso genético e em conseqüência exerçam pressão sobre os machos, favorecendo características consideradas desejadas pelas fêmeas. Estes papéis podem ser invertidos, como ocorre com certas espécies de grilos e titegonídeos. Nestas espécies, o macho é disputado pelas fêmeas, que competem avidamente pelo grande espermatóforo gerado pelo macho. Os machos, incapazes de gerarem outro espermatóforo em um curto espaço de tempo, repudiam as fêmeas mais leves e transferem seu espermatóforo para fêmeas mais pesadas (Alcock 1993, Andersson 1994). Apesar das evidências geradas por vários trabalhos, as causas para a existência das diferenças sexuais não são completamente conhecidas.

O principal avanço nas pesquisas em seleção sexual é a demonstração que a escolha da fêmea e o cruzamento preferencial são responsáveis pela manutenção de muitas características sexuais secundárias. Exemplos são vários, tais como feromônios em mariposas, canto em grilos, sapos e aves, cores conspícuas e outros ornamentos visuais em borboletas, peixes e aves (Andersson 1994 e referências contidas). Na maior parte, Darwin estava correto: a escolha das fêmeas favorece características sexuais secundárias conspícuas nos machos, mesmo nas espécies monógamas. Porém, ele subestimou a importância da competição dos machos, pois não somente armas físicas como chifres e cornos, mas também muitos sinais e ornamentos podem ser favorecidos pela competição, independente ou em concerto com a escolha das fêmeas. Em alguns casos, a competição parece ser o principal fator de seleção, como demonstrado para a evolução do tamanho corporal dos machos (Eberhard e Cordero 1995, Eberhard 1996). Alguns ornamentos sexuais secundários não são somente favorecidos pela escolha das fêmeas ou competição entre machos, mas por outros mecanismos, como variações comportamentais, acasalamento preferêncial, *background* evolutivo, aprendizado, *imprinting*, efeitos alométricos e histórias de vidas dos organismos (Andersson 1994).

Apesar de grande parte dos estudos de seleção sexual ser de natureza experimental, muitos trabalhos teóricos (modelagens matemáticas) foram feitos acerca deste tema (O'Donald 1980). Cada um deles aborda efeitos ou situações particulares envolvidas nos processos que envolvem escolha e competição por recursos reprodutivos, pois o número de situações em que pode ocorrer seleção varia enormemente. Assim, não existe um modelo matemático geral para processos evolutivos por seleção natural ou sexual, somente alguns mais consagrados que outros.

Como mencionado anteriormente, os machos de *D. i. alcionea* são, em média, maiores do que as fêmeas, sendo a variância na medida das asas de machos menor do que a variância encontrada para as fêmeas (Haag *et. al.* 1993, Paim 1995). Isso sugere a existência de uma seleção estabilizadora mais forte atuando sobre o tamanho das asas dos machos quando comparado com as fêmeas. Muitos trabalhos indicam que a variabilidade de uma característica morfológica está inversamente relacionada ao efeito sobre a sobrevivência e reprodução (ver exemplos em Freeman e Herron, 2001). Todavia, a correlação inversa entre variabilidade e aptidão darwiniana não significa necessariamente que uma característica pouco variável tenha papel relevante sobre sucesso reprodutivo (Soulé 1982).

Desta forma, é possível supor que haja uma elevada competição intrassexual em *D. i. alcionea*, supostamente com machos maiores obtendo acesso mais facilmente às fêmeas, deslocando os menores durante a corte e cópula, a exemplo do que ocorre em *Heliconius hewitsoni* (Deinert *et. al.* 1994). Nesta espécie, ocorre *pupal mating* (cópula de machos adultos com fêmeas prestes a emergir, ou com fêmeas recém emergidas e ainda não totalmente aptas ao vôo). Estes autores verificaram que os machos que pousam nas pupas são aqueles de maior tamanho de asas e maior tamanho corporal. Apesar de ser um efeito indireto, indivíduos de asas maiores estariam sendo positivamente selecionados. Um dado que poderia corroborar esta hipótese é a existência de uma correlação positiva entre o tamanho das asas e a quantidade de nitrogênio alocada para a reprodução em *D. iulia* (Boggs 1981a, 1981b). Neste caso, possuir asas maiores seria a prova de que possuem um espermatóforo maior, maior resistência, autonomia e capacidade de vôo. Como conseqüência, possuir asas maiores seria um sinal de maior investimento na reprodução.

As informações acima expostas indicam que vários mecanismos envolvendo a reprodução de *D. i. alcionea* pemanecem desconhecidos, os quais envolveriam desde aspectos gerais da biologia reprodutiva, até possibilidades de seleção intra e intersexual, escolha críptica da fêmea, bem como explicações para a origem do dimorfismo sexual invertido e a pequena variância em medidas morfométricas das asas dos machos quando comparados às das fêmeas. Esta tese foi elaborada para abordar estes problemas, utilizando-se de diferentes métodos para responder algumas destas questões.

OBJETIVOS

Embora o gênero *Heliconius* tenha sido extensa e intensamente estudado do ponto de vista ecológico, genético-evolutivo, nas interações com suas plantas hospedeiras, biologia reprodutiva e especiação, outros heliconíneos não têm sido estudados com tal grau de detalhamento; este é o caso do gênero *Dryas* e sua única espécie em toda a região Neotropical, *D. iulia.* Deste modo, este estudo tem por objetivo geral:

1. Apresentar evidências comportamentais, de desenvolvimento, genéticas e populacionais que expliquem os padrões morfológicos do dimorfismo sexual em *D. i. alcionea.*

Objetivos específicos:

1. Analisar a variação sazonal do tamanho das asas em diferentes populações e a influência das variações nutricionais e climáticas sobre tamanho de machos e fêmeas.
2. Estimar a herdabilidade para características ligadas ao tamanho das asas.
3. Caracterizar o dimorfismo sexual através de diferentes técnicas morfométricas
4. Descrever o comportamento sexual de machos e fêmeas e os fatores que levam ao sucesso copulatório dos machos.
5. Determinar se o tamanho dos machos tem algum papel no sucesso copulatório.

CAPÍTULO 2

ANALYSIS OF MATING BEHAVIOR AND SOME POSSIBLE CAUSES OF MALE COPULATORY SUCCESS IN *DRYAS IULIA ALCIONEA* (LEPIDOPTERA: NYMPHALIDAE, HELICONIINAE).

Artigo submetido para publicação no periódico *Journal of Ethology*

Analysis of mating behavior and some possible causes of male copulatory success in Dryas iulia alcionea (Lepidoptera: Nymphalidae, Heliconiinae).

Nicolás O. Mega[1] and Aldo M. de Araújo[2]

[1] *Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul.*
[2] *Departamento de Genética, Universidade Federal do Rio Grande do Sul.*

Abstract: In this study we examine the *mating behavior* of *Dryas iulia* and the acceptance and rejection mechanisms of females during courtship activity. An ethogram of the mating behavior was organized, based on 100 hours of observation in insectarium. Several different behaviors were catalogued and separated into two behavioral repertories (pre-coupling, post-coupling). The behavioral sequence of mating behavior was also analyzed using a total of 53 pairs of *D. iulia*. The courtship activities involved interactions between the sexes in three sequential phases: aerial, ground and spermatic transfer phase. In 49% of the observations the courtship activities led to copulation. The mean time to the occurrence of the first interaction between males and females did not differ between the interactions with and without copulation. The behaviors of females and males between the two groups were analyzed and differences were found between behavioral activities of both sexes. The results indicate that the males' insistence on courtship does not influence their copulatory success, and that females have a decisive role in the occurrence of copulation. The analysis of behavioral transitions showed that there are many alternative behavioral routines in interactions with and without copulation. The number of behavioral transitions recorded was smaller in the group where copulation occurred, indicating that males with copulatory success modified their behavior less frequently. Successful males recorded more transitions with a probability of occurrence greater than 0.4, and their behavioral activity was also less reticulated. Analysis with the stereotyping index showed that situations in which copulations occurred were more stereotyped than those without the occurrence.

Keywords: mating behavior, courtship, Heliconiinae, *Dryas iulia*, behavioral sequence.

Introduction

In recent years, several papers have been published on the behavior, ecology and genetics of Heliconiinae butterflies (e.g., Davies and Bermingham 2002, Flanagan et al. 2004, Jiggings et al. 2005, Joron et al. 2006, Mavárez et al. 2006). Just a few of them deal specifically with courtship and mating behavior (Rutowski and Schaeffer 1984, McMillan et al. 1997, Naisbit et al. 2001, Jiggings et al. 2004, Kronforst et al. 2007); to our knowledge, only two articles were published on Heliconiinae mating behavior: Crane (1955), for *Heliconius erato hydara*, and Rutowski and Schaffer (1984), for *Agraulis vanillae*. Information on courtship behavior is essential in any attempt to evaluate the evolution of dimorphism and selective consequences on variation between sexes.

Dryas iulia Fabricius is a butterfly that presents sexual dimorphism regarding wing size and coloration; the males are more colorful and larger than the females (Brown 1981, Haag and Araújo 1994). In Lepidoptera, females are generally larger than the males and this difference is commonly associated with oviposition activity (Rutowski 1997, Wiklund 2003). Few studies so far have shown the existence of inverted sexual dimorphism in butterflies, with males larger than females (e.g., Wiklund and Kaitala 1995). Studies performed with various animal species show evidence of selection mechanisms involving the evolution of sexual dimorphism, generally relating the differences found to reproductive success, behavioral ecology and development (Andersson 1994, Wiklund 2003, Blanckenhorn 2007).

Here, we describe the mating behavior of *Dryas iulia alcionea,* a butterfly widely distributed in Brazil and in many areas of South and Central America. First, relevant matting behavioral acts of males and females were observed and described, and after words, the behavioral sequence of courtship was analyzed. We also compared the differences between successful and unsucceful courtship using quantitative methods. The evolution of mating behavior in Heliconiinae is briefly discussed.

Materials and Methods

Experiment material and testing conditions:

Experimental population was produced by crossing the offspring of field-caught butterflies. Crossings were conducted with butterflies from distant geographical localities to avoid inbreeding effects (population 1 - 30°02’15”S, 51°01’08”W, population 2 -

29°38’39”S, 53°46’34”W). Larvae were reared on fresh passionflower leaves (*Passiflora suberosa*) under controlled conditions (25ºC and 24h light). After emergence of the adults, butterflies were separated by sex and kept in outdoor cages (3 x 3 x 3 m) to be used in the experiments. This design ensured that all butterflies had no previous sexual experience**.** During captivity, adults were fed *ad libitum* with a moisture of distilled water and honey (3:1).

All observations were made in an outdoor flight cage similar to those described above, always between 10 am and 2 pm, at a temperature of 25-32ºC, only on sunny breezy days. Temperatures outside this range, high winds and cloudy conditions decreased flight activity to allow for efficient testing.

Ethogram and Behavioral sequences

To make an ethogram for mating behavior, 100h of observations in the outdoor cages were made. Groups of butterflies, varying from 1-4 individuals per sex, were chosen randomly among those bred in the laboratory and placed in cages to interact for 1 hour. The observation method used was the focal-animal (Altmann 1974). The age or prior experiences of the butterflies used were not controlled, but butterflies used in the observations ranged from 2-20 days old. Behavioral acts of males and females directly involved in mating were recorded and described.

The sequence of mating behavior was described using the all occurrences sampling method (Altmann 1974). A single virgin female, 2-4 days old, was released to one virgin male, 5-7 days old. A total of 53 pairs were observed. Individuals were used just once to prevent effects of the experience. Observations lasted one hour per pair, with a 10-minute delay before the beginning of behavioral record, allowing butterflies to accommodate themselves to the cage conditions and experimental manipulation.

Quantitative analyses of behavioral sequences were done using three different methods. First, a multivariate non-parametric analysis of variance (NPMANOVA) was used to test the differences between successful and unsuccessful courtships and the univariate Mann-Whitney test to compare specific behavioral differences between response variable. Each behavioral act previously described in the ethogram was taken as variables for NPMANOVA. In order to prevent deviations in statistics, behavioral acts with low frequencies were excluded from the analysis. The NPMANOVA was applied according to

Finch (2005), using a transformation of Pillai's trace into χ^2. Second, to obtain information about most probable behavioral transitions of males during successful and unsuccessful courtships, observation sessions were transformed into a first-order transition matrix, where rows represent the preceding act and columns the subsequent act in the sequence. Self transitions (repetition of a single behavior) were not recorded, since their inclusion in the matrices may obscure the importance of transitions between behaviors (Slater and Ollason 1973, Baker and Cardé 1979, Girling and Cardé 2006). A probabilistic analysis of behavioral sequences was performed with the EthoSeq software (Japyassú et al. 2006), where the most probable transitions were used to build behavioral flowcharts, diagrams summarizing the information about the sequence of behavioral acts. The diagrams were mounted and edited using Microsoft PowerPoint 2002. Third, a stereotypy index (SI) (Haynes and Birch 1984) was used to provide additional information about the differences between successful and unsuccessful courtships. SI was calculated from summed individual transition probabilities for each category of sequences, as described in Girling and Cardé (2006).

Results

1. The ethogram for mating behavior

Behavioral acts associated with mating behavior were catalogued into two different repertories: pre-coupling (table 1A) and post-coupling repertory (table 1B). The number of behavioral acts in each repertory was 18 and 8, respectively. The males showed a higher number of mating behavioral acts when compared to females, including both repertories (21 against 10 – table 1). Fifteen behavioral acts were exclusive to males and four behavioral acts exclusive to females, all of them responses to male copulation attempts (table 1). During observations, males exhibited intense flight activity through the cage, interspersed with short periods of rest. Males spent most of their time chasing other butterflies and courting females. The flight activity of the females had a shorter duration when compared to males. Although not quantified, females spent most of their time flying through vegetation. These observations suggest that the main strategy of searching for mating in *Dryas iulia* is the patrolling of males.

2. Behavioral sequence of mating behavior

During observations, a few behavioral acts previously recorded during ethogram elaboration were not registered and were suppressed from the behavioral sequence description and further analyses (*wing spreading, wing clapping* and *kicking*). We have described the behavioral sequence of courtship in three phases, based on the location of the individuals in relation to the substrate and to each other.

2.1. Aerial phase:

Courtship begins during the aerial phase and has a mean duration of 66.2±81.8 s. This stage starts when both male and female are flying, and involves just a few behavioral acts. The male locates the female during flight and start to chase her (*flight pursuit* behavior). Then, they start to flight in spiral trajectories (*spiraling flight* behavior). The female usually takes an escape route and after that she alights on the substrate; males were never observed stopping the *spiraling flight* before the females. During the aerial phase males can be very persistent, ceasing their activities only after the female has alighted.

2.2. Air-ground phase:

The air-ground phase begins with the female alighting on the substrate and male hovering over her, and it has a mean duration of 67.5±88.7 s. This phase is the most complex of the entire courtship process, showing the highest number of behavioral acts and transitions. It is characterized by the male attempts to approach the female and to perform genital coupling. With the female alighted, the male starts to perform short flights over her (*overflight* behavior), which then changes to a standstill flight (*hovering* behavior). Generally the female responds to this action by pressing her wings against the substrate (*wing pressure* behavior) and raising her abdomen (*abdomen raising* behavior). If the intensity of the male's activity increases, the female's response also becomes more intense. This body posture of female creates a spatial barrier by preventing the male from trying the genital coupling, and it seems to signal non-receptivity for copulation. During non-receptivity signaling of females, courting males may intensify their courtship, by alighting on females' wings (*alighting on wings* behavior). Females generally react to this by kicking the male (*kicking* behavior), but they can also fly way. When female flights again, she is promptly chased by the male. Sometimes the male may grabs on female's wings, knocking her off the substrate. During free fall, the female fly away and is chased by the male again. Sometimes, both male and female fall directly to the ground. During the courtship, receptive females usually relax their non-receptivity position and promptly shut her wings (*wing shutting* behavior). By relaxing the pressure of the wings against the substrate and lowering the abdomen the female stimulates the male to try genital coupling, which may alights on the side of female (*parallel alighting* behavior). This moment seems to be crucial for the copulatory success of the male, since it is when the female changes her body posture and allows the male's approach, and consequentely the genital coupling. Copulation begins with the male bending his abdomen towards female direction (*abdominal bending* behavior). The attempt at genital coupling is not always successful. After the female acceptance, if the male attempts genital coupling for a long time without success, the female may return readily to the non-receptivity posture. Eventually, very excited males may try to perform genital coupling with the head of the female. In this case, the female promptly changes her posture to a rejection position. The males usually react to female behavioral modification, exhibiting aggressive courting behaviors (*touching with the proboscis*, *pushing with the head* and *wing lever*).

2.3. Ground phase:

During ground phase, genital coupling occurs and the spermatic transfer begins. It is the longest phase of the mating (122.2±21.0 min), and it can be characterized by the opposite posture of the male towards the females and by the great immobility of the couple at the mating site. This stage begins after genital coupling. Then, the male rotates his body assuming an opposite posture position in relation to female (*coupled rotation* behavior). During rotation, the male closes his wings and left the female wings and coupled genitals protected between his wings (*wing protection* behavior). After rotation, the male exhibited some wing flapping, which starts at high frequency and gradually slow down (*post-coupling flapping* behavior). After this sequence the couple stays immobile at the mating site (*post-coupling resting* behavior). If disturbed, the male may carry the female to another place in a short distance flight (*post-coupling flight* behavior).

In all of the 23copulas observed, mating was invariably ended by the male. The male opens his wings at a 45^{o} angle with the substrate and stays in this position for a few moments. After, he performs some wing flapping and decouples himself from the female and then walks a few centimeters on the substrate. This observation suggests that the duration of the transfer phase is determined by the male. After genital separation, the butterflies fly away and may exhibit some wing flapping (*post-coupling wing flapping* behavior). Next, both butterflies exhibit a long roosting behavior (*post-coupling roosting* behavior). After this phase, the male generally returns to its foraging activity before the female.

3. Successful courtships versus unsuccessful courtships

A total of 53 courtship events were observed, 49% of which resulted in successful mating. There was a high variability between successful and unsuccessful courtships, but no statistical difference was found between the groups with respect to the time of first male-female interaction (10.6±8.6 min and 9.3±7.9 min, respectively; t=-0.609, p=0.545). Mating occurred 20.9±13.4 minutes after the first contact between the butterflies.

The NPMANOVA revealed significant quantitative differences between successful and unsuccessful courtships, both for male courting activity (χ^2=34.616, df=20, p=0.047) and for female response to courting males (χ^2=23.880, df=14, p=0.047). Males and females behave differently regarding to some behavioral acts (table 2). Among the females, the *wing pressure, abdomen raising, overflight* and *flight pursuit* behavior were performed significantly more often where no copulation took place, suggesting that these are behavioral acts signalize non-receptiveness. The *wing shutting* behavior was more often executed where copulation occurred, and it seems to be very important in signaling receptiveness. Among the males, *hovering* and *parallel alighting* behavior were often performed by very persistent males and were more often executed by the males that did not copulate. These results suggest that insistence of males in courting do not affect mating success. The *parallel alighting, abdominal bending* behaviors were performed most by the males that copulated. These behavioral acts were always displayed right after the female has shut her wings, reinforcing the evidence of the importance of this behavior in signaling acceptance to the courting male.

The analysis of behavioral transition probabilities of males between successful and unsuccessful courtship revealed notable differences (figure 1). The number of transitions recorded was significantly lower in successful courtships (transitions=244, mean=9.4±6.0, N=26) when compared to unsuccessful courtships (transitions=329, mean=12.2±7.8, N=27) (t=2.497, p=0.016). Thus, unsuccessful males changed their behavior more frequently than successful males.

In the behavioral flowchart, it is noted that the set of behavioral transitions of successful (figure 1A) males is less reticulated than the one of unsuccessful males (figure 1B). However, the number of transitions with probabilities greater than 40% was higher in the successful males' diagram (N=11, against N=7 for unsuccessful males). These data suggest that successful male shown behavioral patterns less variable than unsuccessful ones during courtship. The results of the stereotypy indices (SI) analysis confirm this tendency (table 3). The SI was greater in situations where copulation occurred (0.46) than when no copulation occurred (0.32). Likewise, of the 10 behaviors common to both groups, 8 of them presented higher stereotypy values in the group where copulation occurred.

Discussion

Behavioral acts, repertories and sequences

The males have shown a behavioral repertory with more acts when compared to the females. This result is expected, since in most butterfly species it is the male who actively seeks out for mates; females are also less active than males (Scott 1972, Rutowski 1984). Both in nature and in captivity, when they are not feeding, the males of *D. iulia* are chasing other butterflies. Their behavior is typically patrolling mating strategy (Scott 1972).

In *D. iulia*, the typical posture of rejection by the female is characterized by wing pressure against the substrate and the raising of abdomen, which prevents the male from approaching and trying the genital coupling. This rejection behavior is similar to that found in many Pieridae species (Scott 1972). This author reports that the behavior also occurs in *Heliconius erato*, but other studies do not confirm that this behavior exists (Crane 1955; A.L. Klein, personal communication). However, Rutowski and Schaefer (1984) described the same behavior in another Heliconiinae (*Agraulis vanillae*). In certain cases, the males can overcome the rejection behavior after prolonged courting activity, but generally the males are unable to force the females to copulate (Scott 1972, Svärd and Wiklund 1989, Wiklund and Forsberg 1991). The typical female behavioral of acceptance consists of suddenly shutting of wings (*wing shutting* behavior, see table 1). This posture allows the male's approach and the genital coupling.

The *spiraling flight* behavior (table 1A) occurs mainly in interssexual interactions, mostly during courting activity, but some endurance contests may occurs between to passing males. Kemp (2002) describes the occurrence of this behavior in *Hypolimnas bolina*, characterizing it as a dispute among males to defend a territory. Garcias (1983) describes similar behavior for two other species of Heliconiinae, *Agraulis vanillae* and *Euiedes aliphera*, suggesting that it is important to defend foraging sites. *D. iulia*, on the contrary, is not territorial, and it is very tolerant with its co-specifics, to the point of forming communal dormitories, with a lower degree of viscosity than in the case of *H. erato phyllis*, for instance. As far as we know, our description of the *spiraling flight* behavior of *D. iulia* is the first record of occurrence of this type of behavior between different sexes for a species of the Heliconiinae subfamily. The evolution of this behavior in the group is uncertain, but may have originated in the social interactions or defending feeding sites, and later took on a role in the courting behavior. Our observations suggest that spiraling flights play an important role in reproductive activity, probably in stimulating courtship of resistant females.

Another behavioral act, *looping*, was not recorded very often in our observations, and its function is unknown. This behavior is well known in other Nymphalidae (*Danaus gilippus*). It is considered an important stage of chemical stimulation, being essential for copulatory success; the male releases some pheromones close to the female's antennae, until the male manages to achieve a copulatory position (Brower et al. 1965). Since looping was recorded on few occasions during observations, we believe it is not essential for have success in courtship, but some function of chemical stimulation cannot be ruled out.

All the courting activities observed here were started by males during flight. Krebs (1988), studying the mating behavior of *Papilio glaucus* (Papilionidae) found similar results. This result strengthens the hypothesis of patrolling behavior as the priority strategy in mate search.

After genital coupling, during post-copulatory roosting, the male protects the genitals of both sexes between its wings. This immobility may be explained as an attempt to increase the efficiency of spermatic transfer, but also can increase the chances of being found by possible competitors and predators. The *coupled flight* behavior could have been the evolutionary response to this problem. Two chance observations, performed in the field, support this hypothesis. First, it is not unusual to see groups of 2-5 males over a couple that has just begun the mating (transfer phase), disturbing the coupled pair and trying to do genital coupling with the female (*copulatory disturbance* behavior, see table 1B). The copulating male promptly responds by spreading his wings to (*copulatory wing spreading* behavior), and may fly away if there is excessive disturbance by the other males (*postnuptial flight* behavior). This type of behavior can also be observed in *Actinote carycina* (personal observations). Second, copulations in the field usually take place under vegetation, in shady places or others partly lit by the sun shining through the canopy. This behavior may prevent harmful overheating or encounters with visually oriented predators.

All copulations were always finalized by the males. After a few hours in the transfer phase, the male promptly begins to flutter his wings, walk a few centimeters and fly away, leaving the female on the mating site. In fact, few references can be found on the subject. Phelan and Baker (1990) showed that in several species of moths the female is responsible for ending the copulation, beginning to walk while coupled to the male (*drag walk*). The end of copulation is also started by the females in *Precis sp.* (Scott 1972). However, according to Wickman (1985) it is plausible that the males determine the end of copulation in several species, as observed in *Pararge aegeria* and in *Papilio zelicaon* (Shields 1967). The evolutionary implications of this behavior are extremely important, suggesting that a conflict of interests between males and females may be involved in the process. The interests of males and females may diverge in reproduction, where characteristics favored in one sex may be harmful to the other (Chapman et al. 2003). Many recent studies have documented the occurrence of this process in different species (see Arnqvist and Rowe 2005), including Lepidoptera (Andersson et al. 2000), always suggesting that the origin of the conflict is rooted in the rise of anisogamy, as suggested by Parker (1979) and Parker and Partridge (1998). Currently, the sexual conflict is so important in postcopulatory sexual selection in insects, that it is considered a key process in the speciation of some groups (Arnqvist et al. 2000).

The success in courtships

The results of the comparison between successful and unsuccessful courtships revealed that copulatory success depends basically on acceptance by the female. Males that exhibited prolonged courtship activity were no more successful than less insistent males.

Besides, typical copulatory induction behaviors, such as *hovering*, *alighting on wings* and *spiraling flight,* were not recorded more frequently in successful courtships. The only significant association between behavior and copulatory success of the males can be seen with regard to the behavioral acts, *parallel alighting* and *abdomen bending*. However, these behavioral acts are generally exhibited by the males after a female gives signs of her receptiveness. These male behavioral acts appear to be a direct response to female activity, and not their consequence.

The mechanisms underlying mate selection by *D. iulia* females are still uncertain. Brown Jr. (1981) and Haag and Araújo (1994), reported that *D. iulia* presents sexual dimorphism regarding wing pigmentation and body size. The existence of dimorphism suggests the possibility of occurrence of sexual selection during species evolution. Further studies are needed to explain the role of size, color and life histories in the mating process of *D. iulia*.

Evolution of courtship behavioral patterns

When we compare the behavior of courtship and mating in *D. iulia* to the studies performed in other Heliconiinae (*Heliconius erato* - Crane 1955, *Agraulis vanillae* - Rutowski and Schaefer 1984), we see similar behavioral patterns. However, what calls most attention in comparing the species behaviors is the occurrence of some marked differences in behavioral signaling and specific recognition.

The behavioral act *wing clapping* of *D. iulia,* is similar to the wing clap display of *Agraulis vanillae* (Rutowski and Schaefer 1984). Moreover, some differences are observed as regards the female's antenna laid back between the male's wings, which occur in *A. vanillae* and it is not observed in *D. iulia*. Similar behavior is observed in old males of *Heliconius erato*, also without involving the female's antenna stimulation (Crane 1955). It is not know whether this behavior occurs in the other genera of Heliconiinae, as in *Dione*, *Podotricha*, *Neruda*, *Euides*, *Dryadula* and *Phylaethria*. Its origin and function must be further studied to elaborate an evolutionary scenario.

Experiments with models have shown that movement, size and general color are important in courtship in several species (Scott 1972). In *Heliconius,* a series of studies suggests that visual signaling is extremely important for sexual activity (Crane 1955, Emsley 1970, Gilbert 1976, Jiggins et al. 2001, Lutz 2002, Jiggins et al. 2004). Apparently, as in several species of butterflies, the *Heliconius* courtship evolved strongly towards long distance visual signaling. However, little is known about short distance chemical signaling and behavior stereotypy (see Gilbert 1976, Hernández and Benson 1998 and Schultz et al. 2008 for further contributions). Possibly, because of Müllerian mimicry, considerable selective pressure must have occurred on the chemical signaling. This evolutionary scenario does not seem to have occurred in *D. iulia* with such strong selective pressure. The extrusion of female abdominal glands which commonly occurs in *H. erato* (Crane 1955) and is sometimes observed in *Agraulis vanillae* (Rutowski and Schaefer 1984), was not recorded in *D. iulia* during our observations. The occurrence of *pupal mating* in some species of *Heliconius* (Brown, 1981; Hernández and Benson, 1998), and the diversity of mimetic patterns observed in the *H. erato* - *H. melpomene* system (Brown et al. 1974, Brower 1996) support the hypothesis on the importance of chemical signaling in genus *Heliconius*.

Emsley (1970) reported the case of mullerian mimicry between *H. erato phyllis*, *H. melpomene melpomene* and *H. besckei*, where the courtship releaser of the two first species would be the red band, present in the forewings, while the third one would be the yellow bar, present in the hindwings. Similar evidence was found in Lutz (2002). It is possible that the recognition between *H. erato* and *H. melpomene* possesses an important chemical component or some unknown stereotypic behavior not described up to the present moment.

In *D. iulia*, the visual patterns seem important for choosing and recognizing sexual partners, as observed in *Papilio glaucus* (Krebs 1998). It is not unusual to observe *D. iulia* males chasing other species of butterflies of the orange mimetic ring (Garcias 1983), a behavior similar to that observed in *Agraulis vanillae* (Rutowski and Schaefer 1984). Emsley (1963) describes the occurrence, in sympatry, of five other species of Heliconiinae with patterns of orange and black colors for the neotropics. However, many of these species present higher population densities at different times of the year (Garcias 1983). This scenario would make the issue of interspecific recognition of *D. iulia* less problematic, compared to the mimetic rings of *H. erato* and *H. melpomeme.* This does not mean that there is no chemical signaling in *D. iulia*, only that this type of signaling would not be as important as in the mimetic rings of *Heliconius.* The sexual and foraging behaviors between species with the orange mimetic ring are very different, as seen in the sexual behavior of *Agraulis vanillae* (Rutowski and Schaefer 1984) and *D. iulia*. In this case, natural selection might lead to the evolution of stereotyped behaviors that offered a clear signal of the species identity, allowing the females to recognize and to evaluate the courting males.

The rejection posture of *H. erato* involves *fluttering* (Crane 1955) and exhibition of some scent glands, a behavior that is not observed in *D. iulia*. Old males and females behave similarly in the two species, suppressing certain courting behaviors compared to the younger ones, even with significant differences in relation to the reproductive dynamics of the two species (Boggs 1981). In *Pieris protodice*, young females appear to be more selective in choosing their first sexual partner than old females that have a shorter life expectancy (Rutowski 1980). The same pattern was found in *Eurema daira* (Daniels 2007). In some species it is seen that the males prefer young females (Andersson et al. 2005) and females prefer experienced males (Kemp 2002). Casual observations performed in the field and in captivity, suggest that females solicit courtship by stimulating males to court them. This behavior was observed in females of an advanced adult age not inseminated after several days of adult life. In these cases, the female does not show the typical rejection posture, but promptly takes a position of acceptance at the slightest sign of male activity. This behavioral pattern was observed in a few other butterfly species (Scott 1972), and was recently studied in *Eurema daria* (Daniels 2007).

Much work is still to be done to explain the role of visual and chemical signalization and stereotypy of behaviors in the courtship activity and copulatory success in Heliconiinae. We hope our efforts bring some light to the present discussion on behavioral patterns and courtship evolution in Heliconiinae.

Acknowledgments

We are grateful to J. Zanin, G. Pasqualin, A. Carrion, A.A. Ferreira, P.R. Vieira and A.L. Klein for help in keeping larvae and adults fed adequately. We thank S. Callegari-Jacques for the criticism on statistics and A.L. Klein and M. P. Almerão for suggestions on the previous versions of the manuscript. We also thank Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Programa de Apoio a Núcleos de Excelência (PRONEX) for financial support.

References:

Altmann, J. 1974. Observational study of behavior: sampling methods. Behaviour 49(3-4): 227-267.

Andersson, J., Borg-Karlson, A.K., Wiklund, C. 2000. Sexual cooperation and conflict in butterflies: a male-transferred anti-aphrodisiac reduces harassment of recently mated females. Proceedings of Royal Society of London B 267(1450): 1271-1275.

Andersson, M. 1994. Sexual selection. Princeton University Press. New Jersey. 599pp.

Arnqvist, G., Rowe, L. 1995. Sexual conflict and arms races between the sexes: a morphological adaptation for control of mating in a female insect. Proceedings of Royal Society of London B 261(1360): 123-127.

Arnqvist, G., Rowe, L. 2005. Sexual conflict. Princeton University Press, New Jersey.

Arnqvist, G., Edvardsson, M., Friberg, U., Nilsson, T. 2000. Sexual conflict promotes speciation in insects. Proceedings of National Academy of Science of USA 97(19): 10460-10464.

Baker, T.C., Cardé, R.T. 1979. Courtship behavior of the oriental fruit moth (Grapholita molesta): experimental analysis and consideration of the role of sexual selection in the evolution of courtship pheromones in the Lepidoptera. Annals of Entomological Society of America 72: 173-188.

Blanckenhorn, W.U., Dixon, A.F.G., Fairbairn, D.J., Foellmer, M.W., Gibert, P., van der Linde, K., Meier, R., Nylin, S., Pitnick, S., Schoff, C., Signorelli, M., Teder, T., Wiklund, C. 2007. Proximate causes of rensch's rule: does sexual size dimorphism in arthropods result from sex differences in development time? The American Naturalist 169(2): 245-257.

Boggs, C.L. 1981. Selection pressures affecting male nutrient investment at mating in Heliconiine butterflies. Evolution 35(5): 931-940.

Brower, A.V.Z. 1996. Parallel race formation and the evolution of mimicry in heliconius butterflies: a phylogenetic hypothesis from mitochondrial DNA sequences. Evolution 50(1): 195-221.

Brower, L.P., Brower, J.V.C., Cranston, F.P. 1965. Courtship behavior of the queen butterfly *Danaus gilippus berenice*. Zoologica 50(1): 1-39.

Brown Jr., K.S, Sheppard, P.M., Turner, J.R.G. 1974. Quaternary refugia in tropical America: evidence from race formation in *Heliconius* butterflies. Proceedings of Royal Society of London B 187(1088): 369-378.

Brown Jr., K.S. 1981. The biology of *Heliconius* and related genera. Annual Review of Entomology 26: 427–456.

Brown Jr., K.S. 1992. Borboletas da Serra do Japi: diversidade, hábitats, recursos alimentares e variação temporal. In História Natural da Serra do Japi: ecologia e preservação de uma área florestal no sudeste do Brasil. Edited by L.P.C. Morellato. Unicamp/Fapesp, Campinas. pp.142-187.

Brown Jr., K.S., Yepez, F.F. 1984. Los Heliconiinae (Lepidoptera, Nymphalidae) de Venezuela. Boletín de Entomología Venezolana 3(4): 29-76.

Chapman, T., Arnqvist, G., Bahghamm, J., Rowe, L. 2003. Sexual conflict. Trends in Ecology and Evolution 18(1): 41-47.

Crane, J. 1955. Imaginal behavior of a Trinidad butterfly, *Heliconius erato hydara* Hewitson, with special reference to the social use of color. Zoologica 40(16): 167-195.

Daniels, J.C. 2007. Courtship solicitation by females of the barred sulphur butterfly (*Eurema daira*)(Lepidoptera: Pieridae). Journal of Insect Behavior 20(1): 129-135.

Davies, N., Bermingham, E. 2002. The historical biogeography of two Caribbean butterflies (Lepidoptera: Heliconiidae) as inferred from genetic variation at multiple loci. Evolution 56(3): 573–589.

Emsley, M.G. 1963. A morphological study of imagine Heliconiinae (Lepidoptera: Nymphalidae) with consideration of the evolutionary relationships within the group. Zoologica 48(8): 85-131.

Emsley, M.G. 1970. An observation on the use of color for species-recognition in *Heliconius besckei* (Nymphalidae). Journal of Lepidopterists' Society 24(1): 25.

Ferreira, A.A., Garcia, R.N., Araújo, A.M. 2006. Pupal melanization in *Heliconius erato phyllis* (Lepidoptera; Nymphalidae): genetic and environmental effects. Genetica 126(3): 133-140.

Finch, H. 2005. Comparison of the performance of nonparametric and parametric MANOVA test statistics when assumptions are violated. Methodology 1(1):27-38.

Flanagan, N., Tobler, A., Kapan, D., Davison, A., Pybus, O., Planas, S. 2004. Historical demography of Müllerian mimicry in the Neo-tropical *Heliconius* butterflies. Proceedings of National Academy of Science of USA 101(26): 9704–9709.

Garcias, G. 1983. Aspectos da biologia populacional de cinco espécies de heliconiíneos do anel mimético "laranja" (Lepidotera, Nymphalidae). M.Sc. thesis. Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Gilbert, L.E. 1976. Postmating female odor in *Heliconius* butterflies: a male-contributed antiaphrodisiac? Science 193(4251): 419-420.

Girling, R.D., Cardé, R.T. 2006. Analysis of the Courtship Behavior of the Navel Orangeworm, *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), with a commentary on methods for the analysis of sequences of behavioral transitions. Journal of Insect Behavior 19(4): 497-520.

Haag, K.L, Araújo, A.M. 1994. Inbreeding, genetic load and morphometric variation in natural populations of *Dryas iulia* (Lepidoptera, Nymphalidae). Revista Brasileira de Genética 17(1): 35-39.

Haynes, K.F., Birch, M.C. 1984. Mate-locating and courtship behaviors of the artichoke plume moth, *Platyptilia carduidactyla* (Lepidoptera: Pterophoridae). Environmental Entomology 13: 399-408.

Hernández. M.I.M., Benson, W.W. 1998. Small-male advantage in the territorial tropical butterfly *Heliconius sara* (Nymphalidae): a paradoxical strategy? Animal Behaviour 56(33): 533–540.

Japyassú, H.F., Alberts, C.C., Izar, P., Sato, T. 2006. EthoSeq: a tool for phylogenetic analysis and data mining in behavioral sequences. Behavior Research Methods 38(4): 549-556(8).

Jiggins, C.D., Estrada, C., Rodrigues, A. 2004. Mimicry and the evolution of pre-mating isolation in *Heliconius melpomene*. Journal of Evolutionary Biology 17(3): 680–691.

Jiggins, C.D., Naisbit, R.E., Coe, R.L., Mallet, J. 2001 Reproductive isolation caused by colour pattern mimicry. Nature 411(6835): 302–305.

Jiggins, C.D., Mavárez, J., Beltrán, M., McMillan, W.O., Johnston, J.S., Bermingham, E.B. 2005. A genetic linkage map of the mimetic butterfly, *Heliconius melpomene*. Genetics 171(2): 557–570.

Joron, M., Jiggins, C.D., Papanicolaou, A., McMillan, W.O. 2006. *Heliconius* wing patterns: an evo-devo model for understanding phenotypic diversity. Heredity 97(3): 157–167.

Kemp, D.J. 2002. Sexual Selection constrained by life history in a butterfly. Proceedings of Royal Society of London B 269(1498): 1341-1345.

Krebs, R.A. 1988. The matting behavior of *Papilio glaucus* (Papilionidae). Journal of Research on Lepidoptera 26(1-4): 27-31.

Kronforst, M.R., Young, L.G., Gilbert, L.E. 2007. Reinforcement of mate preference among hybridizing *Heliconius* butterflies. Journal of Evolutionary Biology 20(1): 278-285.

Lutz, L. 2002. Contextos fenotípicos multifuncionais: influência do padrão de coloração de uma borboleta impalatável (*Heliconius erato phyllis*) sobre seu sucesso de acasalamento, ritmo diário de atividades e sobrevivência em campo. Ph.D. thesis. Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Mavárez, J., Salazar, C.A., Bermingham, E., Salcedo, C., Jiggins, C.D., Linares, M. 2006. Speciation by hybridization in *Heliconius* butterflies. Nature 441(7095): 868-871.

McMillan, W.O., Jiggins, C.D., Mallet, J. 1997. What initiates speciation in passion-vine butterflies? Proceedings of National Academy of Science of USA 94(16): 8628–8633.

Naisbit, R.E., Jiggins, C.D., Mallet, J. 2001. Disruptive sexual selection against hybrids contributes to speciation between *Heliconius cydno* and *Heliconius melpomene*. Proceedings of Royal Society of London B 268(1478): 1849–1854.

Parker, G.A. 1979 Sexual selection and sexual conflict. In Sexual selection and reproductive competition in insects (ed. M. S. Blum & N. B. Blum), pp. 123-166. NewYork: Academic Press.

Parker, G.A., Partridge L. 1998. Sexual conflict and speciation. Philosophical Transactions of the Royal Society B 353(1366): 261-274

Phelan, P.L., Baker, T.C. 1990. Comparative study of courtship in twelve *Phycitine* moths (Lepidoptera: Pyralidae). Journal of Insect Behaviour 3(3): 303-326.

Rutowski, R.L. 1980. Courtship solicitation by females of the checkered white butterfly *Pieris protodice*. Behavioral Ecology and Sociobiology 7: 113-117.

Rutowski, R.L. 1984. Sexual selection and the evolution of butterfly mating behavior. Journal of Reseach on the Lepidoptera 23(2): 125-142.

Rutowski, R.L. 1997. Sexual dimorphism, mating systems and ecology in butterflies. In The evolution of mating systems in Insects and Arachnids. Edited by J.C. Choe. Library of Congress, Cambrigde. pp 397.

Rutowski, R.L., Schaefer, J. 1984. The courtship behavior of the gulf fritillary, *Agraulis vanillae* (Nymphalidae). Journal of the Lepidopterists' Society 38(1): 23-31.

Scott, J.A. 1972. Mating of butterflies. Journal of Reseach on the Lepidoptera 11(2): 99-127.

Schulz, S., Estrada, C., Yildizhan, S., Boppré, M., Gilbert, L.E. 2008. An antiaphrodisiac in *Heliconius melpomene* butterflies. Journal of Chemical Ecology 34(1): 82-93.

Shields, O. 1967. Hilltopping. Journal of Reseach on the Lepidoptera 6: 69-178.

Slater, J.B., Ollason, J.C. 1973. The temporal patterning of behaviour in isolated male zebra finches: transition analysis. Behaviour 2: 248-269.

Svärd, L., Wiklund, C. 1989. Mass and production rate of ejaculates in relation to monandry/polyandry in butterflies. Behavioral Ecology and Sociobiology 24(6): 395-402.

Wickman, P.O. 1985. Male determine mating duration in butterflies? Journal of the Lepidopterists' Society 39(4): 341-342.

Wilklund, C. 2003. Sexual selection and the evolution of butterfly mating systems. In Butterflies: ecology and evolution taking flight. Edited by C. Boggs et al. University of Chicago Press, Chicago. pp 67-90.

Wiklund, C., Forsberg, J. 1991. Sexual size dimorphism in relation to female polygamy and protandry in butterflies: a comparative study of swedish pieridae and satyridae. Oikos 60(3): 373-381.

Wiklund, C., Kaitala, A. 1995. Sexual selection for large male size in a polyandrous butterfly: the effect of body size on male versus female reproductive success in *Pieris napi*. Behavioural Ecology 6 (1): 6-13.

Table 1. Description of behavioral acts involved in the courtship and mating of *Dryas iulia alcionea*, obtained after 100 hours of observation of groups of 1-4 individuals per sex (method: focal animal). The behavioral repertory was divided into: **A.** pre-coupling and **B.** post-coupling behavioral acts.

A.

Behavioral Act	Sex	Description
Abdomen raising	Females	The female raises the abdomen, keeping it above the line of the hindwings. The angle of the abdomen in relation to the substrate may vary between 30-60°. When approached by other butterflies, she moves her abdomen up and down several times.
Wing shutting	Females	When courted by males, after alighting, the female suddenly shuts its wings, keeps the abdomen below the line of the hindwings and remains motionless.
Wing pressure	Females	If disturbed or courted by a male, a perched female may spread their wings, pressing it against the substrate. This behavior is generally associated with abdomen raising.
Wing spreading	Females	The females remain motionless, with the wings fully opened, usually with the abdomen in the same level of hindwings.
Wing lever	Males	The male stays side-by-side with the female and inserts its wings and head under its wings. The male creates a kind of lever, trying to force the female to close its wings.
Wing clapping	Males	The male alights next to the female and exhibits some wing flapping, followed by body rotations. It ends when male and female face each other, touch antennae, or the female turns the genitalia towards the male and shuts wings, lowering the abdomen.
Abdominal bending	Males	The male stays beside the female and bends the abdomen, seeking the female's genitalia. Repetitive spreading movements of the claspers may occur during the bending, as well wing fluttering.
Hovering	Males	The male hovers over another perched butterfly (5-10 cm away). The hovering butterfly may touch the perched one on its wings and antennae, generally with the forelegs.
Looping	Males	The male performs loops around a flying female and may touch his posterior wings on female antennae. The number of loops performed by the male is variable.
Parallel alighting	Males	The male alights next to another butterfly, generally keeping the body axis parallel to the body axis of the perched butterfly.
Wing separation	Males	When hovered, the perched male moves its wings and exposes the discolored margins of the hindwings.
Pushing with the head	Males	When perched side by side with a female, the male pushes the female with its head. Some touching between male and female antennae may occur during the process.
Touching with the proboscis	Males	The male touches several areas of the female's body with the proboscis, usually wings, genitalia and abdomen. It is exhibited together with some wing fluttering.
Spiraling flight	Males	High speed flight in spiraling patterns initiated by males toward passing females.
Kicking	Males Females	Sudden reaction, jerking, trying to remove the butterfly that perches on its wings. It is usually followed by some wing flapping.
Flight pursuit	Males Females	Agitated quick flight, where one individual pursues the other for a short time (3-7 s) and short distance (10-30cm). Interaction ends when the pursuer changes the flight course. It is common among males during patrolling, and females disturbed by males.
Alighting on wings	Males Females	Alighting on the wings of another butterfly. The most common response to alighting on the wings is kicking behavior or a runaway flight.
Wing flapping	Males Females	When overflight or hovered, butterflies may flap their wing. During flapping, wings are usually spread in a short range (75-90°) and flapped with low frequency (1-3 per second).
Overflight	Males Females	Circular or ellipsoid flights over a perched butterfly. The flying butterfly approaches and flies away several times over the perched one. The perched butterfly generally responds with some wing fluttering.

B.

Behavioral Act	Sex	Description
Post-coupling wing spreading	Males	When disturbed or overflown, the male coupled with female spreads its wings. Generally the forewings are maintained with larger opening angles when compared to hindwings.
Post-coupling wing flapping	Males	Long range (75-90°) and low frequency (1-2 per second) wing flapping. This movement may occur as a reaction to overflying passing butterflies over coupled male.
Coupled rotation	Males	After the occurrence of genital coupling, the male moves laterally until both sexes face opposite directions.
Copulatory protection	Males	After coupled rotation, the male places its wings outside the female's wings, keeping the copulatory plug and the female's abdomen protected between his wings.
Post-coupling flight	Males	When excessively disturbed, a male coupled with a female may flight away carrying her together. The female stays motionless, with her wings closed and legs folded up.
Mating unplug	Males	Separation of the mating pair after copulation. The male begins to flutter its wings, and then release the female. After this, he walks a few centimeters on the substrate, do some wing flapping and than flew away.
Post-coupling wing flapping	Males Females	After unplug, both sexes exhibit a lengthy wing flapping (29-63 s), with large range (75-90°) and low frequency (1-3 p/ second). This behavior lasts longer in males.
Post-coupling roosting	Males Females	After the end of copula, both male and female shut their wings and remain motionless for some minutes (8-47 minutes). It is generally shorter in males.

Table 2. Post-hoc analysis (Mann-Whitney) of NPMANOVA on the behavioral acts carried out during interactions between males and females in successful (N=26) and unsuccessful courtships (N=27). Mean, *U*-value and *P*-value for each pairwise behavioral act tested are shown in the table.

Females				
Behavioral Acts	**Mean**		***U*-value**	***P*-value**
	Successful	**Unsuccessful**		
Wing spreading	0.963	0.692	295.5	0.261
Pressure of wings	2.481	0.538	166.0	**<0.001**
Abdomen raising	2.148	0.385	153.5	**<0.001**
Wing flapping	0.667	0.577	328.5	0.655
Overflight	1,111	0.154	211.5	**0.012**
Hovering	0.370	0.192	339.5	0.687
Flight pursuit	1.111	0.308	257.0	**0.043**
Wing shutting	0.481	1.038	170.0	**<0.001**

Males				
Behavioral Acts	**Median**		***U*-value**	***P*-value**
	Successful	**Unsuccessful**		
Wing clapping	1.222	0.307	212.0	**0.004**
Overflight	0.852	0.423	276.0	0.118
Hovering	2.037	0.731	213.5	**0.009**
Flight pursuit	2.629	2.038	335.0	0.770
Spiraling flight	1.667	1.654	304.5	0.391
Alighting on wings	0.667	0.154	257.5	**0.027**
Parallel alighting	0.667	1.115	212.5	**0.006**
Touching with the proboscis	0.259	0.038	311.5	0.166
Pushing with the head	0.556	0.038	309.0	0.291
Wing lever	0.481	0.115	293.0	0.130
Abdominal bending	0.704	1.346	130.0	**<0.001**
Genital coupling	0.185	1.038	85.5	**<0.001**

Table 3. Analysis of male *Dryas iulia alcionea* courtship behavior, showing stereotypy indices (S separately for successful and unsuccessful courtships. SI was calculated from summed individual transition probabilities for each category of sequences for each transition from one behavioral act to all subsequent behavioral acts.

Behavioral act	**Stereotypy Index (*SI*)**	
	Successful courtships	**Unsuccessful Courtships**
Wing clapping	0.30	0.28
Overflight	0.31	0.42
Hovering	0.50	0.34
Flight pursuit	0.46	0.41
Spiraling flight	0.39	0.33
Alighting on wings	0.33	0.26
Parallel alighting	0.60	0.26
Touching with the proboscis	0.25	0.27
Pushing with the head	0.35	0.30
Wing Lever	0.33	0.20
Abdominal bending	0.71	0.42
Genital coupling	1.00	-
Overall	0.46	0.32

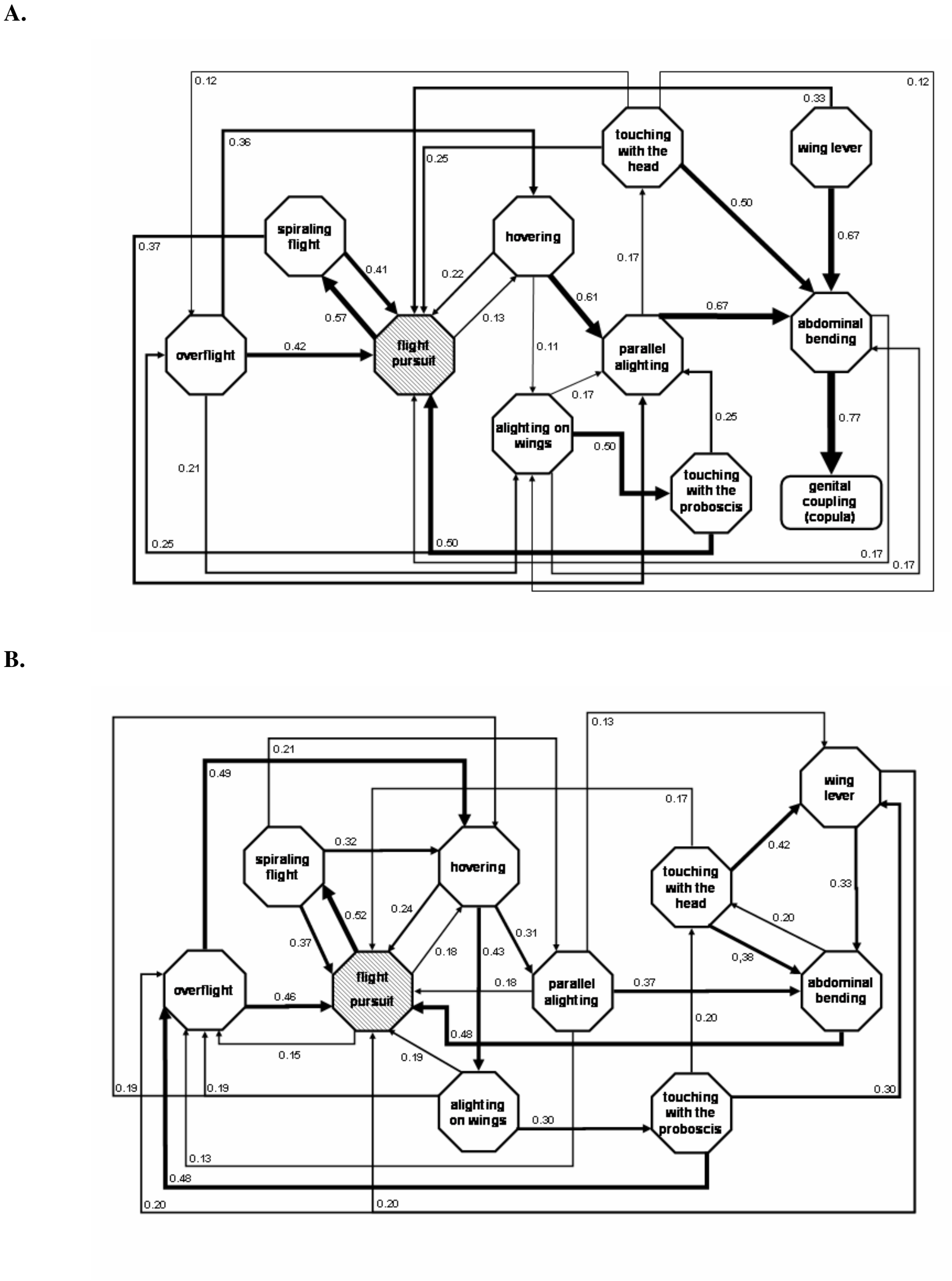


Figure 1. Flowchart of behavioral transition probabilities, calculated from the transition matrix in the courtship of *Dryas iulia alcionea.* The flowchart refers only to male behavior. **A.** successful courtships (N=26). **B.** unsuccessful courtships (N=27). Sequences usually start in the striped octagon. Decimal numbers and corresponding thickness of arrows are conditional probabilities of a particular transition occurring between two behavioral acts. Transitions with a value less than 0.1 are not included to enhance clarity of figures. Descriptions of behavioral acts are listed in table 1.

CAPÍTULO 3

WING SIZE VARIATION IN *DRYAS IULIA ALCIONEA* (LEPIDOPTERA, NYMPHALIDAE): FIELD AND EXPERIMENTAL DATA

Artigo submetido para publicação no periódico *Ecological Entomology*

Wing size variation in *Dryas iulia alcionea* (Lepidoptera, Nymphalidae): field and experimental data

Nicolás O. Mega[1] and Aldo M. de Araújo[2]

[1] *Programa de Pós Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul.*
[2] *Departamento de Genética, Universidade Federal do Rio Grande do Sul.*

Abstract:

The size of an individual and the growth of its body parts are affected by both genetic and environmental factors that operate through complex molecular and physiological mechanisms. The role played by genes on body size has been intensely investigated due to the effects that body size has on fitness. The present study assesses the seasonal variation in adult body size of different populations in southern Brazil. The results show that populations differed as to host plant community and mean seasonal temperatures; and that the variation in *D. iulia* body size exhibited significant correlation with host plant communities and seasonality. In the laboratory, body size was observed to be greatly influenced by host plant and temperature during larval development. Our results suggest that the phenotypic plasticity observed in *D. iulia* populations is modulated mainly by environmental factors. Males are larger and have less phenotypic variation than females, which suggests the occurrence of sexual selection in *D. iulia*.

Keywords: *Dryas iulia*, Heliconiinae, passionflowers, seasonality, body size, temperature, sexual dimorphism.

Introduction:

Body size is a trait that varies considerably with nutrient supplies and the energy allocated in development (West *et al.*, 1999). The size of an individual and the growth of body parts are affected by a series of genetic and environmental factors, which operate through complex molecular and physiological mechanisms (Nijhout, 2003). The role played by genes and by the environment in the determination of body size has been extensively investigated due to their effects on fitness. These are more directly observed in reproduction capabilities, survival, and dispersion ability (Boggs, 1986; Glazier, 2002; Honek, 1993; Torres-Vila et al, 1995; Rodrigues and Moreira, 2002; Van Dyck, 2003;

West *et al*. 1999).

The genetics of body size depends on the quantitative genetic variation observed in a population, which is directly affected by natural selection (Lande, 1980; Falconer, 1989; Merilä *et al.*, 1998; Castillo, 2005), as well as by the genetic correlation with other hereditary characteristics (Roff, 1996). In insects, at least four environmental factors are likely to affect development: temperature, feeding patterns, photoperiod, and competition (Rodrigues and Moreira, 2002, and references therein). Due to the plastic character of development, body size presents considerable intraspecific variations (Nijhout and Wheeler, 1996; Thorne *et al.*, 2006).

Dryas iulia alcionea (Lepidoptera, Nymphalidae) is a widely distributed species, being found in roughly all the continental portion of South America (Brown and Yepez, 1984). Also, it presents significant adaptive plasticity, being able to live in the most varied habitats (Paim *et al.*, 2004). The larvae use several species of passionwines as host plants, preferably plants of subgenus *Passiflora* (formerly Plectostemma) (Brown and Milke, 1972; Perico and Araújo, 1991; Brown, 1992).

The natural populations of *D. i. alcionea* are relatively large and show consistent body size variation across different sites (Haag and Araújo, 1994) and seasons (Paim, 1995). Nevertheless, the reasons for the differences observed have not yet been explained. *Heliconius erato*, another Heliconiinae species, exhibits a significant variation in adult body size within and across populations (Benson *et al.*, 1976; Rodrigues and Moreira, 2002), as well as significant seasonal variations (Rodrigues and Moreira, 2004). These works suggest that the main factor explaning body size variation observed in *H. erato* is the change in quality and availability of host plants. Body size is also mentioned as being temperature-dependent, with low larval development temperatures leading to smaller adults.

The present study evaluates the seasonal variation in *D. iulia alcionea* adult body size in four different populations of *D. i. alcionea* located in southern Brazil, and the likely reasons behind this variation. The differences related to host plants, in each site, were evaluated in terms of the *Passiflora* plant community structure. Climate variations between seasons were measured through the mean temperatures and total rainfall. The effects of environmental factors (host plant and temperature) on development and body size were analyzed in the laboratory.

Materials and Methods

Sampling sites

Samples were collected from four sites located at Rio Grande do Sul State: 1) Estação Experimental de Águas Belas (EAB - 30°02'15"S, 51°01'08"W); 2) Horto Florestal Barba Negra (HBN - 30°21'44"S, 51°13'59"W); 3) Estrada do Perau Velho (EPV - 29°38'39"S, 53°46'34"W), and 4) Morro da Borússia (MDB - 29°52'41"S, 50°16'33"W) (Figure 1).

EAB is located at Viamão municipality, and is a mixed area with fields utilized for agriculture, pastures, secondary patches of the Atlantic Forest, and *Eucalyptus* plantations. The undergrowth of the *Eucalyptus* area consists mainly of shrubs, weeds and some small-sized trees. HBN is located at Barra do Ribeiro municipality and consists of a 19-ha *Eucalyptus* monoculture. The main vegetation consists of plantations of different *Eucalyptus* species. The undergrowth is formed by palms, cactuses and several shrubs, lianas, pteridophytes, and herbaceous species (Rodrigues and Moreira, 2004). EPV is located at Santa Maria municipality. The site is a country road that links the Central Lowland of the state of Rio Grande do Sul and the southern portion of the Southern Brazil Plateau. The vegetation present in the site is essentially a secondary northern stretch of the Atlantic Rainforest. MDB is located at Osório municipality, on the eastern slope of the Southern Brazil Plateau. Local vegetation is part of the Atlantic Rainforest *stricto sensu*, though of secondary succession.

Structure of host plant communities

To determine the structure of the plant communities used by *D. i. alcionea*, a survey of the relative occurrence and abundance of *Passiflora* species was conducted in the sampling sites. Surveys were carried out only once during the spring 2006, the season in which vegetative structures of passionwines grow more intensely (Escobar, 1988; Cervi, 1997). Samplings were done follow transects of 300m, counting shoots as sample unit. Richness (S), evenness (E), and the Shannon diversity index (H) were estimated for each passionwine community. Similarity between the structures of communities was determined by calculation of simple Euclidian distances using the software SPSS 15.0 (SPSS Inc., Austin, Texas, USA). A dendrogram was constructed based on the distance matrix using the UPGMA grouping method (Sneath and Sokal, 1973) and the software MEGA 4.0 (Tamura *et al.*, 2007).

Measurement of climatic changes

In order to assess the seasonal variation in weather, macroclimate variables (rainfall, maximum and minimum mean temperatures) were obtained from the Centro de Metereologia Aplicada da Fundação Estadual de Pesquisa Agropecuária (FEPAGRO), the Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA/Agritempo), the Instituto Nacional de Pesquisas Espaciais (INPE-CEPTEC), and the Centro de Metereologia da Companhia Aracruz Celulose S.A. (Barra do Ribeiro, RS). The climatic data from the years sampled were compared to historic means (1961-2007) to detect climatic abnormalities in the sites studied.

Seasonal variation in adult size

Seasonal variation in adult wing size was assessed for the four sites described. Samplings were conducted between February 2006 and May 2007, once every season. For the sake of collection representativeness in terms of typical seasonal conditions, collections were always carried out between 30 and 60 days after the solstice or equinox. For each site, the collection area chosen were 300-m-long trails or pathways along the vegetation communities. Samples were conducted using two entomological nets for 4-h collection periods, always between 10 a.m. and 2 p.m. on sunny days, with temperatures between 15 and 32°C and mild breeze. Under adverse weather, collections were discontinued and resumed another day, under fair weather, at the same hour of the day before when the interruption occurred, thus meeting the collection time schedule per site and season.

The adults captured were brought to the laboratory and kept at -20°C upon analysis. All specimens were sexed and measured. As wing size is considered a good predictor of body size for Lepidoptera (Miller, 1977; Haukioja, 1993), forewing length (FL) was used as a body size measure. Wings of the butterflies collected were dissected, placed on a styrofoam slab, pressed under a transparent glass slide and measured using a 0.01-mm-precision digital caliper. Forewing length was measured on both the left and right wings using the anatomical landmarks class I (Monteiro and Reis, 1999). The anatomical landmarks used were the forewing insertion on the thorax and the R4 vein terminus.

Initially, measurements obtained were tested for normality (Kolmogorov-Smirnov test) and compared to verify the existence of asymmetry between the right and the left sides (*t*-test). After, the FL measurements were analyzed by the factorial ANOVA, the factors being population, sex and season of collection. To compare the differences between populations and seasons, one-way ANOVA was used. Multiple comparisons were carried out using *post-hoc* tests (Tukey HSD). Differences in size between sexes were compared through a *t*-test.

Associations between local and phenotypical factors in the collection sites

The Mantel test was used for matrix correlation to analyze the possible associations between geographic, climatic, structural and phenotypical factors in the collection sites. Distance matrixes were generated for the sites considering the geographic distance, the climatic distance, the host plant community structure distance, and morphological distance in terms of FL. The geographic distance matrix was generated using the geodesic distance calculated through geographic coordinates, with the software *GeoTigger* (version 2008, available in www.tigra.com.br/geoTiggra). The climate matrix was generated by the Euclidean distance, using the mean temperature records and rainfall records for each season. The host plant community structure matrix was also generated by simple Euclidean distance, using the occurrence and relative abundance of *Passiflora* species. The phenotypical matrix was generated by calculating the squared differences between the FL means for each site in the different seasons. Paired comparison was used to evaluate the correlation between distance matrixes, with 10,000 permutations used to calculate the statistical significance.

Effects of environmental variation in adult body size

1. D. i. alcionea stocks

Laboratory tests were conducted using a stock of females captured in nature and individually kept in outdoor cages (3 x 3 x 3 m) containing some host plants (*P. suberosa*, *P. misera*, and *P. capsularis*), near the Departamento de Genética, Universidade Federal do Rio Grande do Sul. Females were fed once a day with a mixture of honey and water (3:1) and monitored for oviposition. Eggs were collected daily, being individually placed in plastic vials and taken to the laboratory, where the treatments specific to each experimental procedure were conducted.

2. Host plant effect

The influence of the feeding source on adult body size was assessed under controlled conditions in the laboratory (25±1°C, 24L:0D photoperiod). Stock larvae were observed during development in two different treatments: feeding with *P. suberosa* only, and feeding with *P. misera* only. Larvae were observed daily and food was available *ad* *libitum*. After adult emergence, 48 individuals were randomly selected to form 4 groups with 12 individuals of each sex per treatment.

3. Effect of temperature

The influence of temperature on adult body size was evaluated under controlled conditions in the laboratory. Stock larvae were taken to the laboratory and individually placed in plastic vials. Larvae were fed daily *ad libitum* (*P. suberosa*), and underwent one of two temperature regimens: 17±1°C or 25±1°C (24L:0D photoperiod). After adult emersion, 100 individuals were randomly selected to form 4 groups with 25 individuals of each sex per treatment.

4. Measurements and statistical analysis of the experiments with host plant and temperature

Wing measurements of individuals tested were conducted identically to the procedures taken for individuals collected from nature. Measurements were tested for normality (Kolmogorov-Smirnov test) previous to using the two-way ANOVA (random model). All cases were normally distributed. The differences between sexes and between treatments were analyzed using multiple *t* tests.

Results

Host plant community

The most frequent passionwine species in the studied areas were *P. suberosa*, *P. misera*, and *P. capsularis*. The least frequent were *P. alata*, followed by *P. edulis*, and *P. tenuifila*. The passionflower community differed across some of the communities studied (Table 1). MDB, in the Atlantic Rainforest *strictu sensu*, was the site with the highest passionwine richness. EPV was the site with the smallest population richness, with one species only (*P. suberosa*). Despite the fact that EPV is located in a western prolongation of the Atlantic Rainforest, the diversity index for the site is smaller than those observed for the populations present in the *Eucalyptus* cultivation area (EPV, H = 0.0; EAB, H = 0.971). The site with the greatest passionflower diversity index was MDB (H = 1.214).

Figure 2 shows the dendrogram constructed for the distance between *Passiflora* communities in the different sites, and reveals two consistent clusters. The first cluster groups EAB and EPV, and accumulates a smaller amount of diversity as compared to the second cluster, formed by HBN and MDB.

Measurements of climatic variations

In general the macroclimate variations observed in the collection period followed the historic temperature means (Figure 3). As for the temperatures, winter and spring 2006, as well as summer 2007 presented temperatures that were slightly higher as compared to the historic means.

Rainfall varied greatly across the seasons, as well as across the years sampled. As for rainfall, seasons do not have a steady pattern in southern Brazil, with rains falling evenly in all seasons. In Rio Grande do Sul State, the only trend observed is for summer and autumn to be drier than spring and winter. However, during the samplings conducted in 2006, the seasons' rainfall was below the historic mean. The seasons in 2007 revealed an excess rainfall as compared to the historic data. The largest differences between historic means and the records collected during sampling periods were seen for winter 2006 (draught) and summer and autumn 2007 (excess rainfall), in EAB and HBN.

Seasonal variation in wing size

A three-way ANOVA was applied to compare body size variation between populations, seasons, and sexes. As for the populations, statistically significant differences were observed between the different collections ($F = 2.611$; $p < 0.049$). The *post-hoc* test revealed the occurrence of two statistical FL mean groups: (1) populations in EAB, MDB, and HBN, and (2) MDB, HBN, and EPV (Table 3A). The highest FL means were observed in EPV, whose population presented only one host plant species (*P. suberosa*), while the lowest means were seen in EAB. Intermediate FL values were observed in MDB and HBN. The Mantel test did not reveal any significant correlation between community structure and FL variation (Table 4). Therefore, the differences in body size found between the populations collected in EAB and EPV cannot be explained only by the host plant community structure, especially because the main host plant in these two sites is *P. suberosa*.

The seasonal variation in adult body size between the sexes, for the different populations and seasons is shown in Figure 4. In total, 849 individuals were captured (EAB = 193, MDB = 120, HBN = 336, EPV = 200). The largest number of individuals captured was recorded in autumn collections (N = 329), while the lowest number was seen in spring samplings (N = 10). FL measurements were normally distributed ($z = 1.233$, $p = 0.101$), and no significant differences were observed in asymmetry within males and females

(females, $t = 0.292$, $p = 0.802$; males, $t = -0.119$, $p = 0.906$), thus we decided to analyze and discuss the results only for left wings.

Regarding the seasons, statistically significant differences were observed for the different sample collections ($F = 19.086$; $p < 0.001$). The *post-hoc* test revealed the existence of three FL mean statistical groups, which corresponded to the following seasons: (1) winter, (2) autumn and spring, and (3) summer (Table 3B). The Mantel test did not detect any correlation between climate and phenotypical variations (Table 4). Yet, when the effect of temperature and rainfall are analyzed separately, significant correlations were observed between body size and temperatures ($r_{MinTemp} = 0.555$, $p = 0.007$; $r_{MaxTemp} = 0.689$, $p < 0.001$), though not between body size and rainfall ($r_{Rainfall} = 0.325$, $p = 0.140$). These results suggest that adult body size is directly affected by variations in seasonal temperatures.

Similarly, statistically significant differences were observed between sexes ($F = 36.064$; $p < 0.001$: Table 2). Males were consistently larger than females (males, 41.60 mm, N = 636; females 40.00 mm, N = 213) and sexual differences were consistent in each collection season (Figure 3).

Correlation between local and phenotypical factors in the study sites

The Mantel test results are shown in Table 4. No statistically significant correlations were observed between any of the distance matrixes in the pairwise comparisons. These results suggest the independence of the four sets of distances calculated and that the results of each distance matrix cannot be explained by any other distance data set.

Host plant effects

The results for two-way ANOVA revealed statistically significant effects of host plant on wing size (F=22.470; $p<0.001$). Significant differences were observed in wing size between sexes (F=35.762; $p<0.001$; males larger than female) and between host plant treatments ($F = 27.896$; $p<0.001$; fed with *P. misera* larger than *P. suberosa*), but no significant interaction was seen between sex and treatment ($F = 3.756$; $p = 0.059$). The results of multiple comparisons are shown on table 5A. Males were always larger than females, and individuals fed with *P. suberosa* were always smaller then those fed with *P. misera* (table 5A).

Effects of temperature

The two-way ANOVA results revealed statistically significant differences between individuals treated at 17°C and at 25°C (F = 22.354; $p < 0.001$). Adult body size was smaller in the individuals reared at 17°C as compared to 25°C (F = 22.849; $p < 0.001$) with females being smaller than males (F = 39.274; $p < 0.001$) and a significant correlation between sex and treatment was observed (F = 4.938; $p < 0.029$). The results of the multiple comparisons between treatments and sexes are shown in Table 5B. Size of females did not differ statistically between treatments, while for males such difference was of close to 5%. These results point to the possible influence of temperature on the development of *D. i. alcionea*, especially that of males, with lower temperatures leading to adults of smaller wing sizes.

Discussion

Host plant effects on body size

The results of the present study corroborate the hypothesis that food availability varies for different sites and may influence the development of *D. i. alcionea* larvae, as discussed by Rodrigues and Moreira (2002, 2004) for *H. erato phyllis*. In Insecta, several studies have shown that body size is directly influenced by nutrition conditions under which organisms develop (McNamara and Houston, 1996; Thorne *et al.*, 2006, and references therein). Similarly, the quality and availability of food are crucial to the development of butterflies (Rodrigues and Moreira, 2004). Perico and Araújo (1995) studied the acceptance by *H. erato* and *D. iulia* on different *Passiflora* species and discovered that females of *D. iulia* shows marked oviposition preference for *P. suberosa*, *P. misera*, *P. capsularis*, and *P. elegans*, when compared to *P. caerulea*, *P. alata*, *P. edulis*, and *P. tenuifila* (65% and 9% preference, respectively). *D. iulia* larvae generated high proportions of viable adults when fed with *P. capsularis*, *P. suberosa*, *P. misera*, *P. elegans*, and *P. actinia*, and low proportions when fed with *P. edulis*. *Passiflora alata*, *P. tenuifila*; *P. caerulea* were lethal to *D. iulia* larvae. Therefore, as regards the utilization of these passionwine species as food resource, the discussion in this study has to be limited to the five species more readily accepted by *D. iulia*

Our results indicated that *D. iulia* adults are larger when reared on *P. misera* as compared to *P. suberosa*. Périco and Araújo (1995) showed that *D. iulia* larvae fed with *P. capsularis* and *P. misera* developed into heavier pupae, when compared to larvae fed with other species, while *H. erato* pupae fed with *P. misera* and *P. elegans* reach heavier pupa weight. Rodrigues and Moreira (2004) have demonstrated that *H. erato* larvae fed with *P. suberosa* generate adults with shorter wing length when compared to larvae fed with *P. misera*. Therefore, it would be logical to suppose that the use of certain *Passiflora* species as host plants leads to the adults with larger body sizes. Our results corroborate these hypotheses, showing that *D. iulia* larvae fed with different host plants develop into adults of different body sizes. The reasons why different host plants cause variation in body size in Heliconiinae species it is not known and further studies must be done to clarify this issue.

HBN and MDB populations presented intermediate *D. iulia alcionea* body size. As mentioned earlier, these populations were formed, apart from *P. suberosa*, by large amounts of *P. misera* and *P. capsularis*, host plants that lead to high development rates in *D. iulia*. Nevertheless, these two species are not avaliable in colder seasons, as they are deciduous species (Rodrigues and Moreira, 2004); thus, is only *P. suberosa* left as food resource. Although they present different phenologies (Cervi, 1977) these plant species can be found in similar habitats in the sites sampled. When *P. misera* and *P. capsularis* are defoliated, there is virtually only *P. suberosa* left as food resource, apart from other less abundant species. This food scarcity may cause perceptible effects on body size of resident *D. iulia* populations, which could explain the intermediate mean values in populations collected in MDB and in HBN.

Mean body size observed in EAB and EPV cannot be explained only in the light of the structure of the host species community during the season of vegetative growth of passionflowers. In EPV, the only species found is *P. suberosa*, and in EAB it is the only one available in large amounts. Yet, EPV is found in an area in which food resources for larvae are kept relatively constant throughout the year, as it is a natural environment area. EAB is located in an area that constantly undergoes anthropic influence due to the non-planned cutting of *Eucalyptus*, and thus may suffer resource scarcity as a consequence of constant environmental changes. As the food amount is directly linked to adult body size, the difference between sizes for the different populations may be explained based on the availability of *P. suberosa*.

Wiklund (1984) suggests that the choice for one host plant by a female depends on plant abundance and apparency, as well as on the female's ability to find the host plant. Nevertheless, plant abundance may not be used as a preference criterion (Perico and Araújo, 1995). Rodrigues and Moreira (2004) also suggest that the adoption of one *Passiflora* species as host plant by *H. erato* depends not only on the innate preference of the species and the plant's nutritional quality, but also on the host plant phenology. The same authors suggest that *P. misera* affords higher growth rates and larger adult body sizes. When available, this species is always used preferentially as compared to *P. suberosa*, despite of the corresponding abundances. During winter, when *P. misera* is unavailable due to defoliation, *H. erato* switches to *P. suberosa*, as it affords inferior growth rates and smaller adult body sizes. The same may occur for *D. iulia*, suggesting that the variation in adult body size could at least partly be explained by host plant phenology.

Adult body size is a very important trait in an animal's life history (West *et al.*, 1999), since quality and amount of food ingested during the growth phase are important factors for body development of the several insect species. Thus, it is expected that the variation in the properties of plant species used as food resource leads to important changes in the life history of many species, as demonstrated for some herbivorous insects (Derr *et al.*, 1981; Scriber and Slansky, 1981; Smiley and Wisdom, 1985; Bernays and Chapman, 1994; Rodrigues and Moreira, 2002; Rodrigues and Moreira, 2004; Thorne *et al.*, 2006).

Influence of the climatic conditions on body size

A strong correlation between adult body size and mean temperatures of larval development was observed in the present study. When body size of butterflies collected from different sites and different seasons are compared, it is possible to observe that larger body sizes coincide with hotter periods, while smaller body sizes are seen in the colder seasons. These results are corroborated by the tests conducted in the laboratory. These patterns may be explained by two aspects of developmental biology of *D. iulia.* First, during hot seasons, large amounts of passionflower with growing vegetative structures are available, which increases the supply of quality food resources to larvae. Second, higher temperatures afford higher metabolic rates, which make larvae increase their overall intake of food.

Cook (1961) showed that the environmental effect on larval development may greatly influence body size of natural *Panaxia dominula* (Arctiidae) populations. Chapman (1998) argues that body development in insects is affected by environment temperature, with lower temperatures increasing the organism's growth rate thus leading to larger body sizes. Nevertheless, for many species this pattern is not observed. It is well known that host plant quality influences growth rates, molting and development times (Stamp, 1990; Thompson, 1988; Périco and Araújo; 1995; Reavey and Lawton, 1991; Nijhout, 2003; Rodrigues and Moreira, 2004). In a study of the natural *D. iulia* populations, Paim (1995) showed that body size of the adults collected in summer is larger than that observed for adults collected in winter. The same was observed for *H. erato*, even in situations simulated in laboratory (Rodrigues and Moreira, 2004). Our results also confirm this trend in *D. iulia*: higher temperatures lead to larger adult body sizes.

Size differences between sexes

Apart from the differences in size observed for the distinct host plant populations and seasons, this study also showed that size differences between males and females were consistent in all samplings where both sexes were collected and analized. Haag and Araújo (1994) and Paim (1995) found similar results in studies with other natural *D. iulia* populations.

In spite of being described as a species with sexual dimorphism regarding wing color (Brown, 1981; DeVries, 1987) and although Haag and Araújo (1994) have found differences in wing size between males and females, no study so far published classifies *D. iulia* as a species with sexual dimorphism in terms of body size. Body size sexual dimorphism is known for several Lepidoptera species, and a general rule states that the females are larger than the males. This dimorphism is generally associated to reproductive costs (Rutowski, 1997), as females invest a larger amount of energy in egg production (Boggs, 1981). Yet, this does not seem to be the case of *D. iulia*, for which sexual dimorphism occurs the other way round, considering the rule observed for Lepidoptera.

Nevertheless, it is possible to hypothesize over another role for the sexual dimorphism in the species. In this sense, the first evidence is given by the population structure and by the dispersal ability of *D. iulia*, which is a species with an isolation by distance model of population structure (Haag *et al.*, 1993). In this case, the inheritance of wing size is important for the dispersal ability of individuals. The second evidence comes from the sexual behavior of *D. iulia*, in which males are typically patrolling (Mega and Araújo, in preparation). Males of species with this kind of behavior actively search for females for mating (Wilkund, 2003). Larger wings may afford better chances of successful mating, since this characteristic increases the spreading ability of males. On the other hand, females could be selected to develop wings optimized for oviposition activity, leading to the patterns of wing variation observed in nature. Additional studies are needed to test these hypotheses.

Conclusion

Based on the results of the present study, we found evidences that wing size variation is influenced mainly by the availability and quality of host plants resources in the different sites investigated. Seasonal variations in body size are related to differences on temperatures during larval development, as well as to the relative abundance of host plant species avaliable during seasons. Given that there is a significant genetic component determining wing size (Mega and Araújo, in preparation), it is possible that the patterns of wing size variation found may be also caused by inbreeding and genetic erosion during periods of low population size, as demonstrated by Haag e Araújo (1994) under crossing experiments with $F > 0$. The causes of the differences observed between sizes of males and females cannot be explained by the results of the present study.

Acknowledgements

The authors are grateful to A.A. Ferreira, A. Carrion, A.L. Klein, G. Pasqualin, J. Zanin, and P.R Vieira for the assistance in feeding adult insects and in the maintenance of the stocks of immatures. Special thanks to A.L. Klein, P.R. Vieira, and A.A. Ferreira for field collections, to G.R.P. Moreira for suggestions on the experimental method, and to A.L. Klein and A.F. Quadros for the critical reading of the manuscript. We also thank The Instituto Brasileiro do Meio Ambiente e Recursos Renováveis (IBAMA), the Secretaria Estadual do Meio Ambiente (SEMA), the Fundação de Pesquisa Agropecuária do Estado do Rio Grande do Sul, (FEPAGRO), and Aracruz Celulose S.A. for the permission to conduct collections in the study areas. Financial support was granted by Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Programa de Apoio a Núcleos de Excelência (PRONEX).

References

Benson, W.W., Brown Jr., K.S. and Gilbert, L.E. 1976. Coevolution of plants and herbivores: passion flower butterflies. Evolution 29: 659-680.

Bernays, E.A. and Chapman, R.F. 1994. Host-plant selection by phytophagous insects. Chapman & Hall, New York, 312pp.

Boggs, C.L. 1981. Nutritional and life-history determinants of resource allocation in holometabolous insects. Am. Nat. 117: 692- 709.

Boggs, C.L. 1986. Reproductive strategies of female butterflies: variation in and constrains on fecundity. Ecol. Entomol. 11: 7-15.

Brown Jr., K.S. 1981. The biology of *Heliconius* and related genera. Ann. Rev. Entomol. 26: 427-456.

Brown Jr., K.S. 1992. Borboletas da Serra do Japi: diversidade, hábitats, recursos alimentares e variação temporal. In História Natural da Serra do Japi: ecologia e preservação de uma área florestal no sudeste do Brasil. Edited by L.P.C. Morellato. Unicamp/Fapesp, Campinas. pp.142-187.

Brown Jr. K.S. and Mielke O.H.H. 1972. The heliconians of Brazil (Lepidoptera: Nymphalidae). Part II. Introduction and general comments, with a supplementary revision of the tribe. Zoologica 57: 1–40.

Brown Jr., K.S. and Yepez, F.F. 1984. Los Heliconiinae (Lepidoptera, Nymphalidae) de Venezuela. Boletín de Entomología Venezolana 3(4): 29-76.

Castillo, R.C. del. 2005. The quantitative genetic basis of female and male body size and their implications on the evolution of body size dimorphism in the house cricket *Acheta domesticus* (Gryllidae). Gen. Mol. Biol. 28(4): 843-848.

Cervi, A.C. 1997. Passifloraceae do Brasil. Estudo do gênero *Passiflora* L., subgênero *Passiflora*. Fontqueria 45: 1-92.

Chapman, R.F. 1998. The insects: structure and function. Cambridge University Press, Cambridge, 788pp.

Cook, L.M. 1961. Influence of larval environment on adult size and fecundity in the moth *Panaxia dominula* L. Nature 192: 282.

Derr, J.A., Alden, B. and Dingle, H. 1981. Insect life histories in relation to migration, body size, and host plant array: a comparative study of *Dysdercus*. J. Anim. Ecol. 50: 181-193.

DeVries, P.J. 1987. The butterflies of Costa Rica and their natural history (Papilionidae, Pieridae, Nymphalidae). Princeton University Press. 327pp.

Escobar, L.K. 1988. Passifloraceae. Flora de Colombia, 10: 1-138.

Falconer, D.S. 1989. Introduction to quantitative genetics. Longman, Harlow, 438pp.

Glazier, D.S. 2002. Resource-allocation rules and the heritability of traits. Evolution 561(8): 1696-1700.

Haag, K.L and Araújo, A.M. 1994. Inbreeding, genetic load and morphometric variation in natural populations of *Dryas iulia* (Lepidoptera, Nymphalidae). Rev. Brasil. Genet. 17(1): 35-39.

Haag, K.L, Araújo, A.M and Zaha, A. 1993. Genetic structure of natural populations of *Dryas iulia* (Lepidoptera, Nymphalidae) revealed by enzyme polymorphisms and mtDNA RFPLs. Bioch. Genet. 31: 447-458.

Haukioja, E. 1993. Effects of food and predation on population dynamics. In: Caterpillars: ecological and evolutionary constrains of foraging. Eds. N.E. Stamp & T.M. Casey. pp 425-447. Chapman & Hall, New York.

Honêk, A. 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos 66(3): 483-492.

Lande, R. 1980. Sexual dimorphism, sexual selection and adaptation in polygenic characters. Evolution 34: 292-305.

McNamara, J.M. & Houston, A.I. 1996. State-dependent life histories. Nature 380: 215–221.

Merilä, J., Sheldon, B.C. and Ellegren, H. 1998. Quantitative genetics of sexual dimorfism in the collared flycatcher, *Ficedula albicollis*. Evolution 5(3): 870-876.

Miller, W.E. 1977. Wing measure as a size index in Lepidoptera: the family Olethreutidae. Ann. Entomol. Soc. Am. 70: 253-256.

Monteiro, L.R. and Reis, S.F. 1999. Princípios de morfometria geométrica. Holos Editora, Ribeirão Preto, 188pp.

Nijhout, H.F. 2003. The control of body size in insects. Develop. Biol. 261: 1–9.

Nijhout, H.F. and Wheeler, D.E., 1996. Growth models of complex allometries in insects. Am. Nat. 148: 40–56.

Paim, A.C. 1995. Polimorfismo enzimático e variação morfológica em uma população natural de *Dryas iulia* (Fabr. 1775) (Lepidoptera; Nymphalidae). Dissertação de Mestrado. Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Paim, A.C., Kamisky, L.A and Moreira, G.P.R. 2004. Morfologia externa dos estágios imaturos de heliconíneos neotropicais. IV. *Dryas iulia alcionea* (Lepidoptera, Nymphalidae, Heliconinae). Iheríngia, Serie Zoológica, 94(1): 25-35.

Perico, E. and Araújo, A.M. 1995. Suitability of host plants (Passifloraceae) and their acceptance by *Heliconius erato* and *Dryas iulia* (Lepidoptera; Nymphalidae). Evol. Biol. 5: 59-74.

Reavey, D. and Lawton, J.H. 1991. Larval contribution to fitness in leaf-eating insects. In: Reproductive behaviour of insects: individuals and populations. Eds. W.J. Bailey & J. Ridsdill-Smith, Chapman & Hall, London, pp 239-329.

Rodrigues, D. and Moreira, G.R.P. 2002. Geographical variation in larval host-plant use by *Heliconius erato* (Lepidoptera: Nymphalidae) and consequences for adult life history. Braz. J. Biol. 62(2): 321-332.

Rodrigues, D. and Moreira, G.R.P. 2004. Seasonal variation in larval host plants and consequences for *Heliconius erato* (Lepidoptera: Nymphalidae) adult body size. Austral Ecology 29:437-445.

Roff, D.A. 1996. The evolution of genetic correlations: an analysis of patterns. Evolution 50: 1392-1403.

Rutowski, R.L. 1997. Sexual dimorphism, mating systems and ecology in butterflies. In The evolution of mating systems in Insects and Arachnids. Edited by J.C. Choe. Library of Congress, Cambridge. 397pp.

Scriber, J.M. and Slansky, F. Jr. 1981. The nutritional ecology of immature insects. Annu. Rev. Entomol. 26: 183-211.

Smiley, T.C. and Wisdom, C.S. 1985. Determinants of growth rate on chemically heterogeneous host plants by specialist insects. Biochem. Syst. Ecol. 13: 305-312.

Sneath, P.H.A. and Sokal, R.R. 1973. Numerical taxonomy. The principles and practice of numerical classification. Freeman, San Francisco, 573 pp.

Stamp, N.E. 1990. Growth versus molting time of caterpillars as a function of temperature, nutrient concentration and the phenolic ritin. Oecologia 82: 107-113.

Tamura, K., Dudley, J., Nei, M. and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24: 1596-1599.

Thompson, J.N. 1988. Evolutionary ecology of the relationship between oviposition preference and the performance of offspring in phytophagous insects. Entomol. Exp. Appl. 47: 3-14.

Thorne, A.D., Pexton, J.J., Dytham, C. and Mayhew, P.J. 2006. Small body size in an insect shifts development, prior to adult eclosion, towards early reproduction. Proc. R. Soc. B 273: 1099–1103.

Torres-Vila, L.M., Stockel, J. and Roehrich, R. 1995. Le potentiel reproducteur et ses variables biotiques associées chez le mâle de l'Eudémis de la vigne *Lobesia botrana*. Entomol. Exp. Appl. 77(1): 105-119.

Van Dick, H. 2003. Mate Location: a matter of design? Adaptive morphological variation in the speckled wood butterfly. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, 739pp.

West, G.B., Brown, J.H. and Enquist, B.J. 1999 The fourth dimension of life: fractal geometry and allometric scaling of organisms. Science 284, 167–169.

Wiklund, C. 1984. Egg-laying patterns in butterflies in realtion to their phenology and the visual apparency and abundance on their host plants. Oecologia 63: 23-29.

Wiklund, C. 2003. Sexual selection and the evolution of butterfly mating systems. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, 739pp.

Table 1. *Passiflora* community in studied sites. Species occurrence is shown as approximate percentages. Collection sites: Estação Experimental de Águas Belas (EAB); Horto Florestal Barba Negra (HBN); Estrada do Perau Velho (EPV); Morro da Borússia (MDB). Indexes: (*S*) Species richness, (*E*) Species evenness, (*H*) Sannon diversity, (*D*) Simpson diversity.

	Populations and species occurrence (%)			
Species	EAB	HBN	EPV	MDB
P. actinia	0	0	0	3
P. alata	1	0	0	5
P. caerulea	5	5	0	7
P. capsularis	0	0	0	39
P. edulis	1	0	0	1
P. elegans	2	2	0	0
P. misera	0	37	0	0
P. suberosa	91	55	100	45
P. tenuifila	0	1	0	0
S	5	5	1	6
E	0.252	0.604	-	0.677
H	0.406	0.971	0.0	1.214

Table 2. Three-way ANOVA results for *D. iulia alcionea* forewing length measurements for the different populations, seasons and sex.

Efect	***F***	**df**	**p**
Population	2.611	3	0.049
Season	19.086	5	0.000
Sex	36.064	1	0.000
Population * Season	3.172	13	0.000
Population * Sex	0.231	3	0.875
Season * Sex	1.810	5	0.108
Population * Season * Sex	0.635	9	0.767

Table 3. Statistical clusters generated by the *post-hoc* test (Tukey HSD) for the three-way ANOVA for forewing length (FL). (**A**) Seasons, (**B**) Populations.

A.

		Statistical clusters		
Sample	**N**	**1**	**2**	**3**
Winter 2006	17	37.45		
Spring 2006	10		40.78	
Autumn 2007	329		40.82	
Autumn 2006	261		40.86	
Summer 2006	155			42.18
Summer 2007	77			42.25
Sig. (p)		1.000	0.052	0.999

B.

		Statistical clusters	
Population	**N**	**1**	**2**
EAB	193	40.90	
MDB	120	41.07	41.07
HBN	336	41.21	41.21
EPV	200		41.44
Sig. (p)		0.263	0.151

Table 4. Mantel test results for pairwise comparisons between geographic, climate, host plant community, and phenotype distance matrixes.

Comparison	**r**	**p**
Geography x Climate	-0,061	0,457
Geography x Comm. structure	-0.172	0.585
Geography x Phenot. distance	0.496	0.217
Comm. structure x Climate	-0.002	0.503
Comm. structure x Phenotype	0.534	0.162
Climate x Phenotype	-0.222	0.706

Table 5. Forewing length means (FL) of *Dryas iulia* obtained from the laboratory tests about the effects of environmental factors on body size. **A.** Effect of host plant (*P. suberosa* x *P. misera*, N = 48, 12 larvae per sex per treatment). **B.** Effect of temperature (17 ± 1°C x 25 ± 1°C, N = 100, 25 larvae per sex per treatment).

A.

		Treatment		**Statistics**		
		P. suberosa	***P. misera***	**Mean**	***t***	**p**
Sex	**Females**	39.33	42.05	40.70	-4.579	<0.001
	Males	42.32	43.58	42.95	-2.718	0.016
Statistics	**Mean**	40.83	42.82	*41.83*	-	-
	t	-5.750	-2.787	-	-	-
	p	<0.001	0.011	-	-	-

B.

		Treatment		**Statistics**		
		17ºC	**25ºC**	**Mean**	***t***	**p**
Sex	**Females**	39.99	40.80	40.40	-1.514	0.138
	Males	41.27	43.48	42.37	-6.541	<0.001
Statistics	**Mean**	40.63	42.14	*41.38*	-	-
	t	-3.665	-5.089	-	-	-
	p	0.001	<0.001	-	-	-

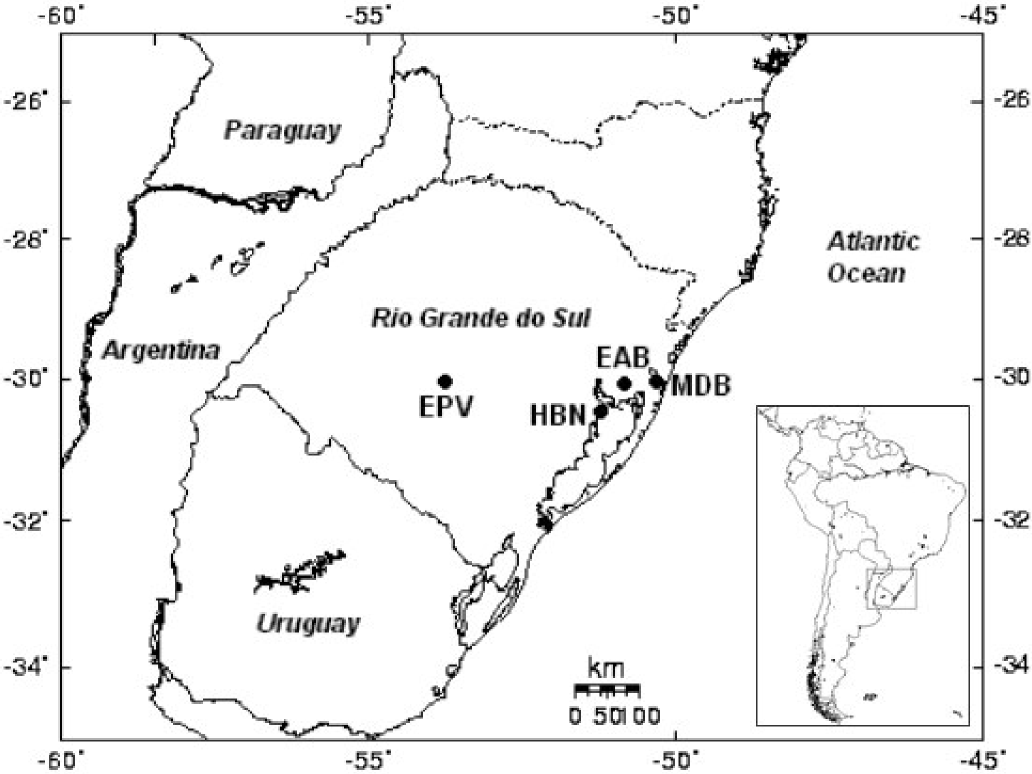


Figure 1. Geographical location of the study sites in the state of Rio Grande do Sul. Estação Experimental de Águas Belas (EAB); Horto Florestal Barba Negra (HBN); Estrada do Perau Velho (EPV); Morro da Borússia (MDB).

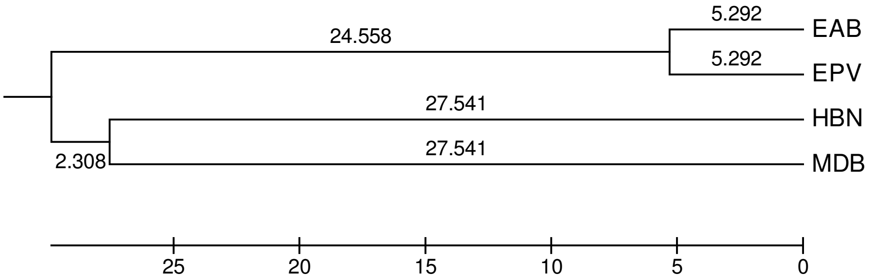


Figure 2. Relationship among *Passiflora* species communities present in the four studied sites. Estação Experimental de Águas Belas (EAB), Horto Florestal Barba Negra (HBN), Estrada do Perau Velho (EPV), Morro da Borússia (MDB). Dendrogram constructed by UPGMA method on the Euclidean distances among communities. Numbers above branches and the ruler under the dendrogram represent the distance between groups.

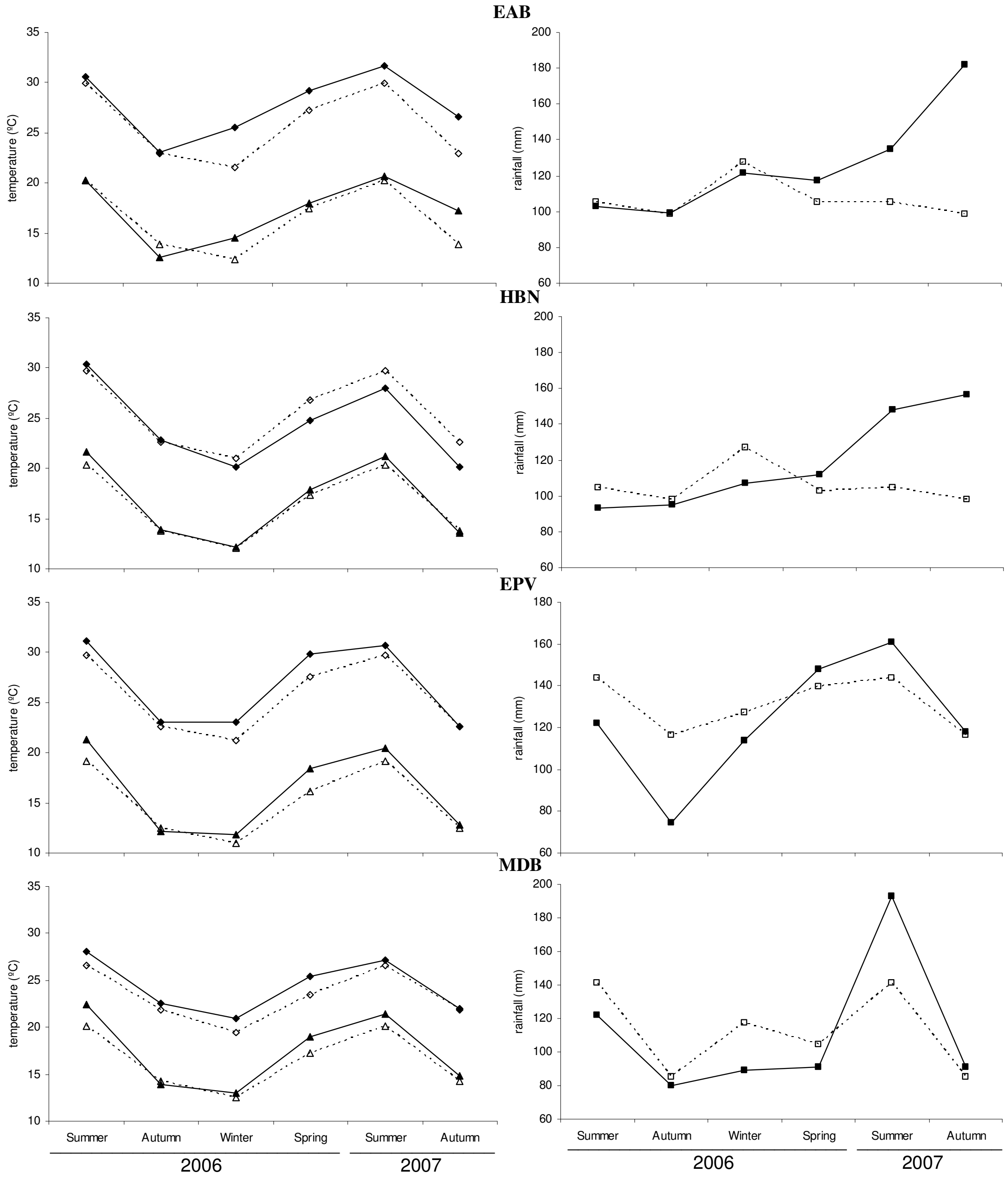


Figure 3. Observed and expected macroclimate variations for the four studied sites, throughout field study. Estação Experimental de Águas Belas (EAB), Horto Florestal Barba Negra (HBN), Estrada do Perau Velho (EPV), Morro da Borússia (MDB). The first column represents the variation in temperature means (°C), the second column represents the variation in total rainfall (mm). Solid lines represent the climate variations observed in the sampling period; dotted lines represent the historic climate patterns expected; lozenges represent the variations in maximum temperature means; triangles represent the variations in minimum temperature means; squares represent the variations in rainfall.

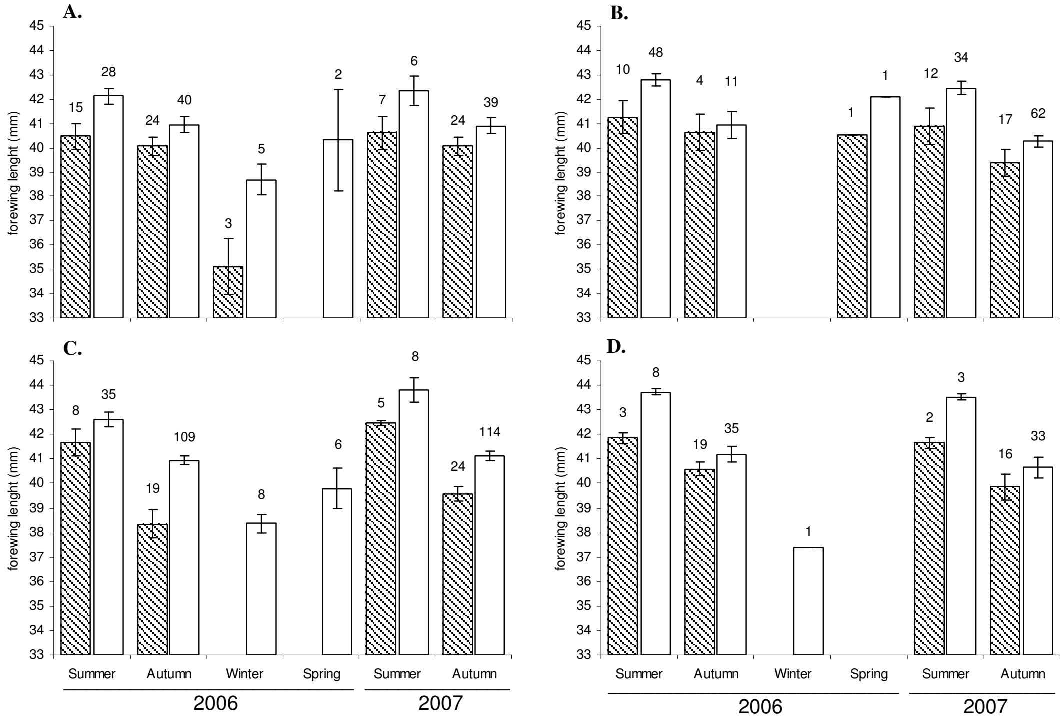


Figure 4. Wing size variation (mean±standard error) in *Dryas iulia alcionea* throughout the field study in the four studied sites. **(A)** Estação Experimental de Águas Belas (EAB), **(B)** Horto Florestal Barba Negra (HBN), **(C)** Estrada do Perau Velho (EPV), **(D)** Morro da Borússia (MDB). Striped bars: females. White bars: males. Standard error of the means is shown on top of bars. Numbers above bars represent total number of individuals sampled on each occasion.

CAPÍTULO 4

HOW HERITABLE IS WING SIZE IN *DRYAS IULIA ALCIONEA* (LEPIDOPTERA, NYMPHALIDAE)? INFERENCES ON DEVELOPMENT AND SEXUAL SELECTION.

Artigo aceito para publicação no periódico *Genetica.*

How heritable is wing size in *Dryas iulia alcionea* (Lepidoptera, Nymphalidae)? Inferences on development and sexual selection.

Nicolás Oliveira Mega[1] and Aldo Mellender de Araújo[2]

[1]*Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul.*
[2]*Departamento de Genética, Universidade Federal do Rio Grande do Sul.*

Abstract: Body size varies according to nutrient supply and energy available during development. It also depends on the quantitative genetic variation in a population and the action of evolutionary forces. We analyzed the genetic correlation, variation and heritability of eight morphological traits of *Dryas iulia* wings. Analysis of wing size indicated significant differences between sexes. Measurements were always larger in males, although females showed greater variation. Significant genetic correlations were found between all morphological traits within each sex, and for some traits between sexes. High mean genetic correlation was found for wing traits within each sex, but mean genetic correlation between sexes was low. Regression analysis revealed significant genetic effects of both progenitors' wing size on offspring wing size, suggesting no differential contribution of dam or sire. Results also suggest that forewing and hindwing evolution is independent.

Key-words: sexual dimorphism, heritability, genetic correlations, body size, sexual selection, Heliconiinae.

Introduction:

The size of an individual and growth of their body parts is affected by genetic and environmental factors, operating through complex molecular and physiological mechanisms (West et al., 1999, Nijhout, 2003). The role of genes and environment in determining body size has been investigated due to its effect on fitness (West et al., 1999; Glazier, 2002). Its main effects are perceived directly on reproduction, survival and dispersal (Boggs, 1986; Honek, 1993; Torres-Vila et al., 1995; Rodrigues and Moreira, 2002, Van Dyck, 2003).

In genetic terms body size depends on the quantitative genetic variation within a population, which is directly affected by natural selection and genetic correlation with other hereditary characteristics (Lande, 1980; Falconer, 1989; Roff, 1996; Merilä et al. 1998; Castillo, 2005). In insects, at least four environmental factors can affect development: temperature, quality and type of food, photoperiod and competition (Rodrigues and Moreira, 2002). Due to the plasticity of development in body size there is great intraspecific variation (Nijhout and Wheeler, 1996; Thorne et al. 2006).

Dryas iulia alcionea is a butterfly species with wide geographical distribution and it can be found in virtually all the continental portion of South America (Brown Jr and Yepez, 1984). This species has great adaptive plasticity and can live in several kinds of habitats (Paim et al., 2004). Their larvae use different species of passionwines as host plants, preferably plants of the subgenus *Passiflora* (formerly *Plectostemma*) (Brown Jr and Milke, 1972; Perico and Araújo, 1991; Brown Jr, 1992). Natural populations of *D. i. alcionea* are relatively large and have considerable variation in size among localities and seasons (Haag and Araújo, 1994; Paim, 1995). However, nothing is known about the influence of genetic variation on body size in this species.

This study analyzes the phenotypic variation and heritability of eight wing size traits of *D. i. alcionea* (Lepidoptera, Nymphalidae). As the size of the wings is considered a good predictor of body size for Lepidoptera (Miller, 1977; Haukioja, 1993) and has been used successfully to analyze the variation in size of natural populations (Haag and Araújo, 1994; Paim, 1995; Rodrigues and Moreira 2002, 2004), all characteristics analyzed in this study involved wing measurements.

Material and Methods:

Butterfly Stocks:

To estimate the heritability of body size in *D. i. alcionea*, broods (N=14) were reared under controlled conditions. Females were caught in the wild and brought to an open air insectary to lay eggs. Adults were kept in cages measuring 2.5 x 2.5 x 3.0 m and received daily a mixture of water and honey (3:1); each cage had plenty of vegetation and host plants for oviposition (*Passiflora suberosa*, *P. misera* and *P. capsularis*). Eggs were collected daily and put individually in translucent plastic cups (8.5 cm high and 7.5 cm diameter). Preadult development was followed under controlled conditions (24h light, 25±1ºC) and larvae were fed with *P. suberosa* leaves *ad libitum.* This procedure ensures that the body size of progenitors is manly related to their genetic potential and not to environmental variations, except for maternal effects. Females and males obtained from these stocks were crossed in the insectary and used as parental generation to the different broods reared. To avoid inbreeding effects on the F1 body size, all matings were controlled (F = 0). The rearing method used for F1 followed the same procedure applied to the progenitors.

Wing Measurements

The wings of the progenitor butterflies and their offspring were dissected, digitized with a tabletop scanner (HP ScanJet 2400), and measured using *Image J* software (version 1.38x, available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD). Linear distances were taken on the images of wings nearest to 0.01mm. The standard error of the mean was ±0.012 when the forewing length of same wing was repeatedly measured (n=20, mean=43.55mm, s.d.=0.049). Eight variables of the wings were measured: forewing length (FL), forewing width (FW), forewing area (FA), forewing perimeter (FP), hindwing length (HL), hindwing width (HW), hindwing area (HA) and hindwing perimeter (HP). In order to avoid redundancy in the presentation and discussion of the data, only the measures concerning right wings were analyzed. The linear measures were taken always between type-I landmarks (see Monteiro and Reis, 1999; Zelditch et al., 2004), to ensure homology between the distances measured (figure 1). All wing measures were scaled with the same scale of reference (236 pixels = 100 mm, at a 300 dpi resolution).

Statistical Analysis

Body Size Dimorphism

A multivariate analysis of variance (MANOVA) was performed for the eight wing traits among the 14 broods to detect sexual dimorphism. Additional *t*-tests were conducted to detect which measured traits differed between sexes. The coefficient of variation ($CV=\sigma/\mu$) was also estimated for each trait.

Genetic Correlations

Genetic correlations between wing traits were calculated using Pearson linear correlations, which were performed separately for each sex and between sexes. Since environmental influence was partially controlled, it is expected that linear correlations between mean phenotypes represent mainly the genetic correlations between wing traits (see Lynch and Wash, 1998). To verify genetic independence of hindwing and forewing morphological traits, mean genetic correlations were computed for each group of wing traits and compared between sexes. Multiple comparisons between pairwise genetic correlations were compared using *t*-tests corrected with False Discovery Rate Method (Benjamin and Hochberg, 1995)

Heritability Analysis

Heritability (h^2) was calculated separately for each sex for all wing traits measured, using the conventional method of offspring-parent regression (Falconer, 1989). First, the average value of daughters for each wing trait was regressed on the dam value; a second regression was performed between the average values of sons on the sire values. Standard errors of the estimates were calculated according to Roff (1997). To eliminate the effects of size on the phenotypic variation allometric equations were used to correct measures by the body weight of each individual, as described in Lynch and Walsh (1998).

Results

Body Size Dimorphism

Overall, 14 sibships were used in this study, totaling 332 individuals (females = 148, males = 184). The number of butterflies in each sibling group ranged from 13 to 33, with an average of 24 individuals. A slight excess of males was observed, which did not differ statistically from the expected proportion of 1:1. The analysis of body size dimorphism indicated differences between sexes (MANOVA Wilks's $\lambda = 0.695$; $F_{(8,323)}=17.708$; $p<0.001$). Statistical differences were observed for all traits analyzed (table 1). Wing trait measurements were always larger in males, although females showed more variation than males.

Genetic Correlations

For each sex, genetic correlations were significant at the 0.05 level for all pairwise correlations (table 2a, b). The mean genetic correlation for morphological traits of female wings was 0.890, and for males 0.869. Between sex correlations showed that significant genetic correlations were observed for 12 of the 64 pairwise comparisons (table 2C), suggesting that several wing traits are independent and then could be subjected to different selection regimes. Although not always significant, mean genetic correlations between females x males of the same wing showed higher values when compared with traits from different wings (table 3). These results suggest independent evolution of female and male traits, allowing sexual dimorphism to evolve. Also, both hindwings and forewings seem to correspond to different evolutionary modules.

Heritability Analysis

Regression analysis revealed significant additive genetic effects of parent wing size on the offspring wing size (table 4). Significant heritability values were observed for all wing traits, both for dam on daughters and sire on sons. Overall, sire showed a higher mean genetic contribution on sons (0.536) when compared to dam genetic contribution on daughters, suggesting an uneven genetic contribution of parents on F1. The highest additive genetic contribution was observed for HL of sire on sons (0.854) and lowest for FW of sire on sons (0.192).

Discussion

Genetic correlations, wing development and the evolution of sexual dimorphism

Significant genetic correlations were found between all morphological traits in each sex, and for some traits between sexes. A high mean genetic correlation was found for female and male wing morphological traits, but the mean genetic correlation between females vs. males was found to be lower. For female x male analysis, genetic correlations among morphological traits of the same wing showed higher values when compared with morphological traits from different wings. Some level of correlation between hindwing and forewing measurements is expected due to allometric effects of body size. Thus, these results suggest genetic and developmental independence of hindwing and forewing morphological traits, as described for other insects (Tautz and Sommer, 1995; Wolpert et al. 2000). Positional information on body segments and developmental compartmentalization led to the evolution of serially repeated elements and the emergence of serial homology (French, 1996). In these compartments, serial homologues were able to acquire site-specific developmental regulation and this allowed them to diverge morphologically (Lawrence, 1992). Compartmentalization of the wing also reduced the developmental correlation among pattern elements (Nijhout, 2001). Since metamery is a common trait to all insects, independent development of hind and forewing is expected to have evolved in *D. i. alcionea* as well.

According to Castillo (2005), when genetic correlations between traits are small, independent evolution between them is possible. Likewise, when the same situation is found between sexes, the independent evolution of female and male traits is possible and sexual dimorphism can evolve (Lande 1980, Lynch and Walsh 1998). If genetic correlation between sexes is high, genetic variance would be maintained in the population as a result of a tradeoff between body size and development time (Simmons and Ward 1991). Depending on the sign, genetic correlations between two traits can either facilitate or impede evolution. A conflict arises when two negatively genetic correlated traits are both selected in the same direction (Lynch and Walsh, 1998), which seems not to be the case of *D. i. alcionea* wing traits. A positive genetic correlation between two traits causes an evolutionary constraint that connects both characters, since no change in either trait can occur without change in the other. Thus, the results presented here suggest the occurrence of an independent evolution of hindwing and forewing, as well as the possibility of evolution of sexual dimorphism in *D. i. alcionea.*

Wing trait measurements in this study were always larger in males, although females showed more variation than males. These results suggest the action of stabilizing selection or sexual selection on wing size in males. In general, sexual selection is stronger in males than in females (Andersson, 1994). As a result of selection, genetic variance in traits selected can be eroded; this seems to be the case of *D. i. alcionea.* Indeed, if genetic correlation between sexes is small, and females are not the target of selection, some genetic variation could be restored in the next generations of males by recombination (Castillo, 2005). This could be one of the reasons why we do not find only one male standard size in nature. Other reasons for such a variation could be due to phenotypic plasticity and resource allocation priorities during development.

Genetic influence on body size and evolution

The results presented here suggest significant influence of genetics in the adult body size of *D. i. alcionea*. This result is not expected, since phenotypic characteristics that are highly influenced by the life history of the organism tend to have low heritability (Mousseau and Roff, 1987). Many studies suggest that characteristics that are less important in the reproductive function have secondary preferences in the allocation of resources during development (see discussion on Glazier, 2002). This pattern is very common in ectotherms (Mousseau and Roff, 1987) and may be extremely important for species with indirect development, such as the holometabolous insects.

In the sexual dimorphic butterflies *Inachis io* and *Araschnia levana* adult size is larger in females and influenced by environmental changes of light and temperature (Windig, 1999). In *I. io*, heritabilities for adult size are low for long-day spring forms, but larger and significantly different from 0 for summer and short-day spring forms. In *A. levana*, heritabilities for adult wing size are around 30%. For both species, it is suggested that genetic and environmental variation affects adult body size. In non-dimorphic butterfly *Heliconius erato phyllis*, a species closely related to *D. i. alcionea*, the body size of adults has a heritability value around zero (Rodrigues and Moreira, 2002). The body size of *H. e. phyllis* is highly variable within and between populations, and there are also significant seasonal variations, suggesting that the main reason for body size plasticity in nature is the variation of environmental conditions during development.

In insects, body size is very important for fitness (Nylin and Gotthard, 1998). Female fecundity generally shows a strong positive relationship to adult body mass (Honek, 1993). In males, body size can be important in male-male competition for females (Goldsmith and Alcock, 1993) as well as for female choice of mates (Gilburn and Day, 1994), and it can also affect the male's mobility and ability to find females (Tammaru et al., 1996). There is often a positive relationship between male size and success in sperm competition in Lepidoptera (Carroll, 1994). Thus, when some life history trait shows high heritability value, it is expected that it is subjected to strong evolutionary constraints. The fact that females of most insects are larger than males, suggests that the fecundity advantage of large female size typically is more important for fitness than the advantages of large size in males. However, *D. i. alcionea* shows an inverted sexual dimorphism, which suggests a greater importance for large size in males.

It is known that many species of *Heliconius* do not present extensive long-rage dispersal and that there is no sexual dimorphism in many of these species (Ehrlich and Gilbert, 1973; DeVries, 1987; Oliveira and Araújo, 1992). *H. e. phyllis* has a very small home range and their populations have patterns of genetic variability that fit the island model of population structure (Silva and Araújo, 1994). The same pattern is not found in *D. i. alcionea*. The population genetic variability of *D. i. alcionea* and its dispersal ability suggest the existence of populations isolated by distance (Haag and Araújo, 1994). Possibly as an effect of different population characteristics between the genera *Heliconius* and *Dryas*, the selective pressure on the size of the wings could have acted differently in the evolution of the genetic inheritance of wing traits between these groups.

Van Dick (2003) suggests that adaptive morphological changes can influence the mate location strategies. *D. i. alcionea* can be considered a patrolling species, as defined by Scott (1972), since the males actively search for females to mate. Thus, in aerodynamic terms, males with a larger wing span could cover greater distances more easily and at lower energy costs, obtaining adaptive advantages in mate search and further copulation.

In conclusion, the significant values of the wing size heritability in *D. i. alcionea*, found in this study, suggest some importance of wing size in the reproductive biology of the species, especially for males.

Acknowledgments

We would like to thank A.A. Ferreira, A. Carrion, A.L. Klein, G. Pasqualin, J. Zanin and P.R. Vieira for helping keep the caterpillar and butterfly stocks. We also thank A.L. Klein for the wing digitalizations and assistance with wing measurements and two anonymous reviewers for some important suggestions that improved the text. Financial support was provided by Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Programa de Apoio a Núcleos de Excelência (PRONEX).

References

Benson WW, Brown Jr KS, Gilbert LE (1976) Coevolution of plants and herbivores: passion-flower butterflies. Evolution 29:659–80.

Boggs CL (1986) Reproductive strategies of female butterflies: variation in and constrains on fecundity. Ecol Entomol 11:7-15.

Brown Jr KS (1992) Borboletas da Serra do Japi: diversidade, hábitats, recursos alimentares e variação temporal. In História Natural da Serra do Japi: ecologia e preservação de uma área florestal no sudeste do Brasil. Edited by L.P.C. Morellato. Unicamp/Fapesp, Campinas. pp142-187.

Brown Jr KS, Mielke OHH (1972) The Heliconians of Brazil (Lepidoptera: Nymphalidae). Part II. Introduction and general comments, with a supplementary revision of the tribe. Zoologica 57:1–40.

Brown Jr KS, Yepez FF (1984) Los Heliconiinae (Lepidoptera, Nymphalidae) de Venezuela. Bol Entomol Venezolana 3:29-76.

Carroll AL (1994) Interactions between body size and mating history influence the reproductive success of males of a tortricid moth, Zeiraphera canadensis. Can J Zool 72:2124–2132.

Castillo RC del (2005) The quantitative genetic basis of female and male body size and their implications on the evolution of body size dimorphism in the house cricket Acheta domesticus (Gryllidae). Gen Mol Biol 28:843-848.

DeVries PJ (1987) The butterflies of Costa Rica and their natural history (Papilionidae, Pieridae, Nymphalidae). Princeton University Press, Princeton.

Falconer DS (1989) Introduction to quantitative genetics. Longman, Harlow.

French V (1996) Segmentation (and eve) in very odd insects embryos. BioEssays 18:435-438.

Gilburn AS, Day TH (1994) Evolution of female choice in seaweed flies: Fisherian and good genes mechanisms operate
in different populations. Proc R Soc London B 255:159–65.

Glazier DS (2002) Resource-allocation rules and the heritability of traits. Evolution 561:1696-1700.

Goldsmith SK, Alcock J (1993) The mating chances of small males of the cerambycid beetle Trachyderes mandibularis
differ in different environments (Coleoptera, Cerambycidae). J Insect Behav 6:351–360.

Haag LH, Araújo AM (1994) Inbreeding, genetic load and morphometric variation in natural populations of Dryas iulia (Lepidoptera, Nymphalidae). Rev Brasil Genet 17:35-39.

Haag LH, Araújo AM, Zaha A (1993) Genetic structure of natural populations of Dryas iulia (Lepidoptera, Nymphalidae) revealed by enzyme polimorphims and mtDNA RFPLs. Bioch Genet 31:447-458.

Honêk A (1993) Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos 66:483-492.

Lande R (1980) Sexual dimorphism, sexual selection and adaptation in polygenic characters. Evolution 34:292-305.

Lawrence PA (1992) The making of a fly: The Genetics of Animal Design. Blackwell Scientific Publications. Oxford.

Lynch M, Walsh B (1998) Genetics and analysis of quantitative traits. Sinauer Associates. Sunderland.

Merilä J, Sheldon BC, Ellegren H (1998) Quatitative genetics of sexual dimorfism in the collared flycatcher, Ficedula albicollis. Evolution 5:870-876.

Miller WE (1977) Wing measure as a size index in Lepidoptera: the family Olethreutidae. Ann Entomol Soc Am 70:253-256.

Monteiro LR, Reis SF (1999) Princípios de morfometria geométrica. Holos Editora, Ribeirão Preto.

Mousseau TA, Roff DA (1987) Natural selection and the heritability of fitness components. Heredity 59: 181-197.

Nijhout HF (2001) Elements of butterfly wing patterns. J Exp Zool 291: 213–225.

Nijhout HF (2003) The control of body size in insects. Develop Biol 261:1–9.

Nijhout HF, Wheeler DE (1996) Growth models of complex allometries in insects. Am Nat 148: 40-56.

Nylin S, Gotthard K (1998) Plasticity in life-history traits. Ann Rev Ent 43: 63–83.

Oliveira DL, Araújo AM (1992) Studies on the genetics and ecology of Heliconius erato (Lepidoptera: Nymphalidae). IV: Effective size and variability of the raylets in natural populations. Rev Bras Genet 15:789-799.

Paim AC (1995) Polimorfismo enzimático e variação morfológica em uma população natural de Dryas iulia (Fabr. 1775) (Lepidoptera; Nymphalidae). Master Degree Dissertation. Departamento de Genética, Universidade Federal do Rio Grande do Sul, Porto Alegre.

Paim AC, Kamisky LA, Moreira GPR (2004) Morfologia externa dos estágios imaturos de heliconíneos neotropicais. IV. Dryas iulia alcionea (Lepidoptera, Nymphalidae, Heliconinae). Iheríngia Zool 94:25-35.

Perico E, Araújo AM (1995) Suitability of host plants (Passifloraceae) and their acceptance by Heliconius erato and Dryas iulia (Lepidoptera; Nymphalidae). Evol Biol 5:59-74.

Rodrigues D, Moreira GRP (2002) Geographical variation in larval host-plant use by Heliconius erato (Lepidoptera: Nymphalidae) and consequences for adult life history. Braz J Biol 62:321-332.

Rodrigues D, Moreira GRP (2004) Seasonal variation in larval host plants and consequences for Heliconius erato (Lepidoptera: Nymphalidae) adult body size. Austral Ecol 29:437-445.

Roff DA (1996) The evolution of genetic correlations: an analysis of patterns. Evolution 50:1392-1403.

Roff DA (1997) Evolutionary quantitative genetics. Chapman and Hall, New York.

Rutowski RL (1997) Sexual dimorphism, mating systems and ecology in butterflies. In The evolution of mating systems in Insects and Arachnids. Edited by J.C. Choe. Library of Congress, Cambrigde. pp 257-271.

Scott JA (1972) Mating of Butterflies. J Res Lepid 11:99-127.

Silva LA, Araújo AM (1994) The genetic structure of Heliconius erato populations. Rev Bras Genet 17:19-24.

Simmons LW, Ward PI (1991) The heritability of sexually dimorphic traits in the yellow dung fly Scathophaga stercoraria (L.). J Evol Biol 4:593-601.

Tammaru T, Ruohomaki K, Saikkonen K (1996) Components of male fitness in relation to body size in Epirrita autumnata (Lepidoptera, Geometridae). Ecol Entomol 21:185–192.

Tautz D, Sommer RJ (1995) Evolution of segmentation genes in insects. Trends Genet 11: 23-27.

Thorne AD, Pexton JJ, Dytham C, Mayhew, PJ (2006) Small body size in an insect shifts development, prior to adult eclosion, towards early reproduction. Proc R Soc B 273:1099–1103.

Torres-Vila LM, Stockel J, Roehrich R (1995) Le potentiel reproducteur et ses variables biotiques associées chez le mâle de l'Eudémis de la vigne Lobesia botrana. Entomol Exp Appl 77:105-119.

Van Dick H (2003) Mate Location: a matter of design? Adaptive morphological variation in the speckled wood butterfly. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, pp 353-366.

West GB, Brown JH, Enquist BJ (1999) The fourth dimension of life: fractal geometry and allometric scaling of organisms. Science 284:167–169.

Wiklund C (2003) Sexual selection and the evolution of butterfly mating systems. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, pp 67-108.

Wolpert L, Beddington R, Brockes J, Jessell T, Lawrence P, Meyerowitz E (2006) Principles of Development, 3th edition. Oxford University Press USA, New York.

Zelditch M, Swiderski D, Sheets DH, Fink W (2004). Geometric Morphometrics for Biologists: A Primer. Elsevier Academic Press, San Diego.

Table 1 - Mean (± standard deviation) and the coefficients of variation of the eight morphological wing traits in females (N=148) and males (N=184) of *Dryas iulia alcionea.* Area measured in mm^2, other traits in mm. Significance values are from one-tailed *t*-test (330 degrees of freedom) of differences between sexes

	Mean ± s.d.					**Coefficient of Variation**			
Wing Trait	**Females**	**Males**	***t***	**p**		**Females**	**Males**	***t***	**p**
FL	40.35 ± 2.28	41.91 ± 2.08	-6.491	<0.001		0.056	0.049	1.694	0.045
FW	16.32 ± 1.15	16.67 ± 1.00	-2.953	0.003		0.070	0.059	2.071	0.019
HL	21.93 ± 1.48	22.71 ± 1.38	-4.954	<0.001		0.067	0.061	1.338	0.091
HW	19.32 ± 1.30	20.23 ± 1.21	-6.539	<0.001		0.067	0.060	1.410	0.079
FA	458.53 ± 54.87	489.78 ± 49.09	-5.470	<0.001		0.120	0.100	2.260	0.012
FP	92.35 ± 5.29	95.81 ± 4.66	-6.332	<0.001		0.057	0.049	2.085	0.019
HA	344.49 ± 43.54	376.21 ± 43.50	-6.599	<0.001		0.126	0.116	1.129	0.129
HP	70.06 ± 4.52	73.11 ± 4.33	-6.249	<0.001		0.064	0.059	1.095	0.137

Table 2 - Genetic correlations between the eight wing morphological traits of *Dryas iulia alcionea.* **a.** Females (N=148). **b.** Males (N=184). **b.** Females x Males (N=332). For table 2A and 2B all correlations are significant at 0.05 level. For table 2C, significant values for pairwise correlations at 0.05 level are shown in bold face. All multiple comparisons were adjusted by False Discovery Rate Method.

a. Females

	FL	**FW**	**HL**	**HW**	**FA**	**FP**	**HA**	**HP**
FL	1.000	0.905	0.767	0.921	0.961	0.981	0.792	0.805
FW		1.000	0.774	0.921	0.970	0.944	0.842	0.847
HL			1.000	0.854	0.799	0.792	0.862	0.867
HW				1.000	0.941	0.935	0.888	0.893
FA					1.000	0.988	0.850	0.856
FP						1.000	0.823	0.833
HA							1.000	0.994
HP								1.000

b. Males

	FL	**FW**	**HL**	**HW**	**FA**	**FP**	**HA**	**HP**
FL	1.000	0.810	0.725	0.897	0.923	0.953	0.791	0.791
FW		1.000	0.673	0.848	0.933	0.885	0.795	0.784
HL			1.000	0.846	0.750	0.741	0.859	0.858
HW				1.000	0.906	0.892	0.900	0.893
FA					1.000	0.982	0.847	0.838
FP						1.000	0.829	0.827
HA							1.000	0.995
HP								1.000

c. Females x Males

	Female Trait							
Male Trait	**FL**	**FW**	**HL**	**HW**	**FA**	**FP**	**HA**	**HP**
FL	**0.740**	0.648	0.059	0.661	**0.702**	**0.706**	0.414	0.434
FW	0.406	0.522	0.065	0.416	0.412	0.397	0.255	0.263
HL	0.189	0.004	**0.796**	0.196	0.064	0.102	**0.675**	**0.680**
HW	0.414	0.532	0.206	**0.681**	0.480	0.435	**0.664**	**0.678**
FA	0.614	0.585	0.028	0.597	0.592	0.604	0.384	0.392
FP	0.642	0.502	0.005	0.529	0.570	0.608	0.301	0.316
HA	0.133	0.360	0.491	0.483	0.228	0.166	**0.688**	**0.716**
HP	0.107	0.301	0.473	0.430	0.177	0.125	0.636	**0.672**

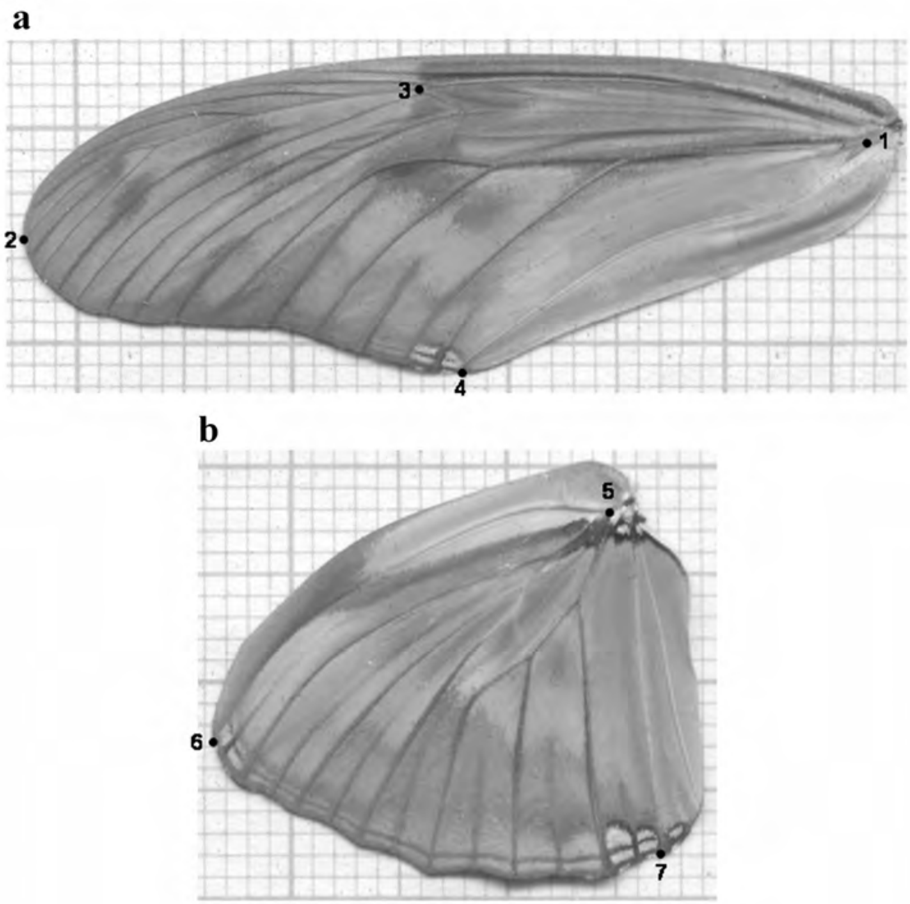
Table 3 - Mean genetic correlation among hindwing and forewing morphological traits of female and males of *D. iulia alcionea*, calculated for the genetic correlations shown in table 2c.

	Female Trait	
Male Trait	**Hindwing**	**Forewing**
Hindwing	0.578	0.320
Forewing	0.239	0.573

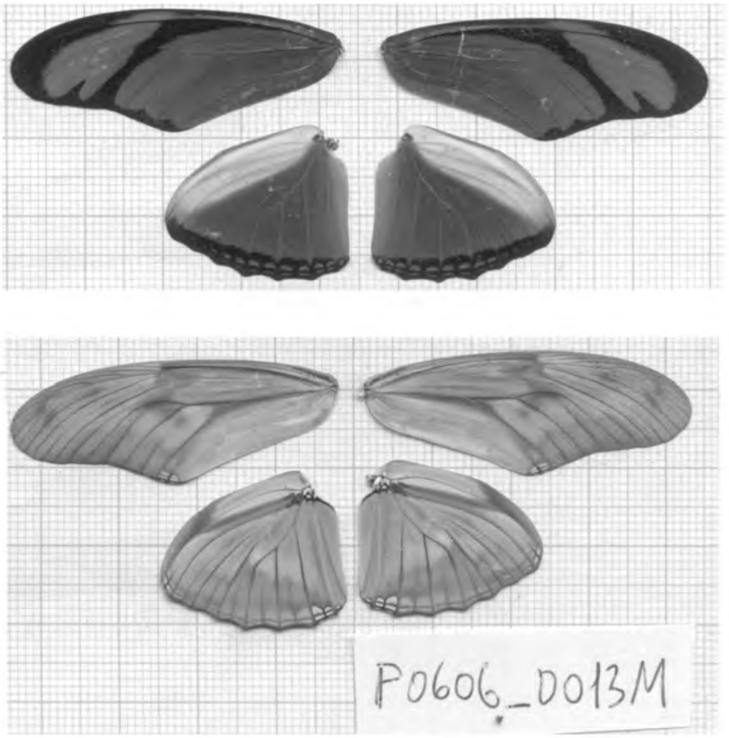
Table 4 - Values for the heritability (± s.e.) estimated for each wing trait of *Dryas iulia alcionea* by the conventional method of linear regression. Heritability was estimated for dam value against daughters midvalue (Dam x Daughters), and sire value against sons midvalue (Sire x Sons). All measurements were log transformed and corrected by log body weight. FL, forewing length; FW, forewing width; HL, hindwing length; HW, hindwing width; FA, forewing area; FP, forewing perimeter; HA, hindwing area; HP, hindwing perimeter.

Trait	Parent x Offspring	h^2 ± s.e.	*t*	Sig.	Trait	Parent x Offspring	h^2 ± s.e.	*t*	Sig.
FL	*Dam x Daughters*	0.626±0.022	28.454	<0.0001	**FA**	*Dam x Daughters*	0.548±0.040	13.700	<0.0001
	Sire x Sons	0.468±0.030	15.600	<0.0001		*Sire x Sons*	0.480±0.054	8.888	<0.0001
FW	*Dam x Daughters*	0.546±0.022	24.818	<0.0001	**FP**	*Dam x Daughters*	0.606±0.020	30.300	<0.0001
	Sire x Sons	0.192±0.034	5.646	0.0001		*Sire x Sons*	0.642±0.026	24.692	<0.0001
HL	*Dam x Daughters*	0.304±0.034	17.882	<0.0001	**HA**	*Dam x Daughters*	0.310±0.058	5.344	0.0002
	Sire x Sons	0.854±0.042	8.940	<0.0001		*Sire x Sons*	0.532±0.056	9.500	<0.0001
HW	*Dam x Daughters*	0.336±0.024	14.000	<0.0001	**HP**	*Dam x Daughters*	0.426±0.028	15.214	<0.0001
	Sire x Sons	0.654±0.032	19.234	<0.0001		*Sire x Sons*	0.464±0.028	16.570	<0.0001

Fig.1 Landmarks used as reference for wing linear measurements. In the forewing **(a)**, length was measured between landmarks 1 and 2, and width between landmarks 3 and 4. In the hindwing **(b)**, length was measured between landmarks 5 and 6, and width between landmarks 5 and 7. Landmarks: **(1)** bifurcation of Cu vein with A1 vein; **(2)** encounter of vein R3 with the margin of forewing; **(3)** origin of vein M1 **(4)** encounter of vein 1A with the margin of the forewing; **(5)** bifurcation between the veins Sc + R1 and R3; **(6)** encounter of vein Sc + R1 with the margin of the hindwing; **(7)** encounter of vein 1A with the margin of the hindwing.



CAPÍTULO 5



ANALYSIS OF WING SEXUAL DIMORPHISM IN *DRYAS IULIA ALCIONEA* (LEPIDOPTERA, NYMPHALIDAE) USING TRADITIONAL AND GEOMETRIC MORPHOMETRICS

Artigo a ser submetido para o periódico *Neotropical Entomology*

Analysis of wing sexual dimorphism in *Dryas iulia alcionea* (Lepidoptera, Nymphalidae) using traditional and geometric morphometrics.

Nicolás O. Mega[1], André L. Klein[2] e Aldo M. de Araújo[3]

[1]*Programa de Pós Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul.*
[2]*Curso de Ciências Biológicas, Universidade Federal do Rio Grande do Sul.*
[3]*Departamento de Genética, Universidade Federal do Rio Grande do Sul.*

Abstract:

Species and individuals can differ in several aspects of their phenotype, and such differences are potencial targets of natural and sexual selection. In these cases, body shape and size might be seen as a result of resource allocation priority for the energy assimilated during development. Here, we analyze sexual dimorphism of *D. iulia alcionea* wings using traditional and geometric morphology-based methods. The analyses were performed on forewing and hindwing using linear measures (length, width, area and perimeter) and geometric descriptors of shape (partial and relative warps scores) and size (centroid size) derived form anatomical landmarks. Both traditional and geometric morphometric analysis revealed high significant sexual dimorphism of size and shape. Males were larger than females and exhibit wings narrower and more elongated than females. Males seem to have an aerodynamic wing shape designed to flight in a straight line through long distances, while females seem to have an aerodynamic shape optimized to hovering flight. The results presented are also supported by behavioral and ecological differences between males and females. The sexual dimorphism could be connected with different habits of life and indirectly related to reproductive functions. Also, the less pronounced variability of male traits suggests the action of selection on male wing traits.

Key-words: sexual dimorphism, wing morphometrics, Heliconiinae, aerodynamics efficiency.

Introduction:

Species and individuals can differ in several aspects of their phenotype, including morphology, physiology and behavior (Van Dick 2003) and such differences may potencial targets of natural and sexual selection. In butterflies, the wings can be subject to selection regarding defense strategies, thermoregulation, and flight patterns during courtship (Di Mare and Corseuil 2004a, 2004b). Lepidopterans exhibit a broad range of mate location strategies and mating systems (Deinert 2003) and its aspects and morphological traits have been intensely studied in the later years (additional references, see Rutowisky 1997 and Wiklund 2003). In these cases, body shape and size might be seen as a result of resource allocation priority for the energy assimilated to grow, survive and reproduce (Harder 1985, Barbault 1988, Freeman and Herron 2001, Glazier 2002).

Until recently, most morphometric analysis applied multivariate statistics to sets of measured distances of organisms. Such approach is referred to as traditional morphometrics (Rohlf and Marcus, 1993). In the late 1980s, new morphometric tools were developed from Kendall's theory of shapes and his definition of geometric shape (Mutanen and Pretorius 2007). The method was based in the use of coordinates of landmarks, which are superimposed and subject to the standard multivariate analysis (Zelditch et al. 2004). Such approach is referred to as geometric morphometrics and allowed researchers to eliminate size effects from shape analysis.

Dryas iulia alcionea is a butterfly that presents subtle sexual dimorphism regarding the color of the wings and size (Brown Jr. 1981, Haag and Araújo 1994). In Lepidoptera, females are generally larger than the males and this difference is commonly associated with oviposition activity (Rutowski 1997, Wiklund 2003). Few studies so far have shown the existence of inverted sexual dimorphism in butterflies (for instance, Wiklund and Kaitala 1995). Many evolutionary ecological studies have documented sexual dimorphism in morphology or behavior (Berwaerts et al 2006). Such studies have shown evidences of selection on the evolution of sexual dimorphism, generally related to reproductive success, behavioral ecology and development (Andersson 1994, Wiklund 2003, Blanckenhorn et al 2007).

The aim of this study is to analyze sexual dimorphism of *D. i. alcionea* wings using morphology-based methods, including both traditional and geometric morphometrics. We discuss briefly some aspects of aerodynamics efficiency and sexual selection possibly acting on the evolution of sexual dimorphism in this butterfly spicies.

Material and Methods:

Specimens and Wing Measurements

Dryas iulia alcionea specimens were captured in natural populations of Rio Grande do Sul State, South Brazil, or reared in laboratory from eggs collected from females captured in the wild and kept in captivity. The wings were dissected and digitalized (300 dpi resolution) with a table scanner (HP ScanJet 2400). Measurements and landmark plotting were always done in the right forewing and hindwing, using anatomic landmarks from type-I (Monteiro and Reis 1999) as reference, to ensure homology between the distances measures and landmark positioning.

Traditional morphometrics

Four wing measurements were taken from 928 butterflies (239 females, 689 males), both on forewing and hindwing: length, width, area and perimeter. Linear distances were taken directly on the wings using a digital caliper, nearest 0.1mm. The standard error of the mean was ±0.012 when the forewing length of same wing was repeatedly measured (n=20, mean=43.55mm, s.d.=0.049). Area and Perimeter were measured on the digital images using the computer graphics program *Image J* (version 1.38, available at http://rsb.info.nih.gov/ij; developed by Wayne Rasband).

The wing measurements for hind and forewing were compared between sexes by a MANOVA and paired *t*-tests performed to each wing trait separately. Variability on each wing trait was measured using the coefficient of variation (CV). In order to reduce dimensionality of the data set, Principal Components Analysis (PCA) was done using all eight wing measurements together, using the software PAST (version 1.68). Sexual dimorphism over size and shape was evaluated by comparing PC1 and PC2 scores, using a *t*-test in the software SPSS 15.0.

Geometric morphometrics

Twenty-one landmarks were defined on the ventral face of the forewing and 19 on the hindwing (figure 1). The landmark digitalizations were carried out directly on the digital images of 103 females and 120 males (N=223) reared under laboratory conditions, using TPS2DIG software (Rohlf, 2004). The digitized landmark data were subject to Procrustes superimposition (Rohlf and Slice, 1990) using IMP CoordGen6f. The centroid size of each configuration prior to superimposition was used as the size variable. The partial and relative warp scores constitute the shape variables and were used in all subsequent shape analysis.

The wing size sexual dimorphism for fore and hindwing was investigated by applying a *t*-test to the centroid size, using SPSS 15.0. The wing shape sexual dimorphism was tested by a MANOVA on the shape variables. Principal Component Analysis (PCA) on the covariance matrix derived from the partial warp scores of generalized least-squares (GLS) Procrustes superimposed data was done, using the IMP PCAGen6 and PAST 1.68 programs. Multiple t-tests were performed for each PC generated by PCA, using SPSS 15.0.

To represent shape change as deformation among landmarks we used thin plate spline (TPS) interpolation function (Bookstein 1991). Sex-specific consensus configurations were calculated with TPSRELW software (Rohlf 2004) and represented with the aid of deformation grids (figure 1b, d). Wing deformations were represented using relative warps, plotting the mean form of each sex against consensus configuration for both sexes along the first two relative warp axes. The significance of deformations was tested by a MANOVA, using SPSS 15.0.

Results

Traditional morphometrics

The results of MANOVA revealed significant differences between males and females, both for forewing (F=59911.76, $p<0.001$) and hindwing (F=84010.91, $p<0.001$). The results of t-tests on the eight wing variables showed significant differences between sexes for all wing traits measured (table 1); generally females showed greater variation than males, for linear and area measures. Males were significantly larger than the females and the average percentage size difference between the sexes was approximately 5%. The largest difference between sexes was for hindwing (10%), while the smaller difference was found for the length of forewing (2%).

The result of the PCA for forewing showed that 99.99% of the variation found in the sample can be explained by the first two principal components (PC1 99.96% and PC2 0.03%) and that the most important load counting for PCA was AAA (0.994, table 2). Similar results were obtained for hindwing PCA. The result of the PCA for hindwing was very similar to that observed for forewing PCA (PC1 99.98% and PC2 0.01%), with the most important load counting for PCA related to AAP (0.994, table 2). The PCA plot (PCA1 x PCA2) for both forewings and hindwings, show that the differences accumulated in the two first principal components are small, generating large overlapping between males and females (Figure 2). However, males and females differ significantly for the first two principal components, both for forewing (PCA1 (size), t=-8.455, $p<0.001$; PCA2 (shape), t=6.794, $p<0.001$) and hindwing (PCA1 (size), t=-12.711, $p<0.001$; PCA2 (shape), *t*=-4.368, p<0.001). Traditional morphometrics analysis suggests that the sexes have a moderate sexual dimorphism for size and shape of wings.

Geometric morphometrics

The analysis of wing size revealed significant differences between sexes (table 3). Males were significantly larger than females for both forewing (*t*=-3.729, p<0.001) and hindwing (*t*=-4.273, p<0.001). Again, females showed more size variation when compared to males, for both forewing and hindwing (table 3).

The results of MANOVA revealed highly significant differences between shape of males and females, both for the forewing (*F*=36.69, p<0.001) and the hindwing (*F*=29.66, p<0.001). Figure 3 shows the PCA plot for the most significant morphological differences of hindwing and forewing, calculated using shape variables. In the hindwing, the first three principal components accounted for 63.2% of all observed morphometric variation (first 33.58%, second 18.26% and third 11.36%). In the forewing, the first three principal accounted for 66.58% of all observed morphometric variation (first 33.64%, second 18.13% and third 14.81%).

Comparisons of TPS deformations of hindwing and forewing for both sexes in relation to overall consensus configuration are shown in figure 4 and 5. Visualizations of TPS revealed that the forewing of males has a narrow and elongated form when compared to females' forewing. In the posterior part of forewing, between landmarks 14-21, this tendency changes and males presents forewing with higher convexity forms. The hindwing deformations had lower magnitude between sexes, when compared to forewing deformations. Major differences are found for the bind forces acting on landmark 3 and for the compression across landmarks 7-19. This last deformation makes male's hindwing more elongated than female's ones.

Discussion

Tradicional vs. Geometric Morphometrics

We have shown that traditional morphometrics as well as geometric morphometrics analysis were able to identify sexual dimorphism on *D. iulia alcionea* wings. Both methods produce accurate identifications of sexual differences regarding wing size and shape, which can not always be done by visual identification. Moreover, our results indicate that geometric morphometrics provides a more powerful tool to analyze and represent shape differences. The mathematical methods used in geometric morphometric analysis allow separating size and shape during investigation, increasing the power statistics on the recognition of form change (Bookstein 1981, Zelditch et al. 2004). However, traditional morphometrics are especially useful to deal with data on form when size variation is reduced within groups, or when considerable size differences is present among the groups analyzed. It is highly recommended to use both traditional and geometric methods combined, since together they may produce more powerful results (Mutanen and Pretorius 2007).

The sexual dimorphism in D. iulia alcionea

Both traditional and geometric morphometric analysis revealed size and shape sexual dimorphism in *D. iulia alcionea*. Males seem to be larger and less variable then females for all wing traits, both on forewing and hindwing. The shape of males' wings are narrower and more elongated than females' wings, with males showing less shape variability, as observed for size. These results raise the possibility of the action of some kind of selection on males' wings. At least two major causes could have acted to produce the patterns found: selection based on aerodynamics efficiency and female mate preference.

The insect flight performance depends on the thoracic muscles, articulation between thorax and wing, and the wings themselves that generate the aerodynamic forces. The size and shape of the wings are very important to flight, since they are responsible for insect flight trajectories and body kinematics (Dudley 2000). Two composite measures, wing loading and aspect ratio, which respectively describe wing area and forewing elongation, are known to influence butterfly acceleration ability (Dudley 2000, Berwaerts et al 2002, Di Mare and Courseil 2004b). The wing loading varies according to body mass and wing area, while the aspect ratio depends on the size and the surface of the wings. According to Dudley & Srygley (1994) the speed of flight can be estimateased on the index of narrowness. In butterflies, sexual differences in body and wing shape have been interpreted in terms of the different types of flight that are likely to impose different demand on aerodynamic performance (Wickman 1992, Rutowski 1997, Berwaerts et al 2006). Thus variation in size and shape between males and females could play an important role on aerodynamics and sexual behavioral flight differences.

It is well known that forewing length plays an important role in aerodynamics and speed of flight, and that the hindwings are more closely related to handling and direction of flight (Dudley 2000, Berwaerts et al 2002, Di Mare and Corseuil 2004b). Narrow and elongated wings are suitable for long displacements and are generally associated to insects with gliding flight (Srygley and Chai 1990; Srygley 1994, 1999). This type of wing generally offers lower average speeds and is associated with species from open habitats. Wider wings, tending to an elliptical shape, are capable of sustaining a higher weight per unit area. This type of wing allows an individual to take off and accelerate very fast, also providing an accurate beaten flight with great maneuverability, that would be advantageous in small spaces (Hildebrand 1995, Tennekes 1996, Di Mare and Corseuil 2004b).

The results of shape analysis on the wings of *D. iulia* suggest that the males have wings optimized to flight long distances in open areas without many obstacles. Similarly, the wings of females are optimized to support large body masses with efficiency and agility in places with limited space, allowing high maneuverability. These comparisons are in line with the sexual behavior observed in the field. Males of *D. iulia* patrol and actively seek for females to mate. Females are generally seen in the middle of the vegetation in slow and beaten flights during oviposition (Mega and Araújo, in preparation). The results presented here suggest that the differences between males and females could be attributed to a difference of intersexual niche or ecological sexual dimorphism as suggested for some Papilionidae species (Di Mare and Corseuil 2004a). If we think in terms of aerodynamics efficiency, males seem to have an aerodynamic shape optimized to flight in a straight line through long distances, while females seem to have an aerodynamic shape optimized to hovering flight.

The results presented are also supported by behavioral and ecological differences between males and females. The sex differences could be connected with different habits of life and indirectly related to reproductive functions (Rutowski 1997). That seems to be the case of *D. iulia*. Males and females of *D. iulia* have differences on ecology and foraging behavior, as previously mentioned. Female spend most of their active time alternating between oviposition and feeding flights, whereas males spend most of their active time locating mates. Similar behavioral patterns are observed in *Pararge aegeria* (Berwaerts et al 2006). Such ecological sex differences could be the result of adaptations to foraging differences between sexes and may first arise either by sexual selection or by natural selection, and later on being enlarged by natural selection for separation in diet or other aspects (Lande 1980, Roff 1986, Andersson 1994). Imperfect genetic correlations between sexes allow natural selection to create sexual dimorphism (Slatkin 1984, Roff 1996).

We have showed that males are larger than females and that variability of the characteristics studied were always lower in males when compared to females. This suggests the action of sexual selection on the wings of males, which could be explained by the preference of females by males with larger wings, or by competition between males over mates or fertilizations. There is now much evidence that females often choose their mate, and that choice could favor some male traits (Andersson 1994). The exact ways in which female choice selects for such traits are still debated and so are the ways in which female preferences evolve. Some male traits may evolve simply because they make it easier for females to find the male, but sometimes these traits may represent male quality and ability (Kemp 2002).

Mate choice by one sex therefore usually implies in competition over mates in the other sex, even if rivals never meet each other. If females are the limiting resource, competition among males occurs when mating by one individual makes females harder to come by for other males. Direct struggles over females may occur, with competitors trying to exclude each other. These struggles do not always involve fight, but can involve scrambles, endurance rivalry and contests. This competition over mates can take several forms and favor a wide range of attributes. In butterflies, these struggles generally are not aggressive and may favor the evolution of visual, chemical and locomotory organs (Thornhill & Alcock 1983, Wickman & Wiklund 1983; Rosenberg & Enquist 1991; Kemp 2000, Berwaerts et al 2006). Although contests are very common among butterflies, indirect competition could occur among males. If some trait on males increases the success in locating mates or copulation, this trait may be selected by intrasexual competition and favored by evolution. If we think in flight performance, wing size and form could be selected by nature and/or by females, improving mating and reproductive success on males.

Acknowledgements

We would like to thank A.A. Ferreira, A. Carrion, A.L. Klein, G. Pasqualin, J. Zanin e P.R. Vieira for helping in keeping the caterpillar and butterfly stocks. We also thank to L.E. Schmidt and R. Fornel for the assistance with geometric morphometrics and criticism on the early versions of the manuscript. Financial support was provided by Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Programa de Apoio a Núcleos de Excelência (PRONEX).

Referências

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey. 624 pp.

Barbault, R. 1988. Body size, ecological constraints, and the evolution of life-history strategies, p. 261-286. In: M.K. Hecht; B. Wallace & G.T. Prance (Eds). Evolutionary Biology. Plenum, New York.

Berwaerts, K.; Van Dyck, H., Aerts, P. 2002. Does flight morphology relate to flight performance? An experimental test with the butterfly *Pararge aegeria*. Functional Ecology, 26:484–491.

Berwaerts, K.; Aerts, P.; Van Dyck, H. 2006. On the sex-specific mechanisms of butterfly flight: flight performance relative to flight morphology, wing kinematics, and sex in *Parage aegeria*. Bio. Journal of the Linnean Society, 89: 675-687.

Blanckenhorn, W.U.; Dixon, A.F.G.; Fairbairn, D.J.; Foellmer, M.W.; Gibert, P.; van der Linde, K.; Meier, R.; Nylin, S.; Pitnick, S.; Schoff, C.; Signorelli, M.; Teder, T.; Wiklund, C. 2007. Proximate causes of Rensch's rule: does sexual size dimorphism in arthropods result from sex differences in development time? American Naturalist, 169(2), 245 - 257.

Bookstein, F.L. 1991. Morphometric tools for landmark data: Geometry and Biology. Cambridge, Cambridge University Press, 455 pp.

Brown Jr., K.S. 1981. The biology of Heliconius and related genera. Annual Review of Entomology 26: 427–456.

Deinert, E.I. 2003. Mate location and competition for mates in a pupal mating butterfly. Pp. 91–108 in Boggs, C. L., Watt, W. B. & Ehrlich, P. E. (eds). Butterflies: ecology and evolution taking flight. The University of Chicago Press, Chicago, 739pp.

Di Mare, R.A.; Corseuil, E. 2004a. Morfometria de Papilioninae (Lepidoptera, Papilionidae) ocorrentes em quatro localidades do Rio Grande do Sul, Brasil. III. Análise da forma das asas através de marcos anatômicos. Revista Brasileira de Zoologia, 21(4): 847-855.

Di Mare, R.A.; Corseuil, E. 2004b. Morfometria de Papilioninae (Lepidoptera, Papilionidae) ocorrentes em quatro localidades do Rio Grande do Sul, Brasil. II. Relação entre partes do corpo, aerodinâmica de vôo e tipos de asas. Revista Brasileira de Zoologia, 21(4): 833-846.

Dudley, R. 2000. The biomechanics of insect flight. Princeton, Princeton University Press, 476pp.

Freeman, S.; Herron, J.C. 2001. Evolutionary Analysis (2nd edition). Prencitece Hall, Ipper Saddle River, NJ, 704 pp.

Glazier, D.S. 2002. Resource-allocation rules and the heritability of traits. Evolution 561(8): 1696-1700.

Haag, K.L; Araújo, A.M. 1994. Inbreeding, genetic load and morphometric variation in natural populations of Dryas iulia (Lepidoptera, Nymphalidae). Rev. Brasil. Genet. 17(1): 35-39.

Harder, L.D. 1985. Morphology as a predictor of flower choice by Bumble Bees. Ecology, 66 (1): 198- 210.

Hildebrand, M. 1995. Análise da estrutura dos vertebrados. São Paulo, Atheneu Editora, 700p.

Kemp, D.J. 2000. Contest behaviour in territorial male butterflies: does size matter? Behav Ecol 11:591–596.

Kemp, D.J. 2002. Butterfly contests and flight physiology: why do older males fight harder? Behav Ecol 13:456–461

Lande, R. 1980. Sexual dimorphism, sexual selection and adaptation in polygenic characters. Evolution 34: 292-305.

Rohlf, F.J. 2004. TPS Series, Department of Ecology and Evolution, State University of New York, Stony Brook, New York.

Rohlf, F.J.; Marcus, L.F. 1993. A revolution in morphometrics. Trends in ecology and evolution, 8: 129-132.

Monteiro, L.R.; Reis, S.F. 1999. Princípios de morfometria geométrica. Holos Editora, Ribeirão Preto, 188pp.

Mutanen, M.; Pretorius, E. 2007. Subjective visual evaluation vs. traditional and geometric morphometrics in species delimitation: a comparison of moth genitalia. Systematic Entomology, 32(2): 371-386.

Roff, D.A. 1986. The evolution of wing dimorphism in insects. Evolution, 40: 1009-1021.

Roff, D.A. 1996. The evolution of genetic correlations: an analysis of patterns. Evolution 50: 1392-1403.

Rohlf, F.J. 1990. Morphometrics. Annual Review of Ecology and. Systematics, 21: 299-316.

Rohlf, F.J., Slice, D.E. 1990. Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Zool.*, 39:40-59.

Rosenberg, R.H.; Enquist, M. 1991. Contest behaviour inWeidemeyer’s admiral butterfly Limenitis weidemeyerii (Nymphalidae): the effect of size and residency. Animal Behavior, 42: 805–811.

Rutowski, R.L. 1997. Sexual dimorphism, mating systems and ecology in butterflies. In The evolution of mating systems in Insects and Arachnids. Edited by J.C. Choe. Library of Congress, Cambrigde. 397pp.

Slatkin, M. 1984. Ecological causes of sexual dimorphism. Evolution 38: 622-630.

Srygley, R.B. 1994. Locomotor mimicry in butterflies? The associations of positions of centres of mass among groups of mimetic, unprofictable prey. Phil. Trans. R. Soc. Lond. B 343: 145-155.

Srygley, R.B. 1999. Locomotor mimicry in Heliconius butterflies: contrast analyses of flight morphology and kinematics. Phil. Trans. R. Soc. Lond. B 354: 203-214.

Srygley, R.B.; Chai, P. 1990. Flight morphology of Neotropical butterflies: palatability and distribution of mass to the thorax and abdomen. Oecologia, Berlin, 84: 491-499.

Tennekes, H. 1996. The Simple Science of Flight from Insects to Jumbo Jets. Cambridge, MT Press, 132p.

Thornhill, R.; J. Alcock, 1983. The Evolution of Insect Mating Systems. Harvard University, Cambridge, 547pp.

Van Dick, H. 2003. Mate Location: a matter of design? Adaptive morphological variation in the speckled wood butterfly. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, 739pp.

Wickman, P-O. 1992. Sexual Selection and butterfly design – a comparative study. Evolution, 46: 1525-1536.

Wickman, P-O.; Wiklund, C. 1983. Territorial defence and its seasonal decline in the speckled wood butterfly (Pararge aegeria). Animal Behavior, 31: 1206–1216.

Wiklund, C. 2003. Sexual selection and the evolution of butterfly mating systems. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, 739pp.

Zelditch, M.; Swiderski, D.; Sheets, D.; Fink W. 2004. Geometric Morphometrics for Biologists: a primer. Elsevier Academic Press, London, 443pp.

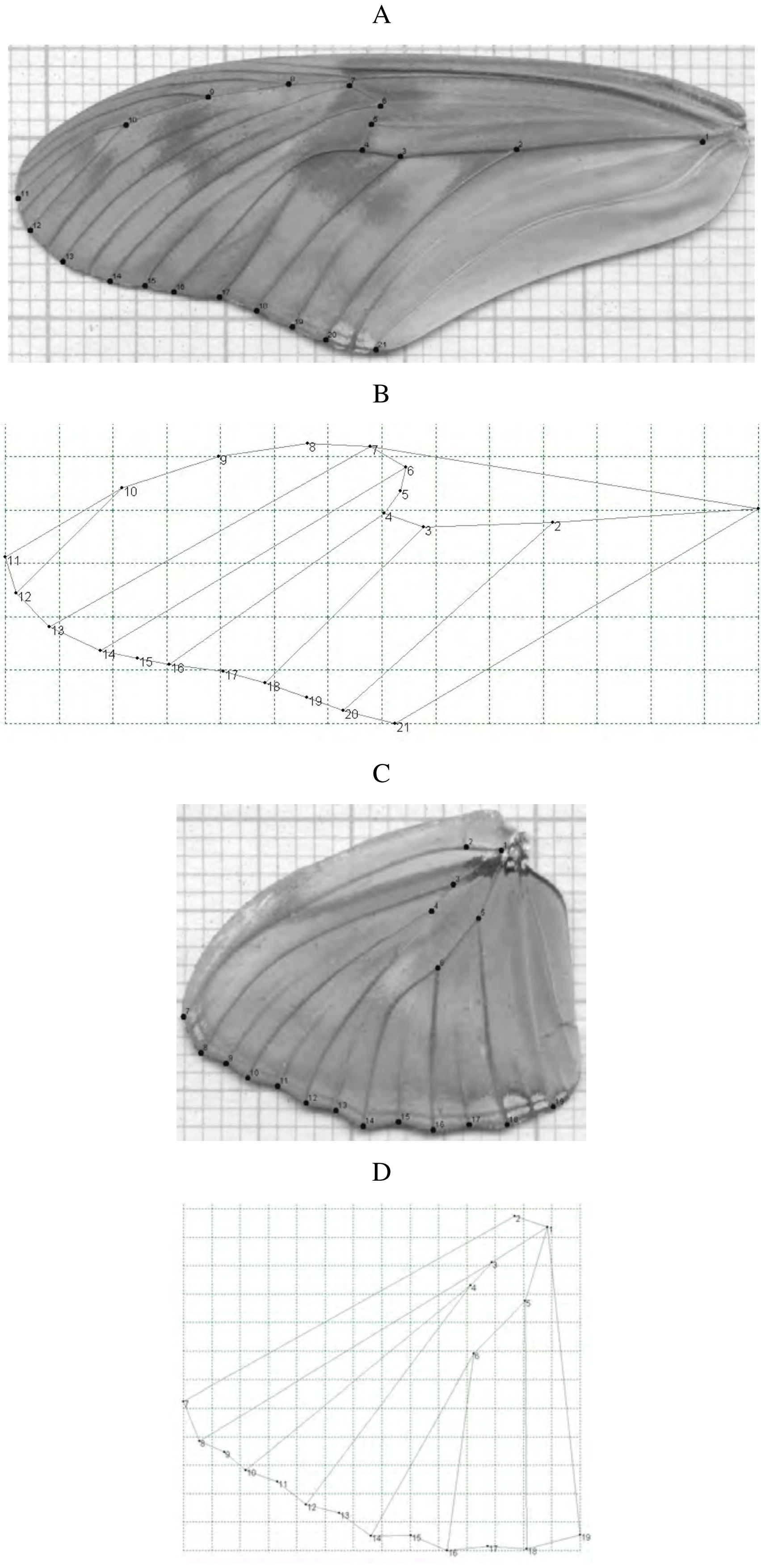
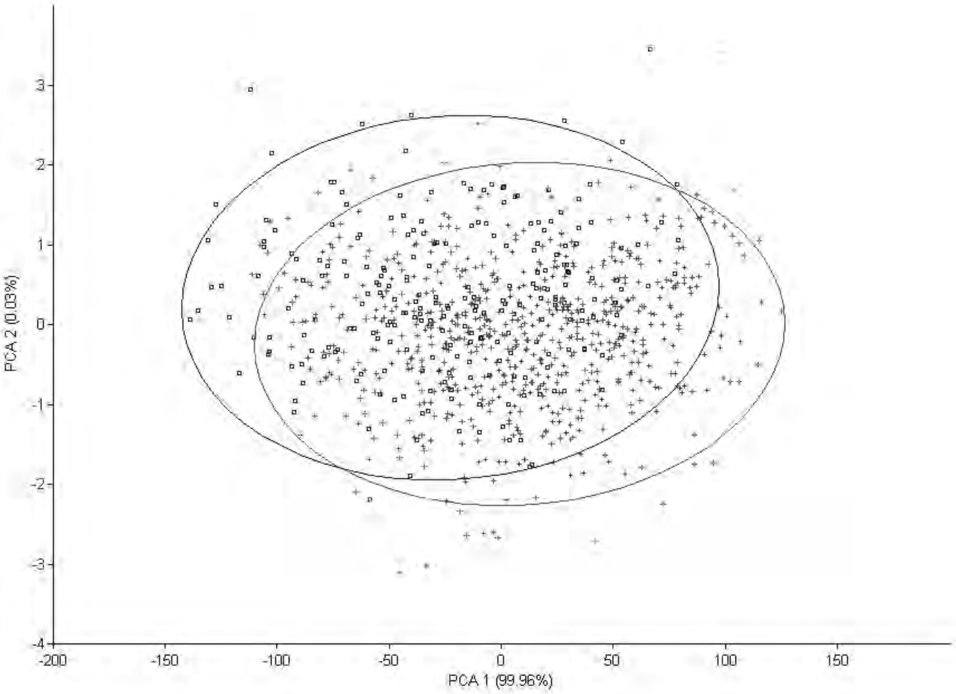

A
B
C
D

Figure 1. Landmarks and geometric representations of *D. iulia alcionea* wings used in the geometric morphometric analysis. **A.** and **B.,** forewing; **C.** and **D.**, hindwing.

A.


PCA 2 (0.03%)
3
2
1
0
-1
-2
-3
-4
-200
-150
-100
-50
0
50
100
150
PCA 1 (99.96%)

B.

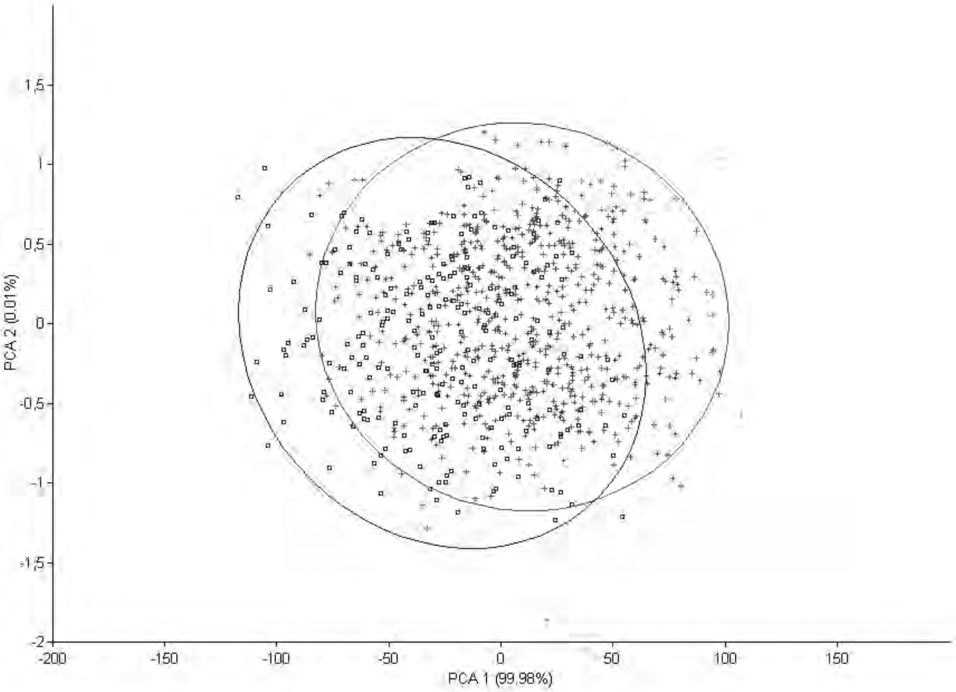
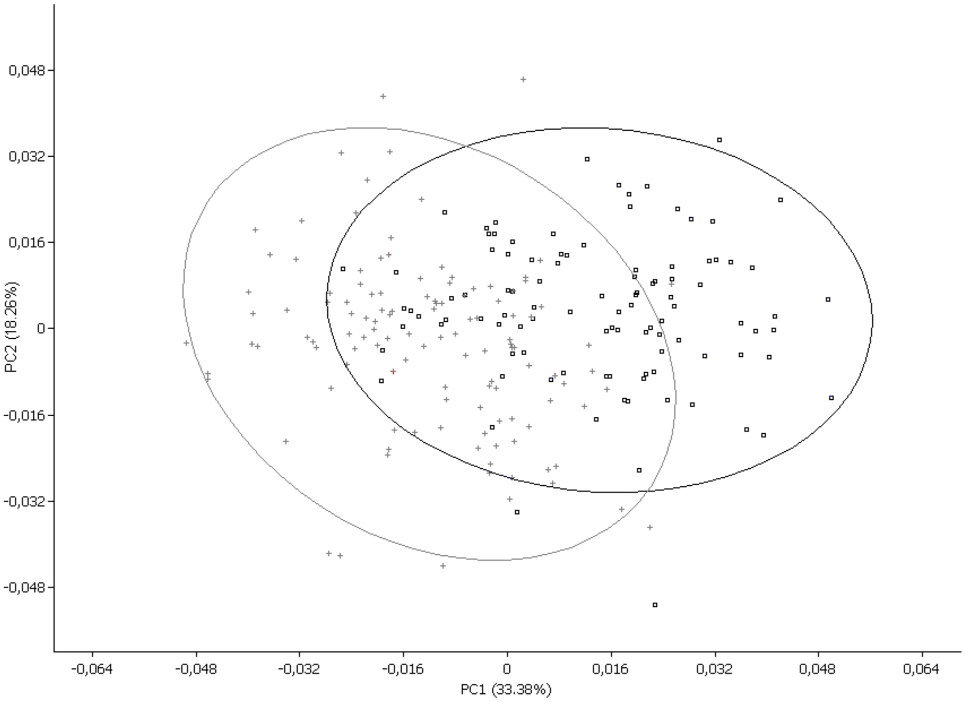

PCA 2 (0.01%)
1,5
1
0,5
0
-0,5
-1
-1,5
-2
-200
-150
-100
-50
0
50
100
150
PCA 1 (99.98%)

Figure 2. Principal Components Analysis plot of the linear wing traits of *D. iulia alcionea*. **A.** Forewing, **B.** Hindwing. The differences accumulated in the two first principal components are showed below each axis. Ellipses around data represent the 95% confidence interval for the data set. Black squares, females; gray crosses, males.

A


0,048
0,032
0,016
0
-0,016
-0,032
-0,048
PC2 (18.26%)
-0,064
-0,048
-0,032
-0,016
0
0,016
0,032
0,048
0,064
PC1 (33.38%)

B

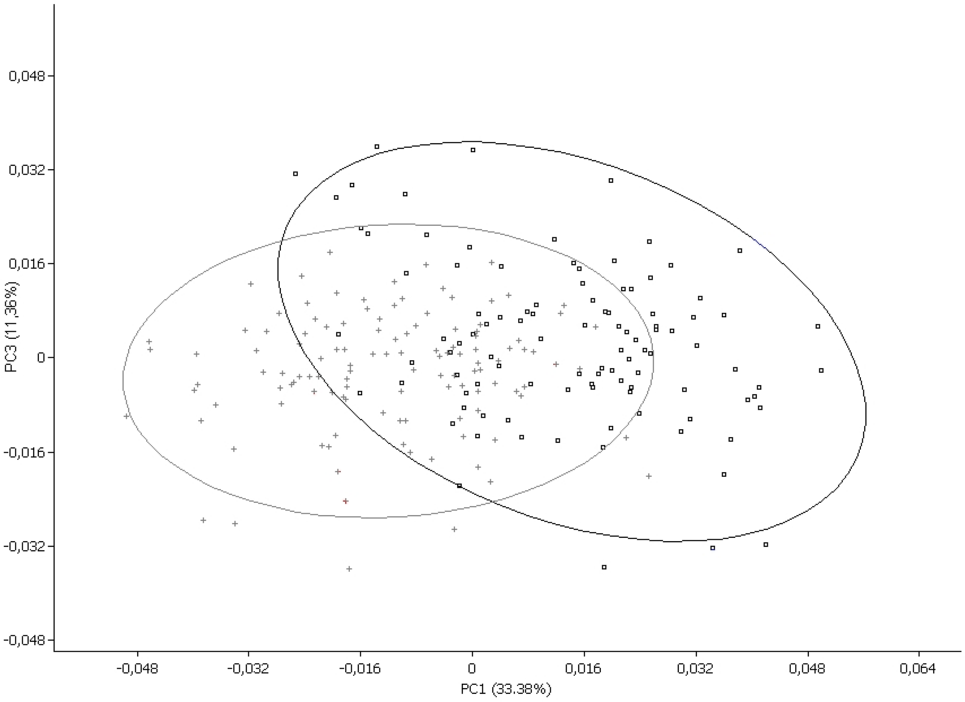
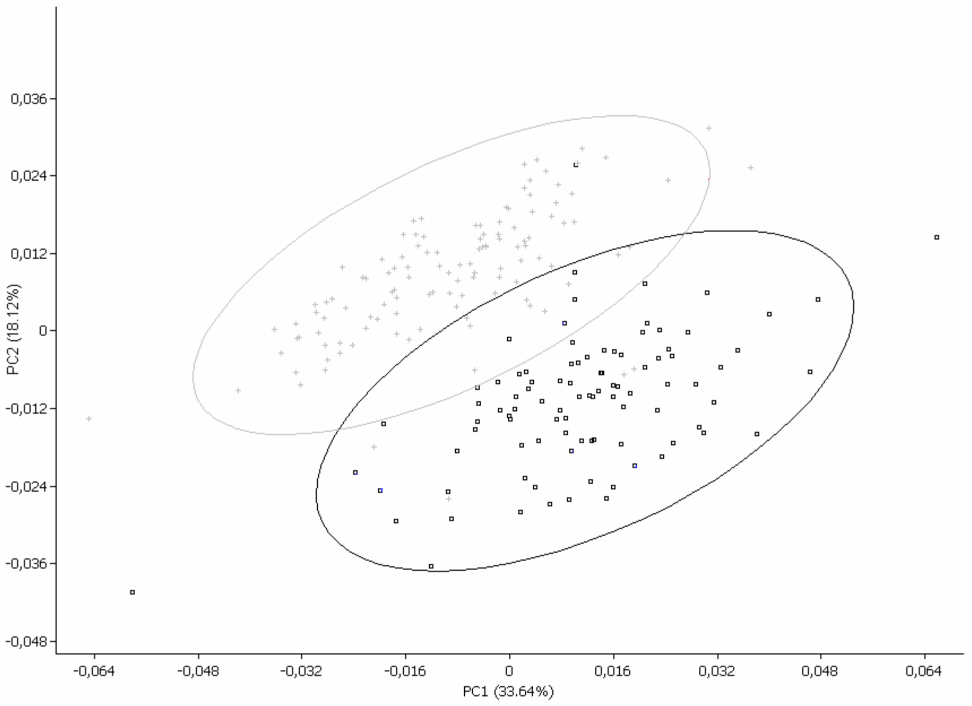

0,048
0,032
0,016
0
-0,016
-0,032
-0,048
PC3 (11,36%)
-0,048
-0,032
-0,016
0
0,016
0,032
0,048
0,064
PC1 (33.38%)

Figure 3. Principal Components Analysis plot of the most significant principal components (PC) of the shape variables of *D. iulia alcionea* forewing. **A.** PC1 *vs.* PC2; **B.** PC1 *vs.* PC3. The differences accumulated on each principal component are showed below each axis. Ellipses around data represent the 95% confidence interval for the data set. Black squares, females; gray crosses, males.

A


0,036
0,024
0,012
0
-0,012
-0,024
-0,036
-0,048
PC2 (18.12%)
-0,064
-0,048
-0,032
-0,016
0
0,016
0,032
0,048
0,064
PC1 (33.64%)

B

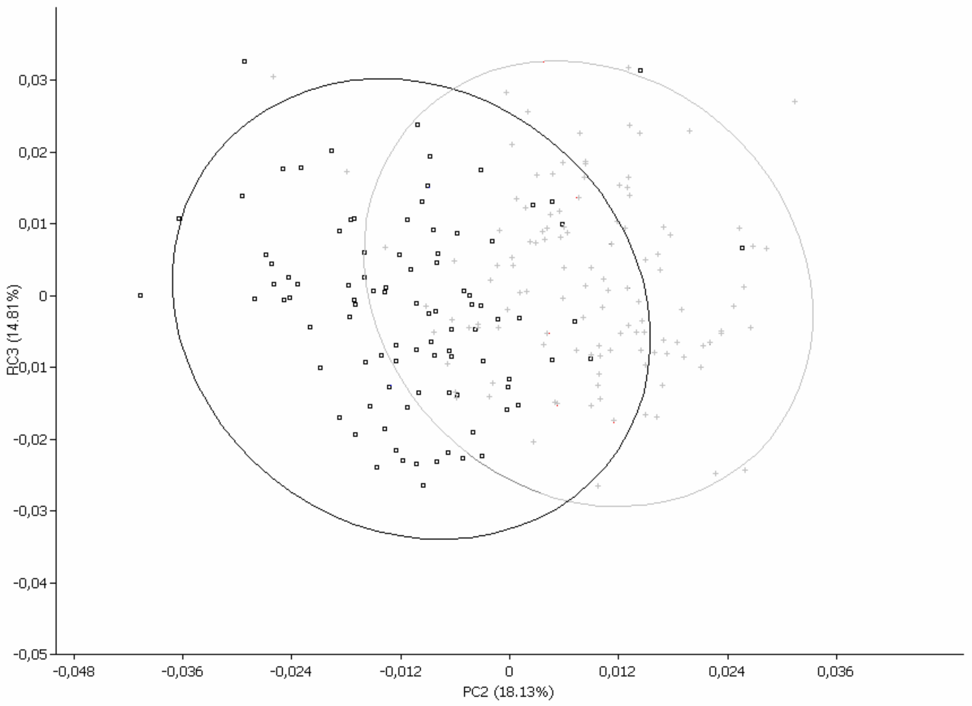

0,03
0,02
0,01
0
-0,01
-0,02
-0,03
-0,04
-0,05
PC3 (14.81%)
-0,048
-0,036
-0,024
-0,012
0
0,012
0,024
0,036
PC2 (18.13%)

Figure 4. Principal Components Analysis plot of the most significant principal components (PC) of the shape variables of *D. iulia alcionea* hindwing. **A.** PC1 *vs.* PC2; **B.** PC2 *vs.* PC3. Ellipses around data represent the 95% confidence interval for the data set. Black squares and ellipse, females; gray crosses and ellipse, males.

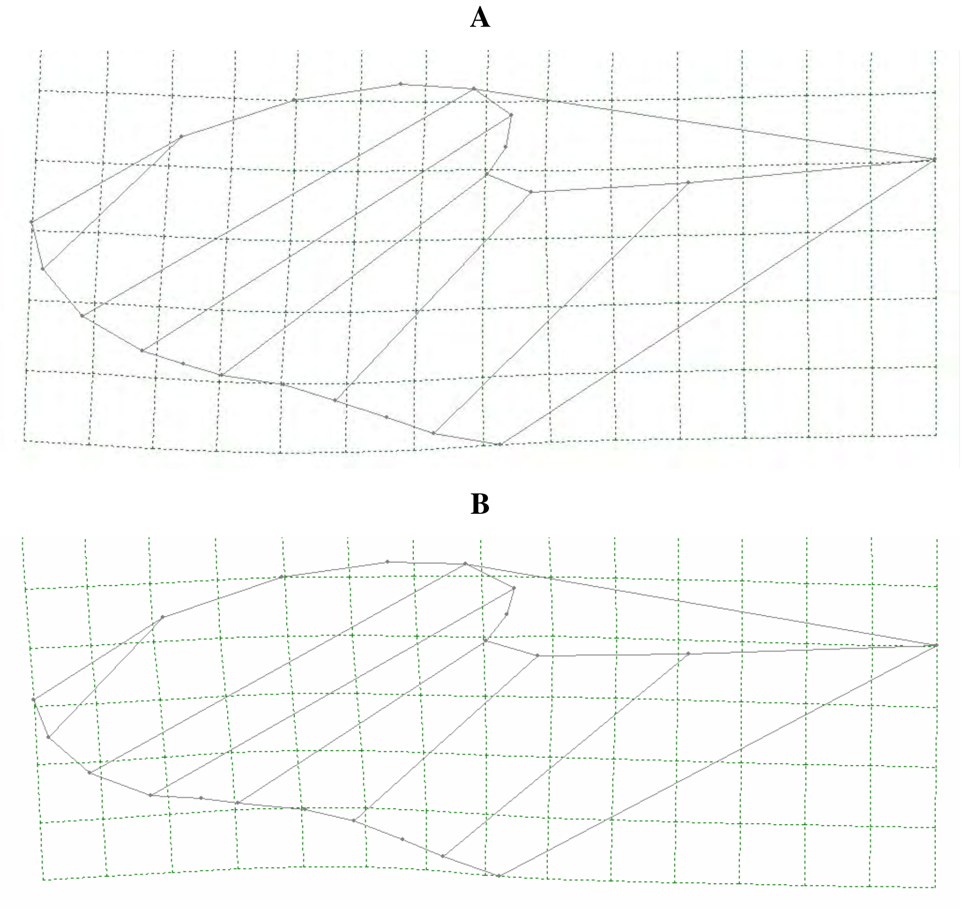


Figure 5. Forewing *D. iulia alcionea* wing deformations in relation to a consensus shape after relative warp analysis. **A.** female, **B.** male.

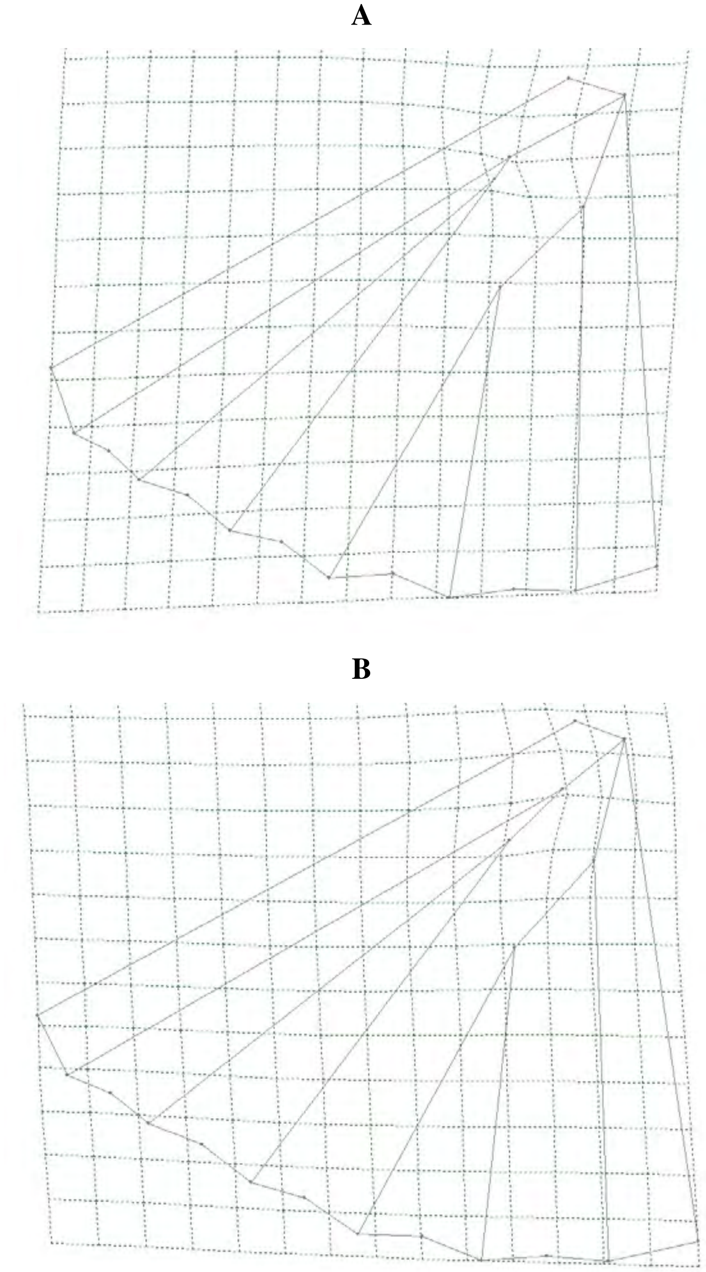


Figure 6. Hindwing *D. iulia alcionea* wing deformations in relation to a consensus shape after relative warp analysis. **A.** female, **B.** male.

Table 1. Wing measurements (mean ± standard deviation) on males and females *D. iulia alcionea* wings (N= 928; 689 males and 239 females). Linear distances were taken directly on the wings using a digital caliper, nearest 0.1mm. All differences between sexes were significant at $p<0.001$. CV, coefficient of variation.

A.

Forewing trait	Sex	Mean ± s.d.	CV
Length (mm)	Female	39.804 ± 1.995	0.050
	Male	41.270 ± 2.003	0.049
Width (mm)	Female	16.196 ± 1.004	0.062
	Male	16.556 ± 0.917	0.055
Area (mm^2)	Female	448.679 ± 48.203	0.107
	Male	479.167 ± 47.967	0.100
Perimeter (mm)	Female	91.249 ± 4.699	0.051
	Male	94.551 ± 4.629	0.049

B.

Hindwing trait	Sex	Mean ± s.d.	CV
Length (mm)	Female	21.930 ± 1.248	0.057
	Male	22.751 ± 1.189	0.052
Width (mm)	Female	19.210 ± 1.098	0.057
	Male	20.127 ± 1.054	0.052
Area (mm^2)	Female	340.639 ± 36.625	0.108
	Male	376.111 ± 37.258	0.099
Perimeter (mm)	Female	69.724 ± 3.786	0.054
	Male	73.147 ± 3.644	0.050

Table 2. Principal Components Analysis loadings plot of the linear measurements of *D. iulia alcionea* forewing and hindwing.

Forewing		Hindwing	
Wing Trait	PCA loadings	Wing Trait	PCA loadings
CAA	0.041	CAP	0.029
LAA	0.018	LAP	0.027
AAA	0.994	AAP	0.994
PAA	0.096	PAP	0.098

Table 3. Centroid size (mean ± standard deviation) on males and females *Dryas iulia* wings (N= 223; 120 males and 103 females). Measures were done using IMP CoordGen6f in landmark configuration prior to superimposition of 21 landmarks of the forewing and 19 on the hindwing. All differences between sexes were significant at p<0.001. CV, coefficient of variation.

	Sex	N	Mean ± s.d.	CV
Forewing	Females	103	1130.754 ± 59.750	0.053
	Males	120	1158.448 ± 49.581	0.043
Hindwing	Females	94	915.864 ± 52.937	0.058
	Males	119	946.304 ± 50.579	0.053

CAPÍTULO 6

WING SIZE AND MATING BEHAVIOR IN *DRYAS IULIA ALCIONEA* (LEPIDOPTERA, NYMPHALIDAE): ARE LARGER MALES MORE SUCCESSFUL IN ACHIEVING COPULATION THAN SMALLER ONES?

Artigo a ser submetido para o periódico *Journal of Insect Behavior*

Wing size and mating behavior in *Dryas iulia alcionea* (Lepidoptera, Nymphalidae): are larger males more successful in achieving copulation than smaller ones?

Nicolás O. Mega[1] and Aldo M. de Araújo[2]

[1]*Programa de Pós Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul.*
[2]*Departamento de Genética, Universidade Federal do Rio Grande do Sul*

Abstract: Female receptivity in butterflies is influenced by a multitude of factors that vary among females, and is frequently affected by the quality and persistence of courting males. Here we investigate whether wing size of males of *D. iulia alcionea* plays an important role in female choice through single and binary-choice experiments. We also tested the age-specific sexual latency of both sexes and the peak of sexual activity during the initial days of adult life. The results indicate that males and females differ with respect to the period sexual latency imago emersion. Females became sexually active more readily than males, but sexual activity lasted significantly less. Our results suggest that wing size does not play a role in male copulatory success. Males of different sizes have the same copulatory success and do not behave differently in relation to receptive females. The receptivity of the females do not differ to males with different wing-span. Three scenarios could explain the observed patterns of dimorphism and its evolution: selection of ecological sex differences, intrassexual selection and size-dependent parental investment. The mechanisms underlying *D. iulia alcionea* females mate choice are still uncertain.

Key-words: female choice, mating success, sexual selection, wing size, Heliconiinae

Introduction:

Female receptivity in butterflies and moths is influenced by a multitude of factors that vary among females, and is frequently affected by the quality and persistence of courting males (Wedell, 2005). The females decision about whether mating or not is essentially influenced by the result of internal physiological mechanisms during male courtship. Females are generally reluctant to mate and do so only after appropriate stimulation by males (Ringo 1996). Males often engage in courtship display prior to mating, which can function as an aphrodisiac to stimulate copulation. The display of courting males may be target of strong selection, since females usually to analyze male quality and viability (Thornhill & Alcock 1983, Andersson 1994).

In recent years, several studies have stressed the importance of pheromones to mate choice in insects, since they are predominantly used for intraspecific communication. The sex pheromones in Lepidoptera are involved for attraction of mates over long distances, but also can play a role in courtship behavior (Constanzo & Monteiro 2007). They may convey information about the prospective mates, such as quality and quantity of nuptial gifts, developmental stability, dominance status, body size, degree of relatedness, genetic quality and viability (Smith 1983, Thornhill 1992, Moore et al 1997, Wedell 2005). Other studies have inferred the importance of visual cues in mate recognition and acceptance in butterflies, like movement, size and wing color (Scott 1972, Andersson 1994 and references therein). In the genus *Heliconius,* a series of works suggests that visual signaling is extremely important for sexual activity (Crane 1955, Emsley 1970, Gilbert 1976, Jiggins et al. 2001, Lutz 2002, Jiggins et al. 2004). Apparently, in several species of *Heliconius* butterflies courtship evolved strongly towards long distance visual signaling (Jiggins et al 2001).

The body size of an individual is affected by genetic and environmental factors that operate through complex molecular and physiological mechanisms and directly influence fitness (West et al., 1999, Glazier, 2002, Nijhout, 2003). Its main effects are noticed directly on reproduction, survival and dispersal (Boggs, 1986; Honek, 1993; Torres-Vila et al., 1995; Rodrigues & Moreira, 2002, Van Dyck, 2003) and so can be considered an excellent indicator of male quality in mate choice.

Several studies have shown that females often choose their mate, favoring conspicuous male traits (for a review, see Andersson 1994). In Lepidoptera, selection on male size has been studied in *Phrynagida californica*, *Ephestia elutella* and *Heliconius sara*, among others (Mason 1969, Phelan & Baker 1986, Hernandez & Benson 1998). It has been suggested that body size can be decisive in succeeding territorial disputes, influencing fecundity and providing information on parental investment on offspring. If females prefer males with some special trait like those and if males differ in their range of attraction, females that search for and compare several males and mate with the most effective signaler should also tend to bear sons with higher than average mating success, leading to a evolutionary process known as runaway selection (Fisher 1915, 1930). In these cases, the evolution on the male’s trait is only stopped by natural selection.

Not only chemical and visual factors influencing mate choice and copulatory success affect mating behavior of individuals, but also the endogenous reproductive maturation state of young adults, which ensure they are ready to reproduce (Wedell 2005). After emergence of adults, some butterflies pass through a period of sexual latency, where many organs are not completely functional (Chapman 1998). At this stage, also called teneral, adult insects devote its time to feeding (Kirkton & Schultz 2001). In some Odonata, the function of this period is to complete sexual maturation and the acquisition of the adult color, mass and fat reserves. Males demand more fat for flying, while females may divert some of its reserves to egg manufacturing (Córdoba-Aguilar & Cordero-Rivera 2005).

Dryas iulia Fabricius is a nonterritorial butterfly that presents sexual dimorphism regarding wing color and size, in which the males are more colorful and larger than the females (Brown Jr. 1981, Haag and Araújo 1994, Mega & Araújo, submitted). Males and females also differ greatly in their foraging behavior; males spend most of their time searching for females, while females are frequently observed among vegetation searching for oviposition sites. Our previously field studies pointed out that the variability of wing size in males are always lower than the variability in females, apparently as if subjected to the action of stabilizing selection on male size (Mega & Araújo, submitted). Another possible reason could be the action of sexual selection on male wing size, played by female choice or by intrassexual competition among males.

Here we investigate whether wing size of male butterflies plays an important role in female choice. We also analyzed the period of sexual latency for both sexes and the peak of sexual activity during the initial days of adult life.

Material and Methods:

Butterfly stocks and test conditions

Experimental population was produced by crossing F1 of field-caught butterflies. Crossings were conducted with butterflies from different geographical localities to avoid inbreeding effects. Larvae were fed *ad libitum* on fresh leaves of passionflower (*Passiflora suberosa*) under controlled conditions in the laboratory (25ºC and 24h light). After emergence of the adults, butterflies of different sexes were kept apart in outdoor cages (3 x 3 x 3 m) to be used in the tests. This design ensured that all butterflies used had no previous sexual experience**.** During captivity, adults were fed with a moisture of distilled water and honey (3:1). All tests were performed in an outdoor flight cage similar to those described above, between 10 am and 2 pm at a temperature of 25-32ºC, only on sunny days and no wind. Observations lasted one hour per test, and both males and females were not used more than once, to avoid experience effects.

Determining the sex-specific age latency of sexual activity:

The sex-specific age latency of sexual activity of males and females in the first ten days of adulthood was tested using the butterflies from the stock as explained above. Virgin males with different ages (1-10 days) were placed in contact with a two-day virgin female and observed for one hour. Males were considered sexually active after displaying courting behavior to females; as soon as this has occurred observations were interrupted. Ten different males were used for each one of the 10 days of adulthood (n=100). To determine the age of receptiveness of females, virgin females with different ages of adulthood (1-10 days) were placed in contact with experienced males of at least ten days of adult life. It was considered receptive the female who exhibited signs of male acceptance (wing shutting – Mega & Araújo, submitted) when courted by males. Ten different females were used for each one of the 10 days of adulthood (n=100). Both procedures allowed the establishment of frequency distributions (courting for males and receptiveness for females), which were tested independently by a Kolmogorov-Smirnov Goodness of Fit for discrete ordinal scale (Zar 1996).

Copulatory success, behavioral differences between males and the response of females

To determine whether the size of male influences its copulatory success, we performed both single and binary-choice tests. In the single-choice tests, one male and one female were placed together in the same cage. Prior to each test, forewing length of the individuals were measured. Males were considered “large” when they were at least 10% larger than females, and “small” when they were at least 10% smaller than females (n=30 for small males, n=30 for large males). In the binary-choice tests two males (one small and one large) were placed together in the same cage with one female (n=30). All butterflies used in the experiments were within the period of sexually active adult life (females 2-4 days, males 5-8 days). Male copulatory success was computed as the number of males that were able to copulate with females and analyzed by with a χ^2-test (with Yates correction) in comparison with males that do not copulate. We also did a heterogeneity χ^2 (with Yates correction) to test whether the results obtained in the single and multiple-choice tests could be considered homogeneous.

The differences between courtship display of small and large males were compared by inspecting the frequencies of behavioral acts of males in each trial. The ethogram used for quantify courtship display was previously described by Mega & Araújo (submitted); here we include a short version of the table there presented (table 1). Each behavioral act listed in that table was taken as variables for a nonparametric multivariate analysis of variance (NPMANOVA), first to compare large and small males within each experimental design (single-choice and multiple-choice) irrespective of copulation success. Secondly, a comparison was made between large and small males regarding to copulatory success (successful and non-successful). The same procedure was used to analyze the response of females to the courting males of different sizes. NPMANOVA was applied according to Finch (2005), using a transformation of Pillai's trace into χ^2.

Results

Sexual latency and receptiveness

The age-specific sexual latency of males and females varied significantly in the first 10 days of adulthood ($D_{females}$=9.908, $0.02<p<0.05$; D_{males}=13.82, $0.005<p<0.01$). Females tested have proved to be receptive to the experienced males since the first day of adulthood. However, females were more receptive between the second and fourth days of adulthood, reducing considerably their receptiveness after the fifth day (figure 1A). There were no signs of receptivity of females after the eighth day. Males were more active in courting females after the fifth day of adulthood. After this day, over 80% of males tested showed intense courtship activity (figure 1B).

Copulatory success, behavioral differences between males and the response of females

The results of copulatory success of large and small males for single and binary-choice are shown in table 2A, B. In the single-choice tests, small males had higher frequency of copulatory success when compared with large males, however these differences were not statistically significant (χ^2_{Yates}=1.816, df=1, p=0.178). In the binary-choice tests males of different sizes had exactly the same copulatory performance (χ^2_{Yates}=0.267, df=1, p=0.718). Heterogeneity test revealed that results from single and binary-choice are homogeneous (χ^2_{Yates}=0.139, df=1, p=0.709), so results of both experimental designs can be considered consistent.

The analysis of NPMANOVA showed no significant differences between large and small males, both for single-choice (χ^2=6.655, df=20, p=0.998) and binary-choice tests (χ^2=7.801, df=20, p=0.993). Males with different sizes also did not show differences regarding copulatory success (Single-choice - without copula, χ^2=8.216, df=20, p=0.990; with copula, χ^2=16.925, df=20, p=0.678; binary-choice - without copula, χ^2=11.264, df=20, p=0.939; with copula, χ^2=12.362, df=20, p=0.903). The response of females to males of different sizes did not differ significantly in any of the experimental designs used (Single-choice - χ^2=10.816, df=16, p=0.821; multiple-choice - χ^2=11.387, df=16, p=0.785). Females showed the same response to males of different size when the occurrence of copula was concerned (single-choice - without copula, χ^2=10.322, df=16, p=0.849; with copula, χ^2=8.550, df=16, p=0.931; multiple-choice - without copula, χ^2=7.260, df=16, p=0.968; with copula, χ^2=8.442, df=16, p=0.935).

Discussion

Sexual activity, reproductive maturity and mating strategies

Our results clearly indicate that males and females differ with respect to the period of sexual latency in their first ten days of adult life. Females become sexually active more readily than males, although their sexual activity (receptivity) lasts significantly less than that of males. As the main strategy of searching for mating in *D. iulia* is the patrolling of males, the difference of sexual maturity between the sexes could be explained partly for the time needed to complete hardening and accommodation structures involved in the flight activity. The teneral stage of flying insects does not prevent individuals from play their basic activities for body maintenance, as searching for food and shelter against bad weather, but hampers the achievement of very complex activities, such as the courtship. *Dryas iulia alcionea* females do not actively search for males and do not depend directly from courtship to mate, so the full development of the flight apparatus and the associated muscles are not a limiting factor. The maturity of female gonads also would needs to be taken into account, since the existence of a *bursae copulatrix* and a spermateca guarantee that the stock of sperm is ready to be used when the eggs are mature (Drummond 1984). Sperm storage is almost ubiquitous in insects and it is often stated that females need only mate a single time in order to obtain enough sperm to fertilize one the ova they will produce during their lifespan. Also, many insect females become increasingly unreceptive to mating as they age and will kick out males attempting to gain any genital contact (Simmons 2001). Thus, the investment strategy of the female seems to be focused on a quickly achieving of sperm and then invest primarily in oviposition, which could be considered a long-term reproductive investment. The larger initial period of sexual latency of males, coupled with the low investment in spermatofore production and the extension of sexual activities until the end of his life, could be interpreted as both short and medium-term reproductive investment. This larger initial latency in sexual activities, ensure the full development of structures involved in flight and courtship. It would also favor the full development of gonads, ensuring that the production of spermatofores would contain a larger amount of viable gametes, able to fertilize females' eggs. This hypothesis is supported with results obtained with laboratory rearing and observation of adult activities in captivity, where females bred in captivity live longer than males bred under the same conditions (females, 44 days; males, 31 days).

On the other hand, even taking into account that *D. i. alcionea* has a great dispersal ability, the asynchronic sexual maturation between sexes could be a way to avoid inbreeding. This later possibility is grounded in a former study with this butterfly where estimates of the genetic load were done (Haag & Araújo, 1994).

The physiological mechanisms that promote the change from latency to plain sexual activity are far from being understood. However, several factors have been identified, including control by portions of central nervous system, diet, hormones, visual cues, pheromone and cheamical communication (Ringo 1996, Simmons 2001, Rutowski 2003, Wedell 2005). In insects, the ontogeny of receptivity of females is coordinated with the temporal pattern of mating and in most of species females go through cycles of receptivity and unreceptivity. They usually begin adulthood unreceptive and later develop an initial receptivity, which is generally lost quickly after mating. Postmating receptivity can arise after mating or later in adult life which again declines sharply after a new mating (Ringo 1996). For males, many authors have stressed that biophysiological constraints are important in behavioral changes and contest ability of males (see discussion on Kemp 2002). The importance of life-history constraints and energetic intake must be taken into account when interpreting this kind of processes (see Kokko 1997 and references therein). In *Drosophila*, testis growth occurs predominantly during teneral stage so sperm production requires long pre-reprodutive periods (Pitnick 1993).

Male size and differences in copulatory success

The results presented here suggest that males of different sizes have the same copulatory success and that, apparently, large and small males do not behave differently towards sexually active females. As for female receptiveness, it was not observed preference by males with larger wing span. Thus, the results presented in this study strongly suggest that wing size does not matter in copulatory success of males as far as female choice is concerned. It should be reminded, however, that the experimental design used here was not able to isolate chemical cues, so we can not discard them as important. Nothing is known either about the preference of female based on UV-reflectance, as demonstrated for *Bicyclus anynana* (Robertson & Monteiro, 2005). Probably, the cues used for discrimination include both chemical and visual signals, as demonstrated for other butterflies (Silberglied 1984, Yamashita 1995, Rutowski 1997). Theoretical models suggest that female preference for multiple traits may evolve when the cost of choice is low and when different signals indicate distinct components of quality (Van Doorn & Weissing 2004).

Independent of which cue is used to choose a mate, mating usually contains an element of discrimination, at least with respect to species identity, otherwise mismatings between different species should be more common than they are (Andersson 1994). A female indicates her receptivity by allowing or helping the male to fertilize her eggs. Thus, fertilization seems at least partly controlled by female behavior. Among the acceptable signals, sexual selection should favor those which most effectively stimulate the recipients, like intensity and persistence, or conspicuous signals (Ryan 1990a, 1990b). It has long been known that scent is used for mating (Jacobson 1972, Lloyd 1979, Thornhill 1979, Greenfield 1981, Grant 1987). Many studies have inferred the use of chemical cues as an additional signal to visual cues during mating in Lepidoptera (Jiggins et al. 2001, Fordyce et al 2002, Contanzo & Monteiro 2007). Rutowski (1997) states that no chemical signals produced by a female butterfly are known to attract or affect the behavior of males at a distance of more than a few centimeters. Instead, male butterflies typically locate females by looking for them.

Even assuming that other factors may influence the choice of *D. iulia* females, they can not explain the patterns of wing size variation found for males and females. At least three scenarios, which are not mutually exclusive, could explain the observed patterns of wing dimorphism and its evolution: selection of ecological sex differences, intrassexual selection and size-dependent parental investment.

First, ecological differences between sexes seem to explain some sex dimorphic traits used for feeding and foraging in many species. Ecological sex differences may evolve under different ecological pressures if sexes compete over some resource. Ecological sex differences may first arise owing to sexual selection, later on being engarled by natural selection (Andersson 1994). If we think in aerodynamics efficiency, *D. iulia* males seem to have a wing shape optimized to flight through long distances with high energetic efficiency, mainly for mating behavior, while females seem to have a wing shape optimized to hovering flight, essentially for inspection of host plants and oviposition. This possibility matches with field observations. Males spend most of their time searching for females, while females spend it searching for host plants for oviposition (Mega & Araújo, unpublished). In *D. iulia*, due to notable differences in behavioral ecology of each sex, selection may act differently on males and females allowing sexual dimorphism to evolve.

Second, butterfly flight performance depends on the thoracic muscles, articulation between thorax and wings, and the wings themselves that generate the aerodynamic forces. The wing form is very important to flight, since they are responsible for the air trajectories and body kinematics (Dudley 2000). Males with different wing sizes would have distinct flight capabilities, particularly for acceleration, maneuverability and resistance. Males with smaller wing-span have wings tending to an elliptical shape, a type of wing that favors quick and explosives flights with short duration. This type of wing is commonly found in species with territorial behavior. Males with large wing-span, on the contrary, tend to have wings in triangular format, which favors prolonged flights with little energy waste. This type of wing is commonly found in species with patrolling behavior. Another Heliconiinae, *Heliconius sara*, can either guard small dispersed territories and court passing females (resident males) or search actively for females and pupa (patrolling males). Small males seem to have advantage on guard territories, but large males have more advantage in locate female pupae, which attract males pheromonally (Deinert et al 1994, Hernandez & Benson 1998). Since *D. iulia* males do not show resident behavior, large males may have advantage in mating when they search actively for females, as shown for *H. sara*. As wing size is inherited (Mega & Araújo, submitted), it is expected that larger males have larger offspring, and population may evolve toward large male wing size.

Third, males with large wing-span may signal that they have a big body size. Since organs of many insects grow isometrically to their body size, it is expected that males with larger testes are capable of producing a larger sperm when compared to smaller males, as described for several species of *Drosophila* (Pitnick et al 1995). The same pattern is expected for sperm volume. Thus, larger males would be able to fertilize a larger number of eggs. Indeed, sperm possibly have a secondary, nutritive function in some species (Thornhill & Alcock 1983). Butterfly males may produce seminal fluids that provide females with extra nutrition before egg laying (Boggs & Gilbert 1979, Boggs 1981). In some Orthoptera, females may increase egg laying quantity and speed as an effect of nutrients received from spermatophore (Butlin et al 1987). Once again, evolution may indirectly favor large males.

Mating therefore usually implies competition over mates in, at least, one of the sexes, even if rivals never meet each other (Andersson 1994). Competition over mates can take several forms and favor a wide range of attributes (Thornhill & Alcock 1983, Wickman & Wiklund 1983; Rosenberg & Enquist 1991; Kemp 2000, Berwaerts et al 2002, Berwaerts et al 2006). The mechanisms underlying mate choice by *D. iulia alcionea* females are still uncertain, as well as the traits that favor male copulatory success.

Acknowledgements

We would like to thank A.A. Ferreira, A. Carrion, A.L. Klein, G. Pasqualin, J. Zanin e P.R. Vieira for helping in keeping the caterpillar and butterfly stocks. We also thank F. Costa for suggestions in the initial experimental design. Financial support was provided by Conselho Nacional de Desenvolvimento Tecnológico (CNPq) and Programa de Apoio a Núcleos de Excelência (PRONEX).

References:

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton, New Jersey. 624 pp.

Berwaerts, K., Van Dyck, H., Aerts, P. 2002. Does flight morphology relate to flight performance? An experimental test with the butterfly Pararge aegeria. Functional Ecology, 26:484–491.

Berwaerts, K., Aerts, P., Van Dyck, H. 2006. On the sex-specific mechanisms of butterfly flight: flight performance relative to flight morphology, wing kinematics, and sex in Parage aegeria. Bio. Journal of the Linnean Society, 89: 675-687.

Boggs, C.L. 1981.Pressures Affecting Male Nutrient Investment at Mating in Heliconiine Butterflies. Evolution, 35: 931-940.

Boggs, C.L. 1986. Reproductive strategies of female butterflies: variation in and constrains on fecundity. Ecol. Entomol. 11: 7-15.

Boggs, C.L. & Gilbert, L.E. 1979. Male contribution to egg production in butterflies: evidences for transfer of nutrients at matting. Science, 206: 83-84.

Brown Jr., K.S. 1981. The biology of Heliconius and related genera. Annual Review of Entomology 26: 427–456.

Butlin, R.K., Woodhatch, C.W. & Hewitt, G.M. 1987. Male spermatophore investment increases female fecundity in a grasshopper. Evolution 41, 221-225.

Chapman, R.F. 1998. The Insects: Structure and Function. Cambridge University Press, Cambridge, 788pp.

Contanzo, K. & Monteiro, A. 2007. The use of chemical and visual cues in female choice in the butterfly Bicyclus anynana. Proceedings of the Royal Society B, 274: 845-851.

Córdoba-Aguilar, A. & Cordero-Rivera, A. 2005. Evolution and Ecology of Calopterygidae (Zygoptera: Odonata): Status of Knowledge and Research Perspectives Neotropical Entomology 34(6):861-879.

Crane, J. 1955. Imaginal behavior of a Trinidad butterfly, Heliconius erato hydara Hewitson, with special reference to the social use of color. Zoologica 40(16): 167-195.

Deinert, E. I. 2003. Mate location and competition for mates in a pupal mating butterfly. Pp. 91–108 in Boggs, C. L., Watt, W. B. & Ehrlich, P. E. (eds). Butterflies: ecology and evolution taking flight. The University of Chicago Press, Chicago, 739pp.

Deinert, E.I., Longino, J.T. & Gilbert, L.E. 1994. Mate competition in butterflies. Nature, 370:23 – 24.

Drummond, B A. 1984. Multiple mating and sperm competition in the Lepidoptera. In: Smith, R. L. (ed.), Sperm Competition and the Evolution of Animal Mating Systems, Academic Press, New York, pp. 291–370.

Dudley, R. 2000. The biomechanics of insect flight. Princeton, Princeton University Press, 476pp.

Emsley, M.G. 1970. An observation on the use of color for species-recognition in Heliconius besckei (Nymphalidae). J. Lepid. Soc 24(1): 25.

Finch, H. 2005. Comparison of the Performance of Nonparametric and Parametric MANOVA Test Statistics when Assumptions Are Violated. Methodology 1(1):27-38.

Fisher, R.A. 1915. The evolution of sexual preference. Eugenics Review 7: 184-192.

Fisher, R.A. 1930. The Genetical Theory of Natural Selection. Clarendon Press, Oxford.

Fordyce, J.A., Nice, C.C., Forister, M.L. & Shapiro, A.M. 2002. The significance of wing pattern diversity in the Lycaenidae: mate discrimination by two recently diverged species. Journal of Evolutionary Biology 15, 871-879.

Gilbert, L.E. 1976. Postmating female odor in Heliconius butterflies: a male-contributed antiaphrodisiac? Science 30 July 1976: 193 (4251): 419 - 420.

Glazier, D.S. 2002. Resource-allocation rules and the heritability of traits. Evolution 561(8): 1696-1700.

Grant, G.G. 1987. Copulatory behaviour of spruce budworm, Choristoneura funiferana (Lepidoptera: Tortricidae): experimental analysis of the role of sex pheromone and associated stimuli. Annals of Entomological Society of America, 80: 78-88.

Greenfield, M.D. 1981. Moth sex pheromones: An evolutionary perspective. Florida Entomologist, 64: 4-17.

Haag, L.H & Araújo, A.M. 1994. Inbreeding, genetic load and morphometric variation in natural populations of Dryas iulia (Lepidoptera, Nymphalidae). Rev. Brasil. Genet. 17(1): 35-39.

Hernández, M.I.M. & Benson, W.W. 1998. Small-male advantage in the territorial tropical butterfly Heliconius sara (Nymphalidae): a paradoxical strategy? Animal Behaviour 56: 533-540.

Honêk, A. 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. Oikos 66(3): 483-492.

Jacobson, M. 1972. Insect Sex Pheromones. Academic Press, New York.

Jiggins, C.D., Estrada, C., Rodrigues, A. 2004. Mimicry and the evolution of pre-mating isolation in Heliconius melpomene. Journal of Evolutionary Biology 17(3): 680–691.

Jiggins, C.D., Naisbit, R.E., Coe, R.L., & Mallet, J. 2001 Reproductive isolation caused by colour pattern mimicry, Nature 411 (6835): 302–305.

Kemp, D.J. 2000. Contest behaviour in territorial male butterflies: does size matter? Behavioral Ecology 11:591–596.

Kemp, D.J. 2002. Butterfly contests and flight physiology: why do older males fight harder? Behav Ecol 13:456–461.

Kokko, H. 1997. Evolutionarily stable strategies of age-dependent sexual advertisement. Behavioral Ecology and Sociobiology, 41: 99-107.

Kirkton, S.D. & T.D. Schultz. 2001. Age-specific behavior and habitat selection of adult male damselflies, Calopteryx maculata (Odonata: Calopterygidae). Journal of Insect Behaviour, 14: 545-555.

Lande, R. 1980. Sexual dimorphism, sexual selection and adaptation in polygenic characters. Evolution 34: 292-305.

Lloyd, J.E. 1979. Mating behavior and natural selection. Florida Entomologist, 62: 17-34.

Lutz, L. 2002. Contextos fenotípicos multifuncionais: influência do padrão de coloração de uma borboleta impalatável (Heliconius erato phyllis) sobre seu sucesso de acasalamento, ritmo diário de atividades e sobrevivência em campo. Ph.D. thesis. Department of Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Mason, L.F. 1969. Mating selection in the California oak moth (Lepidoptera: Dioptidae). Evolution 23: 55-58.

Moore, P. J., Reagan-Wallin, N. L., Haynes, K. F. & Moore, A. J. 1997 Odour conveys status in cockroaches. Nature 389, 25.

Nijhout, H.F. 2003. The control of body size in insects. Develop. Biol. 261: 1–9.

Pitnick, S. 1993. Operational sex ratios and sperm limitation in populations of Drosophila pachea. Behavioral Ecology and Sociobiology 33:383-391.

Pitnick, S., Spicer, G.S. & Markow, T.A. 1995. How long is a giant sperm? Nature 375, 109.

Phelan, P.L. & Baker, T.C. 1986. Male-size-related courtship success and intersexual selection in the tobacco moth,Ephestia elutella. Journal Cellular and Molecular Life Sciences 42, 1291-1293.

Ringo, J. 1996. Sexual Receptivity in Insects. Annual Review of Entomology, 41: 473-494.

Robertson, K.A. & Monteiro, A. 2005. Female Bicyclus anynana butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. Proceedings of the Royal Society of London B, 272: 1541-1546.

Rodrigues, D. & Moreira, G.R.P. 2002. Geographical variation in larval host-plant use by Heliconius erato (Lepidoptera: Nymphalidae) and consequences for adult life history. Braz. J. Biol. 62(2): 321-332.

Roff, D.A. 1986. The evolution of wing dimorphism in insects. Evolution, 40: 1009-1021.

Rosenberg, R.H. & Enquist, M. 1991. Contest behaviour in Weidemeyer's admiral butterfly Limenitis weidemeyerii (Nymphalidae): the effect of size and residency. Animal Behavior, 42: 805–811.

Ryan, M.J. 1990a. Signals, species, and sexual selection. American Scientist 78: 46-52.

Ryan, M.J. 1990b. Sexual selection, sensory systems and sensory exploitation. Oxford Surveys in Evolutionary Biology 7: 157-195.

Rutowski, R.L. 1997. Sexual dimorphism, mating systems and ecology in butterflies. In The evolution of mating systems in Insects and Arachnids. Edited by J.C. Choe. Library of Congress, Cambrigde. 397pp.

Rutowski, R. L. 2003. Visual ecology of adult butterflies. In: C. L. Boggs, W. B. Watt, and P. R. Ehrlich (eds), Butterflies: Ecology and Evolution Taking Flight. University of Chicago Press, Chicago. Pgs. 9-25.

Scott, J.A. 1972. Mating of Butterflies. Journal of Reseach on the Lepidoptera 11(2): 99-127.

Silberglied, R. E. 1984 Visual communication and sexual selection among butterflies. In The biology of butterflies (ed. R. I. Vane-Wright & P. E. Ackery), pp. 207–223. London: Academic Press.

Simmons, L.W. 2001. Sperm Competition and Its Evolutionary Consequences in the Insects. Princeton University Press, Princeton, 448 pp.

Slatkin, M. 1984. Ecological causes of sexual dimorphism. Evolution 38: 622-630.

Smith, B.H. 1983. Recognition of Female Kin by Male Bees through Olfactory Signals. Proceedings of the National Academy of Sciences of the United States of America, 80: 4551-4553.

Thornhill, R. 1979. Male and females sexual selection and the evolution of mating systems in insects. In. M.S. Blum and N.A. Blum eds. Sexual Selection and Reproductive Competition in Insects, 81-121. Academic Press, New York.

Thornhill, R. 1992. Female preference for the pheromone of males with low fluctuating asymmetry in the Japanese scorpionfly (Panorpa japonica: Mecoptera). Behavioral Ecology 3: 277-283.

Thornhill, R. & J. Alcock, 1983. The Evolution of Insect Mating Systems. Harvard University, Cambridge, 547pp.

Torres-Vila, L.M., Stockel, J. & Roehrich, R. 1995. Le potentiel reproducteur et ses variables biotiques associées chez le mâle de l'Eudémis de la vigne Lobesia botrana. Entomol. Exp. Appl. 77(1): 105-119.

Trivers, R.L. 1972. Parental investment and sexual selection, p.136-179. In: B. Campbell (Ed.). Sexual Selection and the Descent of Man. Chicago, Aldine, 378p.

van Dick, H. 2003. Mate Location: a matter of design? Adaptive morphological variation in the speckled wood butterfly. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, 739pp.

van Doorn, G.S. & Weissing, F.J. 1994. The evolution of female preferences for multiple indicators of quality. The American Naturalist, 164: 173-186.

Yamashita, K. 1995. Comparison of visual cues in mating behavior of four species of swallowtail butterflies, pp 133-144. In J. Scriber, Y. Tsubaki, R. Lederhouse, eds. Swallowtail Butterflies: Their Ecology and Evolutionary Biology. Gainesville, FL: Scientific Publishers.

Wedell, N. 2005. Female receptivity in butterflies and moths. Journal of Experimental Biology, 208: 3433-3440.

West, G.B., Brown, J.H. & Enquist, B.J. 1999 The fourth dimension of life: fractal geometry and allometric scaling of organisms. Science 284, 167–169.

Wickman, P-O. 1992. Sexual Selection and butterfly design – a comparative study. Evolution, 46: 1525-1536.

Wickman, P-O. & Wiklund, C. 1983. Territorial defence and its seasonal decline in the speckled wood butterfly (Pararge aegeria). Animal Behavior, 31: 1206–1216.

Wiklund, C. 2003. Sexual selection and the evolution of butterfly mating systems. In Butterflies: Ecology and the evolution taking flight. Ed. C. Boggs, W.B. Watt e P.R. Ehrlich. University Press, Chicago, 739pp.

Zar, J.H. 1996. Biostatistical Analysis, 3rd ed. Prentice Hall, New Jersey, 662pp.

Table 1. *Dryas iulia alcionea* behavioral acts of used for quantitative analysis courtship display of males of different size (**A**) and the response of females for their activity (**B**). Reproduced with modifications from Mega & Araújo (submitted).

A.

Males	
Behavior Act	**Meaning**
Wing clapping	courtship
Overflight	female recognition
Hovering	courtship
Flight pursuit	courtship
Spiraling flight	courtship
Alighting on wings	induce female fight
Parallel alighting	copulation attempt
Pushing with the proboscis	courtship
Touching with the head	courtship
Wing lever	force copulation position
Abdominal bending	copulation attempt

B.

Females	
Behavior Act	**Meaning**
Wing spreading	courtship solicitation
Pressure of wings	male rejection
Abdomen raising	male rejection
Wing flapping	male rejection
Alighting on wings	courtship solicitation
Overflight	courtship solicitation
Hovering	courtship solicitation
Fight pursuit	male rejection
Wing shutting	male acceptance

Table 2. Copulatory success (the number of males that were able to copulate with females) of large and small males of *D. iulia alcionea*. **A.** single-choice, **B.** binary-choice. Values in each table cell represent the number of males that copulated (successful courtships) or did not copulate (unsuccessful courtships) with females during trials. Results of χ^2-*tests* for each experimental design are shown below the tables.

A.

	Successful Courtships	Unuccessful Courtships	Total
Large Males	11	17	28
Small Males	16	12	28
Total	28	28	56

$\chi^2_{(1)Yates} = 1.816; p = 0.178$

B.

	Successful Courtships	Unuccessful Courtships	Total
Large Males	7	8	15
Small Males	7	8	15
Total	14	16	30

$\chi^2_{(1)Yates} = 0.267; p = 0.718$

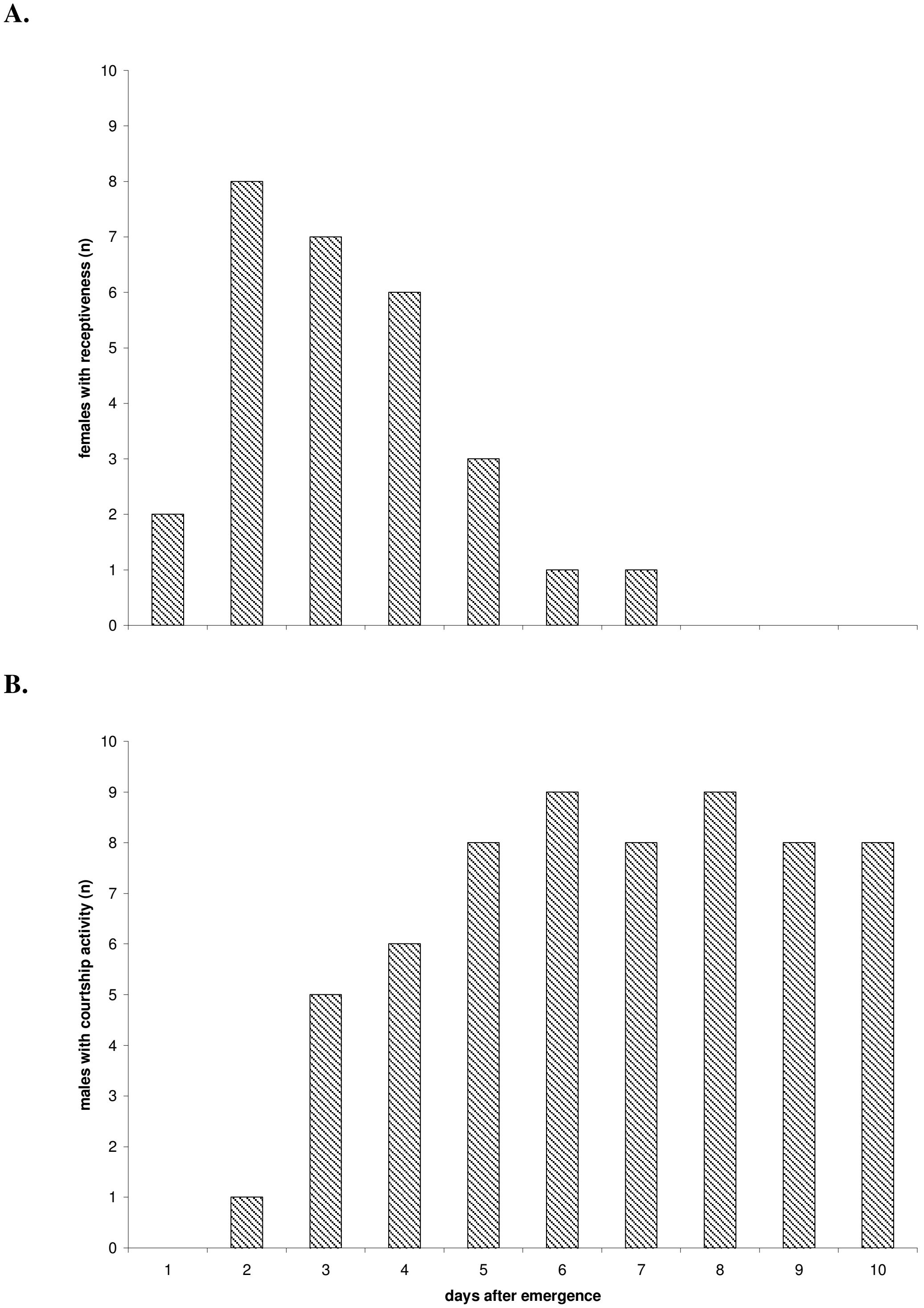


Figure 1. Receptiveness (females, **A**) and courting activity (males, **B**) in sexual naïve individuals of *D. iulia alcionea* tested in the first 10 days of adulthood. Each age category was tested with 10 replicates and frequencies computed as the total number of individuals that were sexual active. Females were tested with at least 10-day experienced males; males were tested with 2-day receptive virgin females.

CAPÍTULO 7

DISCUSSÃO FINAL

DISCUSSÃO

Ao longo desta tese, foram analisadas e discutidas algumas das diferenças sexuais encontradas em *D. i. alcionea*. As principais resultados e conclusões desta tese foram:

1. Os machos são maiores do que as fêmeas a apresentam menor variação de características ligadas ao tamanho das asas.
2. A forma das asas dos machos parece ser adaptada para vôos de longa duração, a forma das asas das fêmeas adaptada para vôos curtos com alta manobrabilidade.
3. O repertório comportamental de acasalamento dos machos é maior do que o das fêmeas e diretamente relacionado a tentativa de efetuar cópula.
4. Os machos buscam e cortejam ativamente as fêmeas.
5. As fêmeas parecem ter um papel decisivo no sucesso copulatório dos machos, já que a intensidade do cortejo dos machos parece não influenciar o sucesso copulatório.
6. Os machos com padrões comportamentais menos variáveis parecem ter maior sucesso copulatório.
7. Os machos grandes e pequenos se comportam da mesma maneira frente a fêmeas receptivas, sugerindo que não existem estratégias alternativas para compensar efeitos causados por tamanhos corporais diminutos.
8. O tamanho dos machos não influencia a escolha das fêmeas.
9. Não há disputa direta entre machos, com comportamento agonístico.
10. O tamanho das asas é uma característica com um moderado fator genético.
11. A correlação genética entre as características das asas dos machos e fêmeas é imperfeita ($r \neq 1{,}0$), o que permitiria ou indicaria a ação de seleção sobre o dimorfismo sexual de tamanho encontrado para as características das asas.
12. A correlação entre características das asas anteriores e posteriores é baixa, havendo uma grande independência genética entre as características dos dois conjuntos de asas.
13. O tamanho apresenta significativa variação fenotípica entre populações e estações do ano, sendo fortemente influenciado pelo recurso alimentar e temperatura durante a fase de desenvolvimento.

Para explicar o dimorfismo sexual encontrado em *D. i. alcionea* com base nos resultados encontrados, uma série de fatores deve ser considerada. Um bom ponto de partida pode ser a própria existência do dimorfismo na espécie e suas implicações para a evolução da espécie. Em Lepidoptera, uma variedade de diferenças morfológicas, além das gônadas e genitálias, é comumente encontrada. Essas incluem diferenças na coloração e forma das asas, tamanho e proporção corporal, e presença de estruturas produtoras e receptoras de sinais (Rutowski, 1997). O tamanho e a natureza dessas diferenças podem ser explicados basicamente de duas formas: (1) como resultado de pressões seletivas atuando sobre diferenças pré-existentes entre machos e fêmeas ou (2) através de pressões de seleção diferentes atuando independentemente em cada sexo (Slatkin 1984, Shine 1989, Mueller 1990).

Talvez, o tamanho corporal seja a diferença sexual mais comum e conspícua entre machos e fêmeas de muitas espécies. Em Lepidoptera, as fêmeas são geralmente maiores que os machos. A explicação desse desvio em favor das fêmeas seria a relação direta encontrada entre o tamanho corporal e sucesso reprodutivo. Fêmeas maiores seriam capazes de produzir um maior número de ovos, o que refletiria em uma prole maior. Essa vantagem poderia ser manifestada não somente no número de ovos, mas na qualidade dos mesmos, produzindo uma prole de melhor qualidade ou viabilidade. Darwin (1871) chamou esse processo de hipótese da vantagem de fecundidade. Alguns trabalhos sugerem que o mesmo efeito poderia ser observado entre machos, de modo que indivíduos grandes transfeririam uma quantidade maior de esperma e obteriam maior sucesso reprodutivo (para discussão completa, ver Simmons 2001). Contudo, a evolução de tamanhos corporais muito avantajados não pode progredir indefinidamente, devido a efeitos atuantes na viabilidade do organismo.

Alguns modelos mostram que a estabilidade do tamanho corporal é promovida pelas relações de custo e benefício que atuam sobre a variação de tamanho existente em grupos de indivíduos (ver discussão em Andersson 1994). Efeitos limitantes são comumente impostos a organismos muito grandes em termos de viabilidade, além de serem limitados por fatores genéticos, filogenéticos, fisiológicos e de desenvolvimento (Blanckenhorn 2000, 2005). Essas limitações acabam sendo equilibradas diferencialmente entre os sexos, resultando no dimorfismo sexual de tamanho. O modelo do equilíbrio diferencial é amplamente aceito para diversas espécies, contudo requer a demonstração completa dos custos e benefícios do tamanho corporal (Arak 1988, Andersson 1994, Blanckenhorn 2005, figura 1).

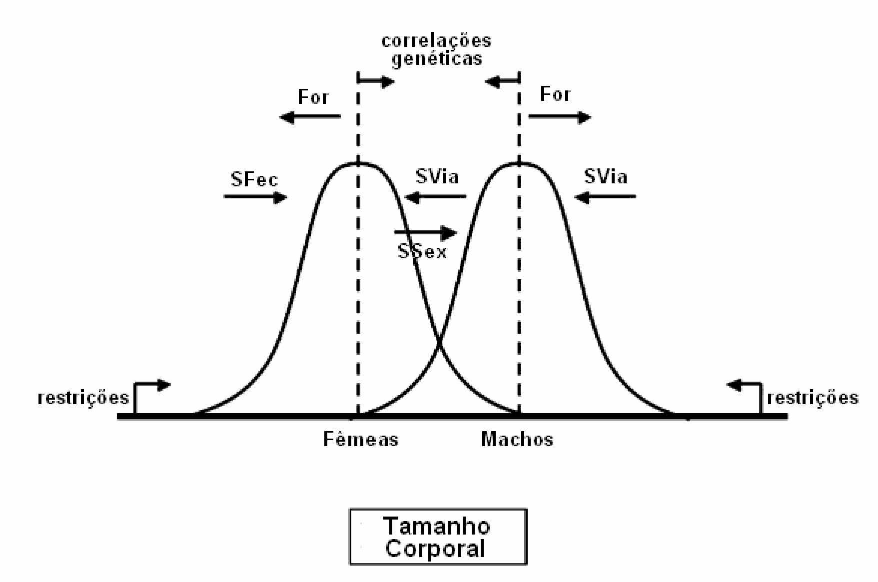


Figura 1. Modelo de equilíbrio diferencial para a evolução do dimorfismo sexual de tamanho em uma dada espécie (reproduzido com modificações de Blanckenhorn 2005). Distribuições do tamanho corporal para o caso onde os machos são maiores que as fêmeas. A seleção para fecundidade (SFec) tende a selecionar para aumento de tamanho nas fêmeas e a seleção sexual (SSex) para o aumento de tamanho nos machos. A seleção de viabilidade (SVia) seleciona para o menor tamanho corporal em ambos sexos. A especialização para o forageamento (For) pode selecionar para tamanhos divergentes entre machos e fêmeas. Se essas pressões seletivas equilibram diferencialmente nos sexos, o dimorfismo sexual de tamanho surge numa dada espécie.

O modelo do equilíbrio diferencial (figura 1) sugere que o dimorfismo sexual de tamanho afeta as histórias de vidas (crescimento, desenvolvimento, maturidade reprodutiva, expectativa de vida, etc), mas também indica que é afetado por ela. O mesmo é valido para ecologia (habitat, escolha alimentar, ect.) e comportamento (forageamento, reprodução, ect.) (Blanckenhorn 2000, 2005). Nesse contexto, o dimorfismo sexual de tamanho pode ser pensado como um epifenômeno resultante de forças seletivas relacionadas, que atuam diferentemente sobre machos e fêmeas (Blanckehorn 2005).

O maior problema de qualquer modelo que estude a evolução de uma certa característica é a incapacidade de determinar se, num dado ponto do tempo, as diferenças observadas entre os sexos são a causa ou conseqüência da evolução. Além disso, o que nós enxergamos agora pode muito bem ser uma conseqüência de pressões seletivas ambientais que foram muito diferentes no passado se comparadas com o presente. Connell (1980) chamou esse efeito de “fantasmas do passado da evolução”.

Voltando na questão abordada pelo modelo da figura 1, tamanho corporal, a seleção atuante sobre os machos (SSex), selecionando positivamente machos de maior tamanho corporal, poderia ser causada essencialmente pela preferência das fêmeas por machos maiores (escolha da fêmea) ou por disputas entre machos, que favorecendo o indivíduo maior em detrimento no menor (competição entre machos).

No caso da escolha das fêmeas, o benefício causado pela a escolha seria positivo tanto para machos como para fêmeas. Em curto prazo, machos maiores, que possuiriam maior capacidade de transferência espermática, deixariam uma prole mais numerosa quando comparados com machos menores. Do outro lado, as fêmeas aumentariam suas chances de manter alta a viabilidade de seus ovos. Em longo prazo, devido às correlações genéticas existentes entre os sexos, o aumento de freqüência de machos grandes acarretaria num maior tamanho corporal das fêmeas. Estas seriam favorecidas pelo aumente sentido na fecundidade, característica comumente associada ao tamanho corporal. Desta forma, a evolução da característica dos machos, escolhida pelas fêmeas, acabaria sendo associada diretamente a própria preferência (runaway selection - Fisher 1930). Em *Dryas iulia alcionea*, esse não parece ser a situação mais ajustada para explicar o dimorfismo sexual de tamanho. Apesar de terem sido encontrados sinais de seleção atuando sobre a morfologia dos machos (capítulo 5) e um componente genético significativo altamente correlacionado entre os sexos, influenciado o tamanho corporal (capítulo 4), não foram encontrados sinais de preferência das fêmeas com base no tamanho corporal (capítulo 6).

Na evolução direcionada pela competição entre machos, os exemplos mais comuns encontrados fazem menção à evolução de ornamentos ou de estruturas envolvidas diretamente na disputa entre machos. Essas disputas não necessariamente envolvem interações agonísticas e evolução de características utilizadas diretamente para subjugar seus adversários, apesar de muitos estudos apresentarem suas discussões direcionadas no sentido de uma evolução armamentista entre machos (Andersson 1994). Além do surgimento de chifres, presas, esporas e outras armas, alguns ornamentos, *displays* comportamentais e padrões de coloração poderiam ser considerados armas psicológicas nas disputas entre machos, conforme sugerido por Fisher (1930). Em algumas espécies de borboletas territoriais, o tamanho das asas pode ter efeitos significativos no sucesso copulatório, como sugerido por Hernandéz e Benson (1998). Em espécies não territoriais, o tamanho das asas e sua relação com o sucesso reprodutivo permanece não determinado. Contudo, é bem conhecido que espécies não territoriais geralmente procuram ativamente por parceiros para acasalar. Qualquer característica que aumente a eficiência da procura por parceiros poderia ser selecionada positivamente. Assim, disputas entre machos poderiam ocorrer mesmo que os concorrentes nunca se encontrem. O sucesso do vencedor seria simplesmente baseado na sua vantagem em encontrar e cortejar as fêmeas. Essa hipótese encaixa muito bem nos resultados encontrados com *D. i. alcionea*. Machos apresentam uma forma de asas aparentemente otimizada para vôos de longa duração, com alta eficiência energética, enquanto fêmeas possuem asas otimizadas a vôos que requerem mais explosão e manobrabilidade (capítulo 5). Esses resultados parecem ser corroborados pelas diferenças comportamentais observadas entre machos e fêmeas, onde machos gastam maior parte do seu tempo em atividades de dispersão, enquanto as fêmeas procuram por plantas hospedeiras para oviposição (capítulo 2). Nos experimentos de sucesso copulatório, onde foram usados machos com diferentes envergaduras de asas, não foram observadas diferenças de sucesso entre machos grandes e pequenos (capítulo 6). Contudo, esses resultados não entram em conflito com a hipótese sugerida, já que o confinamento em um espaço reduzido (gaiolas de $27m^3$) não acarretaria em efeitos sentidos na capacidade de dispersão. Haag e Araújo (1994) mostram que populações geograficamente distantes possuem alta similaridade genética, sugerindo que a vagilidade da espécie seja grande. Esses resultados estão de acordo com diversas observações realizadas em campo e tentativas de experimentos de marcação e recaptura (Brown Jr. 1981; A.C. Paim, comunicação pessoal; N.O. Mega observações pessoais).

Uma atenção especial, além das possíveis vantagens copulatórias, deve ser dada aos resultados observados nas análises de morfometria das asas de *D. i. alcionea* e ao seu significado evolutivo (capítulo 5). Uma hipótese alternativa poderia explicar a evolução do dimorfismo sem o envolvimento de processos relacionados à seleção sexual: seleção para diferenças sexuais ecológicas. A natureza desse processo evolutivo seria embasada nas diferenças sexuais comportamentais observadas na alimentação e forageamento. Pressões seletivas diferenciais seriam observadas para machos e fêmeas, conforme previsto no modelo da figura 1 (For). Neste caso, os sexos tenderiam a sofrer desvios de nicho ecológico para evitar competição por recursos alimentares. Na prática, seria muito difícil separar efeitos causados for seleção ecológica e seleção sexual. Shine (1989) afirma que diferenças sexuais causadas por diferentes hábitos de vida são de alguma forma, mas não totalmente, relacionadas a funções reprodutivas. Machos e fêmeas de *D. i. alcionea* alimentam-se essencialmente de néctar de flores, mas diferem parcialmente nos seus horários de atividade. Fêmeas iniciam suas atividades de vôo diárias alguns minutos antes dos machos, encerrando suas atividades mais cedo (N.O. Mega, dados não publicados). Mesmo assim, encontros entre machos e fêmeas freqüentemente correm próximos a plantas utilizadas como fonte alimentar. Joron (2005) mostrou a existência de segregação de habitat em outro Heliconiinae, *H. numata*, apesar de discutir seus dados para a importância da manutenção do mimetismo müleriano e não em termos de seleção ecológica.

Apesar da genialidade dos trabalhos de Darwin (1859, 1871), ele subestimou o papel da escolha da fêmea na evolução do dimorfismo sexual, assim como a maior parte dos pesquisadores nas décadas de 30-40. Eberhard (1985, 1991, 1993), décadas mais tarde, esclareceu a importância do papel da escolha das fêmeas, principalmente no que se refere à escolha críptica. Essa escolha é particularmente importante em espécies poligâmicas, abrindo a discussão para o nível de competição espermática. *D. i. alcionea* é tida como uma espécie poligâmica facultativa (Brown Jr. 1981, Garcias 1983), podendo ter encontrado até quatro espermatóforos dentro de uma única *bursae copulatrix* (Mega et al., em preparação). Por esse motivo, não pode ser descartada a possibilidade de alguma escolha das fêmeas durante os processo de acasalamento. Aparentemente, a insistência dos machos (capítulo 2) e o tamanho corporal (capítulo 6) não influenciam a escolha das fêmeas. Contudo, machos que apresentam menor variação comportamental durante atividades de cortejo parecem obter maior sucesso copulatório, sendo menos rejeitados por fêmeas virgens em idade reprodutiva (capítulo 2).

Mesmo que não tendo sido mostrado nessa tese algum mecanismo claro pelo qual as fêmeas de *D. i. alcionea* escolhem seus parceiros, é esperado que alguma espécie de discriminação deva ocorrer, pelo menos no que diz respeito à identidade da espécie. Se isso não ocorresse, confusões de identidade entre espécies seriam muito mais freqüentes do que comumente observado (Andersson 1994). Na grande maioria das espécies, a fêmea indica de alguma forma a sua receptividade, permitindo ou ajudando o macho a fertilizar seus ovos (Andersson 1994). Controversamente, em insetos, essa não parece ser a regra. Rutowski (1997) afirma que fêmeas de borboletas não exibem nenhuma espécie de sinal indicando sua receptividade, mas que as diferenças de "display" comportamental entre machos seriam devidas a diferenças existentes na natureza dos sinais usados pela escolha das fêmeas. A complexidade dos padrões comportamentais dos machos seria muito variável, podendo ser extremamente simples, como exibido por muitos Pieridae (Scott 1972), ou extremante elaborado como o exibido por *Danaus gilippus* (hairpencilling, Brower et al. 1965). Esses displays poderiam ser minuciosamente analisados pelas fêmeas e usados como referência para sua escolha. Em *D. i. alcionea*, alguma espécie de escolha das fêmeas parece ocorrer, conforme descrito no capítulo 2, onde as fêmeas subitamente modificam sua postura de rejeição e permitem aproximação dos machos. Assim, a fertilização parece ser, ao menos parcialmente, controlada pelo comportamento das fêmeas, apesar de que não tenhamos identificado claramente qual fator propicia o sucesso copulatório dos machos.

Finalmente, um ponto não abordado nessa tese, mas importante para a discussão do da evolução do tamanho corporal e dimorfismo sexual, é a questão relativa ao tempo de desenvolvimento das formas imaturas e protandria. A grosso modo, o tamanho corporal seria um produto direto do efeitos dos genes em função do ambiente. Mantendo-se constante as condições genéticas e ambientais, o tamanho acaba sendo influenciado diretamente pela taxa de crescimento e tempo de desenvolvimento. Assim, um certo tamanho corporal seria desenvolvido alterando a velocidade ou o tempo de desenvolvimento. Em algumas espécies, machos podem se beneficiar por acelerar seu desenvolvimento, emergindo antes das fêmeas e antes que outros machos, obtendo vantagens reprodutivas. As vantagens poderiam seriam obtidas basicamente na redução do período teneral, o que garantiria a plena funcionalidade das gônodas no momento da emersão das fêmeas (Blanckenhorn 2005). A observação dos diferentes tempos de maturação sexual, descrita no capítulo 2, abre a possibilidade para uma eventual vantagem reprodutiva dos machos se a ocorrência de protandria em *D. i. alcionea* fosse confirmada. Contudo, para que o dimorfismo sexual de tamanho continuasse a ser desviado em favor dos machos, as formas imaturas dos machos deveriam apresentar um taxa de desenvolvimento muito mais acelerada quando comparados às fêmeas. Se considerarmos que os machos são, em média, aproximadamente 10% maiores do que as fêmeas, e que uma fêmea leva em torno de 30 dias para se desenvolver em imago, e que as taxas de desenvolvimento não difere entre os sexos, dois ovos contendo embriões de diferentes sexos, que foram ovipositados no mesmo momento, teriam uma defasagem de aproximadamente três dias entre a emergência dos imagos. Para que ocorra protandria, com reais efeitos sobre a redução da defasagem no período de amadurecimento sexual entre machos e fêmeas, a taxa de crescimento de um imaturo macho deveria ser acelerada em 20%. Essas taxas são muito elevadas para um sistema apresentando padrões de dimorfismo como os observados em *D. i. alcionea*.

Como surgiu o dimorfismo em *D. i. alcionea* é uma pergunta muito difícil de ser respondida. Wiklund e Forsberg (1991) e Blanckenhorn (2005) sugerem que abordagens filogenéticas podem esclarecer os padrões observados entre as espécies de um grupo, mas não esclarecem satisfatoriamente os mecanismos ou processos que levam a esses padrões. Por outro lado, estudos pontuais, efetuados somente com uma espécie, acabam sendo estudos fenomenológicos, investigando as conseqüências ao invés das causas (Ding e

Blanckenhorn 2002). Nesta tese, não existem evidências suficientes para responder essa pergunta. Algumas suposições podem ser feitas se olharmos para os padrões de dimorfismo sexual em Heliconiinae. Em geral, pode ser notado que nos ramos evolutivos mais basais são encontrados os dimorfismos sexuais mais marcantes. Por exemplo, no grupamento filético formado pelos gêneros *Dryas, Dryadula, Philaetria*, bem como no agrupamento formado pelos gêneros *Dione* e *Agraulis* (Penz 1999, Penz e Peggie 2003) são encontradas notáveis diferenças relativas com relação ao dimorfismo das asas. Já nos ramos mais derivados (gêneros *Heliconius* e *Euides*), essas diferenças não são observadas. Possivelmente, e evolução do mimetismo nos ramos filogenéticos mais derivados tenha sido tão importante que alterou de alguma forma os padrões ancestrais de dimorfismo.

As correlações genéticas imperfeitas ($r \neq 1,0$), um pré-requisito para que o dimorfismo sexual possa evoluir, foram observadas em *D. i. alcionea* (capítulo 4). Em média, as correlações genéticas observadas entre machos e fêmeas, para as características das asas foram de aproximadamente 0,65. Isso significa dizer que, por exemplo, se a seleção (SSex, figura 1) aumenta o tamanho das asas dos machos em 1 mm a cada 100 gerações, o tamanho das asas das fêmeas aumenta somente 0,65 mm no mesmo período. Em um tempo profundo, essa diferença de ganho no tamanho corporal acabaria gerando um dimorfismo sexual exagerado. A evolução do dimorfismo só seria estabilizada se houvesse uma perda substancial de variabilidade genética em função da seleção por viabilidade (SVia, figura 1). Tamanhos corporais muito avantajados trariam grandes demandas energéticas, tornando inviável a sua manutenção. Além isso, outras pressões seletivas, como, por exemplo, a ação de predadores atraídos por indivíduos grandes e chamativos, que poderiam causar um efeito semelhante à seleção por viabilidade. O efeito líquido dessas duas pressões seletivas causaria seria uma seleção estabilizadora, gerando um certo grau de erosão genética nas populações. Essa hipótese estaria de acordo com os resultados mostrados no capítulo 5 e com trabalho de Haag e Araújo (1994), descrevendo a baixa variabilidade interpopulacional de *D. i. alcionea.* Assim, grandes variações de tamanho corporal em populações naturais poderiam ser explicadas essencialmente por histórias de vida, como mostrado para *H. erato* (Rodrigues e Moreira 2004), ou pela prioridade na alocação de recursos, que favorece a manutenção corporal e a reprodução em detrimento do tamanho corporal (Glazier 2002). Seria impossível determinar se as diferenças sexuais em *Dryas iulia alcionea* surgiram pelas correlações genéticas imperfeitas, ou se as correlações imperfeitas sugiram por causa de do dimorfismo.

REFERÊNCIAS

Alcock J (1993) Animal Behavior. 5ed. Sinauer Associates Inc. Sunderland, Massachusetts.

Andersson M (1994) Sexual Selection. Princeton University Press. New Jersey. 599pp.

Andersson S and Dobson HE (2003) Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. J Chem Ecol 29:2303-2318.

Arak A (1988) Sexual dimorphism in body size: a model and a test. Evolution 42: 820-825.

Benson WW, Brown Jr KS, Gilbert LE (1975) Coevolution of plants and herbivores: passion flower butterflies. Evolution 29:659–80.

Biezanko CM and Freitas RG (1938) Catálogo dos insetos encontrados na cidade de Pelotas e seus arredores. Fascículo I. Lepidópteros. Contribuição a o conhecimento da Fisiografia do Rio Grande do Sul. 32pp.

Biezanko CM and Link D (1972) Nomes populares dos lepidópteros no Rio Grande do Sul. Boletim Técnico D.F. 4. Imprensa Universitária, Santa Maria.

Blanckenhorn WU (2000) The evolution of body size: what keeps organisms small? Q Rev Biol 75:385-407.

Blanckenhorn WU (2005) Behavioral Causes and Consequences of Sexual Size Dimorphism. Ethology 111:977-1016.

Blanckenhorn WU, Dixon AFG, Fairbairn DJ, Foellmer MW, Gibert P, van der Linde K, Meier R, Nylin S, Pitnick S, Schoff C, Signorelli M, Teder T and Wiklund C (2007) Proximate Causes of Rensch's Rule: Does Sexual Size Dimorphism in Arthropods Result from Sex Differences in Development Time? Am Nat 169:245-257.

Boggs CL (1981a) Nutritional and life-history determinants of resource allocation in holometabolous insects. Am Nat 117:692- 709.

Boggs CL (1981b) Selection pressures affecting male nutrient investment at mating in Heliconiine butterflies. Evolution 35:931-940.

Boggs CL (1986) Reproductive strategies of female butterflies: variation in and constrains on fecundity. Ecol Entomol 11:7-15.

Boggs CL and Gilbert LE (1979) Male contribution to egg production in butterflies: evidences for transfer of nutrients at matting. Science 206:83-84.

Borges SS (2005) Padrão natural, preferência alimentar e performance das larvas de *Dryas iulia alcionea* (Lepidoptera: Nymphalidae) em relação à idade da folha de *Passiflora suberosa* (Passifloraceae). Dissertação de Bacharelado em Ciências Biológicas, Universidade Federal do Rio Grande do Sul, 36 pp.

Brower LP, Brower JVC and Cranston FP (1965) Courtship behavior of the queen butterfly, *Danaus gilippus berenice*. Zoologica 50:1-39.

Brown Jr KS (1979) Ecologia geográfica e evolução em florestas neotropicais. Dissertação de Livre Docência, UNICAMP, Campinas, 265p.

Brown Jr KS (1981) The biology of *Heliconius* and related genera. Ann Rev Entomol 26:427-456.

Brown Jr KS (1992) Borboletas da Serra do Japi: diversidade, hábitats, recursos alimentares e variação temporal. In: Morellato LPC (ed) História Natural da Serra do Japi: ecologia e preservação de uma área florestal no sudeste do Brasil. Unicamp/Fapesp, Campinas. pp.142-187.

Brown Jr KS and Mielke OHH (1972) The heliconians of Brazil (Lepidoptera: Nymphalidae). Part II. Introduction and general comments, with a supplementary revision of the tribe. Zoologica 57: 1–40.

Burns JM (1968) Mating frequency in natural populations of skippers and butterflies as determined by spermatophore counts. Proc Natl Acad Sci USA 61:852-859.

Cervi AC (1997) Passifloraceae do Brasil. Estudo do gênero *Passiflora* L., subgênero Passiflora. Fontqueria 45:1-92.

Connell JH (1980) Diversity and the coevolution of competitors, or the ghost of competition past. Oikos 35:131-138.

Darwin C (1859) On the origin of species by means of Natural Selection (reprinted in 2003). Broadview Press, Buffalo, 630pp.

Darwin C (1871) The descent of man and selection in relation to sex (reprinted in 2004). Penguin Putanam Inc., New York, 864pp.

Davies N and Bermingham E (2002) The historical biogeography of two Caribbean butterflies (Lepidoptera: Heliconiidae) as inferred from genetic variation at multiple loci. Evolution 56:573–589.

Deinert EI, Longino JT and Gilbert LE (1994) Mate competition in butterflies. Nature, 370:23-24.

DeVries PJ (1987) The butterflies of Costa Rica and their natural history (Papilionidae, Pieridae, Nymphalidae). Princeton University Press. 327pp.

Ding A and Blanckenhorn WU (2002) The effect of sexual size dimorphism on mating behavior in two dung flies with contrasting dimorphism. Evol Ecol Res 4:1-15.

Drummond III BA (1984) Multiple mating and sperm competition in the Lepidoptera. In: Smith RL (ed) Sperm Competition and the Evolution of Animal Mating Systems, Academic Press, New York, pp 291–370.

Dunlap-Pianka H, Boggs C and Gilbert LE (1977) Ovarian dynamics in Heliconiine Butterflies and skippers. Science 197:487-490.

Eberhard WG (1985) Sexual selection and animal genitalia. Harvard University Press, Cambrigde.

Eberhard WG (1991) Copulatory courtship and cryptic female choice in insects. Biol Rev 66: 1-31.

Eberhard WG (1993) Evaluating models of sexual selection: genitalia as a test case. Am Nat 142: 564-571.

Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Priceton.

Eberhard WG and Cordero C (1995) Sexual selection by cryptic female choice. Trends Ecol Evol 10:493-496.

Emsley MG (1963) A morphological study of imagine Heliconiinae (Lepidoptera: Nymphalidae) with consideration of the evolutionary relationships within the group. Zoologica 48:85-131.

Ehrlich PR and Raven PH (1964) Butterflies and plants: a study in coevolution. Evolution 18:586-608.

Estrada C and Jiggins CD (2002) Patterns of pollen feeding and habitat preference among *Heliconius* species. Ecol Entomol 27:448-456.

Fisher RA (1930) The Genetical Theory of Natural Selection. Clarendon Press, Oxford.

Freeman S and Herron JC (2001) Evolutionary Analysis. Prentice Hall, Ipper Saddle River.

Garcias G (1983) Aspectos da Biologia Populacional de Cinco Espécies de Heliconiíneos do Anel Mimético “laranja” (Lepidoptera, Nymphalidae). Dissertação de Mestrado em Genética. Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Glazier DS (2002) Resource-allocation rules and the heritability of traits. Evolution 561:1696-1700.

Gröehs RW (2000) Comportamento de larvas de primeiros instares de *Dryas iluia alcionea* (Lepidoptera; Nymphalidae), sobre folhas de *Passiflora suberosa* (Passifloraceae). Dissertação de Bacharelado em Ciências Biológicas – ênfase ambiental. Universidade Federal do Rio Grande do Sul, Porto Alegre. 12 pp.

Haag KL, Araújo AM and Zaha A (1993) Genetic structure of natural populations of Dryas iulia (Lepidoptera, Nymphalidae) revealed by enzyme polymorphisms and mtDNA RFPLs. Bioch Genet 31:447-458.

Haag KL and Araújo AM (1994) Inbreeding, genetic load and morphometric variation in natural populations of Dryas iulia (Lepidoptera, Nymphalidae). Rev Bras Gen 17:35-39.

Hernández MIM and BensonWW (1998) Small-male advantage in the territorial tropical butterfly Heliconius sara (Nymphalidae): a paradoxical strategy? Anim Behav 56:533-540.

Joron M (2005) Polymorphic mimicry, microhabitat use, and sex-specific behaviour. J Evol Biol 18:547-556.

Kingan TG, Bodnar WM, Raina AK, Shabanowitz J. and Hunt DF (1995) The loss of female sex pheromone after mating in the corn earworm moth *Helicoverpa zea*: identification of a male pheromonostatic peptide. Proc Natl Acad Sci USA 92:5082-5086.

Kirkton SD and Schultz TD (2001) Age-specific behavior and habitat selection of adult male damselflies, Calopteryx maculata (Odonata: Calopterygidae). J Ins Behav 14:545-555.

Krebs, J.R. and Davis, N.B. 1993. An Introduction to Behavioral Ecology. 3 ed. Blackwell Scientific Publications. Oxford.

LaMunyon CW and Eisner T (1993) Postcopulatory sexual selection in an arctiid moth (*Utetheisa ornatrix*). Proc Natl Acad Sci USA 90:4689–4692.

Mega NO (2004) O comportamento de larvas de *Dryas iulia alcionea* (Lepidoptera: Nymphalidae): implicações ecológico-evolutivas. Dissertação de Mestrado, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Mega NO and Araújo AM (2008) Do caterpillars of *Dryas iulia alcionea* (Lepidoptera, Nymphalidae) show evidence of adaptive behavior to avoid predation by ants? J Nat Hist 42:129-137.

Mueller HC (1990) The evolution of reversed sexual dimorphism by sexual selection: the separate effects of intrasexual selection and intersexual selection. Evolution 44:315-331.

O'Donald P (1980) Genetic models of sexual seletion. University Press. Cambrigde.

Paim AC (1995) Polimorfismo enzimático e variação morfológica em uma população natural de *Dryas iulia* (Fabr. 1775) (Lepidoptera; Nymphalidae). Dissertação de Mestrado. Universidade Federal do Rio Grande do Sul, Porto Alegre, RS.

Paim AC, Kamisky LA and Moreira GPR (2004) Morfologia externa dos estágios imaturos de heliconíneos neotropicais. IV. *Dryas iulia alcionea* (Lepidoptera, Nymphalidae, Heliconinae). Iheríngia Zool 94:25-35.

Parker GA (1979) Sexual selection and sexual conflict. In: Blum MS and Blum NA (eds) Sexual selection and the Reproductive Competition in Insects. Academic Press, New York, pp 123-166.

Penz CM (1999) Higher level phylogeny for the passion-vine butterflies (Nymphalidae, Heliconiinae) based on early stage and adult morphology. Zool J Lin Soc 127:277-344.

Penz CM and Peggie D (2003) Phylogenetic relationships among Heliconiinae genera based on morphology (Lepidoptera: Nymphalidae). Systc Entomol 28:451-479.

Perico E and Araújo AM (1991) Suitability of host plants (Passifloraceae) and their acceptance by *Heliconius erato* and *Dryas iulia* (Lepidoptera; Nymphalidae). Evol Biol 5:59-74.

Rodrigues D and Moreira GRP (2004) Seasonal variation in larval host plants and consequences for Heliconius erato (Lepidoptera: Nymphalidae) adult body size. Aust Ecol 29:437-445.

Rutowski RL (1997) Sexual dimorphism, mating systems and ecology in butterflies. In: Choe JC (ed) The evolution of mating systems in Insects and Arachnids. Library of Congress, Cambrigde. pp 397.

Scott JA (1972) Mating of Butterflies. J Res Lepid 11:99-127.

Shine R (1989) Ecological causes for the evolution of sexual size dimorphism: a review of the evidence. Q Rev Biol 64:419-461.

Simmons LW (2001) Sperm Competition and Its Evolutionary Consequences in the Insects. Princeton University Press, Princeton, 448 pp.

Slatkin M (1984) Ecological causes of sexual dimorphism. Evolution 38:622-630.

Sneath PHA. and Sokal RR (1973) Numerical taxonomy. The principles and practice of numerical classification. Freeman, San Francisco, 573 pp.

Soulé M (1982) Allometric variation I. The theory and some consequences. Am Nat 120:751-764.

Swanson HF and Monge-Najera J (2000) The effects of methodological limitations in the study of butterfly behavior and demography: A daily study of *Vanessa atalanta* (Lepidoptera: Nymphalidae) for 22 years . Rev Biol Trop 48: 605-614.

Thornhill R and J Alcock (1983) The Evolution of Insect Mating Systems. Harvard University, Cambridge, 547pp.

Vaidya VG (1969) Investigations on the role of visual stimuli in the egg-laying and resting behaviour of *Papilio demoleus* L. (Papilionidae, Lepidoptera). Anim Behav 17:350-355.

Wilklund C (2003) Sexual Selection and the Evolution of Butterfly Mating Systems. In: Boggs CC, Watt WB and Ehrlich PR (eds) Butterflies: Ecology and Evolution Taking Flight. University of Chicago Press, Chicago. pp 67-90.

Wiklund C and Forsberg J (1991) Sexual Size Dimorphism in Relation to Female Polygamy and Protandry in Butterflies: a Comparative Study of Swedish Pieridae and Satyridae. Oikos 60:373-381.

ANEXO 1

A PHENOL-FREE PROTOCOL FOR EFFICIENT AND RAPID DNA EXTRACTION FROM INSECTS, SPIDERS AND ISOPODS

Artigo a submetido para revista *Brazilian Archives of Biology and Technology*

A phenol-free protocol for efficient and rapid DNA extraction from insects, spiders and isopods

Nicolás O. Mega[1] and Luis Fernando Revers[2]

[1]*Programa de Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul.*
[2]*Centro Nacional de Pesquisa de Uva e Vinho, Empresa Brasileira de Pesquisa Agropecuária.*

Abstract: We developed a rapid and efficient method to obtain high quality DNA from small samples of arthropod tissues without using phenol, an organic contaminant in DNA and RNA preparations. We also used insoluble polyvinylpolypirrolidone to increase DNA efficiency extraction and further pureness after cellular lysis. It was possible to obtain intact DNA from all samples tested; pure DNAs were obtained, with the absence of proteins. The purity of DNA was checked spectrophotometrically and it usefulness was evaluated in an AFLP essay, showing that DNA recollected was suitable for utilization in sensible molecular biology techniques. The method presented here will enable researchers to do DNA-based studies of arthropods, generating lower amounts of toxic waste when compared with phenol-chloroform methods.

Key-words: high quality arthropod DNA, DNA extraction, phenol-free protocol.

Introduction

The analysis of genetic variation using DNA fingerprints and sequences has become an important tool to access evolutionary history, diversity in genetic improvement programs and identification of cell and virus strains, as well as ecological functions and molecular biological routines. All these procedures require the extraction of DNA from a variety of tissues and cell cultivations and it is an important step in research projects, allowing the archival of material for actual and further studies. Techniques for DNA extraction have been described for all Kingdoms of life, as well for virus (Sambrook and Russell 2001).

The quality of DNA extraction depends on the protocol followed. Likewise, the suitability of a DNA isolation method depends on the DNA source, because there are great differences in the substances present in the biological material (Reineke et al. 1998). Some DNA extraction protocols are very efficient to recollect DNA from tissue samples, removing residual substances from the DNA preparations; moreover some of then are not so efficient in the removal of proteins or other organic contaminants, causing enzymatic inhibition in further molecular analysis. In these cases, the use of protocols for DNA purification is required, which can be time and fund consuming.

Reineke et al. (1998) evaluated several DNA extraction protocols with insect material and determined the suitability of the resulting DNA for AFLP analysis. The study also investigated the effect of additional clean-up steps on the improvement of DNA quality. They concluded that the best protocols suitable for AFLP analysis use phenol or phenol:chloroform solvents, and additional purification steps are recommend. CTAB-based methods, as described in Moeller et al. (1992), demonstrated to provide DNA of equal quality, without using hazardous chemicals

In this study we describe a rapid and efficient method for high quality DNA extraction from small samples of freshly and frozen insect tissues. This method is CTAB-based and do not use phenol, an organic contaminant in DNA and RNA preparations, which can represent a health risk to researchers. We also use polyvinylpolypirrolidone (PVPP), and insoluble organic polymer, to increase DNA efficiency extraction and further pureness after cellular lysis, which is a protocol commonly used for plant DNA extractions (Lodhi et al. 1994; Lefort and Douglas 1999).

Materials and Methods

Organisms and tissue preparations

Five insects, two spiders and three isopods species were used as biological samples for DNA extraction. All specimens were collected from nature, identified and weighted (table 1). The body part of each sample used for DNA extraction is shown in table 1. Each sample was frozen in liquid nitrogen and immediately grinded in 1.5 ml sterile microcentrifuge tubes and stored below -70°C. Tissue samples were thoroughly crushed into a very fine powder, but not grinded very heavily in order to avoid shearing DNA.

DNA extraction: Grinded tissues were mixed with insoluble polyvinylpolypirrolidone (PVPP) (Sigma, P6755) in a proportion of 100mg of PVPP per 1g of grinded tissue, 600µL of extraction buffer (50mM Tris pH 8.0, 20mM EDTA pH 8.0, 1.1M NaCl, 0.4M LiCl, 1% CTAB, 2% PVP40 (Sigma), 0.5% Tween 20, 0.2% β-mercaptoethanol) in 1.5 micro centrifuge tubes and mixed thoroughly. The tubes were incubated at 60°C for 25 minutes and inverted each 10 minutes for good solubilization of the grinded tissues. The tubes were cooled at room temperature and 600µL of chloroform:isoamyl alcohol mixture (24:1) were added. The tubes were then mixed gently by inversion to form an emulsion, during 4 minutes. After that the tubes were centrifuged (Eppedendorf Centrifuge 5415R) at 10600g for 5 minutes at room temperature. The aqueous phase present in each tube (~500µL) was transferred to a new 1.5µL microcentrifuge tube very gently, to avoid DNA fragmentation. To each tube was added 250µL of 5M NaCl and 750µL of cold (-20°C) isopropanol. The solution was kept in the freezer at -20ºC for 20 minutes. The samples were then centrifuged (Eppedendorf Centrifuge 5415R) at 10600g for 10 minutes at room temperature. After the centrifugation, the supernatant was poured off and the pellets washed with cold (4°C) 76% ethanol (1000µL). After the washing step, the samples were spun quickly and the excess of ethanol removed with a micropipette. Washed DNA pellets were dried by leaving the tubes uncovered in a 37°C stove for 20 minutes. Samples were dissolved in 50µL TE (10mM Tris HCl ph 8.0, 0.1 mM EDTA pH 8.0) and treated with 1µL of RNAase A (10mg/mL) per 100µL of DNA solution at 50°C for 20 minutes.

Quality and quantification tests

Integrity of the DNA samples was analised in 0.8% ethidium bromide-stained agarose gels, using 1Kb Ladder (Fermentas) as control for DNA shearing. Visual patterns of DNA samples were photographed under UV light and photodocumented using a Stratagene EagleEye II still video system. The DNA samples were quantified using a spectrophotometer (GeneQuant PRO, Amersham Biosciences) in the absorbance spectrum for nucleic acids (260 ηm). To access DNA purity, 230 ηm and 280 ηm readings were recorded and compared with 260 ηm readings. The 230 ηm reading can indicate the presence of organic impurities and buffer contaminants, once phenols, carbohydrates, peptides, urea, thiocyanates, β-mercaptoethanol, humic acids and other buffer components absorb light at this wavelength (Yeates et al. 1998, Luebbehusen 2004, Hansen et al. 2007). The 280 ηm reading indicate the presence of proteins originated from the cells where the nucleic acids were extracted (Yeates et al. 1998, Hansen et al. 2007), once tyrosine, tryptophan and phenylalanine amino acids absorb at this wavelength (GeneQuant PRO, Amersham Biosciences). We considered pure samples exhibiting 260/230 and 260/280 ηm wavelength rates among 1.7-2.2. Background correction was done using the 320 ηm wavelength, compensating the effects of turbidity and high absorbance buffer solution.

We have also validated the quality of some DNA samples by an AFLP assay (Vos et al. 1995), a sensitive multi-step molecular technique to low quality and impure DNA samples. The DNA samples used in this test were from *Dryas iulia* specimens generated in a controlled breeding experiment. Seven tissue samples were tested, including four siblings, the two progenitors and an external control male. The selective amplification step of the AFLP assays were performed using six set of primers EcoRI+3 and MseI+3 nucleotides (EcoRI +ACG / MseI+CTC, EcoRI+ACC / MseI+CGC, EcoRI+AGG / MseI+CCG, EcoRI+ACT / MseI+CCC, EcoRI+ATG / MseI+CCA and EcoRI+ATA / MseI+CCT) and visualized in silver stained 6% polyacrilamide denaturing gels according to Creste et al. (2001). We considered good quality DNA the samples that exhibit clear and reliable AFLP patterns.

Results and Discussion

It was possible to obtain intact DNA from all samples tested. DNA extraction samples exhibited a transparent to light pigmentation aspect. Coloration may vary in one of the following colors: light yellow, light pink, light purple and light brown. The intensity of pigmentation can vary in relation to the amount of tissue used for the DNA extraction, as well with tissue and species utilized. The concentration of these pigments seams to be not associated to DNA purity or quality. Some difficult in dilution of the DNA pellets may occur, due high concentration of DNA obtained. If necessary, samples can be diluted in higher volumes of TE to facilitate DNA elution. We recommend volumes among 50-200µl of TE to maintain high concentration of the stock samples.

In the 0.8% ethidium bromide-stained agarose gels, the DNA samples revealed strong and clear patterns (fig.1). The DNA-shearing effect of the extraction procedure wasn't prominent for any sample. Only low smearing fragments were observed and this low shearing effect is expected, since the tissue samples were mechanically grinded. The RNAse treatment was efficient to eliminate RNA in the samples, once no RNA was observed in the electrophoresis of the DNA samples.

The table 2 shows the results for DNA concentration and purity. The yield of DNA recollected from the tissue samples used varied and a significant correlation between the weight of tissue sample used and the quantity of DNA recollected was observed, as expected ($r_{\text{Pearson}} = 0.842$; $p<0.001$). All samples revealed pure quality DNA in relation to the absence of proteins, but only four samples (Bg, Bs, Pa and Ds_{20}) were considered free of organic/buffer contaminants (Table 3). It is interesting to note that there is a strong correlation between the weight of tissue used for DNA extraction and 260/230 ηm wavelength rate of the sample ($r_{\text{Pearson}} = 0.692$; $p<0.001$). This result suggests that the volume of buffer used was higher than needed in the samples exhibiting traces of buffer components (deviations from 1.7-2.2 260/230 ηm wavelength rate). No significant amounts of proteins were detected in the samples, showing that its removal was very efficient using the protocol presented here, without the necessity of using phenol combined with chloroform.

Figure 2 shows the DNA samples utilized in AFLP essay. Samples revealed good quality, as obtained for the other specimens tested. Wavelength absorbance readings and wavelength rates did not differ significantly from the sample of *Dryas iulia* shown in table 2. The figure 3 shows the AFLPs patterns obtained for seven DNA samples used in the AFLP essay. From the six combinations of primers tested, four exhibited a great number of *loci* with good visualization. They generated 238 loci, been 219 of them polymorphic. These results validated the quality of DNA samples obtained with the protocol presented here, once the AFLP technique is very sensitive to poor quality DNA samples (Schondelmaier et al. 1996, Reineke et al. 1998, Cervera et al. 2000).

Some care must be taken to maximize the yield and quality of the DNA extractions. First, the tissue sample must be completely dried before the grind procedure starts. The formation of ice crystals can prejudice the grinding procedure. Second, excess or lack of grinded tissue can generate samples with excess of proteins and organic compounds, or residual buffer components, respectively. Although, the residual buffer components of the protocol presented here did not interfere significantly the AFLP technique. Excess of tissue also can cause over pigmentation of DNA samples. The pigmentation doesn't affect the quality of DNA, but cellular debris does (personal observations). According to our observations we suggest the use of grinded tissue samples ranging between 0.01-0.20g. The best results were obtained using 0.06 g of grinded tissue as starting sample.

The protocol presented here constitute a rapid method for DNA extraction that needs small samples of arthropod tissue to recollect nucleic acids from freshly, frozen or low term stored samples. It is simple, cheap and can be completed in only a few hours, without generating waste containing phenol contaminants. Once it is difficult to obtain high quality DNA from plants, requiring robust an efficient protocols, we decide to apply the same method currently employed in plants to arthropods. The combination of PVPP/PVP seems to provide a proficient method to isolate arthropod tissue and cellular debris from the aqueous phase containing nuclei acid solution, by forming a compact and consistent interface. The use of an initial buffer combining different salts and detergents, capable of promote cellular lysis and solubilization without cause DNA degradation, provide and efficient method to access good quality genetic material, without the necessity of use phenol during the procedure.

We conclude that the protocol presented here is an excellent tool to obtain high quality DNA, without the necessity of a further step of purification of the template DNA. We believe that the method presented here will enable researchers to study arthropod DNA quickly and at lower costs using molecular biology techniques, generating lower amounts of toxic waste when compared with phenol-chloroform methods.

Acknowledgments

We are grateful to Gisele Passaia, Alessandra Russi and Fernanda Sbeghen for providing laboratory bear and support. We also like to thank Aline Quadros, Lisandra Robe, Luis Ernesto Schmidt and Wilson Morandi Jr. for providing live specimens for DNA extraction. This study was partially funded by grant provided by CNPq. Financially support was provided CNPq, EMBRAPA Uva e Vinho and FAPERGS.

References

Cervera, M.T., Cabezas, J.A., Simon, B., Martinez, J.M. 2000. Genetic relationships among biotypes of Bemisia tabaci (Hemiptera: Aleyrodidae) based on AFLP. Bull. Entomol. Res. 90: 391–396.

Creste, S., Tulmann-Neto, A., Figueira, A. 2001. Detection of single sequence repeat polymorphisms in denaturing polyacrilamide sequecing gels by silver staining. Plant. Mol. Biol. Rep. 19: 299-306.

Hansen, T.O, Simonsen, M.K., Nielsen, F.C, Hundrup Y.A. 2007. Collection of blood, saliva, and buccal cell samples in a pilot study on the danish nurse cohort: comparison of the response rate and quality of genomic DNA. Cancer Epidemiol Biomarkers Prev. 16: 2072-2076.

Lefort, F., and Douglas, G.C. 1999. An efficient micro-method of DNA isolation from mature leaves of four hardwood tree species Acer, Fraxinus, Prunus and Quercus. Ann. For. Sci. 56: 259–263.

Lodhi, M.A., Ye, G.N., Weeden, N.F., and Reisch, B.I. 1994. A simple and efficient method for DNA extraction from Grapevine cultivars and Vitis species. Plant. Mol. Biol. Rep. 12: 6-13.

Luebbehusen, H. 2004. The Significance of the 260/230 Ratio in Determining Nucleic Acid Purity [online]. Avaiable from http://www.bcm.edu/mcfweb/?PMID=3100 (accessed on 7 November 2007).

Moeller. E.M., Bahnweg, G., Sandermann, H., and Geiger, H.H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucl. Acid. Res. 22: 6115-6116.

Reineke, A., Karlovsky, P., and Zebitz, C.P. 1998. Preparation and purification of DNA from insects for AFLP analysis. Ins. Mol. Biol. 7: 95-99.

Sambrook, J., and Russell, D.W. 2001. Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Woodbury, N.Y.

Schondelmaier, J., Steinrücken, G., and Jung, C. 1996. Integration of AFLP markers into a linkage map of sugar beet (*Beta vulgaris* L.). Plant breed. 115: 231-237.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper. M., and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. Nucl. Acid. Res. 23: 4407-4414.

Yeates, C., Gillings, M.R., Davison, A.D., Altavilla, N., Veal, D.A. 1998. Methods for microbial DNA extraction from soil for PCR amplification. Biol. Proc. Online. 1: 40-47.

Table 1. Biological samples used in the DNA extractions. The number in parenthesis indicates the quantity of specimens or body parts used for DNA isolation.

Class	**Order**	**Species**	**Tissue used**	**Tissue weight (g)**
Insecta	*Lepidoptera*	*Dryas iulia alcionea*	Head and thorax (1)	0.0342
		Heliconius erato phyllis	Head and thorax (1)	0.0297
		Grapholita molesta	Whole body (1)	0.0050
		Grapholita molesta	Whole body (4)	0.0199
	Diptera	*Anastrepha fraterculus*	Whole body (1)	0.0137
		Anastrepha fraterculus	Whole body (3)	0.0273
		Drosophila immigrans	Whole body (1)	0.0025
		Drosophila immigrans	Whole body (20)	0.0540
Aracnida	Araneae	*Paratrechalea azul*	Cephalothorax (1)	0.1419
		Paratrechalea ornata	Cephalothorax (1)	0.0828
Crustacea	Isopoda	*Benthana cairensis*	Whole body (1)	0.0295
		Balloniscus glaber	Whole body (1)	0.0418
		Balloniscus sellowi	Whole body (1)	0.0297

Table 2. Analysis of concentration and purity for de DNA samples obtained. The DNA concentration is show in ηg/μl. Wavelength Absorbance in the different wavelength values were measured by the optical density (A) of the sample in relation to a control (ultrapure sterile distilled water). The value of the Wavelenght Rate indicates the purity of the sample: values among 1.7 e 2.2 indicate ultrapure samples (α=0.05); deviation from these values indicate samples with perceptive presence of buffer/organic components (260/230 ηm) and proteins (260/230 ηm). Dilutions used in quantification were made using ultrapure sterile distilled water, optimizing 260ηm readings to best accuracy of the spectrophotometer (0.1-0.8A). Numbers in brackets indicate the quantity of specimens used; bold numbers indicate samples with perceptible excess of contaminants.

Species	***DNA Concentration (ηg/μl)***	***Dilution Factor***	***Wavelength Absorbance (A)***				***Wavelength Rates***		***Extraction Efficiency (g DNA/ g tissue)***
			230 ηm	**260 ηm**	**280 ηm**	**320 ηm**	**260/230 ηm**	**260/280 ηm**	
A. fraterculus (1)	640	100x	0.103	0.137	0.076	0.009	**1.4**	1.9	0.0023
A. fraterculus (2)	1545	100x	0.256	0.336	0.188	0.027	**1.3**	1.9	0.0028
B. cairensis	955	100x	0.140	0.204	0.120	0.013	**1.5**	1.9	0.0029
B. glaber	877	100x	0.112	0.183	0.107	0.008	1.7	1.9	0.0019
B. sellowi	690	100x	0.087	0.142	0.081	0.004	1.7	1.9	0.0021
D. iulia	1050	100x	0.203	0.233	0.140	0.023	**1.2**	1.9	0.0040
D. immigrans (1)	185	10x	0.345	0.377	0.195	0.007	**1.1**	2.0	0.0037
D. immigrans (20)	5580	200x	0.263	0.570	0.284	0.012	2.2	2.0	0.0093
G. molesta (1)	148	10x	0.378	0.323	0.192	0.027	**0.8**	1.8	0.0015
G. molesta (4)	785	100x	0.112	0.164	0.093	0.007	**1.5**	1.8	0.0020
H. erato	885	100x	0.146	0.186	0.109	0.013	**1.3**	1.8	0.0033
P. azul	2150	100x	0.258	0.442	0.241	0.012	1.7	1.9	0.0014
P. ornate	630	100x	0.099	0.134	0.073	0.008	**1.4**	1.9	0.0007

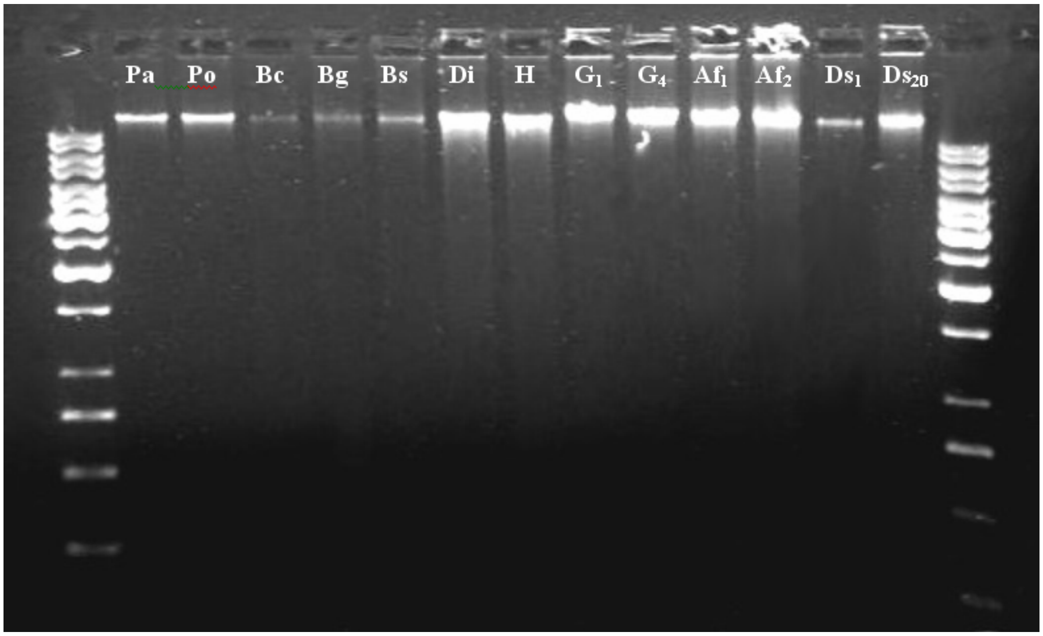


Figure 1. The 0.8% ethidium bromide-stained agarose gel showing DNA samples after DNA extraction from freshly and frozen tissues. **Pa**, *Paratrechalea azul*; **Po**, *Paratrechalea ornata*; **Bc**, *Benthana cairensis*; **Bg**, *Baloniscus glaber*; **Bs**, *Baloniscus sellowii*; **Di**, *Dryas iulia*; **H**, *Heliconius erato*; **G$_1$**, *Grapholita molesta* (n=1), **G$_4$**, *Grapholita molesta* (n=4); **Af$_1$**, *Anastrepha fraterculus* (n=1); **Af$_2$**, *Anastrepha fraterculus* (n=2); **Ds$_1$**, *Drosophila immigrans* (n=1); **Ds$_{20}$**; *Drosophila immigrans* (n=20). DNA patterns (1Kb Ladder) are shown in the far left and far right of the agarose gel. **Pa**, **Po**, **Bc**, **Bg**, **Bs** and **Ds$_{20}$** DNA samples were diluted to be properly observed.

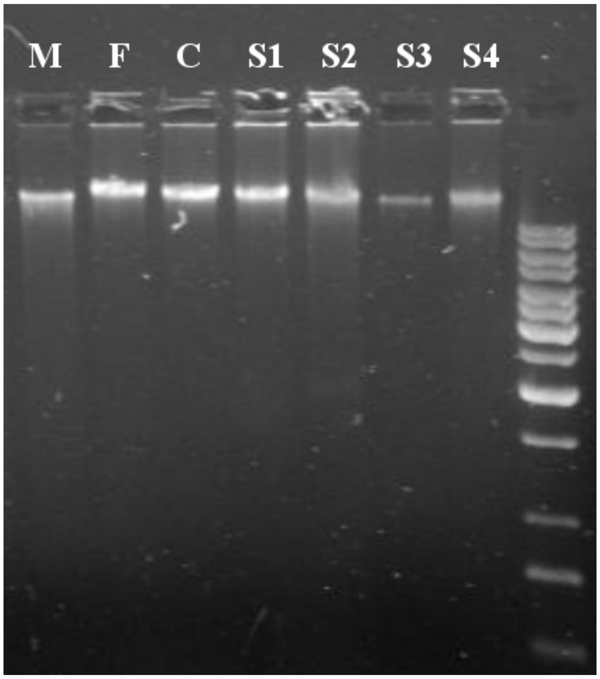


Figure 2. The 0.8% ethidium bromide-stained agarose gel showing DNA samples of *Dryas iulia* used for the AFLP essay. **M**, mother; **F**, father; **C**, external male control; **S1**, sibling 1; **S2**, sibling 2; **S3**, sibling 3 and **S4**, sibling 4. DNA patterns (1Kb Ladder) are shown in the far left. *D. iulia* DNA samples S3 and S4 were diluted 10X to be best visualized on the gel.

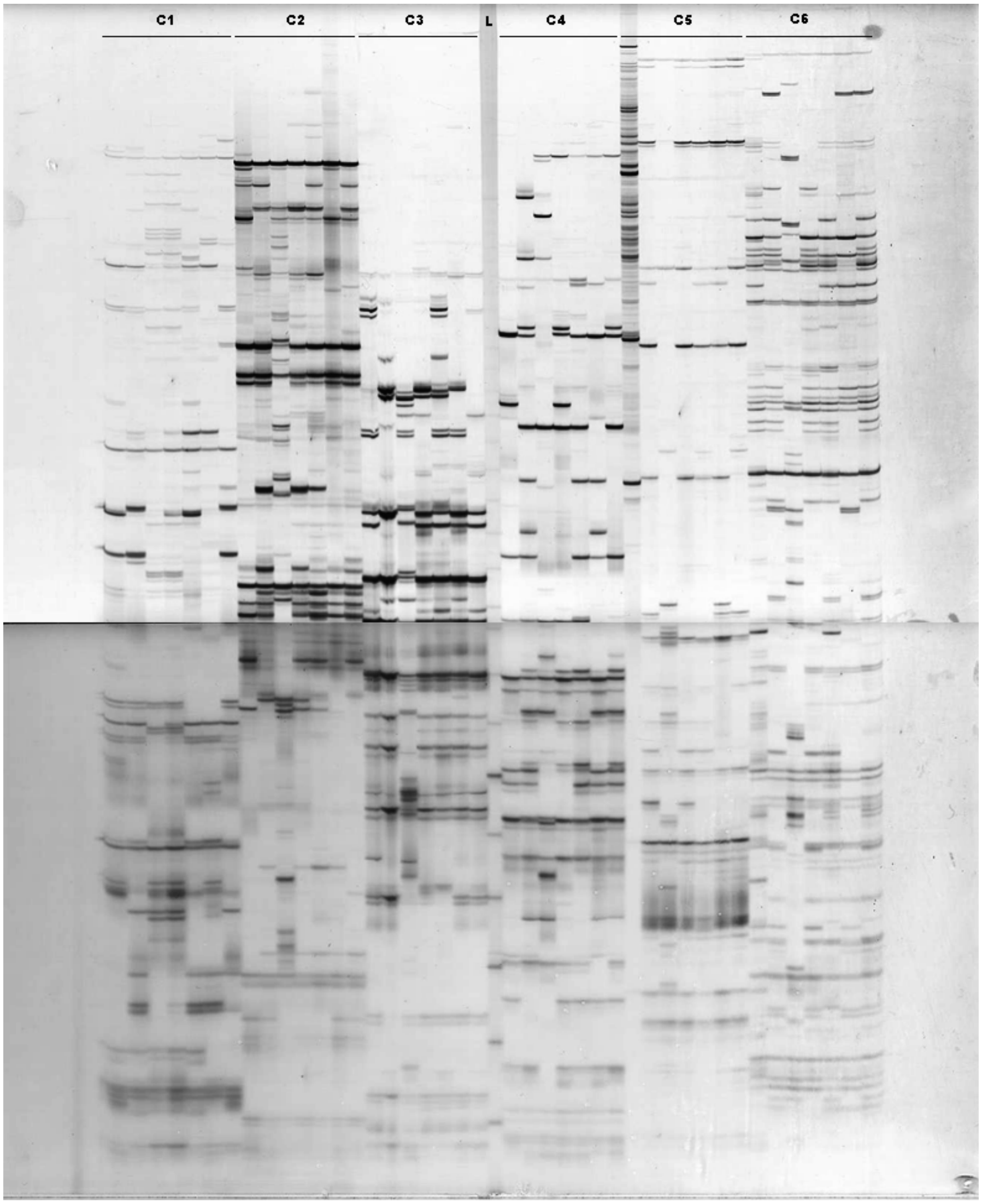


Figure 3. Silver stained 6% polyacrilamide denaturing gel showing the AFLP patterns of DNA samples tested six set of primers. **C1**, EcoRI+ACG / MseI+CTC; **C2**, EcoRI+ACC / MseI+CGC; **C3**, EcoRI+AGG / MseI+CCG; **C4**, EcoRI+ACT / MseI+CCC; **C5**, EcoRI+ATG / MseI+CCA and **C6**, EcoRI+ATA / MseI+CCT; **L**, 1kb Ladder. Sample sequence inside of each set of primer: the two progenitors (fist female, second male), and an external control male and four siblings.

ANEXO 2

INSTALAÇÕES E FOTOS DE CAMPO

Figura 1. Área de coleta no Horto Floresta Barba Negra (HBN), Barra do Ribeiro, RS.

Figura 2. Área de coleta na Estrada do Perau Velho (EPV), Santa Maria, RS.

Figura 3. Area de coleta no Morro da Borússia (MDB), Osório, RS.

Figura 4. Área de coleta na Estação Experimental de Águas Belas (EAB), Viamão, RS.

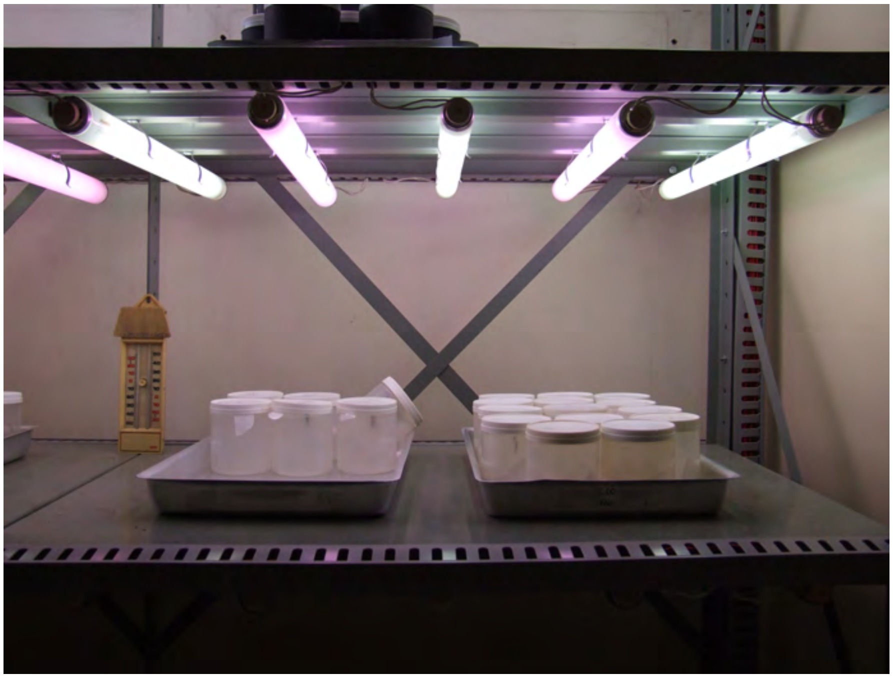


Figura 5. Criação de imaturos em câmara com fotoperíodo e temperatura controladas.

Figura 6. Insetário externo onde foram mantidos os adultos.

Figura 7. Gaiola utilizada para a realização das observações e experimentos de sucesso copulatório.