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LA EVOLUCIÓN DEL TRANSPORTE DE HUEVOS EN "PHYLLOMORPHA LACINIATA" (HET, COREIDAE): UNA APROXIMACIÓN COMPORTAMENTAL, FISIOLÓGICA Y MOLECULAR PARA EXPLICAR SU SIGNIFICADO ADAPTATIVO EN MACHO Y HEMBRAS

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Francisco García González

Bajo la dirección de la doctora

Montserrat Gomendio Kindelan

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La evolución del transporte de huevos en *Phyllomorpha laciniata* (Het., Coreidae)

Una aproximación comportamental, fisiológica y molecular para explicar su significado adaptativo en machos y hembras

Francisco García González

Departamento de Ecología Evolutiva
Museo Nacional de Ciencias Naturales (CSIC)

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Memoria presentada por Francisco García González para optar al grado de Doctor en Ciencias Biológicas.
Dirigida por Montserrat Gomendio Kindelan, Investigador Científico del Consejo Superior de Investigaciones Científicas (Museo Nacional de Ciencias Naturales)

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El Doctorando	*V.º B.º del Director de la Tesis*	*V.º B.º del Tutor de la Tesis*
Francisco García González	Montserrat Gomendio Kindelan	Concepción Ornosa Gallego

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Foto de portada:
Macho de *Phyllomorpha laciniata* portando huevos. Se observan cinco huevos de los cuales dos (los de color negro) están parasitados.

Í N D I C E

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con respecto a uno de los capítulos centrales de la Tesis.

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"En nuestro empeño de concebir la realidad, nos parecemos a alguien que tratara de descubrir el mecanismo invisible de un reloj, del cual ve el movimiento de las agujas, oye el tic-tac, pero no le es posible abrir la caja que lo contiene...podrá imaginar un mecanismo que sea capaz de producir todos los efectos observados; pero nunca estará seguro de si su imagen es la única que los pueda explicar. Jamás podrá compararla con el mecanismo real, y no puede concebir, siquiera, el significado de una tal comparación. Como él, el hombre de ciencia creerá ciertamente que, al aumentar su conocimiento, su imagen de la realidad se hará más simple y explicará mayor número de impresiones sensoriales. Puede creer en la existencia de un límite ideal del saber, al que tiende el entendimiento humano, y llamar a este límite la verdad objetiva"

Albert Einstein y Leopold Infield

"El agua que tocamos en los ríos es la postrera de las que se fueron y la primera de las que vendrán; así el día presente"

Leonardo Da Vinci

A mis padres, a mi hermana y a Esther.

ESTRUCTURA DE LA TESIS

La presente Memoria de Tesis Doctoral está constituida por una **Introducción General** (Capítulo 1), una serie de **Capítulos Temáticos** (Capítulos 2-7), una **Discusión General** (Capítulo 8) y, por último, las **Conclusiones** del trabajo.

La Introducción General expone, en castellano, los antecedentes del tema y revisa el contexto teórico en el que se encuadra la Tesis. Asimismo, en este apartado se plantean los **Objetivos de la Tesis** y se realizan unas breves **Introducciones a los Capítulos temáticos**. En la Discusión General se revisan los resultados obtenidos y su relevancia, todo ello en un contexto integrador.

Cada uno de los Capítulos temáticos reproduce el texto íntegro de manuscritos que han sido enviados para su publicación en revistas científicas internacionales. Por ello, se han presentado en Inglés, el idioma en el que fueron redactados y enviados a dichas revistas. En cualquier caso, la Introducción General realiza una aproximación introductoria a cada uno de estos bloques temáticos y la Discusión General revisa y discute los principales resultados que se han obtenido en dichos Capítulos Temáticos, todo ello en castellano*. Por otra parte, cada uno de los Capítulos incorpora un resumen en castellano.

* Muchos de los términos comunes en Biología fueron, en su origen, definidos en lengua inglesa, y son de difícil traducción al castellano. Sin embargo, una gran parte de los empleados en esta Tesis han sido traducidos de acuerdo a la siguiente propuesta: Soler, M.; Carranza, J.; Cordero Rivera, A.; Moreno, J.; Senar, J. C. y Soler, J. J. 2001. Traducción al español de los términos ingleses más conflictivos utilizados en Etología, Ecología y Evolución. Etología, 9: 43-46 (disponible en http://www.etología.org).

CAPÍTULO 1

Introducción

1. Introducción general y antecedentes

1.1. Adaptación, selección natural y selección sexual

La teoría evolutiva propone que las fuerzas selectivas favorecerán aquellos caracteres que incrementen el éxito reproductivo individual a lo largo de todo el ciclo vital (Darwin, 1859; Endler, 1986). Dicho éxito reproductivo se puede dividir en varios componentes que incluyen supervivencia hasta la edad reproductiva, longevidad en la etapa reproductiva, fecundidad, éxito en la adquisición de pareja y en el apareamiento, y supervivencia de la descendencia (Clutton-Brock, 1988). Para poder establecer que un carácter determinado tiene un valor adaptativo es necesario demostrar que incrementa el éxito reproductivo de los individuos que lo portan (Williams, 1966a; Williams, 1975), y se ha invertido mucho esfuerzo en intentar relacionar caracteres comportamentales, fisiológicos y morfológicos con mejoras en dichos componentes del éxito reproductivo, con el fin de determinar cómo se han adaptado los individuos al medio ambiente en el que viven.

La selección natural favorece aquellos caracteres que mejoran la supervivencia de un individuo. Sin embargo, hay otros caracteres que no sólo no mejoran la supervivencia, sino que con frecuencia la ponen el peligro. Este es el caso de los ornamentos de muchas especies de aves, y de las "armas" de algunas especies de mamíferos, que son caracteres costosos de producir y que, en muchos casos, aumentan la vulnerabilidad frente a predadores. La ventaja que conllevan dichos caracteres es la de aumentar el atractivo frente a las hembras (elección por parte de la hembra) o mejorar la capacidad competitiva entre machos (competencia intrasexual). A este tipo de selección, que favorece aquellos caracteres que mejoran las posibilidades de encontrar pareja y de conseguir cópulas, aún a costa de la supervivencia, se le denomina selección sexual (Darwin, 1871; Andersson, 1994).

En definitiva, un carácter adaptativo es un carácter que confiere ventajas sobre la capacidad de sobrevivir y dejar descendientes, y en este sentido la selección natural y la selección sexual, que son las principales influencias causales del cambio evolutivo, son explicaciones de la adaptación.

1.2. Competencia espermática

La idea de que la cópula era el punto final del proceso de selección de pareja por parte de la hembra, o de la competencia entre los machos por acceder a las hembras, fue la predominante hasta las últimas décadas. Se asumía que las hembras copulaban con un sólo macho, por lo que el éxito en el apareamiento era considerado como equivalente del éxito reproductivo. Sin embargo, la evidencia de que es común que las hembras se apareen con varios machos en un mismo ciclo reproductivo, unido al desarrollo de técnicas para determinar paternidad que han demostrado que las hembras producen descendencia generada por varios machos, ha cambiado radicalmente esta visión (Parker, 1970; Andersson, 1994; Hughes, 1998). Parker (1970) fue el primero que propuso que la selección sexual continúa más allá de la cópula, en forma de competencia espermática, esto es, la competencia entre los gametos de diferentes machos por la fertilización de los óvulos de una hembra. Cuando las hembras se aparean con más de un macho durante un ciclo reproductivo y eyaculados de diferentes machos coexisten en el tiempo en el interior del tracto reproductor femenino existe competencia espermática. La competencia espermática genera presiones evolutivas opuestas en los machos: por un lado, favorece adaptaciones que permiten a los machos anular (por medio de diversos mecanismos) la representación de gametos de machos rivales que se encuentran en el tracto femenino, mientras que por otro favorece adaptaciones que previenen que las hembras se apareen con otros machos o que el esperma de machos rivales futuros anule el suyo propio (Smith, 1984; Birkhead y Møller, 1998; Simmons,

2001). En este sentido, en las especies que presentan competencia espermática ésta ha influido en la evolución de rasgos comportamentales, fisiológicos y celulares, que incluyen un aumento de la producción espermática, una mejora de la calidad del eyaculado, tapones copulatorios, estrategias de guarda de la pareja, o genitalias especializadas para extraer esperma de machos rivales o para situar el eyaculado en las posiciones más ventajosas (Smith, 1984; Birkhead y Møller, 1998; Simmons, 2001).

A nivel intraespecífico la competencia espermática también explica diferencias entre los machos en la inversión en el eyaculado. Una serie de modelos basados en la teoría de juegos ha propuesto que la inversión en eyaculado de los machos de una población varía dependiendo del nivel de competencia espermática y de la situación (favorecida o desfavorecida) que los machos experimenten a la hora de realizar las cópulas. Los modelos teóricos sobre competencia espermática predicen que los machos que están expuestos a un mayor riesgo de competencia espermática (la probabilidad de que un macho se encuentre en competencia espermática por la fecundación de los óvulos de una hembra) deberían invertir más en la producción de espermatozoides (Parker, 1982, 1990a, 1990b, 1998; Parker *et al*., 1997; Ball y Parker, 1998). Esta predicción, y otras resultantes de la teoría de juegos aplicada a la competencia espermática, han sido apoyadas de manera empírica por estudios en varios *taxa*. Un ejemplo representativo se da en el escarabajo coprófago *Onthophagus binodis*. En esta especie de coleóptero los machos pueden seguir dos estrategias alternativas que están asociadas con un dimorfismo en el tamaño. Los machos que alcanzan un tamaño mayor están provistos de unos cuernos prominentes y siguen una estrategia de monopolización de las hembras, mientras que los machos de pequeño tamaño no presentan cuernos y se comportan como "furtivos", es decir, intentan adquirir cópulas con las hembras guardadas por los machos de gran tamaño. Como consecuencia de la estrategia adoptada, los machos pequeños siempre se enfrentan a competencia espermática, mientras que los machos grandes únicamente se enfrentan a competencia espermática cuando algún macho furtivo consigue copular con la hembra guardada. Los resultados obtenidos del análisis de la inversión en eyaculado apoyan de manera contundente las predicciones de los modelos de competencia espermática: los machos pequeños tienen un tamaño relativo de testículo mayor que los machos grandes, y los machos pequeños producen un mayor volumen de eyaculado (Simmons *et al*., 1999).

En resumen, durante los 32 años que han pasado desde el trabajo pionero de Parker (1970), la disciplina de la competencia espermática ha desarrollado un fuerte cuerpo teórico (Parker, 1982, 1990b, 1990a, 1993, 1998; Parker *et al*., 1990; Parker y Begon, 1993; Ball y Parker, 1996; 1997, 1998, 2000; Parker *et al*., 1997), y se ha puesto de manifiesto que la competencia espermática es una importante fuerza selectiva que ha modulado la fisiología, morfología y comportamiento de los organismos (revisión en Birkhead y Møller, 1998; Simmons, 2001). A pesar de que se ha visto que la competencia espermática opera en la mayoría de los grupos animales, los insectos son el mejor modelo para llevar a cabo su estudio. Muchas especies de insectos presentan competencia espermática ya que, primero, las hembras suelen aparearse con machos diferentes en un mismo ciclo reproductivo y, segundo, son capaces de almacenar espermatozoides viables de varios machos que coexisten en el espacio y en el tiempo, durante largos periodos de tiempo. El almacenamiento de los espermatozoides ocurre en la mayoría de especies de insectos, debido a la posesión de órganos especiales denominados espermatecas en donde se mantienen los gametos masculinos, por norma general hasta el momento de la fecundación. Las espermatecas varían en morfología, pero por lo general son modificaciones más o menos complejas de un conducto que desemboca en una pequeña bolsa que puede estar más o menos esclerotizada. Algunas especies de insectos almacenan los

espermatozoides en otros lugares. Por ejemplo, algunos dípteros los almacenan tanto en la espermateca como en el receptáculo seminal, mientras que los odonatos también utilizan la *bursa copulatrix*, un ensanchamiento del tracto reproductor femenino, para realizar un almacenamiento secundario.

La competencia espermática, y por consiguiente, los patrones de paternidad obtenidos por los machos, están determinados por unos mecanismos subyacentes. En insectos estos mecanismos son muy diversos (revisión en Simmons y Siva-Jothy, 1998; Simmons, 2001). Los que ocurren con mayor frecuencia son los siguientes:

(1) Mezcla de esperma ("sperm mixing"): El esperma de los diferentes machos se mezcla en la espermateca femenina y la competencia por la fecundación de los óvulos se resuelve, principalmente, de acuerdo a la representación gamética de cada uno de los machos rivales.

(2) Desplazamiento de esperma por flujo de esperma ("sperm displacement by sperm flushing"): El esperma que se encuentra previamente almacenado en la espermateca es eliminado por el flujo de esperma del macho que insemina en última instancia a la hembra. Este mecanismo está en gran medida determinado por la anatomía femenina y por el volumen del eyaculado transferido.

(3) Desplazamiento de esperma por eliminación de esperma ("sperm removal"): El esperma que se encuentra previamente almacenado es directamente eliminado por el macho que se encuentra en cópula debido a adaptaciones anátomicas de la genitalia. Este mecanismo ocurre principalmente en algunas especies de odonatos, que presentan en el edeago unas estructuras, en algunas ocasiones espinosas, con las cuales el esperma rival es eliminado durante los primeros instantes del acople genital (Waage, 1979).

(4) Pérdida de esperma ("sperm loss"): El esperma de un macho se pierde parcial o totalmente de manera pásiva de la espermateca femenina antes de que otro macho vuelva a inseminar a la hembra.

(5) Estratificación del esperma ("sperm stratification"): El esperma de los diferentes machos que inseminan a una hembra se almacena de manera estratificada en la espermateca femenina de acuerdo al órden con el que accede a este órgano, por lo que, en general, los espermatozoides inseminados por el último macho son los que acceden primero al lugar donde se fecundan los óvulos.

La determinación del mecanismo de competencia espermática que opera en una especie se puede complicar por la combinación de varios de estos mecanismos. Puede ocurrir que ocurra un desplazamiento de esperma incompleto seguido de una mezcla de esperma, o bien puede ocurrir una pérdida de esperma en combinación con algún otro mecanismo, por citar sólo algunos ejemplos. Para discernir los mecanismos implicados en estos casos se hace necesario un análisis detallado de la paternidad obtenida por varios machos que se aparean con una hembra, y el control de factores (intervalos entre cópulas, duración de las cópulas, tamaño de los machos, etc.) que puedan influir en la paternidad observada.

La competencia espermática es, por lo tanto, la continuación de la selección sexual en su vertiente de competencia entre machos, ya que en lugar de ocurrir para conseguir cópulas, ésta sucede después de la cópula y dentro del tracto reproductor femenino para conseguir fecundaciones. Recientemente, Eberhard (1996) ha puesto énfasis en la idea de que también la selección sexual puede continuar después de la cópula en forma de selección por parte de la hembra, si la hembra "selecciona", por medio de diversos mecanismos, el esperma de determinados machos. Thornhill (1983) propuso el término de elección críptica femenina para este proceso debido a que no es posible determinar el efecto de esta elección si el investigador únicamente se basa en la observación de las cópulas que realiza una hembra. En otras palabras, si las hembras favorecen la inseminación o fecundación de sus óvulos, por parte de los espermatozoides de un

macho dado frente a los de otro, este proceso es críptico. En la actualidad se está examinado el alcance de la elección críptica femenina, especialmente por medio de la investigación de incompatibilidades genéticas entre los gametos (Zeh y Zeh, 1996; Zeh y Zeh, 1997; Birkhead, 1998; Tregenza y Wedell, 2000). Con la consideración de la elección críptica femenina el escenario de la selección sexual después de la cópula abarcaría entonces dos facetas: competencia espermática y elección críptica femenina.

1.3. Competencia espermática y cuidado parental

La competencia espermática es, en definitiva, competencia por la fecundación de los óvulos, y por lo tanto, competencia por la paternidad de la descendencia. Los mecanismos de competencia espermática determinan cómo se traducen las cópulas de los machos en éxito a la hora de fecundar. Por lo tanto, la evaluación que los machos hagan de sus posibilidades de generar descendencia dependerá de cuáles sean los mecanismos de competencia espermática. Así pues, si los mecanismos de competencia espermática determinan que sea el último macho en copular el que fecunda los óvulos disponibles, los machos intentarán evitar que otros machos inseminen a las hembras después de ellos, y podrán evaluar sus posibilidades de generar crías según lo exitosos que hayan sido en evitar nuevas cópulas. Si el mecanismo de competencia espermática es el de mezcla de esperma procedente de diferentes eyaculados, el éxito a la hora de fecundar vendrá determinado por la cantidad de espermatozoides transferidos por cada macho, por lo que la evaluación dependerá del número de cópulas conseguidas en relación a otros machos, o de la duración relativa de la cópula si la transferencia de espermatozoides se relaciona positivamente con la duración del acoplamiento.

Una de las preguntas más fascinantes en ecología evolutiva versa sobre la relación entre la confianza de paternidad que tienen los machos y el grado de cuidado parental que proveen a las crías. Se ha sugerido que la confianza de paternidad, definida como la probabilidad media de ser el padre de la descendencia después de la cópula (Alexander, 1974; Simmons, 2001), es un determinante fundamental del cuidado paternal. Esto es así porque se espera que la selección natural favorezca a los individuos que modulan su inversión en el cuidado parental en relación con la paternidad genética, ya que de esta manera no estarían invirtiendo en descendencia que no es suya (Trivers, 1972). Por ello, la competencia espermática influye sobre los niveles de cuidado parental que los machos desarrollen, puesto que la competencia espermática implica que la paternidad en muchos casos no alcance el 100% de la descendencia producida por la hembra tras la cópula, y los mecanismos de competencia espermática determinan la confianza de paternidad (Gwynne, 1984; Wright, 1998).

1.4. Cuidado parental

El cuidado parental, que se define como cualquier forma de comportamiento realizado por los progenitores que incremente la eficacia biológica de la descendencia genética (Clutton-Brock y Godfray, 1991; Clutton-Brock, 1991), ha evolucionado en multitud de *taxa*, desde los insectos hasta los mamíferos. El sexo que provee el cuidado es, en algunas especies la hembra, en otras el macho, y en otras especies ambos sexos. Además, el grado de elaboración del cuidado que se provee es extremadamente variado, desde la inversión en los gametos hasta la asistencia social de la descendencia que ya es adulta, pasando por el cuidado de los huevos y la alimentación de las crías (Clutton-Brock, 1991). El cuidado parental ocurre por norma general en especies en las cuales la descendencia se enfrenta a condiciones bióticas o físicas extremas o peligrosas, puesto que es en estas especies en donde los beneficios, en términos de mejora de la probabilidad de supervivencia de las crías, son elevados (Wilson, 1971; Zeh y Smith, 1985; Tallamy y Wood, 1986;

Clutton-Brock, 1991). El estudio de la evolución del cuidado parental es posible siempre y cuando se identifiquen los beneficios que provee el cuidado para las crías y los costos que supone para los progenitores, puesto que para conocer el valor adaptativo de una estrategia determinada se hace necesario conocer el balance de costos/beneficios a nivel reproductivo (Williams, 1966b; Trivers, 1972; Clutton-Brock, 1991). Fue decisiva en este punto la contribución teórica realizada por Trivers (1972) en la que se concibió el concepto de inversión parental. La inversión parental se define como todo aquello que un progenitor hace por una cría que aumenta las posibilidades de reproducción y supervivencia de ésta, mientras que disminuye la capacidad del progenitor de producir otras crías en el futuro. Este concepto es de extrema importancia pues permite diferenciar entre los beneficios reproductivos derivados del cuidado de las crías, en términos de supervivencia y aumento del éxito reproductivo, y los costos reproductivos que pueden ser medidos por la disminución de la capacidad del progenitor de criar más descendientes.

El cuidado parental en artrópodos está presente en al menos 20 órdenes terrestres (Wilson, 1971; Tallamy y Wood, 1986; Clutton-Brock, 1991), y está representado principalmente en los órdenes de insectos sociales Hymenoptera e Isoptera, y también en Thysanoptera, Embioptera, Coleptera, junto con Heteroptera y Homoptera (ver para estos dos últimos órdenes Odhiambo, 1959; Eberhard, 1975; Smith, 1980; Tallamy y Denno, 1981; Eberhard, 1986; Kudô *et al*., 1989; Tallamy, 2001). A pesar de que el cuidado parental está presente en un gran número de órdenes de insectos, el número de especies que lo presenta es escaso, probablemente debido a que en este grupo se ha seleccionado la producción de un gran número de huevos y los padres pueden hacer poco o nada para proteger o proveer alimento a las crías (Zeh y Smith, 1985; Tallamy y Wood, 1986; Clutton-Brock, 1991). El cuidado paternal exclusivo se encuentra poco representado entre los insectos, aunque tasas altas de predación o parasitismo de los huevos, la ausencia de lugares de ovoposición adecuados, y los beneficios para los machos en relación a la elección de pareja por parte de las hembras, han favorecido probablemente su evolución en algunas especies de insectos (Ridley, 1978; Smith, 1980; Zeh y Smith, 1985; Tallamy, 2000; Tallamy, 2001). Por último, aún menos representado se encuentra el cuidado paternal efectuado como resultado de la ovoposición sobre coespecíficos. Este último se conoce en muy pocas especies de artrópodos: algunas especies de picnogónidos, una especie de opilión y varias especies de heterópteros (Tallamy, 2001). Entre heterópteros el caso más estudiado es de varias especies de chinches acuáticas gigantes de la familia Belostomatinae, en las que el macho recibe puestas de huevos en el dorso y realiza un comportamiento de aireación en la superficie de estos (Smith, 1976; Smith, 1979; Smith, 1997).

Una vez que existe cuidado parental en una especie puede entonces evolucionar el parasitismo intraespecífico de puesta. El parasitismo intraespecífico de puesta se define como el parasitismo que resulta cuando las hembras de una especie depositan sus huevos en los nidos de otros coespecíficos que realizan cuidado parental (ver por ejemplo Petrie y Møller, 1991). Es, por lo tanto, otra estrategia alternativa al cuidado parental para incrementar el éxito reproductivo aumentando la probabilidad de supervivencia de las crías, pero reduciendo o eliminando los costos del cuidado parental (Åhlund y Andersson, 2001). Esta forma de parasitismo aparece en algunos *taxa* de insectos, especialmente insectos sociales (ver revisiones de Field, 1992; Brockman, 1993; Zink, 2000), y es relativamente frecuente en las aves, donde ha sido ampliamente documentado (por ejemplo Yom-Tov, 1980).

1.5. Conflicto sexual

Uno de los aspectos más interesantes relacionados con la competencia espermática y con el cuidado parental es el conflicto entre los intereses de los machos y de las hembras. Se

puede considerar que el conflicto sexual se origina en el mismo momento en que se desarrolla la anisogamia, ya que en este momento la inversión que cada sexo realiza en el zigoto difiere (Parker et al., 1972; Trivers, 1972; Roldan et al., 1992). En especies con anisogamia las hembras invierten energía en la producción de un número reducido de gametos de gran tamaño, mientras que los machos invierten en un gran número de gametos, pero de pequeño tamaño. Esto da como resultado un conflicto sexual que, a menudo, se extiende sobre el cuidado parental postzigótico puesto que (1) Al haber generalmente dos cuidadores potenciales (la hembra y el macho), cada uno de estos se beneficiaría si el otro invirtiera más en cuidado parental, y (2) Es frecuente que haya asimetrías en los costos del cuidado por parte de los dos sexos, favoreciendo la reducción del cuidado de un sexo a expensas del otro sexo (revisión en Westneat y Sargent, 1996).

Las adaptaciones de los machos para afrontar la competencia espermática también dan a menudo como resultado un conflicto entre sexos. Esto es debido a que, frecuentemente, los intentos de los machos de incrementar su éxito reproductivo reducen la eficacia biológica femenina, por lo que entran en conflicto con los intereses de las hembras (ver Stockley, 1997).

1.6. *Phyllomorpha laciniata* como un organismo modelo para el estudio de la competencia espermática y el cuidado parental

Con el presente estudio se pretenden encontrar las respuestas que expliquen, desde un punto de vista adaptativo, el comportamiento de transporte de huevos en *Phyllomorpha laciniata* Villers 1789 (Heteroptera, Coreidae). Esta especie presenta un comportamiento atípico dentro de los insectos terrestres. Las hembras muestran una gran flexibilidad en el lugar de ovoposición: depositan sus huevos sobre la planta hospedadora de la cual se alimentan (generalmente la cariofilácea *Paronychia argentea*) o bien sobre el dorso de otros individuos adultos de la misma especie, tanto machos como hembras (Figura 1). En el caso en el que los huevos sean depositados sobre coespecíficos, estos son transportados hasta la eclosión y consiguiente aparición de las ninfas, que entonces inician vida libre (Bolivar, 1894; Mineo, 1984; Kaitala, 1996; Reguera, 1999).

El comportamiento de puesta sobre coespecíficos en *P. laciniata* fue citado por primera vez en el siglo XIX por Bolivar (1894), y a principios del siglo XX por otros entomólogos o naturalistas que también citaron lugares de recolección (Lambertie, 1902; Royer, 1902; Mayet, 1903; Mingaud, 1903; Jeanell, 1909; Olivier, 1909; Reuter, 1909). La distribución de esta especie de ámbito mediterráneo y su revisión taxonómica fueron estudiadas más recientemente por Vázquez (1985) y Moulet, (1995). Sin embargo, no fue hasta 1984 y la década iniciada en 1990 cuando se realizaron estudios sobre el comportamiento y el ciclo biológico de esta especie (Mineo, 1984; Kaitala, 1996; Reguera, 1999), los cuales han respondido a muchas preguntas desde el punto de vista de su historia natural y ecología, pero que también han puesto de manifiesto la existencia de numerosos interrogantes que, desde el punto de vista evolutivo, tienen un extraordinario interés (ver más adelante). Reguera (1999) inició en el año 1996 los primeros estudios sistemáticos sobre una población natural de *P. laciniata*, y su trabajo ha

Figura 1. Macho de *Phyllomorpha laciniata* portando huevos. El estado de desarrollo de los huevos puede inferirse por su color. Nótese el huevo parasitado, depositado encima de la cabeza, que es fácilmente distinguible por su color negro.

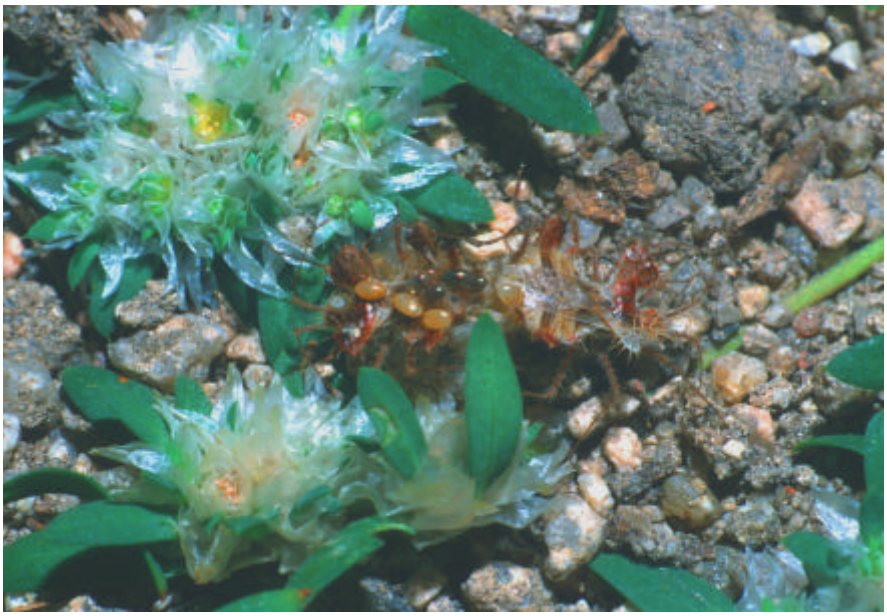

Figura 2. Individuos en cópula. El individuo de la izquierda es el macho y el de la derecha la hembra. Nótese la diferencia en la carga de huevos, que se corresponde con los patrones naturales generales.

Figura 3. Ninfas de *Phyllomorpha laciniata* en su tercer estado ninfal.

sido extremadamente útil para avanzar en la investigación sobre este insecto. El presente trabajo se ha apoyado en estas investigaciones pioneras, a la vez que lo ha hecho en una continuación del estudio sistemático de la población estudiada por Reguera (1999), que permite contrastar datos y obtener una visión temporal amplia de los patrones naturales en esta especie. Paralelamente se han obtenido datos provenientes de otras poblaciones naturales que tienen una gran validez para comprender el sistema y para realizar estudios experimentales.

Con los datos aportados previamente por otros autores (Mineo, 1984; Reguera, 1999), y con los obtenidos durante la realización del presente estudio, se ha podido comprender el ciclo biológico de esta especie. Básicamente, los individuos adultos salen de los refugios invernales, ubicados bajo piedras u hojarasca (Mayet, 1903; Mingaud, 1903; Pierre, 1903; Reuter, 1909; Reguera, 1999), a finales de marzo o principios de abril (fechas para poblaciones de España Central), dependiendo de las condiciones ambientales (ver Reguera, 1999, para una descripción de los hábitats en que se encuentra la especie). Poco tiempo después de que los individuos salgan de sus refugios invernales se inician los apareamientos, que dan lugar a la ovoposición de las hembras (Figura 2). De los huevos depositados emergen las ninfas del primer instar, que tras 5 mudas dan lugar a los nuevos imagos (Figura 3). Esta segunda generación de imagos (adultos emergidos el año en curso) empieza a aparecer entre mayo y julio, dependiendo de las localidades.

Las características que posee esta especie hacen que sea susceptible de ser usada como modelo biológico para el estudio de diferentes aspectos relacionados con la competencia espermática, el cuidado paternal, y la relación

entre ambos. El hecho de que las hembras se apareen con varios machos en un mismo ciclo reproductivo, y de que almacenen los espermatozoides de diferentes machos en la espermateca (órgano femenino de almacenamiento de espermatozoides, ver Figura 1 del Capítulo 4) hasta el momento en el que son usados para la fertilización de los huevos, promueve la existencia de competencia espermática. Por otro lado, una serie de patrones comportamentales y tendencias poblacionales en el transporte de huevos sugieren que este comportamiento podría ser una forma rudimentaria de cuidado paternal (ver más abajo la sección 1.6.2: Transporte de huevos por parte del sexo masculino).

1.6.1. Transporte de huevos por parte del sexo femenino

En *P. laciniata* las hembras no pueden ponerse los huevos sobre ellas mismas, por lo que el caso del transporte de huevos por parte del sexo femenino podría ser un caso de parasitismo intraespecífico. Sin embargo, hay otras hipótesis que podrían explicar el comportamiento de transporte de huevos por parte del sexo femenino. Una de ellas es la selección de parentesco (Hamilton, 1964a; Hamilton, 1964b), bajo la cual las hembras podrían acceder a cuidar huevos de otras hembras si entre ellas existe una estrecha relación de parentesco. La tercera hipótesis que podría explicar este comportamiento es la existencia de una reciprocidad femenina en la puesta y aceptación de huevos (Dugatkin, 1997). Sin embargo, ninguna de estas hipótesis ha sido contrastada hasta la fecha. En cualquier caso, Reguera (1999) demostró que una pequeña proporción de hembras de la población porta huevos y que las hembras muestran una tendencia uniforme a portar un número bajo de huevos. Este patrón se mantiene en los estudios realizados por nosotros (sumando, en total, junto con Reguera (1999), 5 años de estudio y seguimiento de la misma población natural), y podría indicar bien que las hembras aceptan ser "parasitadas" hasta un grado en el que los costos no superan un cierto umbral para el portador, o bien que hay un nivel bajo de parasitismo que los individuos no pueden evitar (Reguera, 1999; Gomendio y Reguera, 2001).

1.6.2. Transporte de huevos por parte del sexo masculino

Las explicaciones adaptativas del transporte por parte de los machos podrían diferir de las aplicables al sexo femenino, puesto que la magnitud del fenómeno es muy superior en el sexo masculino. La literatura científica reciente revela la existencia de una fuerte controversia en relación al valor adaptativo del transporte de huevos por parte de los machos. Algunos autores defienden la idea de que este comportamiento es el resultado de un parasitismo intraespecífico realizado por las hembras (Kaitala et al., 2001). Por otra parte, otros autores sostienen que, a pesar de que el parasitismo intraespecífico pueda explicar parcialmente el transporte de huevos en esta especie, como ocurre con la pequeña proporción de huevos que transporta la población femenina, es improbable que explique los niveles tan altos de transporte de huevos que son observados en la población masculina (Gomendio y Reguera, 2001). Estos últimos autores han sugerido que, dados los altos costos derivados del transporte de huevos para los adultos (Reguera y Gomendio, 1999; Kaitala et al., 2000), es improbable que un macho acepte huevos después de la cópula a no ser que exista alguna probabilidad de que, al menos, alguno de estos huevos haya sido fecundado por él. La hipótesis del cuidado paternal merece ser explorada teniendo en cuenta los siguientes hechos:

(1) Los patrones de transporte de huevos en poblaciones naturales indican que hay diferencias en la existencia e intensidad de este comportamiento entre sexos. Por un lado, mientras que una pequeña proporción de hembras de la población porta huevos, la proporción de machos portando huevos se incrementa poco tiempo después del inicio de la estación

reproductora hasta que prácticamente el 100% de los machos porta huevos (Reguera, 1999). Por otro lado, los machos portan significativamente más huevos que las hembras.

(2) Estudios recientes han mostrado el balance de costos-beneficios derivado del transporte de huevos en *P. laciniata*. Los huevos que son portados por individuos adultos disfrutan de tasas de supervivencia mucho mayores que los huevos que son dejados en planta (Kaitala, 1996; Reguera y Gomendio, 2002). Esto ocurre principalmente porque los huevos que son puestos en la planta sufren de tasas muy altas de parasitismo por parasitoides Esceliónidos de la especie *Gryon bolivari* (Giard) (Mineo, 1979; Mineo, 1984; Reguera y Gomendio, 2002) (Figura 4). Recientemente, se ha detectado que otro parasitoide, un Encírtido del género *Ooencyrtus* puede ser también responsable de la parasitación de los huevos (Virgilio Caleca, comunicación personal), aunque en una frecuencia menor que *G. bolivari*. En cuanto a los costos derivados del cuidado parental se ha visto en estudios experimentales que los individuos portadores sufren tasas de predación mayores que los no portadores (Reguera y Gomendio 1999; Kaitala *et al.* 2000), debido principalmente a una mayor conspicuidad y a que los huevos son depositados generalmente sobre las alas, lo que anula las posibilidades de una huida efectiva de los predadores. La presencia de estos beneficios y costos sugiere que los machos no deberían aceptar huevos a menos que tuvieran alguna probabilidad de aceptar huevos fecundados por ellos.

(3) Este insecto presenta cópulas extremadamente largas, de duraciones medias de entre 20 y 35 horas (Kaitala, 1998; Reguera, 1999). La existencia de unas cópulas tan largas en *P. laciniata* podría apuntar a que los machos intenten bien realizar una guarda de la pareja después del apareamiento, para evitar que otro macho copule con la hembra, o bien maximizar la transferencia de espermatozoides para aumentar la representación espermática en competición. Ambos hechos aumentarían el número de huevos fecundados por el macho que realiza la cópula, y por lo tanto, aumentaría su tasa de paternidad.

Figura 4. *Gryon bolivari* (Hym., Scelionidae), parasitoide de los huevos de *Phyllomorpha laciniata*, y huevos parasitados. Nótese cómo se ve por transparencia al parasitoide adulto que está a punto de emerger de un huevo de *P. laciniata*.

Esto se traduciría a su vez, en una mayor proporción de descendencia genética transportada por un macho que acepte huevos al acabar la cópula. Por lo tanto, los machos podrían estar maximizando su certeza de paternidad con la duración de la cópula, y modular la aceptación de huevos tras la cópula dependiendo de la certeza de paternidad que adquieran.

(4) La aceptación voluntaria de huevos por parte de los machos ante los intentos de ovoposición de las hembras indican que el macho está dispuesto, en algunas ocasiones, a realizar el transporte de huevos (Kaitala y Miettinen, 1997; Miettinen y Kaitala, 2000). Esto se explicaría si el macho tiene relación genética con al menos alguno de los huevos que porta.

(5) Reguera (1999) demostró que poblaciones que diferían en la presión por parasitoides diferían también en la intensidad con la que ocurría el transporte de huevos por parte de los machos, mientras que el transporte de huevos por parte de las hembras, siempre de una magnitud inferior al efectuado por los machos, permanecía invariable. Las diferencias en la flexibilidad con que se manifiesta el transporte de huevos por parte del sexo masculino en poblaciones diferentes podría indicar que este sexo modula la inversión parental dependiendo de las presiones que afecten al balance de costos y beneficios derivados del cuidado.

El objetivo general de esta Tesis es dilucidar

cuál es el valor adaptativo de portar huevos en *P. laciniata*, con el fin de aclarar si dicho comportamiento ha evolucionado como una forma rudimentaria de cuidado parental por parte de los machos, o como una forma de parasitismo por parte de las hembras. Puesto que las hembras de *P. laciniata* depositan algunos huevos sobre plantas y otros sobre individuos, esta especie representa un modelo excepcional que permite comparar los beneficios para los descendientes de recibir cuidado o no, y los costos para los progenitores de proveer dicho cuidado. Este tipo de comparaciones es generalmente imposible en especies donde el cuidado parental está desarrollado, puesto que lo general es que no exista variabilidad ya que todos los descendientes reciben cuidado, y todos los progenitores lo proveen. De hecho los pocos estudios que comparan los beneficios y costos derivados del cuidado para machos y hembras generalmente comparan especies donde sólo los machos cuidan y otras donde sólo las hembras cuidan. El problema al que se enfrentan estos estudios es que estas especies generalmente se diferencian en otros muchos factores que no se controlan. Por lo tanto, la situación de *P. laciniata* donde se da una situación atípica en la que coexisten casos de crías que reciben cuidado con otras que no, y poblaciones con diferentes grados de elaboración de cuidado parental, representa una oportunidad única para estudiar el origen del cuidado parental, el papel de la confianza de paternidad en la disponibilidad de los machos para proveer cuidado a las crías, y el grado de manipulación de las hembras para conseguir el cuidado de sus crías por parte de los machos. El estudio de estas cuestiones requiere el contraste de múltiples teorías e hipótesis en relación con la competencia espermática, el cuidado parental y el conflicto sexual, que se resuelven con análisis de la dinámica de poblaciones naturales, de comportamiento, de fisiología reproductiva, así como con análisis moleculares. Por todo ello, esta Tesis ha adoptado un enfoque multidisciplinar que aborda todos estos niveles.

2. Objetivos

El objetivo central de la Tesis es determinar el significado adaptativo del comportamiento de transporte de huevos en este insecto. Los objetivos concretos han sido los siguientes:

1. Estudiar las poblaciones naturales de *P. laciniata* para conocer el ciclo biológico y los patrones y comportamientos relacionados con su reproducción y con el transporte de huevos.

2. Examinar el papel de la hipótesis del parasitismo intraespecífico y de la aseguración de la paternidad en la explicación de los patrones naturales de transporte de huevos en *P. laciniata*.

3. Contrastar la hipótesis de una preferencia para ovopositar sobre coespecíficos por parte de las hembras debido a la mejora que este comportamiento supondría para la supervivencia de la descendencia.

4. Contrastar la hipótesis que contempla una estimulación de la fecundidad femenina como resultado de la presencia de coespecíficos debido a los beneficios, en términos de eficacia biológica, que esto supondría para las hembras.

5. Determinar el significado adaptativo de las cópulas largas y prolongadas en este insecto por medio del contraste de hipótesis relativas a la guarda de la pareja y a la competencia espermática, y realizar una prueba empírica de los modelos de riesgo de competencia espermática por medio del análisis de la respuesta de los machos a un incremento en dicho riesgo.

6. Desarrollar la aplicación de una metodología molecular para afrontar el problema de analizar relaciones de parentesco y paternidad en este insecto.

7. Aplicar marcadores moleculares en la determinación de las relaciones genéticas entre los huevos y los machos que los portan, y por lo tanto ofrecer datos reales de la existencia o no de cuidado paternal en este insecto.

8. Aplicar marcadores moleculares para determinar los patrones del uso del esperma y así obtener información del mecanismo de competencia espermática que opera en esta

especie.

9. Analizar la relación entre la paternidad resultante de los mecanismos de competencia espermática y la existencia y grado de cuidado paternal realizado.

10. Integrar el conocimiento obtenido en la realización de los objetivos precedentes y deducir, en el marco de la teoría sobre cuidado paternal, competencia espermática, y conflicto sexual, el significado adaptativo del transporte de huevos, especialmente el llevado a cabo por los machos.

3. Introducción breve a los capítulos

Introducción al Capítulo 2. Refutación de la hipótesis del parasitismo intraespecífico de puesta en *Phyllomorpha laciniata*.

La hipótesis de parasitismo intraespecífico de puesta sobre parejas en cópula ("mating pair intraspecific brood parasitism hypothesis") fue propuesta como una de las principales causas que explican los patrones de ovoposición y transporte de huevos en este insecto (Kaitala, 1996). Esta hipótesis propone que un gran número de huevos es depositado sobre parejas en cópula por una hembra ajena a la que se está apareando, aprovechando la supuesta incapacidad de los individuos en cópula a resistir puestas. Dicha hipótesis se basa en que (1) los machos en cópula portan significativamente más huevos que los machos que no están en cópula, y (2) los individuos en cópula en cautividad parecen ofrecer poca resistencia a que una hembra ajena ovoposite sobre ellos (ver por ejemplo, Kaitala y Miettinen, 1997). En estudios posteriores al de su formulación, dicha hipótesis se ha dado por probada y diversos autores han asumido que tiene un papel de gran relevancia en la evolución del transporte de huevos en este insecto (Kaitala, 1998; Härdling y Kaitala, 2001; Katvala y Kaitala, 2001; Tallamy, 2001). Sin embargo, no se ha realizado ningún contraste exhaustivo de esta hipótesis hasta la fecha.

Otra hipótesis alternativa que podría explicar los patrones naturales de transporte de huevos en *P. laciniata* es la "aseguración de la paternidad" ("paternity assurance hypothesis") por parte de los machos. Trivers (1972) predijo que en especies en las cuales los machos realizan inversión parental, el sexo masculino debería de desarrollar adaptaciones que disminuyan sus probabilidades de ser engañados por las hembras y así minimizar el esfuerzo parental empleado en descendientes no genéticos. En el contexto del sistema de *P. laciniata* uno de los mecanismos probables por los que se mejoran las probabilidades de paternidad es la realización de cópulas repetidas. Este insecto nos permite hacer un estudio para discriminar el alcance de la hipótesis del parasitismo intraespecífico y el de la hipótesis de la aseguración de la paternidad, puesto que las predicciones de ambas hipótesis son, en este sistema, diametralmente opuestas.

En el presente trabajo se analizan datos de poblaciones naturales provenientes de un estudio longitudinal en el tiempo, que abarca dos estaciones reproductivas de la especie, para contrastar las dos hipótesis y examinar el papel que tienen en la evolución del comportamiento de transporte de huevos de *P. laciniata*. Los resultados contribuirán a esclarecer si el cuidado paternal puede evolucionar por medio de una explotación femenina del sexo masculino o bien porque los machos tienen cierta confianza de paternidad sobre las crías que cuidan.

Introducción al Capítulo 3. Elección del lugar de ovoposición y estimulación de la ovoposición por presencia de coespecíficos: implicaciones para la eficacia biológica femenina.

Las hembras de especies en las que el cuidado parental no existe o está poco desarrollado pueden incrementar su eficacia biológica si seleccionan los lugares de ovoposición que confieran mayores probabilidades de supervivencia para las crías (ver por ejemplo Jaenike, 1978). Por otra parte, en aquellos casos en los cuales los lugares preferidos para realizar

la ovoposición son escasos o difíciles de encontrar, a las hembras les interesaría ajustar el ciclo reproductivo de tal manera que la ovoposición resulte estimulada frente a la presencia de un sitio idóneo de ovoposición (Papaj, 2000). El ciclo reproductivo femenino depende de un gran número de factores ambientales y sociales, y en algunas especies de insectos y mamíferos se ha demostrado que la presencia de coespecíficos o el apareamiento afecta a la ovulación o a la ovoposición. En todos estos casos, se ha interpretado que la estimulación de los ciclos reproductivos femeninos por coespecíficos beneficia principalmente a los machos, sin embargo, no está claro cómo la fisiología femenina puede estar modulada por los machos sin que esto revierta algún tipo de beneficio sobre las hembras.

El comportamiento en la ovoposición de las hembras de *P. laciniata* constituye una oportunidad única para abordar el estudio de las implicaciones evolutivas de las diferentes estrategias de ovoposición. Esta especie es un modelo ideal debido a que es posible estudiar la selección de lugares de ovoposición a un nivel no sólo intraespecífico sino también intraindividual, puesto que todas y cada una de las hembras de una población adoptan las tres estrategias de puesta a lo largo de sus vidas (huevos sobre la planta hospedadora, sobre un macho o sobre otra hembra).

Como se ha mencionado anteriormente, los huevos de *P. laciniata* que son transportados por un coespecífico tienen mayores probabilidades de supervivencia que los dejados en la planta hospedadora, por lo tanto, las hembras deberían ovopositar con preferencia sobre los coespecíficos para maximizar las tasas de supervivencia de las crías. Además, otra predicción derivada de los beneficios del transporte de huevos es que las hembras experimentarían una estimulación de la ovoposición en presencia de otros individuos para maximizar el número de huevos depositados sobre coespecíficos. En el Capítulo 3 de esta Tesis exploramos en este insecto la existencia de una selección del lugar de ovoposición y la existencia de una estimulación de la ovoposición por presencia de coespecíficos, y discutimos las implicaciones que esto tiene de cara al incremento del éxito reproductivo femenino.

Introducción al Capítulo 4. Ajuste de la duración de las cópulas y del tamaño del eyaculado de acuerdo al riesgo de competencia espermática.

La teoría de la competencia espermática con relación a la inversión en el eyaculado ha matizado la idea tradicional de que los machos emplean la mayoría de su esfuerzo reproductivo en lograr apareamientos, puesto que la asunción de que la producción de espermatozoides está exenta de costos no es correcta (Dewsbury, 1982; Nakatsuru y Kramer, 1982; Olsson et al., 1997). En las especies que presentan competencia espermática, el número de espermatozoides con el que un macho insemina a una hembra debe de resultar de un compromiso entre dos presiones de selección opuestas. Por un lado, el riesgo de competencia espermática favorecerá un incremento en el número de espermatozoides transferido, mientras que por otro lado el incremento en la inversión gamética se hará a expensas de la habilidad de los machos para invertir en apareamientos futuros o en un mantenimiento de la capacidad somática (para, por ejemplo, alimentarse, buscar otra pareja, etc.). En resumen, este compromiso dará como resultado inversiones estratégicas en el eyaculado, de acuerdo a los niveles de competencia espermática a los que se enfrenten los machos.

A pesar de los costos que implica una cópula de larga duración, *P. laciniata* se caracteriza por presentar cópulas extremadamente largas, que en algunas ocasiones alcanzan más de 40 horas. Sin embargo, no se ha realizado ningún estudio hasta la fecha que intente comprender el significado adaptativo de unas cópulas de tanta duración en este insecto. En el Capítulo 4 se examina el sentido adaptativo de las cópulas prolongadas y

de las estrategias de eyaculación en este heteróptero, por medio de la manipulación experimental del riesgo de competencia espermática al que se enfrentan los machos que se encuentran en cópula.

Introducción al Capítulo 5. El desarrollo de una herramienta molecular para analizar relaciones de parentesco y paternidad en *Phyllomorpha laciniata*: extracción de ADN y marcadores AFLP (Amplified Fragment Length Polymorphism).

La pieza clave en el sistema formado por *P. laciniata* es la resolución del problema evolutivo que representa el transporte de huevos por parte de los individuos. La interpretación evolutiva de este comportamiento ha originado una fuerte controversia acerca de su origen como forma de cuidado paternal o como consecuencia del parasitismo intraespecífico (Gomendio y Reguera, 2001; Kaitala et al., 2001). Para comprender el papel del cuidado parental y del parasitismo intraespecífico en este sistema es fundamental determinar con métodos moleculares la paternidad de los huevos portados por los machos.

Se denomina AFLPs (Amplified Fragment Length Polymorphisms) a una técnica que se basa en una doble amplificación, por medio de la PCR (reacción en cadena de la polimerasa), de fragmentos de restricción obtenidos de la digestión del ADN genómico (Vos et al., 1995; Vos y Kuiper, 1997; Mueller y Wolfenbarger, 1999; Gerber et al., 2000). Esta técnica puede hacer posible que el análisis de paternidad en este insecto sea una realidad. Los AFLPs permiten el análisis de muestras de pequeño tamaño, característica que es importante al considerar su uso potencial en *P. laciniata* debido a que únicamente ninfas de primer instar, que son extremadamente pequeñas, pueden ser analizadas (a partir de este instar no sobreviven en cautividad). No existe hasta la fecha ninguna publicación que recoja la existencia de otro tipo de marcadores que sean susceptibles de ser utilizados en esta especie, y el objetivo principal en este apartado de la Tesis fue evaluar varios protocolos de extracción de ADN de *P. laciniata* y encontrar unos cebadores (secuencia corta de nucleótidos que se acopla con la cadena de ADN y provee el extremo 3'-OH en el que la ADN polimerasa comienza la síntesis de ADN) selectivos de AFLPs, que ofrecieran un nivel de polimorfismo genético adecuado para la realización de futuros análisis de paternidad y de parentesco en esta especie.

Introducción al Capítulo 6. Análisis de la paternidad de los huevos portados por los machos.

En los últimos años la aplicación de técnicas moleculares a los estudios de ecología evolutiva ha revolucionado, en gran medida, esta disciplina. El uso de herramientas moleculares para determinar paternidad ha revelado que en sistemas de apareamiento tradicionalmente considerados monógamos, las hembras tienden a aparearse con más de un macho, lo que resulta en que los machos cuidan a menudo de crías que no han generado ellos (Birkhead y Møller, 1992; Møller y Birkhead, 1993; Westneat y Sargent, 1996). La comprensión del cuidado parental, del conflicto sexual, de los sistemas de apareamiento y del comportamiento social ha sido drásticamente alterada y mejorada por la determinación de las relaciones genéticas entre los individuos (ver por ejemplo Hughes, 1998). Los resultados generados mediante el empleo de herramientas moleculares han planteado numerosos interrogantes, entre los que se incluyen la relación entre el cuidado parental y la certeza de paternidad. *Phyllomorpha laciniata* es un modelo idóneo para abordar esta cuestión debido al comportamiento de puesta de huevos sobre coespecíficos, y a los consiguientes beneficios y costos asociados al transporte de huevos.

La utilidad de los AFLPs en análisis de paternidad ha sido únicamente examinada en tres estudios realizados sobre dos especies hasta la fecha; la planta proteacea *Persoonia mollis* (Krauss y

Peakall, 1998; Krauss, 1999) y el pechiazul *Luscinia svecica* (Questiau et al., 1999). Estos estudios pioneros han mostrado que los marcadores AFLPs pueden ser útiles en la determinación de la paternidad. El objetivo de este Capítulo ha sido emplear los marcadores AFLPs como herramienta para realizar análisis de paternidad en *P. laciniata* y contribuir al entendimiento del significado adaptativo del transporte de huevos en esta especie.

Introducción al Capítulo 7. Mecanismos de competencia espermática, confianza de paternidad y evolución del cuidado paternal

Los estudios de competencia espermática han aportado información importante al estudio de la relación entre paternidad y cuidado paternal en especies en las que los machos realizan una inversión parental en las crías (Wright, 1998). Generalmente, en estos estudios el parámetro "paternidad" se ha calculado para dos machos que se aparean en sucesión con una hembra, denominándose P_2 la proporción de descendencia que genera el segundo macho que se aparea (Boorman y Parker, 1976; Simmons y Siva-Jothy, 1998). El estadístico P_2 (o P_n, para el enésimo macho que se aparea con una hembra dada) es de una importancia extrema en el estudio de la competencia espermática. El valor medio de este estadístico para la población o especie, y sus variaciones alrededor de la media, son indicativos de los patrones del éxito en la fecundación en base al estatus del macho según el orden de apareamientos (por ejemplo, primer macho que se aparea versus último macho que se aparea), y por ello, indicativos de los mecanismos de competencia espermática que operan en la especie (Simmons y Siva-Jothy, 1998; Simmons, 2001).

En el presente estudio se analizan los valores de paternidad que adquieren los últimos machos que copulan con hembras que se han apareado en condiciones naturales, y a partir de estos valores se infiere el mecanismo de competencia espermática. El conocimiento de los patrones del uso del esperma es de una importancia extrema en el sistema de *P. laciniata* puesto que es necesario para entender cómo se traduce el comportamiento sexual de los machos en éxito a la hora de fecundar los huevos y, por lo tanto, en la producción de crías, para, finalmente comprender cómo evalúan los machos su certeza de paternidad y cómo influye esto sobre la aceptación de huevos.

En general la relación entre las variaciones en la paternidad y el cuidado paternal es compleja y su existencia o ausencia depende de una serie de factores, como por ejemplo, el compromiso, presente y futuro, entre el esfuerzo dedicado a la reproducción y el dedicado a la supervivencia (ver por ejemplo Westneat y Sherman, 1993). De acuerdo con los modelos teóricos, una disminución en el cuidado paternal en respuesta a una reducción de la paternidad beneficiaría a un macho si: (1) La respuesta de la hembra frente a la "deserción" del macho es un incremento en la inversión maternal en las crías (Trivers, 1972), (2) Si el macho tuviera la certeza de que únicamente reduce el cuidado de las crías que no son suyas, (3) Si los costos de supervivencia para las crías como resultado de la deserción del macho no son elevados (Whittingham et al., 1992), o (4) Si en el futuro la confianza de paternidad del macho fuera a mejorar (Westneat y Sherman, 1993).

Phyllomorpha laciniata constituye un modelo ideal para el estudio de la relación confianza de paternidad-cuidado paternal, puesto que los machos pueden portar huevos de los que son los padres genéticos y otros que no son descendencia genética. Teniendo en cuenta los cuatro casos anteriormente citados que predicen una disminución del cuidado paternal en respuesta a una reducción de la paternidad, en el Capítulo 7 hacemos una revisión de lo que puede ocurrir en esta especie, y en especial del caso en el que las expectativas de paternidad del macho pudieran mejorar en episodios de reproducción futuros, que es el único que hasta el momento queda por resolver en este insecto.

Finalmente, otra pieza clave en el sistema podría ser la existencia de un conflicto sexual

sobre la realización de cuidado parental. En *P. laciniata* el interés de los sexos podría diferir teniendo en cuenta que las hembras se benefician enormemente (vía incremento en su eficacia biológica) de la puesta sobre individuos. Sin embargo, la selección natural favorecería que los machos no cuidaran de huevos que no han sido fecundados por ellos, puesto que el cuidado (transporte de huevos) implica unos altos costos de predación. Por ello, es posible que los intereses evolutivos en ambos sexos, en cuanto al grado del cuidado de los huevos, estén dramáticamente enfrentados. Por ejemplo, los intereses pueden diferir en cuanto a los mecanismos de competencia espermática, puesto que estos mecanismos son los determinantes de la confianza de paternidad que adquieren los machos tras realizar el apareamiento. Este estudio incluye la consideración de un conflicto sexual en la integración del conocimiento recogido hasta la fecha en esta especie, y a la luz de los resultados obtenidos en el Capítulo 7 se discute la importancia de este conflicto en el funcionamiento del sistema.

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CAPÍTULO 2

Refutación de la hipótesis del parasitismo intraespecífico de puesta en *Phyllomorpha laciniata* *

Resumen del Capítulo 2

En las poblaciones naturales de *Phyllomorpha laciniata* Vill. (Het., Coreidae) los individuos adultos de ambos sexos portan huevos, y este comportamiento conlleva unos beneficios en términos de supervivencia para las crías, pero también un costo de riesgo de predación para los adultos. Se ha propuesto que el comportamiento de transporte de huevos en esta especie es el resultado de un parasitismo intraespecífico de puesta, que se define como la puesta de huevos en lugares donde otros individuos coespecíficos cuidarán de la descendencia. Se trata por lo tanto de una estrategia por medio de la cual las hembras explotan el esfuerzo parental de otros individuos e incrementan así su eficacia biológica reduciendo los costos derivados del cuidado parental. Según esta hipótesis el parasitismo intraespecífico se llevaría a cabo cuando las hembras grávidas encuentran a parejas en cópula. Esta hipótesis se basa en que las cópulas en esta especie son largas, en que los individuos en cópula supuestamente pueden ofrecer escasa resistencia a la ovoposición por una hembra grávida, y en que los individuos que están en cópula portan más huevos que los individuos que no se encuentran en cópula. Sin embargo, esta hipótesis no ha sido contrastada hasta el momento y otra hipótesis alternativa debería ser tenida en cuenta: la hipótesis de aseguración de la paternidad. Esta hipótesis propone que los machos que realizan inversión parental deberían desarrollar mecanismos para maximizar su éxito en la fecundación y de esta manera minimizar el esfuerzo parental invertido en descendencia no genética. La hipótesis de la aseguración de la paternidad por cópulas repetidas propone que los machos se aparean

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repetidamente con una misma hembra y aceptan huevos en los intervalos entre las cópulas una vez que se han satisfecho unas determinadas esperanzas de paternidad, puesto que esto incrementa la supervivencia de su descendencia genética. La hipótesis de aseguración de la paternidad por cópulas repetidas en *P. laciniata* arroja predicciones opuestas a las que genera la hipótesis del parasitismo intraespecífico de puesta. Para discriminar ambas hipótesis es importante tener en cuenta que una hembra grávida que encuentra una pareja en cópula realizará la ovoposición indistintamente sobre el macho y la hembra. Esto es debido a que el beneficio para la hembra grávida reside en realizar la ovoposición sobre coespecíficos, independientemente de que sean machos o hembras, y no sobre la planta, donde la probabilidad de supervivencia de los huevos es extremadamente baja.

En el presente Capítulo se examinan ambas hipótesis en el sistema de *P. laciniata* con datos obtenidos a lo largo de dos estaciones reproductivas. Los resultados refutan la hipótesis del parasitismo intraespecífico puesto que: (1) Una mayor proporción de machos porta huevos (casi todos los machos de la población portan huevos, mientras que sólo una pequeña proporción de hembras porta algún huevo), y los machos portan significativamente más huevos que las hembras, (2) Los machos que se encuentran en cópula portan más huevos que los que no están en cópula, pero esta diferencia en la carga de huevos no existe en el sexo femenino, y, además, una mayor proporción de machos en cópula porta huevos mientras que no hay diferencias en la proporción de hembras que portan huevos según se encuentren en cópula o no, y (3) Las diferencias en la carga de huevos entre individuos en cópula y los que no están en cópula reside exclusivamente en los huevos que han sido puestos durante el apareamiento; estas diferencias sólo aparecen en el sexo masculino. Todos los patrones naturales en cuanto al transporte de huevos apoyan la hipótesis de la aseguración de la paternidad por cópulas repetidas, un comportamiento que ha sido observado con anterioridad en el laboratorio. Este estudio, por lo tanto, clarifica el funcionamiento del sistema de *P. laciniata* y refuta una hipótesis que ha sido invocada en múltiples estudios como una explicación general del comportamiento de transporte de huevos en esta especie, puesto que los resultados de campo no apoyan sus predicciones. Este estudio no sólo es de utilidad para avanzar en el conocimiento del comportamiento de *P. laciniata* sino que aporta datos acerca de cómo puede entenderse la explotación del esfuerzo parental por parte de las hembras y la aseguración de la paternidad por parte de los machos en especies con cuidado parental, y cómo puede discriminarse entre ambas estrategias.

A field test of the intraspecific brood parasitism hypothesis in the golden egg bug (*Phyllomorpha laciniata*)

Francisco García-González and Montserrat Gomendio
Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain.

Abstract

Females may increase their reproductive success by manipulating females, who are already investing in their own young, into caring for their offspring, a strategy known as "intraspecific brood parasitism". In this way females exploit other females' parental effort and avoid the costs of parental care. Female golden egg bugs (*Phyllomorpha laciniata*) lay eggs on conspecifics and on plants, but survival is higher when eggs are carried by an adult. It has been suggested that this is a form of "intraspecific brood parasitism", and that females take advantage of the reduced mobility of pairs engaged in copula to dump eggs. The results of our long-term study in a natural population show that nearly all males carry eggs, while a small proportion of females do. This finding does not support the "intraspecific brood parasitism" hypothesis, since females should be equally likely to lay eggs on males and on females when they encounter a mating pair. No differences where found between mating females and single females in the proportion of them carrying eggs, nor in the number of eggs that they were found to carry. Thus, females do not seem to be more vulnerable to egg laying when they copulate. Finally, a greater proportion of mating males carried eggs than single males, and mating males carried more eggs, but differences were due exclusively to the number of recently laid eggs. We suggest that these findings are better explained by paternity assurance, since males suffer predation costs when they carry eggs and they are likely to accept eggs only when certain levels of paternity are met. According to this alternative hypothesis, males will be most likely to accept eggs after copulating with a female, since this will ensure that a proportion of the eggs laid are the male's true genetic offspring. Females also benefit from laying eggs after copulation since it is the best chance they have of having their eggs accepted by another adult. Because paternity is maximised by repeated copulations, once a male has encountered a female they are likely to engage in a sequence of repeated copulations and egg laying attempts, which will result in mating males carrying more recently laid eggs than single males, and in most males in the population carrying eggs, while very few females will.

Keywords: parental care, intraspecific brood parasitism, paternity assurance, *Phyllomorpha laciniata*

Introduction

Parental care is not common among insects, possibly because parents in this group can do little to protect or nurture the offspring and selection favours the production of large numbers of eggs (Zeh and Smith 1985; Tallamy and Wood 1986; Clutton-Brock 1991). Parental care tends to occur in species in which eggs or young face physically harsh or biotically dangerous habitats, because in these species the benefits in terms of improved offspring survival are substantial (Wilson 1971; Eberhard 1975; Ridley 1978; Tallamy and Denno 1981; Zeh and Smith 1985; Tallamy and Wood 1986; Clutton-Brock 1991; Hardin and Tallamy 1992). Exclusive care by males is rare in insects, although heavy predation or parasitation of eggs, the unavailability of suitable oviposition sites and benefits for males regarding female mating

preferences have probably favoured its evolution in some species (Ralston 1977; Ridley 1978; Smith 1980; Zeh and Smith 1985; Tallamy 2000; Tallamy 2001). Parental care, including exclusive paternal care, has been documented in some species of heteropterans and homopterans (Odhiambo 1959; Eberhard 1975; Smith 1976; Ralston 1977; Tallamy and Denno 1981; Eberhard 1986; Kudô *et al.* 1989; Kudô and Nakahira 1993; Mappes and Kaitala 1994).

Once parental care has evolved the possibility of intraspecific brood parasitism arises, i.e. the laying of eggs in places where unrelated adults of the same species will rear the offspring of the parasite, which represents a strategy by which females enhance offspring survival prospects while avoiding the costs of parental care (Brown 1984; Petrie and Møller 1991; Brown and Brown 1998; Åhlund and Andersson 2001). Intraspecific brood parasites, which are known in few insect *taxa* (Müller *et al.* 1990; Field 1992; Brockman 1993; Zink 2000), usually manipulate other females (or a male-female pair) with young into providing care for their offspring, as in the well studied avian *taxa* (see for example Yom-Tov 1980; Müller *et al.* 1990; Petrie and Møller 1991; Field 1992). Theoretically, females could also parasitize the investment of males. In this case, intraspecific brood parasitism would need to be clearly distinguished from paternal care. In many avian species females manipulate their male partners into providing care for unrelated offspring, which are the result of extra-pair copulations (see for example Birkhead *et al.* 1988; Burke *et al.* 1989; Møller and Birkhead 1993; Dixon *et al.* 1994; Hughes 1998). However, this form of "parasitism" has never been considered as "intraspecific brood parasitism" but rather as paternal care with a component of deception.

In natural populations of *Phyllomorpha laciniata* Vill. (Het., Coreidae) most males carry eggs on their backs, and this behaviour entails costs in terms of vulnerability to predators (Reguera and Gomendio 1999; Kaitala *et al.* 2000). It has been suggested that this is the result of intraspecific parasitism by females, who lay eggs on males, particularly while they are engaged in copula (Kaitala 1996). This hypothesis assumes that males are unable to resist laying attempts by females while copulating with other females, and that females take advantage of this vulnerability to lay eggs on unrelated individuals. The reason why females benefit from laying eggs on conspecifics is that egg survival is significantly greater when laid on adults than when laid on plants, where they suffer such high levels of predation rates and parasitation by a wasp that only around 3% of the eggs survive (Reguera and Gomendio 2002).

The main alternative hypothesis to explain egg carrying by males is that they father enough offspring to benefit from the improved survival rates that egg carrying conveys (Gomendio and Reguera 2001). In this scenario males would accept eggs under certain conditions, and egg laying would not be primarily the result of egg laying while males are unable to reject such attempts. Our own results suggest that males carry a variable proportion of genetic offspring (García-González et al., unpublished), a finding which supports this alternative hypothesis.

Since the formulation by Kaitala (1996) of the so-called "mating pair intraspecific brood parasitism hypothesis" (henceforth MPIBP hypothesis) this explanation has been invoked in several publications (Kaitala 1998; Härdling and Kaitala 2001; Katvala and Kaitala 2001; Tallamy 2001). The formulation of the MPIBP hypothesis was based on two lines of evidence. On the one hand, copulation lasts many hours in this species (more than 20 hours on average; see Kaitala 1998 and Reguera 1999), and some observations suggest that mating individuals in captivity can offer little resistance to egg laying females (for example Kaitala and Miettinen 1997). On the other hand, males in copula carry significantly more eggs than single males (Kaitala 1996). However, the fact that mating males are carrying more eggs than single individuals could also be explained by the paternity assurance hypothesis (henceforth PA hypothesis). Parker (1970) was the first to indicate that males should evolve paternity assurance mechanisms which reduce sperm competition, and Trivers (1972) suggested that in

species with paternal investment, males should evolve adaptations to decrease the risk of cuckoldry. Paternity assurance by repeated copulations has been seen to occur in paternally investing species, for example insects (Smith 1979b; Müller and Eggert 1989), birds (Møller 1987; Simmons 1990; Birkhead and Møller 1992), and primates (Møller 1988), and in the golden egg bug this hypothesis suggests that males may increase confidence of paternity by copulating repeatedly with females, thus resulting in females laying eggs on males between repeated copulations.

To test both hypothesis it is worth emphasizing that females lay eggs both on conspecifics and on plants, that eggs on conspecifics enjoy better survival prospects than eggs laid on plants, and that gravid females benefit equally from ovipositing on males or on females because egg survival shows no differences between the sexes (Katvala and Kaitala 2001; García-González & Gomendio, unpublished). In fact, in captivity conditions a female that encounters a mating pair will oviposit indistinctly on the female or on the male (Kaitala and Miettinen 1997).

Therefore, if the MPIBP hypothesis proposed by Kaitala (1996) plays a significant role on the patterns of oviposition and egg carrying in this insect the following predictions should be supported: (1) No sex differences should be observed neither in the proportion of individuals carrying eggs, nor in the average number of eggs carried by each individual, since obviously males and females are equally vulnerable while in copula; and (2) A higher proportion of mating individuals, either males or females, than single individuals should carry recently laid eggs (because of oviposition by an alien female), and no sex differences should be observed in the number of recently laid eggs among mating individuals. On the contrary, if egg load of individuals is mainly explained by the PA hypothesis the following predictions should then be supported: (1) A higher proportion of males than females should carry eggs, and males should carry significantly more eggs than females; and (2) Only a higher proportion of mating males than single males should carry recently laid eggs (because of oviposition by current mating females between repeated copulations), but no differences between mating and single females should be observed. In addition, mating males should carry more recently laid eggs than single males, whereas mating females should carry the same number of recently laid eggs than single females.

Our aim in this paper is to test these predictions to determine whether egg carrying in the golden egg bug is better explained by the mating pair intraspecific brood parasitism hypothesis or by the paternity assurance hypothesis. These findings will improve our understanding of whether care by males can evolve as the result of exploitation by females or whether it is unlikely to evolve when there is no certainty of paternity.

Methods

Field observations and captures were conducted in five close localities of Central Spain: Villaviciosa de Odón, Robledo de Chavela, Colmenar del Arroyo, and Valdemorillo, and El Espinar.

A total of 1464 individuals were observed during years 1998 and 1999 throughout all the reproductive season (middle April to middle August). This sample includes 1244 adults and 220 nymphs in different stages of development.

We did not include in the analyses i) individuals recaptured, which could give rise to pseudorreplication (in Villaviciosa de Odón we carried long-term capture-recapture studies once a week during the whole active season for the two years), and ii) individuals born during the reproductive season, because individuals do not mate and do not accept eggs until the next year (García-González, unpublished). Therefore, the total number of adults analysed was 796 and the total number of matings in which we recorded data on egg-carrying was 76. No significant differences were found in patterns of egg carrying

among different populations, thus we pooled data to increase sample size.

We have followed the methodology used by Kaitala (1996) to assess the stage of development of the eggs. Eggs change in colour as they develop allowing a categorisation according to age (Kaitala 1996). Eggs are white for some hours after being laid, then they turn to yellow, yellow with some orange spots, golden with lots of orange spots, and finally after about 10 days (the exact timing depends on ambient temperature) they eclose (Kaitala 1996; Reguera 1999). Parasitised eggs with Scelionid parasitoids (*Gryon bolivari* Giard) are easily identified. This hymenopteran parasitises recently laid eggs, but parasitation is apparent some days before the emergence of the wasps since the egg turn to black.

Since copulation duration is long in this species (23 hours on average as Kaitala 1998; 32.5 hours on average as Reguera 1999; 11 hours minimum as Mineo 1984), the development stage of eggs carried by mating individuals indicates roughly the time in which the egg was laid with respect to the current mating. Of particular interest to the predictions that will be tested in this paper, is whether eggs carried by mating individuals are white, since this would unequivocally mean that they have been recently laid (either just before or during the current copula). Thus, we distinguished in the analyses recently laid eggs (white eggs) from old eggs (all the others categories), as Kaitala (1996) did. After hatching, the egg shell remains on the individual for some time. In spite of the fact that there are no differences in the number of egg shells carried by mating males or single males (Mann-Whitney U=12650, p=0.66, n mating males: 76, n single males: 344) neither between mating females and single females (Mann-Whitney U=10810, p=0.37, n mating females: 75, n single females: 299), as a conservative measure we included also egg shells in the category of old eggs as Kaitala (1996) and Kaitala (1998) did.

Non-parametric statistics were carried out because of the nature of the dependent variables analysed (Sokal and Rohlf 1981).

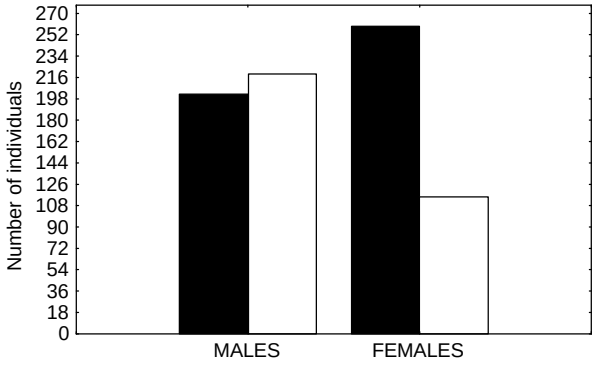

Figure 1. Frequency of males and females carrying eggs (open bar) or without eggs (black bar).

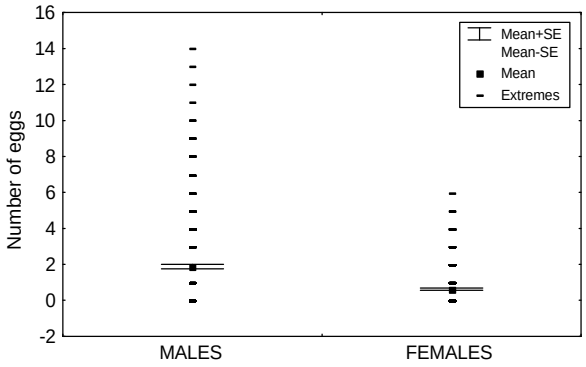

Figure 2. Mean, minimum and maximum number of eggs carried by males and by females.

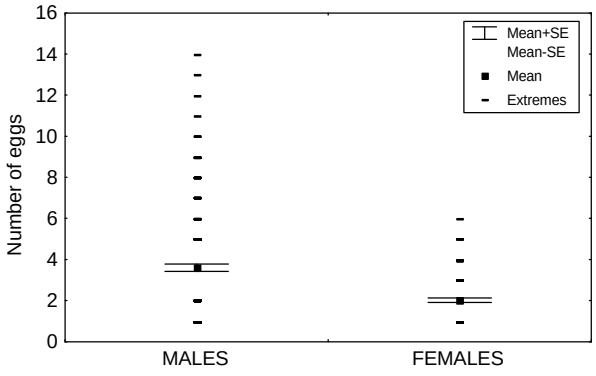

Figure 3. Mean, minimum and maximum number of eggs carried by males or by females that carry eggs.

Results

Prediction 1. The MPIBP hypothesis predicts that males and females should not differ in the proportion of individuals carrying eggs, nor in the average number of eggs carried, since females are equally likely to lay eggs on either sex while a pair is engaged in copula (sex ratio is 1:1 and remains constant throughout all the season; see also Reguera 1999). However, in natural populations a higher proportion of males than females carry eggs (Chi sqared$_{\text{Yates corrected}}$=35.91, d.f.=1, p<<0.001, n=795) (Fig 1) and males carry more eggs than females, either analysing all individuals (Mann-Whitney U test, U=57005.5, p<<0.001, n males: 421, n females: 374) (Fig 2) or analysing only individuals that are carrying at least one egg (Mann-Whitney U test, U=7616.5, p<<0.001, n carrying males: 219, n carrying females: 115) (Fig 3). These results support the predictions of the PA hypothesis.

Prediction 2. The MPIBP hypothesis also predicts that as a consequence of oviposition by alien females on mating pairs, a higher proportion of mating individuals, either males or females, than single individuals should carry recently laid eggs. However, while a higher proportion of mating males than single males carry recently laid eggs (Chi sqared$_{\text{Yates corrected}}$=9.36, d.f.=1, p=0.0022, n=420) (Fig 4), mating females do not carry recently laid eggs in a higher proportion than single females (Chi sqared$_{\text{Yates corrected}}$=0.07, d.f.=1, p=0.8, n=374) (Fig 5). These results strongly support the predictions made by the PA hypothesis. In addition, the MPIBP hypothesis predicts that no sex differences should be observed in the number of recently laid eggs among mating individuals, i. e., mating males and mating females should carry more recently laid eggs than single males and single females, respectively. The number of recently laid eggs is greater in mating males than in single males (Mann-Whitney U test, U=10996.0, p=0.001291, n mating males: 76, n single males: 344), whereas it does not differ between mating and non-mating

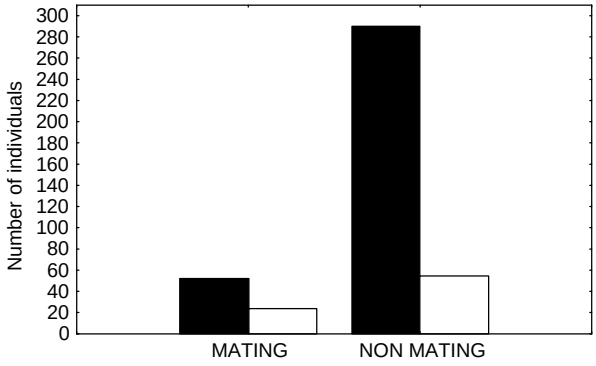

Figure 4. Frequency of mating and non-mating males carrying recently laid eggs (open bar) or without recently laid eggs (black bar).

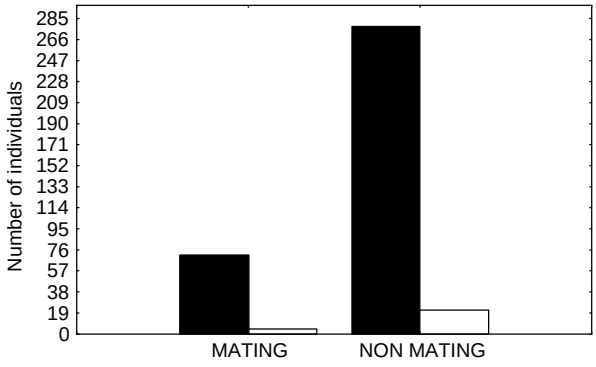

Figure 5. Frequency of mating and non-mating females carrying recently laid eggs (open bar) or without recently laid eggs (black bar).

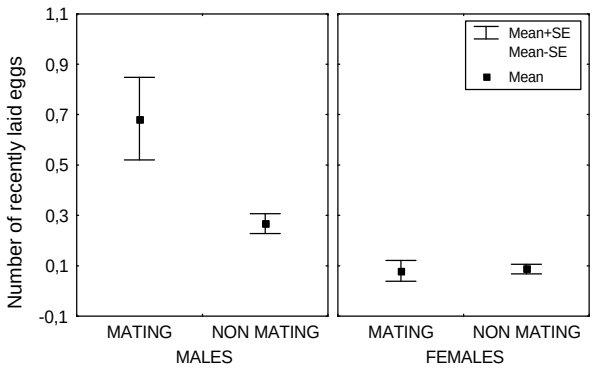

Figure 6. Number of recently laid eggs carried by mating and non-mating individuals.

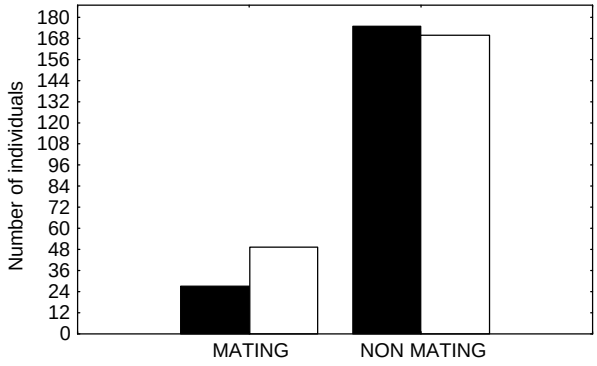

Figure 7. Frequency of mating and non-mating males carrying eggs (open bar) or without eggs (black bar).

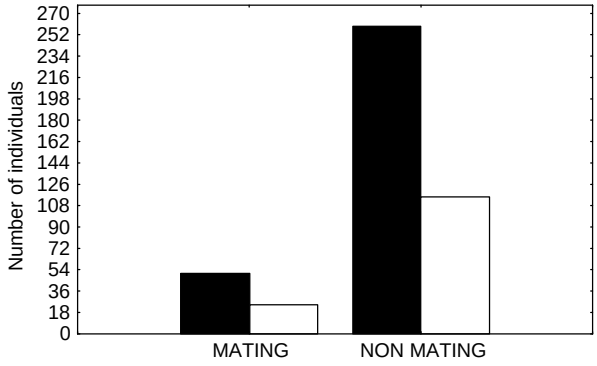

Figure 8. Frequency of mating and non-mating females carrying eggs (open bar) or without eggs (black bar).

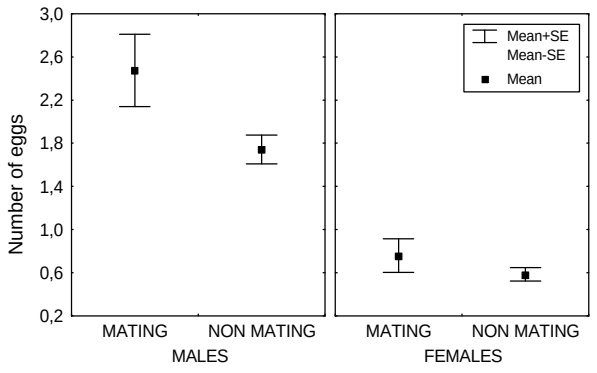

Figure 9. Number of eggs carried by mating and non-mating individuals.

females (Mann-Whitney U test, U=11023.0, p=0.82, n mating females: 75, n single females: 299) (Fig 6). Therefore, results regarding this prediction are also against the MPIBP hypothesis whereas strongly support the PA hypothesis since only males are the recipients of the eggs laid during the mating association.

If we consider total egg load carried by individuals our results do not support the MPIBP hypothesis because a higher proportion of mating males than single ones carry eggs (Chi sqared$_{\text{Yates corrected}}$=5.17, d.f.=1, p=0.023, n total=421) (Fig 7) but this is not the case among females (Chi sqared$_{\text{Yates corrected}}$=0.02, d.f.=1, p=0.9, n total=374) (Fig 8). Moreover, mating males carry more eggs than singles males (Mann-Whitney U test, U=10873.5, p=0.013, n mating males: 76, n single males: 345), but mating females do not carry more eggs than single ones (Mann-Whitney U test, U=10811.5, p=0.56, n mating females: 75, n single females: 299) (Fig 9). The fact that mating males carry more eggs than single ones could be explained, by female preferences to mate with egg carrying males. If this is true, however, we should find differences not in the total number of eggs carried but in the number of old eggs since these eggs are the ones that males were carrying before the current copulation. There are no differences between mating and single males neither in the proportion of males carrying old eggs (Chi sqared$_{\text{Yates corrected}}$=1.46, d.f.=1, p=0.23, n=420) (Fig 10) nor in the number of old eggs carried (Mann-Whitney U test, U=12006.0, p=0.22, n mating males: 76, n non-mating males: 344) (Fig 11). Therefore, differences in total egg load between mating and single males rely exclusively on the number of recently laid eggs as has shown above.

Discussion

Our results from long-term studies in natural populations of *P. laciniata* do not support the hypothesis that egg carrying by adults is the result of egg dumping by females while pairs are in copula, as suggested by the mating pair

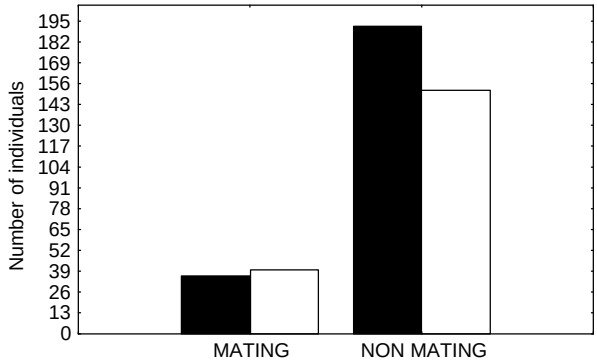

Figure 10. Frequency of mating and non-mating males carrying old eggs (open bar) or without old eggs (black bar).

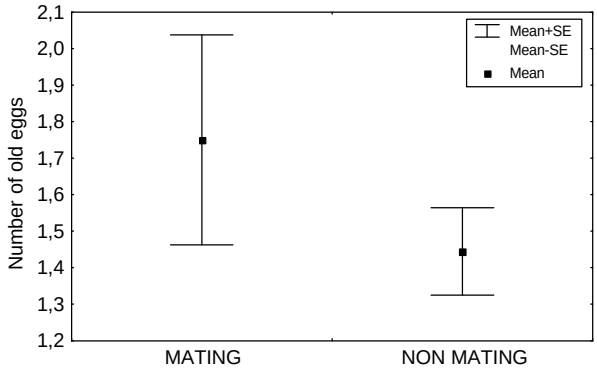

Figure 11. Number of old eggs carried by mating and non-mating males.

intraspecific brood parasitism hypothesis. The main findings against this hypothesis are that more males than females carry eggs in natural populations, and that males carry more eggs than do females. If individuals become more vulnerable to egg dumping while in copula, this should be equally applicable to males and females.

In addition, if parasitic females take advantage of a presumed inability to reject egg-laying attempts while in copula, mating individuals should carry more often eggs than single individuals, as a consequence of oviposition by alien females. Again, no sex differences should exist in this respect. Contrary to these predictions our results show that, among females, no differences where found between mating and single individuals. This could be either because it is wrong to assume that individuals cannot resist laying attempts while in copula, and/or because population densities are too low to enable this strategy to become successful, since encounter rates with copulating pairs are likely to be extremely low. Our long-term studies show that density is very low: data gathered from adults found in an area of 400 m^2 in 44 different days throughout three reproductive periods (years 1998 to 2000), showed densities ranging from 0.005 individuals per square meter to 0.265 individuals/m^2 (mean density=0.042 individuals/m^2, SE=0.007). Despite many observations of matings in the field we have never observed a female laying eggs on copulating individuals. The assumption that alien females exploit mating pairs seems to apply only to captivity conditions: Kaitala and Miettinen (1997) showed, in a study in which mating couples were placed with a single female, that a copulating individual could not resist receiving eggs except by terminating the copulation: in five cases out of 20 (25%) copulation was terminated when a female tried to oviposit on a copulating individual, in 11 cases (55%) a clutch was laid on a copulating female and in 4 cases (20%) on a copulating male (Kaitala and Miettinen 1997). Thus, the MPIBP hypothesis could account for a significant part of the eggs that individuals are carrying in captivity under certain conditions, but seems negligible under natural conditions. This is because, as the experiment by Kaitala and Miettinen (1997) showed, the MPIBP hypothesis does not predict sex differences in the patterns of egg carrying when a large sample of individuals is considered. Individuals in captivity are often closed in limited space at high density, experiments are usually carried with some individuals in a box, so encounter rates between single females and mating pairs may be artificially favoured.

Our results also show that mating males do carry eggs more frequently and in greater numbers than single males. This result could be explained by female preferences to mate with males who are already carrying eggs, either because they have already proven their propensity to care, or because of dilution effects of eggs (see for example Ridley and Rechten 1981; Andersson 1994; Kraak and Weissing 1996; Jennions and Petrie 1997). However, this alternative explanation

can also be rejected because differences between mating and single males are due exclusively to recently laid eggs, which were unlikely to be present when the female made the decision to copulate with that male. In this sense, our data are in agreement with two independent studies that examined female mate choice in this species (Kaitala 1998; Reguera 1999).

We suggest that our results are best explained by the paternity assurance hypothesis, which proposes that, although females benefit equally from laying on males or females, only males accept eggs under conditions in which certain levels of paternity are ensured. Paternity assurance by repeated copulations has been suggested as one of the main forces accounting for the evolution of paternal care in insects (Smith 1980; Zeh and Smith 1985). This mechanism of paternity assurance occurs in the well-known belostomatids, where males carry eggs on their backs too (Smith 1979b; Smith 1979a; Smith 1980) and in the paternally investing burying beetles (Müller and Eggert 1989). Certainty of paternity is likely to be optimal when males copulate repeatedly with the same female. In the golden egg bug system, females may find more acceptance to egg laying when they have already copulated with a male. This is in accordance with other findings that demonstrate that egg laying rate is highest after copulation (García-González & Gomendio, unpublished). Thus, the differences between mating and single males in the number of recently laid eggs could be the result of egg laying by the female currently engaged in copula in the interval between repeated copulations. In this context, it has been observed that females in captivity often remate with the same partner many times (Kaitala 1998); García-González, unpublished).

Patterns of egg carrying in nature suggest that paternity assurance by repeated copulations play an important role explaining egg carrying behaviour in the golden egg bug. Under this scenario, females look for optimal oviposition sites (conspecifics) because of the major benefits in terms of offspring survival (independently of the sex), whereas males search for matings and accept eggs only when the chances of carrying their own offspring are high enough. In this way the costs in terms of increased vulnerability to predators, are compensated by the increased survival of true genetic offspring.

One last consideration is that intraspecific brood parasitism seems to arise when females, who are already providing care to their offspring, are manipulated by other females into caring for additional unrelated young. It seems unlikely that a system could evolve in which females manipulate males, who do not provide care, into caring for unrelated offspring. In a recent paper Härdling and Kaitala (2001) suggest that egg carrying in *P. laciniata* can be the result of mutual cooperation (see Dugatkin 1991; Dugatkin and Alfieri 1991), so that females will mate with males who have already accepted eggs. For such cooperation to evolve a series of assumptions are made which do not fit with our long-term observational data. Thus, it is assumed that individuals live in close mating groups, that remain for a long time together, and in which exchange between groups does not take place. Our long-term data show that no stable groups are formed, that individuals range widely, and that densities are very low, and thus repeated interactions between the same individuals are unlikely. However, it may well be the case that once a male encounters a female, the male attempts repeated copulations, and the female takes the opportunity to try laying several eggs, which are only accepted by the male under these conditions. In fact, Härdling and Kaitala (2001) show that under their theoretical model cooperation can evolve and be stable only if a male has many interactions involving egg-acceptance and mating with the same female. Thus, this form of "cooperation" does not differ in any way from the paternity assurance hypothesis that we have proposed in this paper.

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CAPÍTULO 3

Elección del lugar de ovoposición y estimulación de la ovoposición por presencia de coespecíficos: implicaciones para la eficacia biológica femenina*

Resumen del Capítulo 3

Las hembras de varias especies de insectos han desarrollado preferencias en la elección de los lugares de ovoposición. Estas preferencias han evolucionado siempre que los diferentes lugares de ovoposición a los que puede optar una hembra afecten de forma diferencial la supervivencia de la progenie. La selección de los lugares de ovoposición ha surgido para maximizar la supervivencia de la descendencia, y se espera que esta selección opere especialmente en animales que no realizan cuidado parental o en los que el cuidado de la progenie está poco desarrollado.

Otra forma de incrementar la eficacia biológica femenina por medio de los beneficios para la descendencia es modular el ciclo reproductivo de tal manera que la ovoposición resulte estimulada frente a la presencia de un sitio de ovoposición óptimo. Por otra parte, el ajuste de los eventos reproductivos puede también depender de factores ambientales y/o sociales. Por ejemplo, en algunas especies de mamíferos la presencia de coespecíficos desencadena la ovulación, mientras que en otras la inhibe. En cuanto a los insectos, la presencia de coespecíficos en algunas especies sociales y el apareamiento en una gran variedad de *taxa* estimula la ovoposición.

* Este capítulo reproduce el texto íntegro del siguiente manuscrito enviado para su publicación:

García-González, F. and Gomendio, M. Oviposition site selection and oviposition stimulation by conspecifics in the golden egg bug (*Phyllormorpha laciniata*): implications on female fitness.

Phyllomorpha laciniata es un organismo ideal en el que estos aspectos pueden operar conjuntamente, puesto que el lugar de ovoposición (adulto coespecífico versus planta) tiene un efecto importante sobre la supervivencia de las crías, pero el sustrato óptimo (coespecíficos) no está siempre disponible. Esta especie tiene una alta flexibilidad en la ovoposición que se manifiesta también a nivel intra-individual; una misma hembra puede ovopositar sobre la planta hospedadora o sobre los individuos coespecíficos. Estudios previos han demostrado que la supervivencia de los huevos es claramente mayor cuando estos son puestos sobre coespecíficos. Una predicción derivada de este hecho es que las hembras deberían ovopositar preferencialmente sobre los coespecíficos cuando ello sea factible. Por otra parte, puesto que los adultos coespecíficos constituyen un lugar óptimo de ovoposición, pero que se encuentra con una disponibilidad menor que la opción de la planta hospedadora, una segunda predicción es que las hembras deberían experimentar una estimulación de la ovoposición cuando se encontraran frente a un individuo coespecífico.

En el presente Capítulo hemos analizado estas dos predicciones enfrentando a las hembras a los posibles sitios de puesta y observando el resultado de la ovoposición. Los resultados muestran que cuando los dos substratos de ovoposición se encuentran disponibles, las hembras ovopositan preferencialmente sobre los individuos. La segunda predicción también resulta confirmada puesto que las hembras en presencia de coespecíficos incrementan la tasa de ovoposición con respecto a las que no disponen de individuos sobre los que poner los huevos. La estimulación de coespecíficos es independiente del sexo del individuo que se encuentra presente, lo que tiene importantes implicaciones para el estudio del comportamiento de transporte de huevos en esta especie. Este resultado sugiere que una hembra grávida sale igualmente beneficiada de la puesta sobre individuos de ambos sexos, lo que, por otro lado, se encuentra en concordancia con la ausencia de diferencias en las tasas de supervivencia de huevos que son portados por machos o hembras.

Por último, los resultados obtenidos en la aproximación experimental realizada en el presente Capítulo sugieren que la estimulación de la ovoposición resulta de una aceleración de las tasas de producción y maduración de huevos. Por lo tanto, esta especie presenta un ajuste ovárico frente a la presencia de coespecíficos y no sólo una respuesta en las últimas fases del proceso de ovoposición.

La mayoría de los estudios sobre insectos en los que se ha documentado una estimulación por coespecíficos muestran que ésta resulta de una estimulación que se origina en el apareamiento. Estos estudios han puesto de manifiesto que dicha estimulación es una estrategia masculina que incrementa el éxito reproductivo del macho que realiza la cópula, mientras que supone un coste reproductivo para las hembras, puesto que la estimulación de la ovoposición va, en algunos casos, asociada a una pérdida de la receptividad femenina y de la supervivencia. Sin embargo, el escenario evolutivo de *P. laciniata* parece ser totalmente diferente, puesto que en este sistema son evidentes los beneficios que obtienen las hembras, en términos de eficacia biológica, como resultado de la estimulación de la ovoposición por presencia de coespecíficos, y no es necesaria la cópula para desencadenar la estimulación.

Oviposition site selection and oviposition stimulation by conspecifics in the golden egg bug (*Phyllomorpha laciniata*): implications on female fitness

Francisco García-González and Montserrat Gomendio
Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (C.S.I.C.), Madrid, Spain.

Summary

1. *Phyllomorpha laciniata* Vill. (Heteroptera, Coreidae) females lay eggs on the host plant and on the backs of conspecifics. Since egg survival is greater when eggs develop on the backs on conspecifics, than when laid on plants, we predict that females should prefer to lay eggs on conspecifics. In addition, because conspecifics are a high quality site that represents a limiting resource, females should experience oviposition stimulation upon an encounter with a conspecific.
2. Our results reveal that, when both the host plant and conspecifics are available simultaneously, females lay eggs preferentially on conspecifics. The results also support the second prediction, since females housed with conspecifics lay more than twice the number of eggs than isolated females. Isolated females do not seem to retain eggs, suggesting that oviposition stimulation is the result of an acceleration of egg maturation rates.
3. Other studies have found oviposition stimulation by mating and have suggested that it is the result of male strategies to increase short-term male reproductive success at some cost to females. The evolutionary scenario of our model organism seems to be quite different since females benefit greatly from increasing egg laying when there are conspecifics, because the advantages in terms of offspring survival are likely to translate into substantial increases in female reproductive success.

Key-words: egg-carrying, female reproductive success, ovarian dinamics, oviposition behaviour.

Introduction

Evolutionary theory predicts that selection favours the expression of traits that maximize individual fitness (Clutton-Brock 1988; Parker & Maynard Smith 1990; Rose & Lauder 1996). Among females, offspring survival is one of the most important components of lifetime reproductive success, given the large differences in offspring survival rates between females (Clutton-Brock 1988). In organisms with rudimentary forms of parental care or with no parental care, oviposition site selection should have a considerable impact on female fitness since it often determines to a great extent the chances of survival for offspring. In these species oviposition site preferences should evolve in order to maximize offspring survival. Available evidence shows that females of phytophagous insects and parasitoids place their eggs in sites where their offspring will find enough food resources, high quality food resources or enemy-free spaces (Godfray 1994; Jaenike 1978, 1990; Mangel 1989; Papaj 2000; Papaj & Messing 1996; Thompson & Pellmyr 1991).

In those cases in which preferred oviposition sites are rare or difficult to find, it would be in the females' interest to adjust reproductive cycles so that oviposition is stimulated upon an encounter with a preferred site. Strong evidence suggests that ovarian dynamics in insects responds to variability in host quality and availability in adaptive ways (Papaj 2000). It is well known that female reproductive cycles are influenced by a number of environmental and social factors (Engelman 1970; Wallen & Schneider 2000). Among the latter the presence of conspecifics and mating are known to have strong effects. The presence of conspecifics

may influence the timing of reproductive events in mammals (Bronson 1989; Bronson & Maruniak 1975; Maina & Katz 1999; Schiml, Wersinger & Rissman 2000; Signoret 1980). On the one hand, inhibition of reproduction by conspecific presence is usually related to dominance relationships in group living mammals such as primates and carnivores (Bronson 1989), whereas, on the other hand, ovarian cycle stimulation by the presence of males seems widespread (Bronson 1989; Chemineau 1983; Lindsay *et al.* 1975; Martin *et al.* 1986; McComb 1987; Signoret 1980; Wallen & Schneider 2000). Among insects, the effects of mating on the stimulation of egg production and oviposition are well known (Choe & Crespi 1997; Engelman 1970; Leopold 1976; Thornhill & Alcock 1983; Wigglesworth 1965). In some insects gonadotropic substances transferred by the male during copulation enhance egg maturation or egg laying (Chapman *et al.* 1998; Chen *et al.* 1988; Kubli 1992; Leopold 1976; Loher *et al.* 1981). However, oviposition stimulation by adult conspecific presence has only been reported in a few cases, such as social insects where worker ants, in the absence of the queen, lay more eggs if they are in the presence of other workers (Salzemann & Plateaux 1988), and *Drosophila melanogaster* (Hoffmann & Harshman 1985) where it has been suggested that a "male factor" affects female fecundity in the absence of contact between the sexes, although the mechanisms involved are still unclear.

Benefits resulting from the stimulation of female reproductive cycles by conspecifics have been generally examined from a male perspective. In *D. melanogaster* and other dipteran species the effects of mating upon female reproductive performance seem to enhance male short term reproductive output, while imposing a reproductive cost to females (Chapman *et al.* 1995, 1998; Holland & Rice 1999; Partridge, Green & Fowler 1987; Rice 1996). Thus, stimulation of female reproduction by males has usually been interpreted as benefiting almost exclusively males, who manipulate females so as to increase their reproductive success. Such conflicts of interest between the two sexes have been regarded by some authors as being widespread (Johnstone & Keller 2000; Rice & Holland 1997). It is unclear, however, why female physiology should be influenced by males, if females derive no benefits from such manipulation. One of the few studies to investigate potential benefits to females has suggested that fallow deer females adjust the timing of oestrus to the availability of preferred males and delay ovulation when only undesired males are available (Komers, Birgersson & Ekvall 1999), however further evidence is needed to test this hypothesis.

The golden egg bug (*Phyllomorpha laciniata* Vill) provides a unique opportunity to study the evolutionary implications of different oviposition strategies since females may chose among three alternatives: females can lay their eggs on host plants (*Paronychia argentea*) where they develop unattended, or over the body of conspecific males and females where they are carried until hatching (Bolivar 1894; Kaitala 1996; Reguera 1999). This species, together with the giant waterbugs, are the only insects in which females glue eggs on the backs of conspecifics (Clutton-Brock 1991; Ridley 1978; Smith 1979; Zeh & Smith 1985). While giant waterbugs only lay eggs on males, female golden egg bugs may lay eggs both on males and on other females. This species is an excellent model organism because it is possible to study oviposition choice at intraspecific and intraindividual levels since all females adopt the three laying strategies (eggs on plant, on males and on females) during their reproductive lives.

Eggs of *P. laciniata* carried by an adult have a greater probability of survival than those laid on plants (Gomendio & Reguera 2001; Kaitala 1996; Reguera 1999; Reguera & Gomendio 2002), thus females should oviposit preferentially over conspecifics in order to maximize offspring survival rates. In addition, we predict that females should undergo oviposition stimulation in the presence of conspecific individuals so as to maximize the number of eggs laid on conspecifics. We have explored in the golden egg bug both the existence of oviposition choice and conspecific non-mating oviposition stimulation.

Materials and Methods

We collected 128 individuals of *P. laciniata* in a specific date in two localities in Central Spain, Robledo de Chavela (4 June, 1999; 39 males and 34 females collected) and El Espinar (15 June, 1999; 33 males and 22 females collected) because at that time both populations were at the peak of their reproductive activity. Monitoring of both populations from the beginning of their reproductive cycles allowed us to determine this particular stage. Individuals were placed in small Petri containers (5.5 cm diameter) and kept in constant conditions from then until the end of the experiment (25° C, light from 0800 hours to 2100 hours). Prior to the experiment, eggs were removed from carrying males as well as from carrying females. Throughout all the experimental period individuals were provided daily with fresh branches of the host plant *Paronychia argentea*.

Females can exhibit different oviposition rates, i. e., number of eggs laid during a given period of time, depending on the time since their last copulation (Francisco García-González, unpublished, and this study). Thus, to minimize variation in female oviposition rates, we forced all the females included in the sample into a similar physiological state by placing each female with a male and allowing her to mate once in the laboratory (from now on "pre-experimental copulation"). After the pre-experimental copulation each female was randomly assigned to four treatments: female with the male she previously copulated with (group B, n=7), female with a male other than the one she had copulated with (C, n=7), female with another female (D, n=6), and female with the male she had copulated with and with another male (E, n=4), and along five days we monitored the oviposition rate as well as oviposition site selection (plant vs. conspecific) of each female. Controls consisted of a female maintained under identical conditions but with no other individuals being present at any time (group A, n=11). Some individuals died during the experiment, thus the final number of replicates of each experimental group was lower (see results).

Fecundity may depend on other factors such as female size, female body condition and the existence of previous egg batches. In order to control for these variables we monitored the oviposition rate of each female prior to the beginning of the experiment (number of eggs laid/number of days between their capture and the pre-experimental copulation), and measured female body size and female body condition. Body size was estimated from three length measurements of the right hind tibia using NIH Image 1.60 software (National Institutes of Health, US). Repeatability of these three lengths is 0.99 ($p<0.001$). Females were weighed to the nearest 10^{-4} g on a Sartorius BP 110 S balance (Sartorius AG, Goettingen, Germany), and body condition was inferred as the residuals of body weight regressed by body size. There are no differences among treatments in female oviposition rate prior to the establishment of the experiment (ANOVA: $F_{4,20}=0.7$, $p=0.61$; mean number of eggs=1.05, SE=0.24, n=25), and there are no differences in female weight (ANOVA: $F_{4,23}=0.53$, $p=0.72$) or female body condition (ANOVA: $F_{4,21}=1.1$, $p=0.4$) between the experimental groups.

We also considered the possibility that differences in the number of eggs laid by females could be influenced by the occurrence or lack of copulations in the different experimental groups. To control for this variable in the analyses we checked for copulations three times per day between 0900 hours and 2100 hours with maximum intervals of 6 hours. Copulation in this insect lasts on average for more than 20 hours (Kaitala 1998; Reguera 1999), so it is unlikely that copulations went unnoticed.

Groups were provided with greater surface area of the host plant *Paronychia argentea* than that represented by individuals to make sure that plant availability was not a limiting factor.

In group D (female with other female) it is not possible to distinguish the eggs laid by each female. For this particular group, we have used two approaches to estimate oviposition on plants

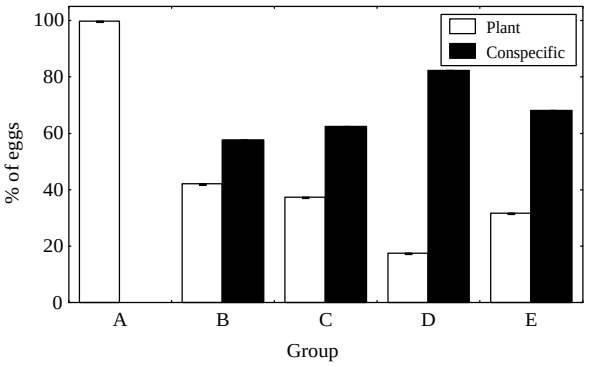
by subject females: (1) Estimating number of eggs that the subject female laid on plant (P_1) according to the proportion of eggs that this same female laid on the recipient female (C_1) out of the total number of laid eggs on conspecific backs, i.e. correcting by fecundity of each female measured as eggs deposited over conspecific: $P_1=(C_1/C_1+C_2)P_{1+2}$, where C_2 is the number of eggs deposited by the recipient female on the subject female and P_{1+2} is the total number of eggs laid on plants by both females, and (2) Estimating P_1 as ½ of the total egg number deposited on plants by the two females. There are no differences in the use of approach (1) or (2) in the analyses, but we think that the first one is more realistic and thus, this approach has been used in all analyses.

We checked for possible "egg-retention" in the female tract by dissecting them at the end of the experiment. We counted full size developed eggs as well as those which had developed to at least half the size of a full grown egg.

We checked that all eggs on plants were firmly glued to the branches or flowers to ensure that they were willingly deposited there and not lost by egg carrying bugs.

Dependent variables were transformed when using parametric statistics using logarithmic or Box-Cox transformations. Homocedasticity was confirmed by using Levene's test (Statsoft, 1996). When parametric assumptions were not fulfilled we used non parametric statistics (Sokal & Rohlf 1981). All test were two tailed.

a)



b)

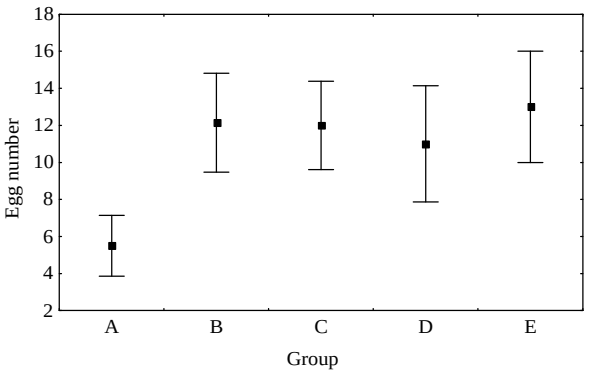


Figure 1. a) Egg allocation of females in the different treatments. A, isolated females; B, females with the male they copulated with; C, females with a male other than the one they copulated with; D, females with another female; E, females with the male they copulated with and with another male. The mean values of individual percentages are shown. b) Mean number of eggs (and standard errors) laid by females in the experimental treatments.

Results

Oviposition site selection

Females housed with conspecifics laid more eggs on adult bugs (66.6%) than on plants (33.3%) (t test for dependent samples: t_{19}=-2.87, p=0.01). Fig. 1a shows that the preference to oviposit on conspecifics emerges in all groups. No differences in the percentage of eggs laid on plants versus conspecifics were found between the experimental groups in which females were housed with different types of conspecifics (i.e. B, C, D and E) (Chi-square test: c2=4.92, d.f.=3, p=0.18).

Oviposition stimulation

The total number of eggs laid in the experimental period differs depending on whether females were isolated or housed with conspecifics. Isolated females laid on average 5.5 eggs (SE=1.6, n=8) during the experimental period, obviously all on plants, whereas non-single females laid on

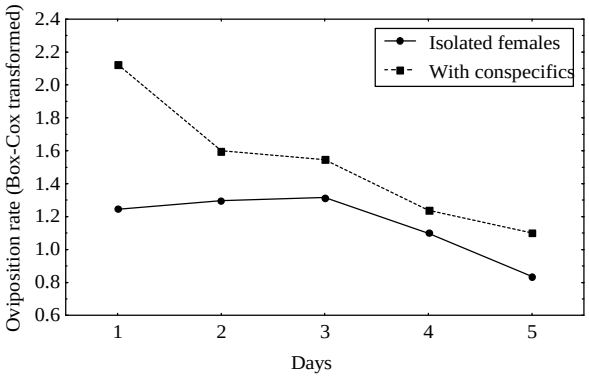


Figure 2: Daily oviposition rates of isolated females and females with conspecifics along the experimental period.

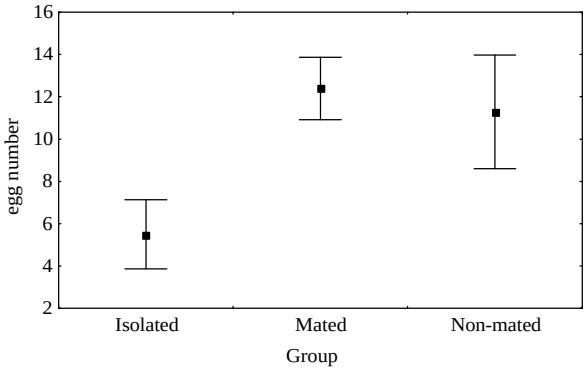


Figure 3: Mean number of eggs (and standard errors) of isolated females (n=8) and females grouped according to whether they copulated (Mated , n=13) or not (Non-mated , n=7) during the experiment.

average 12 eggs (SE=1.3, n=20) (ANOVA: $F_{1,26}$=10.20, p=0.004) (Test 1). The mean number of eggs (± standard errors) laid by females in the groups established were as follows: Group A, 5.5 ± 1.64 (n=8); Group B, 12.14 ± 2.67 (n=7); Group C, 12.0 ± 2.38 (n=6); Group D, 11.0 ± 3.14 (n=4); Group E, 13 ± 3.00 (n=3).

There are significant differences in daily oviposition rates between females in conspecific presence and isolated females when oviposition rate throughout the 5 days is considered for each female (Repeated measures ANOVA: $F_{1,26}$=6.75, p=0.015) (Test 2) (Fig. 2). Oviposition rate decreases with time in both groups ($F_{4,104}$=4.53, p<0.002). As can be seen in Fig. 2, differences lie mainly in the number of eggs laid the first day ($F_{1,26}$=12.5, p<0.002).

Since there is no evidence suggesting that females are "worse" egg-carriers than males (in terms of survival of offspring) we can predict that oviposition stimulation in this species occurs in the presence of both males and females. To test this hypothesis we carried out a planned comparison Anova (Statsoft 1996), in which we used a coefficient of -4 for the group of isolated females and coefficients +1 for the other groups. In doing this, we predicted no differences in the output for groups B, C, D and E, and significant differences between these groups and group A. We detected differences between females in conspecific presence (groups B, C, D and E) and isolated females (group A) (Planned comparison ANOVA: $F_{1,23}$=9.11, p<0.001) (Test 3), and no differences between groups in which females were housed with conspecifics irrespective of whether they were male or female (Fig. 1b).

If oviposition stimulation by conspecific presence is a female strategy aimed at increasing egg survival, there should be no need for mating to take place, at least not in freshly mated females. To test this prediction, we grouped females housed with conspecifics according to whether they had copulated or not (i.e. females housed with females were grouped with females housed with males who had not copulated and this group was labelled "non-mated females"). We compared isolated females, females housed with males who had copulated and "non-mated females", being the overall differences statistically significant (ANOVA: $F_{2,25}$=5.18, p=0.013) (Test 4) (Fig. 3). Significant differences in the number of eggs laid during the experimental period were found between isolated females and "non-mated females" who were housed with conspecifics (Student-Newman-Keuls test, p=0.027). This result shows that non-mating oviposition stimulation is taking place. No differences were found between females in conspecific presence according to whether they had copulated or not (Student-Newman-Keuls test, p=0.54). Tests 1 to 4 keep statistical significance after sequential Bonferroni correction (Rice, 1989).

Egg retention

There are no differences among treatments in the number of fully developed or developing eggs inside the female reproductive tract (Kruskal-Wallis Anova by ranks: H=2.23, d.f.=4, N=25, p=0.7). In addition, there are no differences between females that mated again during the experimental period and females that did not mate after the pre-experimental copulation (Mann-Whitney U test: U=69.5, N mating females=12, N non-mating females=13, p=0.64).

Discussion

The results of this study show that female *Phyllomorpha laciniata* have a strong preference to oviposit on conspecifics. In addition, our results demonstrate that conspecific presence modulates the reproductive performance of this insect. This evidence reveals that oviposition behaviour and, in general, reproductive physiology in the golden egg bug responds to variability in host quality (plants vs. conspecific) and host availability in an adaptive way.

P. laciniata is an ideal model organism to study the evolutionary consequences of oviposition site selection because it shows a unique pattern: females can choose between two radically different oviposition sites, the host plant or conspecifics' backs. The host plant represents an unlimited substratum of "low quality", since eggs suffer high mortality rates, whereas conspecifics represent a "high quality" alternative, i.e. eggs enjoy higher survival rates, which is more difficult to encounter (Reguera & Gomendio 2002). From the point of view of a laying golden egg bug female, oviposition on the back of other individuals has important benefits since eggs carried by conspecifics are less vulnerable to egg predation (mainly by ants) and to a Scelionid parasitoid (Kaitala 1996; Reguera 1999; Reguera & Gomendio 2002). Thus, it is in the females' interest to lay eggs on conspecifics since this will enhance female reproductive success. The importance of oviposition choice has been established for many species of insects and other animals (Godfray 1994; Resetarits 1996; Resetarits & Wilbur 1989; Thompson & Pellmyr 1991), but in these cases the choice is restricted to the quality of specific hosts or oviposition sites and females do not have the opportunity to choose a conspecific as a laying site.

The reason why females also lay eggs on plants, despite the low survival rates, is partly related to the likelihood of encountering a conspecific, and to its willingness to accept eggs. In natural populations, densities are low (ranging from 0.005 individuals per square meter to a maximum of 0.265 indiv./m^2; data gathered from adults found in an area of 400 m^2 in 44 different days throughout three reproductive periods, Francisco García-González, unpublished data) and a willing individual is unlikely to be available every time a female lays an egg. When densities are increased under experimental conditions, females lay a greater proportion of eggs on conspecifics as encounters rates increase (Reguera 1999).

Thus, in natural populations encounters rates may not occur as often as females have eggs ready to be laid, and several lines of evidence suggest that females may not be able to store eggs for long periods of time. *P. laciniata* is a synovigenic insect (that is, females continue to mature oocytes during the adult stage), with physical limitations to store a large egg load (eggs are large in relation to abdomen size, see below). This could force females to oviposit on plants when recipient individuals are difficult to locate or when conspecifics are reluctant to accept eggs. A similar situation takes place in the giant water bug *Abedus herberti* where female egg production continues uninterrupted regardless of whether or not the female encounters a receptive male to mate and to receive her eggs (Smith 1979), even though in this species eggs do not hatch unless brooded by males.

In addition, eggs are laid one by one continuously over the whole breeding season and each egg represents a small proportion of female

lifetime reproductive success. Thus, each decision about where to lay an egg must be balanced against the costs of looking for an optimal site (i.e. a willing conspecific) and the consequent decrease in fecundity (Rosenheim 1999; Rosenheim, Heimpel & Mangel 2000). Given that eggs do have a small probability of surviving in plants, it seems to pay females to lay a proportion of their eggs on plants when conspecifics are not available.

In golden-egg bug populations finding a conspecific where to oviposit seems to be a limiting factor for female fitness, thus females would benefit from increasing egg output upon an encounter with a conspecific. Our findings support this prediction since females housed with conspecifics laid, on average, more than two times the number of eggs laid by isolated females. Our results indicate that the first day of exposure to conspecifics has an immediate effect over oviposition output in females. A more delayed response would be ineffective for female fitness, since individuals do not move in a coordinated way and are unlikely to remain in proximity for long periods of time. From a comparative perspective it is well known that the availability and quality of the host for parasitoids and phytophagous insects often determines the number of eggs laid (Engelman 1970; Papaj 2000; Papaj & Messing 1996; Fournet et al., 2001), and it seems reasonable to predict that oviposition stimulation has evolved when preferred oviposition sites are rare or difficult to encounter.

Stimulation of ovarian dynamics or conspecific stimulation of oviposition by males is widespread, but it is generally the consequence of mating. In mammals, the presence of males or mating may induce ovulation (Bronson 1989; Bronson & Maruniak 1975; Cheminau 1983; Cohen-Tannoudji, Locatelli & Signoret 1986; Lindsay *et al.* 1975; Martin *et al.* 1986; McComb 1987; Schiml *et al.* 2000; Signoret 1980). In some spontaneous ovulators, the luteal phase (preparation of the uterus for implantation) is induced by mating (Dewsbury 1984; Schiml *et al.* 2000). In insects such as grasshoppers, cockroaches, crickets, locusts, fruit flies, and many others, one consequence of mating is the stimulation of oviposition (Eberhard 1996; Engelman 1970; Davey 1967; Lange & Loughton 1985; Leopold 1976; Loher *et al.* 1981). However, non-mating conspecific stimulation is known in very few cases in groups such as mammals (Cohen-Tannoudji *et al.* 1986; McComb 1987) and insects. In the latter, the only documented cases are those ones in which there is a stimulation by conspecific eggs (Monaco, Tallamy & Johnson 1998; Srinivasan *et al.* 1995), those ones in which the presence of cocoons, brood stages, or the worker/brood ratio regulates queen's egg laying in social insects (Gibson & Scott 1990; Wilson 1971), or the cases in which workers of social insects lay more eggs when they are in conspecific presence (Salzemann & Plateaux 1988). Thus, with the exception of social insects, conspecific stimulation in the absence of mating has been rarely reported.

Most of the studies which have looked at the benefits of the stimulation of female reproductive cycles by male presence or mating, have adopted the perspective of the male and have concluded that it is a male strategy aimed at maximising reproductive success, often at some cost to females (Chapman *et al.* 1995; Holland & Rice 1999; Johnstone & Keller 2000; Rice 1996; Rice & Holland 1997). The evolutionary scenario of our model organism seems to be quite different since females benefit greatly from increasing egg laying when there are conspecifics on which to lay eggs. We showed that such stimulation does not require mating, and it occurs both when there are males or females in proximity. This result confirms our expectations since eggs are equally likely to survive on males and females. Thus, we interpret that oviposition stimulation by conspecific presence is a female strategy which has evolved to increase female reproductive success, via its effects on offspring survival.

The last question that we addressed in this study is whether oviposition stimulation in *Phyllomorpha laciniata* is the result of (a) an increase in ovulation or egg maturation rate, or (b) an increase in laying rate of eggs which are already mature. Such alternatives are possible

because in insects ovulation and oviposition may not take place simultaneously. Some insect females can store a batch of mature eggs, until they discover the most suitable place for the eggs (Engelman 1970; Hoffmann *et al.* 1990). We dissected females at the end of the experiments and found that isolated females did not retain eggs, suggesting that conspecific presence stimulates egg maturation. It is worth mentioning that we discarded the possibility that differential egg loads of females at the start of the experiment was a confounding factor, because there were no differences among females in body weight or body condition and it is therefore reasonable to assume that females started the experiment with similar egg loads. These variables explain egg load to a large extent because a female usually contains several eggs in her reproductive tract, and each fully developed egg represents roughly between 2% and 4% of female body weight.

The broad picture that is emerging from the long-term field data, as well as from the experiments carried out, is that female golden-egg bugs benefit greatly from laying eggs on other individuals. However, a proportion of eggs are laid on plants probably because a willing conspecific is not available every time a female lays an egg, an event which takes place almost continuously over several months. Given the marked differences in survival rates between eggs laid on conspecifics and eggs laid on plants, females increase laying rates immediately as a response to the encounter of an individual, irrespective of whether it is a male or a female. This response is likely to increase the chances of laying eggs on conspecifics and thus increase female reproductive success.

From the point of view of the egg recipient the picture is less clear (see Gomendio & Reguera 2001). Carrying eggs is costly for golden egg bugs because it increases predation rates, either because individuals become more easy to detect or because they are less likely to escape (Kaitala, Espadaler & Lehtonen 2000; Reguera & Gomendio 1999). Why then do individuals accept eggs? In natural populations, a low proportion of females carry eggs, and those which do carry very few eggs. We know that females always carry other females' eggs, but it is still unclear if this is a case of intraspecific parasitism, reciprocal altruism or kin selection. Males follow a different trend since all males in natural populations end up carrying eggs, and they carry more eggs than females do. The prevalence of male egg carrying demands an adaptive explanation, since males have the possibility of accepting or rejecting eggs. The evolutionary scenario that emerges is complex due to the conflicts of interest between females who derive benefits in terms of offspring survival and males who suffer survival costs when they carry eggs. A female reproductive strategy which imposes costs on males is uncommon and deserves further study to understand which are the benefits for males which may explain male acceptance.

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CAPÍTULO 4

Ajuste de la duración de las cópulas y del tamaño del eyaculado de acuerdo al riesgo de competencia espermática*

Resumen del Capítulo 4

La competencia espermática es una potente fuerza selectiva que actúa sobre una gran variedad de caracteres relacionados con la reproducción sexual, incluyendo el comportamiento en el apareamiento, la duración de las cópulas, y las características del eyaculado. Varias hipótesis han sido propuestas, en el contexto de la competencia espermática, para explicar la existencia de cópulas largas o prolongadas. Estas hipótesis incluyen la de la guarda de la pareja ("mate guarding"), bajo la cual los machos que se aparean se mantendrían en asociación con la hembra para prevenir que ésta se aparease con machos rivales, y la del "sperm loading", en la cual se predice que el macho que se aparea prolonga la cópula para incrementar la transferencia de espermatozoides y así incrementar su representación gamética en la competencia por la fecundación de los ovulos de la hembra. Estas dos hipótesis comparten la predicción de que los machos prolongarán la cópula en situaciones de riesgo de competencia espermática. Para discriminar entre ambas hipótesis se hace necesario un análisis detallado de los patrones de transferencia de espermatozoides a lo largo de la cópula, y no sólo de las variaciones en la duración de las cópulas debida a diferentes situaciones de riesgo de competencia espermática.

Una serie de modelos teóricos construidos en el marco de la competencia espermática predicen que

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los machos deberían invertir más en la producción de espermatozoides cuando se enfrentan al riesgo de que machos rivales entren en competencia con ellos. En el presente Capítulo exploramos todos estos aspectos en *Phyllomorpha laciniata*, una especie que se caracteriza por presentar cópulas extremadamente largas y sobre la que no se han realizado estudios previos a este respecto. Por otra parte, en esta especie, el conocimiento de las estrategias masculinas para maximizar su éxito en la fertilización es de gran interés para comprender el comportamiento de transporte de huevos por parte de los machos. En este estudio se ha investigado el significado adaptativo de las cópulas largas y prolongadas en este insecto por medio del análisis de la duración de las cópulas y de la transferencia de espermatozoides, bajo situaciones que diferían en cuanto al riesgo de competencia espermática al que eran expuestos los machos durante el apareamiento.

Los datos apoyan las predicciones de los modelos de riesgo: los machos transfieren más espermatozoides cuando están expuestos al riesgo de que exista competencia espermática. Este resultado ha sido previamente observado en otras especies. Sin embargo, hasta la fecha ningún estudio ha puesto de manifiesto que pueda existir una respuesta al riesgo de competencia espermática de tipo aditivo, como la que se ha encontrado en *P. laciniata*: los machos en presencia de machos rivales (grupos experimentales con razones de sexos desviadas a machos) incrementan (1) la duración de la cópula, lo que a su vez incrementa el número de espermatozoides transferidos puesto que los datos demuestran que la transferencia es contínua a lo largo del apareamiento, y (2) la tasa de transferencia de espermatozoides por unidad de tiempo. La relevancia de estos resultados no sólo radica en que apoyan de manera empírica los modelos teóricos de competencia espermática, sino que contribuyen de una manera importante al conocimiento del sistema de *P. laciniata*. Este estudio demuestra que los machos maximizan sus probabilidades de fecundación por medio de cópulas largas y mayores tasas de transferencia. Esto se traduce, probablemente, en que el macho que se aparea adquiere una cierta confianza de paternidad sobre los huevos que son puestos tras la cópula, lo que puede estar en conexión con la aceptación de los huevos por parte de los machos. Por lo tanto, este trabajo constituye una pieza clave en la comprensión del comportamiento de transporte de huevos en esta especie.

Adjustment of copula duration and ejaculate size according to the risk of sperm competition in the golden egg bug (*Phyllomorpha laciniata*)

Francisco García-González and Montserrat Gomendio
Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain.

Abstract

Several hypotheses have been proposed to explain the adaptive significance of prolonged copulations in insects, which include mate guarding and sperm loading functions. We have explored the adaptive significance of the prolonged copulations in the golden egg bug (copulations up to 50 hours), and the effect of an increased risk of sperm competition on ejaculate investment. Our data support predictions derived from sperm competition theory in which males are expected to increase ejaculate expenditure in response to an increased risk of sperm competition. Results show a combined response by males that has not been previously described: males in the presence of rivals increase (i) copulation duration and (ii) the rate of sperm transfer. No relationship is found between male or female size and copulation duration or ejaculate size. Golden egg bug males transfer sperm slowly and gradually throughout copulation, thus an increase in the amount of sperm transferred and the corresponding increase in the male's numerical representation in the female's storage organs could be particularly important in a system in which so few sperm are transferred, and so few sperm are stored by females. In addition, copulation duration may not only serve to increase the total amount of sperm transferred, but may also increase the chances that the female will lay an egg soon after copulation has ended. This could explain why males tend to accept eggs after copulation, since they could be maximising the chances that such eggs are fathered by them, and in this way they would substantially increase the survival rates of their offspring, since eggs laid on plants suffer high mortality rates.

Key words: prolonged copulations, ejaculate size, sperm competition, sperm loading hypothesis, golden egg bug, *Phyllomorpha laciniata*

Introduction

Sexual selection has favoured the evolution of behavioural, physiological and morphological traits in males that increase reproductive success in the face of male-male competition (Andersson, 1994). When females tend to copulate with several males and there is a temporal and spatial overlap of ejaculates from two or more males, a specific type of male-male competition takes place, which is known as sperm competition (Parker, 1970). Sperm competition has been a major selective force shaping many aspects of sexual reproduction, including mate guarding, frequency and duration of copulation, genitalia morphology, testes size, sperm numbers, ejaculate quality, and sperm size (Birkhead and Møller, 1998; Parker, 1970; Simmons, 2001; Smith, 1984).

In the context of sperm competition copulation may serve other functions apart from the obvious of sperm transfer (Simmons, 2001; Thornhill and Alcock, 1983). While in general sperm transfer is accomplished within a few seconds or minutes, some species remain in copula for longer periods. Prolonged copulations are assumed to be costly because they can be energetically expensive, may increase the risk of predation, may increase the probability of disease transmission and may decrease time devoted to other activities (Daly, 1978). Despite such costs prolonged copulations occur in many orders of

insects (Alcock, 1994; Smith, 1984; Thornhill and Alcock, 1983) and several hypotheses have been put forward to explain its adaptive significance. First, the "sperm removal" hypothesis suggests that copula duration could reduce competition with previous ejaculates if males spend longer removing their rivals' sperm (Siva-Jothy, 1987; Siva-Jothy and Tsubaki, 1989). Second, Eberhard (1996) suggested that copula duration may be under female control and that the benefits for females have to do with facilitating cryptic female choice mechanisms. Third, the "in-copula guarding" hypothesis suggests that remaining in copula may function as an extreme form of mate guarding if it prevents the female from remating before oviposition (Alcock, 1994), thus it may reduce or avoid sperm competition with future ejaculates. Finally, the "sperm loading" hypothesis proposes that copula duration may determine the amount of sperm transferred by the male (Dickinson, 1986; Parker et al., 1990), and thus, prolonged copulation may enhance the competitive ability of the ejaculate by increasing sperm numbers (Parker, 1982, 1984, 1993).

High levels of sperm competition have been associated with longer copulations (Alcock, 1994; Alonso-Pimentel and Papaj, 1996; Clark, 1988; McLain, 1989; Sillén-Tullberg, 1981) and such prolonged copulations have generally been interpreted as a mate guarding strategy. However, an increasing number of studies have shown that longer copulations may also imply the transfer of greater sperm numbers (Arnqvist and Danielsson, 1999; Birkhead et al., 1995; Dickinson, 1986; Parker et al., 1990). The hypothesis of sperm loading to explain prolonged copulations under increasing levels of sperm competition is plausible when sperm compete numerically since in this case the most obvious adaptation to sperm competition is selection on males for increased sperm numbers (Eady, 1995; Parker, 1982, 1984, 1993; Wedell and Cook, 1998).

Sperm competition theory suggests that males may respond to current information on sperm competition risk (the probability that a given male will be in direct competition for fertilizations). A male is expected to increase ejaculate expenditure in response to an increased risk of sperm competition (Ball and Parker, 1998; Parker, 1990a, 1998; Parker et al., 1997). Such strategies of ejaculate allocation evolve because the costs of ejaculate production are not trivial (Dewsbury, 1982; Nakatsuru and Kramer, 1982; Olsson et al., 1997) and males are expected to partition their ejaculates optimally (Parker, 1982, 1990a, 1990b; Simmons and Siva-Jothy, 1998). Several intraspecific studies have provided evidence in support of this prediction (Cook and Gage, 1995; Gage, 1995; Gage and Barnard, 1996; Oppliger et al., 1998; Schaus and Sakaluk, 2001; Simmons and Kvarnemo, 1997; Wedell and Cook, 1999a, 1999b), although some have found that males do not react as predicted to an increase in the risk of sperm competition (Birkhead and Fletcher, 1995; Schaus and Sakaluk, 2001; Wedell, 1992). When males adjust sperm transfer to the perceived risk of sperm competition, they may do so by copulating more frequently, by increasing the amount of time spent engaged in sperm transfer, or by increasing the amount of sperm transferred per time unit, however, additive effects between these different mechanisms remain largely unexplored.

Phyllomorpha laciniata is a heteropteran species characterised by an atypical behaviour among insects. Females exhibit a very flexible pattern of oviposition behaviour; they can lay their eggs on host plants (*Paronychia argentea*), where they develop unattended, or on the body of conspecific males and females where they are carried until hatching, when the nymphs start an independent life (Gomendio and Reguera, 2001; Kaitala, 1996; Reguera, 1999). There is an ongoing controversy concerning the evolutionary significance of male egg carrying in this insect. Some authors believe that egg carrying by males is likely to be the result of intraspecific parasitism (Kaitala et al., 2001). However, other authors have argued that, since the costs of carrying eggs are high, males should only accept eggs if by doing so they increase substantially the survival of some of their offspring (Gomendio and Reguera, 2001). Copulation duration is very long in *P. laciniata*, lasting around

20-30 hours on average (Kaitala, 1998; Reguera, 1999) and sometimes reaching 48 hours. However, no previous studies have been conducted on the adaptive significance of the prolonged copulations in this insect, although this information would contribute to improve our understanding of this intriguing system. In natural populations, individuals are likely to experience sperm competition since females mate promiscuously and store sperm in a spermatheca. In this study we examine the response of males to an increased risk of sperm competition.

Methods

Animals and general experimental conditions

Individuals for the experiments were collected in the field. For the experiments on copulation duration we collected 194 individuals of *P. laciniata* (91 males and 103 females) in Colmenar del Arroyo and Robledo de Chavela (Madrid, Central Spain), and in El Espinar (Segovia, Central Spain) on five different dates from the 8th of April to the 10th of May 1999. For the experiments on sperm transfer 175 individuals (88 males, 87 females) were collected in Aldea del Fresno and Robledo de Chavela (Madrid, Central Spain), and El Espinar (maximum distance among these localities is 40 km) on five different days from the 25th of April to the 16th May 2000. Individuals were transported in individual plastic vials to the laboratory in Madrid, placed in small Petri dishes (5.5 cm diameter) and kept at constant conditions (25° C, lights on from 8:00 AM to 9:00 PM). The experiment on copulation duration was carried out from the 16th of April to the 26th of May 1999, and the experiment on sperm transfer from the 3rd of May to the 26th of May 2000.

So far, all attempts to rear this insect in captivity have been unsuccessful. Although we have managed to obtain virgin females from advanced nymphs captured in the field, these adults do not mate until the following year (García-González F, unpublished). For this reason experiments in which virgin females are needed were carried out with virgin females selected among those collected in nature. Virgin females can be found among females collected at the beginning of the reproductive season, though they are present in small numbers. We considered a female as a virgin if: i) she did not lay eggs during at least five days since she was captured in the field ii) she had a normal abdomen (gravid females are recognized by a distended abdomen), and iii) as a indirect measure of the reproductive state of the local population she did not carry eggs in her back. In a series of preliminary studies these three requirements supported the "virgin state assumption" for females collected at the beginning of the reproductive season: females fulfilling these requirements were dissected looking for sperm in the spermatheca and none of them carried sperm.

Throughout all the experimental period individuals were provided ad libitum daily with fresh branches of the host plant *Paronychia argentea* in both sets of experiments. Prior to the experiments, eggs were removed from carrying males as well as from carrying females and individuals were weighed to the nearest 10^{-4} g with a Sartorius BP 110 S balance (Sartorius AG, Goettingen, Germany).

Copula duration

Variation in copulation duration depending on sex ratio conditions was tested in two experiments. In the first one we employed virgin females, whereas in the second one we used non-virgin females. We randomly assigned individuals to different treatments. In the experiment with virgin females (Experiment 1) 22 groups were established; 10 groups with 1 male and 1 female in each container and 12 groups with 2 males and 1 female (sex ratios of 1:1 and 2:1 respectively). In the experiment with non-virgin females (Experiment 2), 20 groups were established; 10 groups with 1 male and 1 female, and 10 groups with 3 males and 1 female (sex ratios of 1:1 and 3:1 respectively). Individuals in groups were kept in

plastic containers (16.5 cm x 16.5 cm x 10.5 cm). Because some individuals died during the experiment the final number of replicates was slightly lower than initially designed.

Males in all groups were placed in the container 2 days before the female to allow them to perceive the presence/absence of rivals, and therefore the risk of sperm competition. Males in groups with sex ratio 2:1 and 3:1 were marked in only one of the multiple lower abdominal chitinous extensions with a little green spot of Tipp-ex (Tipp-ex GmbH and Co. KG) to allow for identification. This facilitated identifying the mating male and monitoring the copula to check that the female does not remate with another different male from the group. Only the first mating of each female was included in the analyses.

We checked for copulations at least four times each day at 9:00, 13:00, 17:00 and 21:00 hours. Copulation in this insect lasts on average for more than 12 hours (23 hours on average as Kaitala, 1998; 32.5 hours on average as Reguera, 1999; 11 hours minimum as Mineo, 1984), so it is unlikely that copulations went unnoticed. In those instances in which the start or the end of a copula was observed, the exact time was registered. Otherwise, to calculate the start and end of each copulation we used the middle point between two intervals.

ANCOVAs and a mixed model of ANOVA were carried out. Male weight was entered as a covariant in the analyses to examine the relationship between male weight and copulation duration. The dependent variable (copulation duration) was Box-Cox transformed to fulfil parametric assumptions (Sokal and Rohlf, 1981). Homogeneity of variances was confirmed by using Levene's test, or Hartley F-max statistic, Cochran C statistic, and the Bartlett Chi-square test (Statsoft, 1996) in the mixed model of Anova.

Sperm transfer-ejaculate size

A total of 37 virgin females were identified among all the females collected. The experiment consisted of placing a virgin female in group of either a male or three males, and determining how many sperm were inseminated after 1, 6 or 12 hours after the beginning of the first copulation. Virgin females were randomly assigned to the different treatments. In groups with sex ratio 1:1 females were distributed among three different experimental groups depending on the time at which copulations were experimentally terminated: 1, 6 and 12 hours. In groups with sex ratio 3:1 females were distributed in groups in which copulations were terminated after 1 and 12 hours.

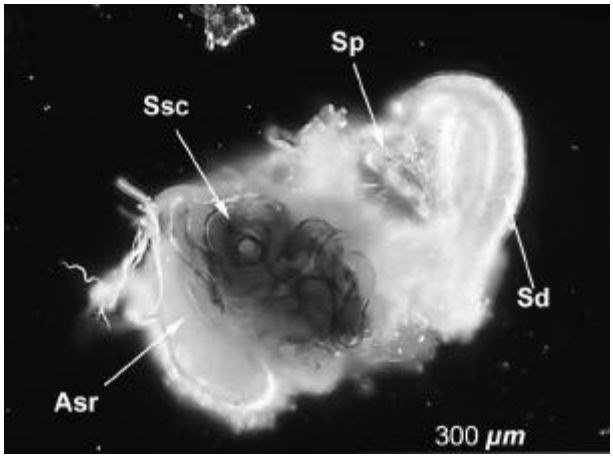

Figure 1. Golden egg bug spermatheca. Asr: Apical seminal receptacle; Ssc: Sclerotized surrounding channel; Sp: Spermathecal pump; Sd: Spermathecal duct (terminology following Pendergrast, 1957, and Moulet, 1993).

All males were kept individually, or together in sets of three, depending on the experimental group, at least 3 days before the introduction of females to perceive the risk of sperm competition. In total, males were at least 7 days in absence of females. We assume this time sufficient to replenish sperm reserves in case they had mated in the field (see for example Arnqvist and Danielsson, 1999).

Males in groups of sex ratio 3:1 were grouped in triads of individuals presenting similar weight. Data on male size was entered as covariant in the analyses (see below) to examine the relationship between male size and ejaculate size.

In studies on the effect of operational sex ratio (OSR, the ratio of the sexually active males in a population to receptive females (Emlen and Oring, 1977)) over variation in traits affected by risk of

sperm competition sex ratio has been usually manipulated by changing the density of just one sex. This could cause OSR to be confounded with that sex's density (Alonso-Pimentel and Papaj, 1996). In order to avoid OSR confounded with the absolute male density this experiment was performed in Petri dishes of 5 cm diameter for groups of sex ratio 1:1 and dishes of 9 cm diameter for groups of sex ratio 3:1. This means a similar male's density in different OSR: 0.042 males/cm^2 in 1:1 and 0.047 males/cm^2 in 3:1.

All groups were set up in the morning and recorded along all day with maximum intervals of 1 hour, except in groups in which the duration of the copulation was designed as 1 hour, that were monitored continually. Copulations that started during the day and continued until night were monitored from 9:00 PM onwards with the aid of red light.

In groups with sex ratio 3:1 the mating male was quickly marked at the beginning of the copulation in one lower abdominal chitinous extension with a little green spot of Tipp-ex (Tipp-ex GmbH and Co. KG) to ensure that there were no takeovers (although no takeovers have been ever seen taking brief periods of time). All matings were carefully observed to ensure penetration had taken place. Copulation was interrupted at 1, 6 or 12 hours from the start as designed depending on the groups. Copulation was instantaneously interrupted by quickly submerging the mating pair in Ethylenglycolmonoethylether (2-Ethoxiethanol) at -80°C. Then, the pair was maintained in 70% Ethanol until female dissection. Spermatheca (Figure 1) was carefully dissected and isolated on a glass slide in a drop of distilled water where it was crumbled with the aid of tweezers. Then, 5 µl of PBS without Ca^{2+} and Mg^{2+} and with 1% Triton was added and spermathecal fragments were stirred during 2 minutes. After adding 5 µl of Propidium Iodide (PI, 0.05 mg/ml) fragments were again stirred during 1 minute and then the sample was spread over an area of 25x15 mm previously drawn in the slide. A few preliminary experiments showed that this method allows dispersal of agglutinated sperm, and also

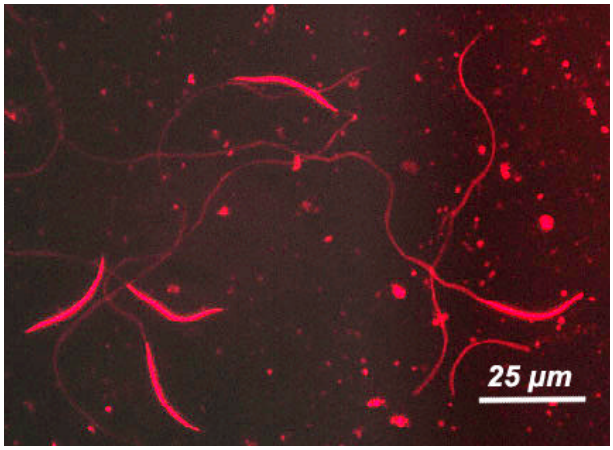

Figure 2. Golden egg bug sperm dyed using Propidium Iodide. Mean head length of *P. laciniata* sperm is 23.3 micrometres (SD=3.16; n= 20 individuals, 160 sperm).

they showed that the numbers of sperm inseminated are usually low. Thus, we did not dilute the suspension and we counted all the sperm in the sample. Preliminary experiments also showed that dilution and counting using a Neubauer Haemocytometer gave unreliable estimates.

This method allowed for a good identification of individual spermatozoa. Occasionally, some sperm clumps were seen but staining with the nuclear dye PI allowed counting of sperm heads (Figure 2 and see Figure 3 for a detailed view of the sperm). The sperm were counted using a fluorescent microscope Axiolab (C. Zeiss, Germany).

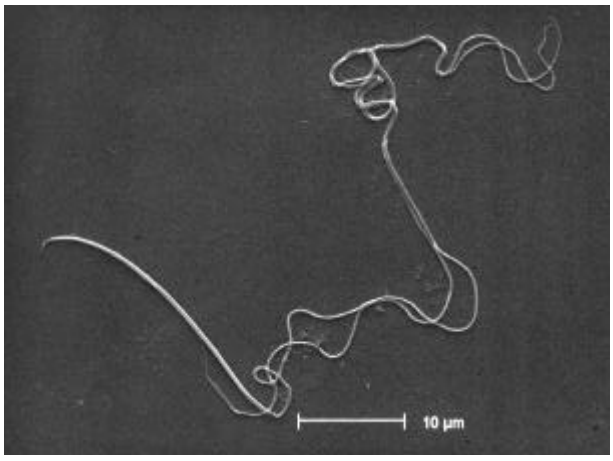

Figure 3. Scanning microscope view of a golden egg bug sperm. Note the biflagellate condition of the sperm, usual in many sperm of *P. laciniata*.

		Copula duration (h)				Male weight (mg)				
	OSR	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.	N
Virgins	1:1	13.32	2.58	7.50	24.00	12.45	0.46	10.50	13.77	7
	2:1	18.67	2.67	8.00	32.00	11.87	0.35	10.33	13.15	9
Non-virgins	1:1	13.61	2.10	4.00	22.00	10.85	0.41	9.00	12.90	8
	3:1	21.18	1.68	12.25	25.38	11.41	0.55	9.10	13.70	7

Table 1. Copulation duration and mating male weight in groups of virgin and non-virgin females under different OSR conditions.

In all cases copulations were experimentally interrupted. Four pairs separated themselves before the desired experimental duration and these were excluded from the analyses.

Since male size could be related to the number of sperm transferred, we controlled statistically this variable in the analyses. At the end of the experiment, males' size was estimated from three length measurements of the right hind tibia. These measurements were carried out capturing the tibia images (tibias were previously prepared on a glass slide) using a stereomicroscope Zeiss (Stemi SV6). Images were captured with a CCD camera (JVC TK-C1381) and tibias measured with NIH Image 1.60 software (National Institutes of Health, US). Repeatability of the three lengths reached R= 0.999 ($p<0.001$).

Male size was entered as a covariant in ANCOVAs to examine the relationship between male size and ejaculate size. Dependent variable (sperm transferred) was log transformed (Sokal and Rohlf, 1981). Homogeneity of variances was confirmed by using Levene's test (Statsoft, 1996). Non parametric test were used when parametric assumptions were not fulfilled.

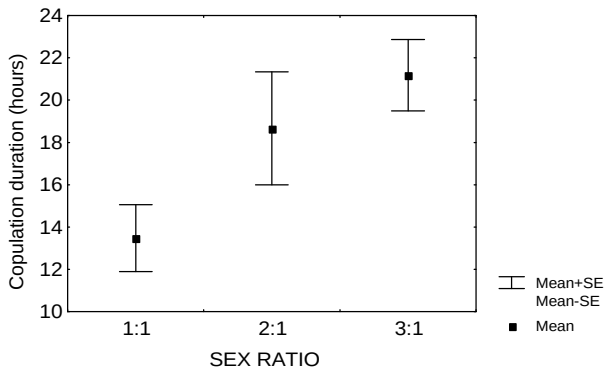

Figure 4. Mean copulation duration (and standard error) under different sex ratio conditions for Experiment 1 (virgin females) and Experiment 2 (non-virgin females) together (see text for details).

Results

Copulation duration

In Experiment 1 copulation duration in groups where sex ratio was 1:1 lasted an average of 13.3 hours, whereas copulation duration in groups where sex ratio was 2:1 lasted an average of 18.7 hours. Copulations were significantly longer in the male biased sex ratio group ($F_{1,13}=5.12$, $p=0.041$) (Table 1). Male weight was entered as covariant ($F_{1,13}=5.86$, $p=0.031$); male weight was higher in the 1:1 group than in the 2:1 group (see Table 1). However, male weight was not correlated with copulation duration ($r=0.41$, $p=0.12$, $n=16$).

There were no significant differences in female weight between groups in Experiment 1 (t test; $t= -1.51$, $d.f.=14$, $p=0.15$, n sex-ratio 1:1=7, n sex ratio 2:1=9). Females did not show a consistent preference to copulate with the largest male present in each group (Chi $sqared_{Yates\ corrected}=1.14$, $d.f.=1$, $p=0.29$, $n=7$). In addition, in the groups where sex ratio was 2:1, mating males (Table 1: mean=11.87 mg, SE=0.35, Min.=10.33, max.=13.15, n=9) were not significantly larger than non-mating ones (mean=11.49 mg, SE=0.26, Min.=10.25, max.=12.8, n=9) ($F_{1,16}=0.76$; $p=0.4$).

In Experiment 2, copulations were also

		Total number of sperm transferred				Male tibia size (10^{-4} m)				
	Time	Mean	SE	Min.	Max.	Mean	SE	Min.	Max.	N
Sex ratio 1:1	1 h	10.00	3.34	1	17	45.93	1.09	42.90	47.95	4
	6 h	27.33	12.99	2	45	43.82	1.57	40.68	45.58	3
	12 h	54.00	19.38	23	108	45.38	2.29	38.82	48.73	4
Sex ratio 3:1	1 h	50.00	13.83	29	88	45.96	2.55	41.70	52.71	4
	12 h	856.33	684.69	29	2215	40.26	1.67	36.95	42.30	3

Table 2. Ejaculate size along copulation duration in conditions of sex ratio 1:1 and 3:1.

significantly longer in the male biased sex ratio ($F_{1,12}$=7.16, p=0.02) (Table 1); copulations in groups where sex ratio was 1:1 lasted an average of 13.6 hours, whereas copulations in groups where sex ratio was 3:1 lasted and average of 21.2 hours. Male weight was non significant in the model ($F_{1,12}$=0.13, p=0.72). Male weight was not correlated with copulation duration in this experiment either (r=0.08, p=0.78, n=15).

There were no significant differences in female weight between groups in Experiment 2 (t test; t= -1.7, d.f.=13, p=0.11, n sex-ratio 1:1=8, n sex ratio 3:1=9). Largest males were not selected preferentially to mate (Chi sqared$_{Yates\ corrected}$=0.33, d.f.=1, p=0.56, n=7). In addition, in the groups with sex ratio 3:1, mating males (Table 1: mean=11.41 mg, SE=0.55, Min.=9.1, max.=13.7, n=7) were not larger than non-mating ones (mean=10.56 mg, SE=0.45, Min.=7.8, max.=14, n=14) ($F_{1,6}$= 2.61; p=0.16). In this case, differences were examined using a mixed model of variance analysis because it was necessary to control by female when analysing differences in male size selection by females. Female was entered as a random factor and male status (mating/non-mating) as fixed factor (Statsoft, 1996).

As no differences were found in copulation duration depending on whether females were virgins or not (sex ratio 1:1, t test, t= -0.10, d.f.=13, p=0.92, n Exp. 1=7, n Exp. 2=8), we pooled the data from the 2 experiments. When all females are analysed together the results show that copulations were significantly longer in male biased sex ratios ($F_{2,27}$=4.11, p=0.028, n sex ratio 1:1=15, n sex ratio 2:1=9, n sex ratio 3:1=7) (Figure 4 and Table 1). The mean copulation duration for all groups was 16.72 hours (SE=1.25, Min.=4 hours, Max.=32 hours). Male weight was non significant in the model ($F_{1,27}$=1.35, p=0.25). Overall, neither male body weight (r=0.20, p=0.287, n=31), nor female body weight were correlated with copulation duration (r=0.29, p=0.11, n=31).

Sperm transfer

In some experiments there was no sperm transfer. These cases were removed from analyses since absence of sperm transfer occurred with similar frequency between treatments (mean percentage of copulations involving no sperm transfer = 30%).

In groups in which sex ratio was 1:1 the numbers of sperm inseminated increased with the duration of copulation ($F_{2,7}$=5.03, p=0.044, n 1 hour=4, n 6 hours=3, n 12 hours=4) (Figure 5 and Table 2). Differences arise between the numbers

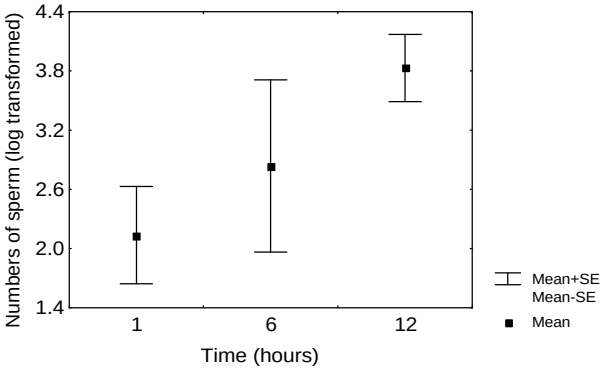

Figure 5. Numbers of sperm inseminated after copulations of 1, 6, or 12 hours, under sex ratio 1:1.

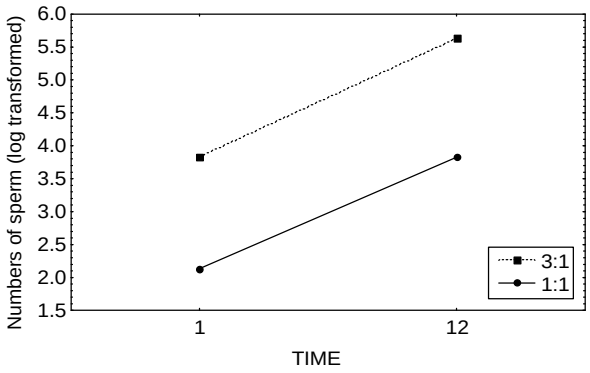

Figure 6. Mean numbers of sperm inseminated after copulations of 1 or 12 hours, under equal or male biased (3:1) sex ratio.

of sperm transferred in copulations lasting 1 hour and copulations lasting 12 hours (Duncan's multiple range test, p=0.032). Male size (hind tibia length) was entered as a covariant in the model ($F_{1,7}$=6.24, p=0.041) (Table 2). There was no association between mating male size and numbers of sperm transferred (r=0.44, p=0.18, n=11).

In groups where sex ratio was 3:1 the mean number of sperm transferred in copulations lasting 12 hours was greater than in copulations lasting 1 hour, but the difference did not reach statistical significance probably due to the small sample sizes and the large variation in sperm transfer observed in copulations lasting 12 hours ($F_{1,4}$=0.73, p=0.44, n 1 hour=4, n 12 hours=3, Table 2). Male size was non significant in the model ($F_{1,4}$=0.34, p=0.59). There was no association between mating male size and ejaculate size (r=-0.55, p=0.2, n=7).

A general model including sperm transfer in groups with sex ratio 1:1 and 3:1 was carried out. In this model, data from copulations lasting 6 hours were excluded since they were only examined under equal sex ratio. The numbers of sperm inseminated increased significantly with the duration of copulations (1 hour vs. 12 hours) ($F_{1,10}$=6.85, p=0.026) and also increased significantly in male biased conditions (1:1 vs. 3:1) ($F_{1,10}$=7.18, p=0.023). Table 2 and Figure 6 show these effects. The interaction between copulation duration and sex ratio was non significant ($F_{1,10}$=0.02, p=0.89). Male size effect was non significant ($F_{1,10}$=0.37, p=0.85). There was no correlation between male size and number of sperm transferred (r=-0.27, p=0.33, n=15) or between female body weight and sperm transferred (r=0.0, p=0.98, n=14).

Five females dissected in the peak of the reproductive period (assumed to have mated multiply in nature) contained an average of 476.6 sperm in the spermatheca (SE=158.1, Min.=71, Max.=947). There were no differences between this number and the sperm in the spermatheca of 12 hours' mated females in male biased sex ratio (Mann-Whitney U test, U=7, p=0.88).

There were no significant differences in female weight between time treatments in the groups of sex ratio 1:1 ($F_{2,8}$=0.14, p=0.87), or in the groups of sex ratio 3:1 ($F_{1,5}$=2.87, p=0.15), or when all data were analysed together (Time effect, $F_{1,16}$=2.60, p=0.13; Sex ratio effect, $F_{1,16}$=2.93, p=0.11; Time x Sex ratio, $F_{1,16}$=1.43, p=0.25).

Discussion

Male golden egg bugs adjust copulation duration and rate of sperm transfer according to the perceived risk of sperm competition. Our results show that the presence of rivals prior and during mating lead to an increase in both (i) copulation duration and (ii) the number of sperm transferred per unit of time. To our knowledge this is the first study to show that males respond to an increase in the perceived risk of sperm competition by combining an increase in the rate of sperm transfer with an increase in the amount of time they spend engaged in sperm transfer. By using both mechanisms simultaneously males maximise the number of sperm transferred and thus the possibilities of fertilization in a context in which sperm competition is likely. Thus, the results of this study support the prediction that males increase ejaculate expenditure when faced with a high risk of sperm competition and reveal a complex underlying mechanism, which has not been described previously.

Our results show that copulations are

unusually long in this species since they last on average 16.7 hours. Previous studies have found even longer copulations (average=23 hours, range 3-53 hours, in Kaitala, 1998, average=32 hours in Reguera, 1999). The experiments carried out in this study also show that copulations are longer when males are in the presence of other males (average of 18.7 h in sex ratio 2:1; average of 21.2 h in sex ratio 3:1) than when males are housed individually with a female (average of 13.5 h). The fact that males tend to prolong copulations in the presence of rivals, may explain the longer durations observed by Reguera (1999) since her experimental conditions included a male biased sex ratio (2:1) and males were, in addition, previously housed in high-density conditions. Prolonged copulations may represent a mate guarding strategy or may be a means by which males increase the amount of sperm transferred (see below); both hypotheses assume that copulation duration is under male control. Golden egg bug males try to force females to copulate, they have claspers with spines in the genitalia possibly having the function of female retention, and when pairs are in copula it is difficult to separate them since they remain firmly attached by the male genitalia. Thus, copulation duration seems to be mostly under male control as it seems to be general in heteropterans (Arnqvist, 1988; Arnqvist and Danielsson, 1999; Carroll, 1991; Sillén-Tullberg, 1981).

Our results show that longer copulations lead to an increase in the numbers of sperm inseminated. Contrary to other heteropterans (see below), sperm transfer occurs throughout copulation. However, males in the presence of rival males achieve an increase in the number of sperm inseminated not only by prolonging copulations, but also by increasing the rate of sperm transfer throughout copulation. While in-copula males inseminate an average of 10 spermatozoa in the first hour when there is no risk of sperm competition, whereas they inseminate an average of 50 spermatozoa when other males are present. After 12 hours, males transfer an average of 54 sperm when housed with a female, while they transfer an average of 856 sperm when housed with other males.

Sexual selection theory provides four different hypotheses to explain prolonged copulations in insects: (1) Sperm removal hypothesis (Siva-Jothy, 1987; Siva-Jothy and Tsubaki, 1989), (2) Cryptic female choice hypothesis, Eberhard (1996), (3) In-copula guarding hypothesis (Alcock, 1994), and (4) Sperm loading hypothesis (Dickinson, 1986) (see introduction for more details).

(1) To our knowledge sperm removal in Heteroptera has been never been documented. The "sperm removal hypothesis" seems unlikely in *P. laciniata* since longer copulations under high sperm competition risk also occur when males copulate with virgin females who have no sperm in the storage organs. (2) Several indirect lines of evidence suggest that females have little control over the duration of copulation in this species, since male genital morphology seems to indicate that males have the ability to retain females in copula, females show no preferences for males based on size (Reguera, 1999) or egg-carrying (Kaitala, 1998; Reguera, 1999), and there is no effect of male or female body weight on copulation duration.

Both mate guarding (3) and sperm loading (4) hypotheses predict longer copulations as sperm competition risk increases. Thus, in order to distinguish between these hypotheses it is necessary to carry out detailed examinations of ejaculate transfer and/or sperm utilization and fertilization success (Simmons, 2001). A sole analysis of copulation duration in the golden egg bug would had supported the mate guarding hypotheses since copulations are longer when rivals are present, and most studies assume that such unusually long copulations are not needed to achieve high sperm transfer. Prolonged copulations are common in other species of the order Heteroptera (Carroll, 1991, 1993; Clark, 1988; McLain, 1980, 1989; Rubenstein, 1989; Sillén-Tullberg, 1981), and longer copulations in male biased sex ratios have been found in *Neacoryphus bicrucis*, *Lygaeus equestris*, *Jadera haematoloma*, *Gerris remigis*, and *Nezara viridula* (Carroll, 1991;

Clark, 1988; McLain, 1980, 1989; Sillén-Tullberg, 1981). The mechanism of gradual sperm transfer observed in *P. laciniata* differs from that described in other heteropterans where complete sperm transfer occurs only minutes after copulation is initiated, e.g. *L. equestris* (Sillén-Tullberg, 1981) and *J. haematoloma* (Carroll, 1991). Prolonged copulations in these species thus seem to be a typical male postinsemination strategy to prevent subsequent matings by females, and the same occurs in *G. remigis* (Clark, 1988, but see below) and *N. bicrucis* (McLain, 1989). However, in *P. laciniata*, longer copulations lead to an increase in the number of sperm inseminated, because sperm transfer takes place throughout copulation, as has been shown to occur in *G. lateralis* (Arnqvist and Danielsson, 1999). Simmons (2001) has noted that in the studies of McLain (1980) and Rubenstein (1989) on *Nezara viridula* and *Gerris remigis*, respectively, prolonged copulations could also be interpreted as a way to increase the number of sperm transferred, since copula duration is associated with fertilization success; in *N. viridula* prolonged copulation reduced the fertilization success of rival males that copulated subsequently while in *G. remigis* the last male's fertilization success was dependent on his copulation duration (Simmons, 2001). The present study clearly reveals that in the golden egg bug prolonged copulations are needed to increase the number of sperm transferred because sperm are inseminated continuously, and thus support the sperm-loading hypothesis.

This study shows that males adjust ejaculate expenditure to the risk of sperm competition (Ball and Parker, 1998; Parker, 1990a, 1998; Parker et al., 1997), and suggest that prolonged copulation and increased rate of sperm transfer in this insect have evolved by direct male-male competition for fertilizations. Other studies have shown that males respond to and increase in sperm competition risk adaptively (see Introduction and reviews of Parker et al., 1997; Simmons, 2001, and Simmons and Siva-Jothy, 1998), but no other study has found a combined response of increasing copulation duration and sperm transfer rate. Production of high numbers of sperm is advantageous in sperm competition contexts when there is complete sperm mixing in the female's spermatheca, or when larger ejaculates are more effective at displacing sperm already stored by the female (Arnqvist and Danielsson, 1999; Dickinson, 1986; Eady, 1995; Parker, 1998; Parker and Simmons, 1991; Wedell and Cook, 1998). A knowledge of the mechanisms of sperm competition in the golden egg bug is needed to understand completely the adaptive significance of the variations in ejaculate expenditure. The fact that sperm are transferred at such a low rate seems to suggest that sperm displacement is unlikely, because when it happens the last ejaculate needs to be large enough to physically displace previous sperm. The golden egg bug spermatheca consists of a small sac-shaped organ, the seminal receptacle or bulb, with a sclerotized channel and a spermathecal pump with separates the spermathecal duct from the sperm store (Figure 1). Multiply mated females store low sperm numbers in the spermatheca (around 477 spermatozoa), which is in accordance with the low numbers of sperm transferred by males. Thus, increased sperm transfer seems to be a male strategy to maximise fertilization success when sperm mixing takes place. Recent evidence shows that sperm precedence patterns support a mechanism of sperm mixing (García-González F and Gomendio M, unpublished). Mean P_2 values, i.e. the proportion of eggs fathered by the second male to mate with a female, are around 0.5 in some heteropteran species (Economopoulus and Gordon, 1972; McLain, 1980, 1985). Other heteropterans shows second male advantage (Arnqvist, 1988; McLain, 1989; Sillén-Tullberg, 1981; Smith, 1979), although success of last males is highly variable in some of them (Carroll, 1991; Rubenstein, 1989; and see for a review Simmons and Siva-Jothy, 1998).

It is surprising that copulation duration is so long in a species with a cryptic phenotype, which has been most likely selected under strong predation pressure. Pairs in copula are likely to be detected more easily by predators and have impaired locomotory capacity (Kaitala and Axén,

2000; Reguera, 1999). The benefits of long copulations may be related to the slow rate at which sperm are transferred which may require a long time to ensure that enough sperm are transferred to ensure fertilization. Males may also benefit from staying physically engaged with the female for long periods if in this way they maximise the chances of being close to the female when the next egg is laid (females lay one egg at a time every day or so throughout the breeding season) and, most importantly, they may maximise the chances that the next egg is fathered by them. Males often accept eggs after copulating with the females, but they incur high costs because they are more vulnerable to predators when carrying eggs (Kaitala and Axén, 2000; Kaitala et al., 2000; Reguera and Gomendio, 1999). Long copulations may be the means by which males try to ensure paternity of the eggs they accept after copulating. It may be important for males to maximise the chances that the female lays the next egg soon after the end of copulation, and that they are able to accept them on their backs, because eggs laid on plants have very low chances of surviving (Reguera and Gomendio, 2002).

The fact that males respond to the presence of rivals by increasing both copulation duration and sperm transfer implies that sperm competition levels are not always high, because otherwise males should maximise ejaculate expenditure in every occasion. It makes sense for males to increase ejaculate expenditure in the presence of other males, if this situation is not the norm. This is in accordance with field data suggesting that population densities are low and when males find females they are unlikely to be surrounded by other competitors. The fact that sperm are transferred slowly over long periods of time suggests that sperm mixing is taking place. The existence of this sperm competition mechanism implies that a greater proportion of sperm present in the sperm storage organs is the best way to maximise fertilization success, and this is precisely what males attempt to achieve by combining an increase in the rate of sperm transfer with the time devoted to it. A large numerical representation could be particularly important in determining fertilisation success when so few sperm are stored by the female as it occurs in this species.

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CAPÍTULO 5

El desarrollo de una herramienta molecular para analizar relaciones de parentesco y paternidad en *P. laciniata* : extracción de ADN y marcadores AFLPs (Amplified Fragment Length Polymorphism)*

Resumen del Capítulo 5

En *Phyllomorpha laciniata* existe una fuerte controversia acerca de la interpretación del origen, evolución, y mantenimiento del comportamiento de transporte de huevos que muestran los individuos adultos. El uso de una herramienta molecular que permita analizar las relaciones de parentesco y paternidad entre los huevos y los individuos que los transportan es esencial para comprender la evolución de este comportamiento.

La técnica de identificación del ADN ("DNA fingerprinting") de reciente desarrollo conocida como AFLPs (Amplified Fragment Length Polymorphisms) está siendo aplicada con éxito en estudios en los que es necesario analizar las relaciones genéticas entre los individuos de una población o especie. La alta fiabilidad de esta técnica unida a otra serie de ventajas hace que sea potencialmente útil para el estudio de la paternidad. Su aplicación en grupos como insectos es, de manera especial, de gran interés, puesto que muchas especies de artrópodos son difíciles de abordar por otras técnicas tradicionales de

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identificación del ADN.

El trabajo presentado en el presente Capítulo ha tenido como objetivo desarrollar unos marcadores AFLP en *P. laciniata*, y evaluar ésta técnica como base para un futuro uso en la determinación de las relaciones genéticas, en especial de las relaciones de parentesco y paternidad, en ésta y otras especies.

Un primer paso para la consecución de este objetivo ha sido la puesta a punto de un método de extracción de ADN de *P. laciniata*. El resultado de una evaluación de siete diferentes métodos de extracción ha mostrado que las extracciones basadas en el CTAB (bromuro de cetiltrimetilamonio) ofrecen ADN en una cantidad y calidad apropiada para realizar AFLPs. Un segundo paso ha sido la evaluación de diferentes parejas de cebadores ("primers") selectivos para obtener los que ofrecieran un nivel de polimorfismo adecuado, además de mostrar resultados claros y reproducibles. Una vez concluido este paso se aplicó la técnica a una muestra de 79 individuos, entre adultos y ninfas. El número de fragmentos obtenido fue de 116, y el grado de polimorfismo de un 92.2%. La fiabilidad de los marcadores AFLPs se ve confirmada por dos hechos: 1. la repetibilidad media fue del 96.6%, y 2. los *loci* obtenidos migraron de manera independiente como se desprende del uso de un índice que analiza la correlación en la migración.

El alto grado de polimorfismo obtenido, sumado a los datos sobre la fiabilidad de la técnica, sugiere que los marcadores AFLPs pueden ser de extraordinaria utilidad en la determinación de la paternidad de los huevos transportados por los individuos de *P. laciniata*. Además, este estudio muestra que los AFLPs pueden ser una herramienta molecular útil en la resolución de preguntas de diversa índole en sistemas de insectos.

DNA extraction and Amplified Fragment Length Polymorphisms (AFLPs) for paternity and relatedness analyses in an egg-carrying insect, the golden egg bug (*Phyllormorpha laciniata*)

F. García-González,[1] Y. Núñez[2], F. Ponz[2], E. R. S. Roldán[1] and M. Gomendio[1]
[1]Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain, [2]Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Ctra. Coruña Km 7.5, 28040 Madrid, Spain.

Abstract

Amplified fragment length polymorphism (AFLP) markers have become a reliable DNA fingerprinting technique for the analyses of genetic relatedness. The potential for its use in systems were no codominant markers are available, or when only small quantities of DNA can be analysed, makes AFLPs a powerful technique to address evolutionary questions in groups such as insects. To solve the evolutionary puzzle represented by egg carrying males and females in the golden egg bug (*Phyllomorpha laciniata*) it is crucial to determine with molecular methods the genetic relatedness between the eggs and the individuals who carried them. We evaluated the suitability of seven DNA extraction protocols to obtain high quality DNA for AFLP analysis in this insect. A CTAB-based protocol provided the best results. On the other hand, primers for AFLP assay in the golden egg bug that offer the most clean and reproducible patterns were determined among multiple combinations of MseI and EcoRI selective primers, and the AFLP method was applied to a sample of 79 golden egg bug individuals including both adults and nymphs. Two AFLP primer pairs generated 116 fragments, being polymorphic 92.2% of them. The repeatability of the method reaches 96.6%, and the use of and index in the correlation in migration for all *loci* shows that *loci* migrate independently. These results suggest that the AFLP technique will prove useful in solving evolutionary questions in insect species.

Keywords: Amplified fragment length polymorphism, DNA extraction, golden egg bug, insects.

Introduction

Evolutionary explanations concerning the origin and maintenance of reproductive strategies and parental behaviour have been dramatically altered by the use of molecular techniques in behavioural ecology studies (Gowaty & Karlin, 1984; Quinn et al., 1987; Burke, 1989; Queller et al., 1993; Avise, 1994; DeWoody et al., 1998; Hughes, 1998). The main reason for this revolution in our way of understanding how animals attempt to maximise their reproductive success is the revelation that in may species males care for offspring who are fathered by other males in the population. This evidence has challenged the view that males should only invest in their true genetic offspring, and has brought to our attention the need to consider conflicts of interest between males and females since both sexes enhance their fitness in different ways (Burke et al., 1989; Birkhead & Møller, 1992; Westneat & Sherman, 1993; Dixon et al., 1994; Westneat & Sargent, 1996; Birkhead & Parker, 1997; Stockley, 1997).

In the golden egg bug (*Phyllomorpha laciniata*) there is a current controversy regarding the significance of egg carrying in relation to paternity of eggs carried by individuals of this species (Gomendio & Reguera, 2001; Kaitala et al., 2001). Females can lay eggs on plants, where they develop unattended, or on conspecifics (either males or females) that carry them until hatching (Kaitala, 1996). The survival of eggs is greatly improved when carried by conspecifics (Reguera &

Gomendio, 2002), but egg carrying represents a significant cost for adults in terms of vulnerability to predators (Reguera, 1999; Reguera & Gomendio, 1999). In natural populations, all males end up carrying eggs, while only a few females carry eggs and, when they do, they carry fewer eggs than do males. Females cannot lay eggs on themselves, so females who carry eggs are always carrying other females' eggs. Thus, egg carrying by females is not a form of parental care and seems to be a case of intraspecific parasitism that affects a small proportion of the female population. Since a much larger proportion of males carry eggs it is a matter of debate whether this is also a form of intraspecific parasitism, or whether males accept eggs because there is a chance that some of the eggs carried will be their true genetic offspring, and thus could be considered as a form of parental care (see Gomendio & Reguera, 2001 and Kaitala et al., 2001). To elucidate the role of parental care and of intraspecific parasitism in this system it is crucial to determine with molecular methods the paternity of the eggs carried by males.

Restriction Fragment Length Polymorphism (RFLP) and PCR amplified microsatellite *loci* are the most frequently used molecular techniques in behavioural ecology studies (Burke, 1989; Westneat, 1990; Queller et al., 1993; Avise, 1994; Schierwater et al., 1994; Pena & Chakraborty, 1994). These techniques require knowledge of the genome under investigation and/or the availability of relative large amounts of DNA. In many *taxa*, particularly among insects, the elucidation of parent-offspring relationships can be a difficult task since no molecular markers such as microsatellites are available in many of these species. The enormous diversity of insect species implies that detailed genetic information about one species is rarely applicable to another (Fagerberg et al., 2001) and only small quantities of DNA are available for analyses given their small body size (a problem which becomes exacerbated when embryos or larvae need to be analysed). Random Amplified Fragment Polymorphism (RAPD) fingerprinting (Welsh & McClelland, 1990; Williams et al., 1990) can overcome these drawbacks and this method has been used for relatedness and paternity analyses (Lewis & Snow, 1992; Lynch & Milligan, 1994), especially in insects (Hadrys et al., 1992, 1993; Hadrys & Siva-Jothy, 1994; Schierwater et al., 1997). However, RAPDs have been criticised due to the occurrence of non-parental bands within a pedigree, raising doubts about its reliability in paternity analyses (Riedy et al., 1992; but see Scott et al., 1992).

Recently, the analysis of Amplified Fragment Length Polymorphisms (AFLPs), a novel technique based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA was introduced by Vos *et al.* (1995). The AFLP technique is a relatively cheap, easy, and fast method to generate hundreds of informative genetic markers (Mueller & Wolfenbarger, 1999). In short, following Vos & Kuiper (1997) and Mueller & Wolfenbarger (1999), this technique has a number of specific advantages: (a) no prior sequence knowledge is required, (b) high marker densities can be obtained with modest efforts, (c) AFLP markers can be detected in almost any background or complexity, (d) repeated AFLP amplifications show near perfect replicability as AFLP amplifications are performed under conditions of high selectivity (high stringency), and (e) it requires minimal amounts of DNA and partially degraded samples can be used. However, it has some limitations: (a) AFLP fingerprints will share very few common fragments when sequence homology is less than 90%, and (b) compared with co-dominant markers, AFLP markers suffer from their general dominant nature (Yan et al., 1999).

AFLP markers have been found to be suitable for the analysis of relatedness and parentage (see for example Maughan et al., 1996; Krauss, 1999; Questiau et al., 1999; Loh et al., 2000; Creswell et al., 2001), especially because AFLP markers are virtually free of artifacts and because comigration of non-allelic fragments occurs at extremely low levels (Mueller & Wolfenbarger, 1999). So far, the utility of the AFLP technique for paternity analyses has been assessed in only a few studies (*Persoonia*

MseI primers	EcoRI primers							
	ACA	**AAG**	**AAC**	**ACT**	**AGG**	**AGC**	**ACG**	**ACC**
CAG	X	X	X	X	X	X	X	X
CAC	X	X	X	X	X	X	X	X
CTG	X	X	X	X	X	X	X	X
CAA	X	X						
CAT	X	X	X	X	X	X	X	X

Table 1. Selective MseI and EcoRI primer combinations tested for *P. laciniata.*

mollis: Krauss & Peakall, 1998; Krauss, 1999; *Luscinia svecica*: Questiau et al., 1999). These pioneering studies have shown that dominant AFLP markers can be useful for an estimation of paternity in *taxa* for which no microsatellite primers are available because, among other reasons, it generates sufficiently large numbers of highly reproducible polymorphic *loci* that are quickly and accurately scored using an automated DNA sequencer.

In order to elucidate whether egg carrying by male golden egg bugs is a form of parental care, or a case of intraspecific parasitism, it is essential to find out whether eggs carried by a male have been fathered by him. The development of a methodology to analyse paternity and parentage in natural and non-natural conditions in both carrying males and females is essential for an understanding of the evolution of egg carrying in this system, and may have important implications for the evolution of parental care in animals. No molecular markers are known in the golden egg bug and only small quantities of DNA are available for analyses (golden egg bug eggs and nymphs are extremely small, and nymphs in captivity do not survive beyond the first instar). The aim of this study was to evaluate several DNA extraction protocols and to find suitable AFLP selective primers for paternity and relatedness analyses in the golden egg bug.

Results and Discussion

The immediate freezing of animals was one important factor concerning DNA yield and quality in all the seven DNA extraction methods tested. A cetyltrimethylammonium bromide (CTAB) based procedure (Method 7 of experimental procedures and Appendix 1) modified from Doyle & Doyle (1987), Weising *et al.* (1995, page 51), Möller *et al.* (1992), and Reineke *et al.* (1998) was the most suitable for DNA extraction in this insect. In spite of the fact that nymphs usually yielded high quality DNA with all the extraction methods tested, adults consistently failed to yield high quality DNA unless Method 7 was used. Reineke *et al.* (1998) carried out the first study concerning the potential use of AFLPs for insect DNA analysis, evaluating six protocols for DNA extraction and determining the suitability of the product for AFLP analysis. Reineke *et al.* (1998) found two suitable methods using phenol or phenol-chloroform extractions and a highly successful method based on a CTAB protocol by Murray & Thompson (1980). This last method was preferred by Reineke *et al.* (1998) since it avoids hazardous treatments, such a phenol extractions. Our results confirm that CTAB based methods are the most recommendable protocols when extracting DNA from the golden egg bug. Phenol-based methods and extraction kits generally yielded low quality adult DNA, whereas Method 6 and more frequently Method 7 resulted in good quality extracts, as revealed by DNA integrity after electrophoresis in agarose gels.

Once that DNA extraction procedure was set up, multiple combinations of MseI and EcoRI selective primers were examined. Thirty different primer combinations were tested (Table 1) and two primer pairs were identified as the most polymorphic, and therefore informative, and the

Primer pair	No. of fragments	Polymorphic fragments	% Polymorphism
Green (MseI-CAT/EcoRI-AAG)	56	50	89.3
Yellow (MseI-CAG/EcoRI-AAC)	60	57	95.0
Total	116	107	92.2

Table 2. Polymorphism for two Amplified Fragment Length Polymorphism primer pairs in a sample of 79 individuals (35 adults, 44 nymphs) of *Phyllomorpha laciniata* from Segovia (Central Spain).

ones which offered the most clean and reproducible patterns: MseI-CAT and EcoRI-AAG (JOE-Green), and MseI-CAG and EcoRI-AAC (NED-Yellow).

Within the study population (79 individuals) these two AFLP primer pairs generated a total of 116 fragments. The mean number of fragments generated per individual was 84.7 (standard error (SE) = 0.86; n = 79; range = 59-98). A total of 107 fragments (92.2%) were polymorphic, as assessed by band absence in at least one individual. The primer pair MseI-CAG/EcoRI-AAC generated the greatest numbers of fragments as well as the greatest number of polymorphic fragments (Table 2).

The rate of repeatability, expressed as the number of repeatable peaks out of the total number of peaks reached 96.6% for the combination of both MseI-CAT/EcoRI-AAG and MseI-CAG/EcoRI-AAC primer pairs, for two sets of six samples processed independently.

Using an index of correlation in migration (IC) for all *loci i* and *j*, with *i* different of *j*, to check for correlation between peaks or between *loci* (Questiau et al., 1999) no correlation in the migration of bands was detected in the two primer pair profiles. A total of 121,660 pairwise comparisons were calculated for Green and 139,514 for Yellow. No value of 0 or 1 were detected for the sum of all individuals from 1 to N within each comparison for *loci i* and *j*, with the exception of comparisons between monomorphic *loci* (15 summatories equal to zero out of 1540 in Green due to comparisons between 6 monomorphic *loci*, and 3 summatories out of 1775 in Yellow due to comparisons between 3 monomorphic *loci*). The average index for Green was IC_{Green}= 0.35±0.21 (mean ± standard deviation, n=1540) and for Yellow was IC_{Yellow}=0.42±0.20 (n=1776). This indicates the independence of the *loci*.

Thus, AFLP analysis in the golden egg bug revealed sets of restriction fragments that could be visualized by PCR without prior knowledge of nucleotide sequence. This technique is robust and reliable because stringent reaction conditions are used for primer annealing. AFLP markers generate high levels of polymorphism in *P. laciniata*. A total of 107 polymorphic fragments obtained with modest efforts have been shown to migrate independently. This high number of genetic markers can be extremely useful to examine relatedness and paternity in the golden egg bug, an insect for which no previous studies on the genetic basis of egg carrying behaviour has ever been published. The genetic relatedness between males and the eggs they are carrying is essential to explain the adaptive value of male egg carrying behaviour, and its likely origin and evolution (Gomendio et al., 2001; Kaitala et al., 2001). So far, and to the best of our knowledge, only one study analysing paternity in animals with the use of AFLP markers has been carried out (Questiau et al., 1999). The methodology developed in our study can be of importance to understanding unresolved evolutionary questions in insect systems such as the golden egg bug, for which no codominant markers exist.

Experimental Procedures

DNA isolation

Genomic DNA was isolated from the thorax of adults previously cleaned from chitinous extensions and from the eggs or the whole body of the nymphs emerged from eggs. Different methods for isolation of genomic DNA from *P. laciniata* were used to find one that provided DNA suitable for AFLP analysis. The following were used (see Appendix 1 for details): (1) phenol-chloroform based extraction (modified from Sambrook et al., 1989) (2) phenol-chloroform based extraction (modified from Towner, 1991) (3) DNAALL-IN-ONE purification kit (BIOTOOLS B&M Labs, S. A., Madrid, Spain): isolation of genomic DNA from animal tissues protocol (4) DNeasy plant kit from QIAGEN (QUIAGEN Ltd, West Sussex, UK) (5) QUIAamp DNA kit for DNA purification from tissues (QIAGEN) (6) Cetyltrimethylammonium bromide (CTAB)-based protocol, modified from Möller et al., (1992) and Reineke *et al*. (1998), and (7) CTAB-based protocol, modified from Doyle & Doyle (1987), Weising *et al.* (1995, page 51), Möller *et al.* (1992), and Reineke *et al.* (1998). Method 7 was used as follows: The tissue was frozen in liquid nitrogen and ground, followed by the addition of 200 ml, for adults, or 100 ml for nymphs, of CTAB buffer (100 mM Tris-HCl, pH 8.0, 20 mM ethylene diamine tetra acetic acid (EDTA), 1.4 M NaCl, 2% CTAB). Proteinase K (to a final concentration of 0.2 mg/ml) was added and samples were incubated at 65°C for 60 min followed by centrifugation at 13000 rpm for 6 min and transfer of supernatant to a new Eppendorf tube. Samples were then washed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 3 min followed by addition of 0.5 vol of 5M NH_4Ac, incubation on ice for 30 min and centrifugation at 14000 rpm for 20 min. After transferring the supernatant to a new Eppendorf tube, 10 ml of RNAse (10 mg/ml) was added and samples were incubated at 37°C for 30 min followed by addition of 2 vol of 100% ethanol and 0.1 vol of NaAc 3M pH 5.2 and subsequent precipitation for 20 min at -80°C. The samples were then centrifuged at 13000 rpm for 10 min and the supernatant was discarded. The precipitate was washed with 200 ml of 70% ethanol and centrifuged at 13000 rpm for 10 min. Finally, after extraction of the supernatant, isolated DNA was diluted in 20 ml of distilled water.

Integrity of DNA was examined by running a 0.8% agarose gel and staining with ethidium bromide.

AFLP analysis

Seventy-nine individuals were processed: 35 adults captured in El Espinar (Segovia, Central Spain) and 44 first-instar-nymphs generated by them. AFLPs were resolved using the AFLP™ Plant Mapping Protocol (Perkin Elmer Applied Biosystems, 1996), but the reactions were performed with half the volume described in the protocol, with the exception of the quantity of DNA and adapters used. In a series of preliminary tests, we verified that the profile of AFLP fragments obtained was identical to that seen using the total reaction volume indicated by the manufacturer.

All products were purchased from Applied Biosystems (Foster City, USA), except enzymes MseI, EcoRI and T4 DNA ligase, which were from New England Biolabs, Inc. (Beverly, USA).

(i) *Restriction of the DNA and ligation of adapters*. Enzymatic digestion with MseI and EcoRI, frequent and rare cutter, respectively, and ligation of adapters were performed simultaneously. Isolated DNA (1.10 ml) was used in a 5.5 ml restriction-ligation reaction at 37°C for 3 h. The reaction was then diluted to 100 ml in TE buffer.

(ii) *Preselective amplification by PCR*. Two microlitres of the diluted restriction-ligation DNA were mixed with 0.5 ml of preselective primers with one nucleotide extension at the 3' end and 7.5 ml of AFLP Core Mix (Perkin-Elmer). PCR was performed as follows: 2 min at 72°C followed by 25 cycles with the following cycle profile: a 1 s DNA denaturation step at 94°C, a 30 s annealing step at 56°C, and 2 min extension step at 72°C. A single step of 60°C for 30 min followed before holding at 4°C. Then, half of the reaction was diluted to 100 ml in TE buffer whereas the other

half was used to verify the successful amplification of target sequences via electrophoresis in a 1.5% agarose gel stained with ethidium bromide.

(iii) *Selective amplification by PCR*. This step uses primers that match the known adapter sequence, plus three selective nucleotides on the 3' end of the MseI primer and three selective nucleotides on the 3' end of the fluorescently labelled EcoRI primers. For each of the two primer pairs the following reaction was performed: 7.5 ml of Core Mix, 0.5 ml of primer MseI, 0.5 ml of primer EcoRI and 1.5 ml of preselective DNA sample from step (ii). A touchdown PCR reaction commenced with one cycle of 94°C for 2 min, 65°C for 30 s, and 72°C for 2 min. In subsequent cycles, the denaturation time was 1 s and the annealing temperature was reduced in 1°C steps to 57°C, followed by 23 cycles at 56°C. A single step of 60°C for 30 min followed before holding at 4°C.

Two microlitres for Green and 4 ml for Yellow selective amplification were added to 15 ml of formamide and 0.5 ml of Genescan-500 ROX-labelled size standard. This mixture was denatured 5 min at 95°C and run on an ABI PRISM 310 Genetic Analyser (Perkin-Elmer). Digitally converted raw data were saved on a computer as samples migrated past the fluorescence detector. Multilocus profiles were visualized using ABI Genescan software.

Data analysis

We considered each fragment as a dominant *locus* with two states: presence or absence. AFLP profiles were scored for the presence/absence of fragments in the 60-300 bp range. Only unambiguous AFLP markers that are easily scored were utilized. The size in base pairs was given by the comigration of a size standard. Two peaks were considered of the same size if they differed by less than 0.5 bp.

Six different samples chosen at random were replicated for the two primer pairs to make an estimate of the error percentage of the AFLP analyses. AFLPs were conducted for each set of six samples separately.

We have used an index of correlation in migration (IC) for all *loci i* and *j*, with *i* different of *j*, to check for correlation between *loci* (Questiau et al., 1999). For this purpose, we calculated all pairwise comparisons between *loci* for all individuals (N), using 2 states for each *locus*: 1 for presence of a peak, 0 for the absence. The index of correlation in migration is:

$$IC = \sum_{n=1}^{N} \left| state_{ith} locus - state_{jth} locus \right| / N$$

A value of one between two fragment positions mean that when a peak appears at the *ith* position, another peak does not appear at the *jth* position, or vice versa. A value of zero mean identical appearance or absence in both *ith* and *jth* position, which could indicate comigration of the two fragments.

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Appendix 1. Outline of the DNA extraction methods tested.

(1) Phenol-Chloroform 1

1. Grind in 200 ml of lysis buffer (10mM TRIS, pH 8.0, 25mM EDTA, 75 mM NaCl) and 50 ml of SDS 10%.
2. Add 2.5 Proteinase K (20mg/ml stock)
3. Add 2 ml RNAse B (10mg/ml stock)
4. Overnight at 37°C in a water bath
5. Add 1 vol of phenol-(10ml)-chloroform-(48ml)-isoamylalcohol-(10ml) mixture.
6. Centrifuge 5 min at 3580 rpm. Take supernatant.
7. Repeat steps 5 and 6.
8. Add 1 vol of chloroform-isoamylacohol.
9. Centrifuge 5 min at 3580 rpm. Take supernatant.
10. Repeat steps 8 and 9.
11. Add 5 ml of 5 M NaCl
12. Add 1 ml of ice-cold 100% EtOH.
13. Incubate on ice for 30 min.
14. Centrifuge 25 min at 17000g (4°C). Remove supernatant.
15. Wash with 1 ml of 70% EtOH.
16. Centrifuge 10 min at 17000g (4°C). Remove supernatant.
17. Suspend in 20 ml of nanopure water.

(2) Phenol Chloroform 2

1. Grind liquid nitrogen without buffer.
2. Supend in 100 ml of extraction buffer (1 ml 1M Tris, pH 7.4, 2 ml 0.5 M EDTA, pH 8, 1 ml 5 M NaCl, 10 ml 20% SDS, 86 ml H2O).
3. Add 20 ml Proteinase K (20mg/ml) and 10 ml 1 M DTT.
4. Incubate 4 h at 50°C in a water bath
5. Add 1 vol of phenol-chloroform.
6. Incubate 5 min on ice.
7. Centrifuge 5 min at 12000 rpm. Take supernatant.
8. Add 1/10 vol NaAc 3 M.
9. Add 2 vol ice cold 100% EtOH.
10. Overnight at 20°C.
11. Centrifuge 30 min at 14000 rpm. Take supernatant.
12. Add 200 ml ice cold 70% EtOH.
13. Repeat step 10. Let samples dry.
14. Suspend in 25 ml nanopure water.

(3) DNAALL-IN-ONE purification kit (Biotools): Isolation of genomic DNA from animal tissues protocol.

(4) DNeasy plant kit (QIAGEN)

(5) QUIAamp DNA kit for DNA purification from tissues (QIAGEN)

(6) CTAB 1

1. Grind in liquid nitrogen without buffer.
2. Suspend in 1 ml of lysis buffer (0.1 M Tris, pH 8.0, 10mM EDTA, 2%SDS, 0.2 mg/ml proteinase K).
3. Incubate 60 min at 58°C.
4. Add 280 ml of 5 M NaCl and 1/10 vol of 10% CTAB.
5. Incubate 10 min at 65°C.
6. Add 1 vol of chloroform:isoamylalcohol (24:1).
7. Incubate 30 min a 0°C
8. Centrifuge 10 min at 13200 rpm (4°C). Take supernatant.
9. Add 0.45 ml of 5M NH_4Ac.
10. Incubate 30 min on ice
11. Centrifuge 30 min at 13200 rpm. Remove supernatant.
12. Precipitate adding 0.55 vol of Isopropanol.
13. Centrifuge 5 min at 13200 rpm (4°C). Remove supernatant.
14. Wash with 200 ml of ice cold EtOH (70%).
15. Centrifuge 10 min at 13200 rpm. Remove

supernatant.

16. Suspend in 20 ml of nanopure water.

(7) CTAB 2

1. Grind in liquid nitrogen without buffer.
2. Suspend in 200 ml, for adults, or 100 ml for nymphs, of CTAB buffer (100 mM Tris-HCl, pH 8.0, 20 mM ethylene diamine tetra acetic acid (EDTA), 1.4 M NaCl, 2% CTAB).
3. Add Proteinase K (to a final concentration of 0.2 mg/ml).
4. Incubate 60 min at 65°C.
5. Centrifuge 6 min at 13000 rpm. Take supernatant.
6. Wash with an equal volume of chloroform:isoamyl alcohol (24:1).
7. Centrifuge 3 min at 13000 .
8. Add 0.5 vol of 5M NH_4Ac.
9. Incubate on ice for 30 min.
10. Centrifuge 20 min at 14000 rpm. Take supernatant.
11. Add 10 ml of RNAse (10 mg/ml).
12. Incubate 30 min at 37°C for 30 min.
13. Add 2 vol of 100% ethanol and 0.1 vol of NaAc (3M pH 5.2).
14. Precipitate 20 min at -80°C.
15. Centrifuge 10 min at 13000 rpm. Discard supernatant.
16. Wash with 200 ml of 70% ethanol.
17. Centrifuge 10 min at 13000 rpm. Discard supernatant.
18. Suspend in 20 ml of distilled water.

CAPÍTULO 6

Análisis de paternidad de los huevos portados por los machos*

Resumen del Capítulo 6

La resolución de los conflictos de intereses que aparecen entre los sexos o entre los individuos que se ven implicados en el cuidado parental necesita del empleo de técnicas moleculares para determinar las relaciones genéticas entre la descendencia y los adultos que efectúan la inversión parental. El significado adaptativo del transporte de huevos en el heteróptero *Phyllomorpha laciniata* ha sido por largo tiempo el objeto de una fuerte controversia. Esta controversia se ha visto cimentada en el desconocimiento de las relaciones genéticas entre los individuos adultos y los huevos que transportan. En el presente trabajo se presenta una primera aproximación al problema de la paternidad de los huevos que portan los machos. Por medio de la técnica conocida como AFLPs se ha determinado la paternidad de los huevos portados por machos que se mantuvieron con hembras bajo condiciones experimentales de diferente razón de sexos ("sex ratio") y densidad.

En total se han realizado AFLPs sobre una muestra de 79 individuos incluyendo ninfas. La frecuencia alélica dominante toma un valor medio de 0.386, calculada para los loci altamente polimórficos en la población de adultos. La probabilidad de exclusión (probabilidad de que un macho elegido al azar en la población pueda ser excluido como el padre de una ninfa dada) alcanza el 98%, o el 88%, dependiendo del número de marcadores de exclusión que se utilicen. La presencia de dos marcadores de exclusión

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(probabilidad de exclusión del 88%) ha sido la utilizada para determinar la exclusión de diferentes machos como padres genéticos de las ninfas.

La mayoría de los huevos fueron fecundados por machos que se aparearon con las hembras antes del comienzo del experimento. Existen diversas explicaciones no excluyentes a este hecho: los machos experimentales realizaron pocas cópulas, realizaron cópulas en las que no hubo transferencia de esperma, o un mecanismo de competencia espermática de mezcla de esperma determina que si las hembras se han apareado con varios machos antes de ser recolectadas en el campo los machos experimentales alcancen una baja representación gamética. Debido a una combinación de estas causas el número de huevos fecundados por los machos experimentales fue relativamente bajo, lo que estaba en contra de una de las presunciones del diseño experimental: que existía precedencia del último macho en copular. Del total de huevos fecundados por los machos experimentales, los machos portaban un 30.8% de descendencia genética (huevos portados por el mismo macho que los fecunda). Los machos portaban más huevos fecundados por ellos mismos que los que se esperarían según un modelo de ovoposición al azar, como se desprende del empleo de métodos de Monte Carlo para simular distribución de huevos aleatoria entre los machos.

Puesto que la proporción de huevos fecundados por machos no experimentales es alta, las hembras no tuvieron la oportunidad de depositar un gran número de huevos sobre los padres genéticos. Por otro lado, las condiciones experimentales de densidad fueron extremadamente altas, lo que favorece la existencia de parasitismo intraespecífico en la puesta de huevos. A pesar de ello, la tasa de paternidad se manifiesta suficientemente alta para considerar que el cuidado paternal puede ser crucial en la explicación del comportamiento de transporte de huevos en *P. laciniata*.

La importancia de este estudio también radica en que demuestra que la técnica de los AFLPs es de gran utilidad para determinar paternidad en este organismo, y en que se desarrolla una metodología para determinar paternidad cuando la madre es desconocida entre una serie de madres potenciales. Esta metodología puede ser de gran utilidad para realizar análisis de parentesco y de paternidad en otros sistemas, ya sea con marcadores AFLP u otros marcadores genéticos.

Paternity analysis in the golden egg bug (*Phyllomorpha laciniata*) using amplified fragment length polymorphisms (AFLPs)

F. García-González,* Y. Núñez†, F. Ponz†, E. R. S. Roldán* and M. Gomendio*
*Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain, †Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Ctra. Coruña Km 7.5, 28040 Madrid, Spain.

Abstract

The evolution of parental care and intraspecific parasitism involve conflicts of interest between mothers and other potential care givers, which can only be understood with the use of molecular techniques to determine the genetic relationship between the young and the adults who contribute to enhance offspring survival. In the golden egg bug (*Phyllomorpha laciniata*) females lay eggs on conspecifics and on plants. The adaptive significance of egg carrying has been the subject of some controversy, but no information is available on the genetic relationship between the eggs and the adult who carries them. We conducted paternity analysis over eggs carried by males housed with field-mated females. Two selective AFLP primer pairs generated a total of 116 unambiguous fragments of which 107 (92.2%) were polymorphic. The mean dominant allele frequency was 0.386. Using the combination of the two primer pairs, exclusion probability was 98% for the detection of one exclusion diagnostic marker, and 88% for the detection of at least two exclusion diagnostic markers. Paternity exclusion based upon at least two diagnostic markers was used. Most of the eggs were sired by males with whom females had mated in the field before the start of our experiment. When we take into account only those eggs sired by males present in the experimental group, 30.8 % were laid on the true genetic fathers. Thus, males seem to carry eggs despite of the fact that a proportion of the young they carry are unrelated, because the benefits in terms of offspring survival are very high. On the other hand, females benefit from laying eggs on any adult, irrespective of the genetic relationship with the young, and for this reason parasitism also plays an important role in this system.

Keywords: Amplified fragment length polymorphism, paternity analysis, paternal care, intraspecific parasitism, golden egg bug, *Phyllomorpha laciniata*.

Introduction

The study of evolutionary ecology has been revolutionised in recent years by the introduction of molecular techniques. Our understanding of parental care, sexual conflict, mating strategies and mating system evolution has been drastically altered by the determination of parentage and genetic relatedness (see for example Gowaty & Karlin 1984; Burke 1989; Avise 1994; Hughes 1998). Traditionally, observational studies had assumed that in most patterns of social organization females were essentially monandrous, and that males and females provide care for their true genetic offspring. The use of molecular techniques to determine paternity revealed that in populations with monogamous mating systems, so-called extra-pair copulations are frequent, and that in polygynous mating systems females also tend to copulate with more than one male. As a result of female polyandry, males often care for unrelated young (Birkhead & Møller 1992; Møller & Birkhead 1993; Westneat & Sargent 1996; Hughes 1998). In addition, intraspecific parasitism by females also results in females caring for other female's

offspring (Yom-Tov 1980; Rohwer & Freeman 1989; Petrie & Møller 1991; Field 1992; Brockmann 1993; Zink 2000). These findings have changed dramatically our views and have highlighted the importance of taking into account conflicts of interest between males and females.

The conceptual changes prompted by these findings have generated a new set of questions, which include the relationship between certainty of paternity and paternal care, and the role played by intraspecific parasitism in shaping female strategies to maximize lifetime reproductive success. It seems to be a widespread phenomenon that in species with paternal care, males look after unrelated offspring as a consequence of female cuckoldry, a fact that challenges the previously held view that paternal care should only evolve when paternity certainty is high. This unexpected finding has stimulated new theoretical models which attempt to explain under which conditions should males care for offspring given the risks of cuckoldry (Whittingham *et al.* 1992; Xia 1992; Westneat & Sherman 1993; Yamamura & Tsuji 1993; Houston 1995), as well as experimental work aimed at understanding how do males assess paternity certainty and how they react to a decrease in levels of paternity certainty (Burke *et al.* 1989; Davies *et al.* 1992; Whittingham *et al.* 1993; Dixon *et al.* 1994; Briskie *et al.* 1998; MacDougall-Shackleton & Robertson 1998; Sheldon & Ellegren 1998; Neff & Gross 2001). Among systems where parental care has already evolved, females not only manipulate males into providing care for offspring which are not their own, but they may also "cheat" other females, by making them look for their offspring, a strategy known as "intraspecific brood parasitism". This behaviour entails laying eggs on another female's nest, thus manipulating the parasitised female into providing care for the parasitic female's offspring (Petrie & Møller 1991; Brockmann 1993). Females who engage in intraspecific brood parasitism greatly improve their lifetime reproductive success (Brown 1984; Møller 1987; Brown & Brown 1998; Åhlund & Andersson 2001). These findings reveal that the costs associated to the provision of care to offspring is a fertile ground for the appearance of conflicts of interest between mothers, and males who may be willing to provide care despite some uncertainty about paternity, as well as other females who may be manipulated into looking for other females' offspring. Understanding these conflicts between the true genetic mother and other potential care givers is essential to explain the evolution of parental care and mating systems, but many questions are still unresolved.

The golden egg bug (*Phyllomorpha laciniata*, Heteroptera, Coreidae) is a good model to study these questions since it shows a unique pattern of oviposition behaviour, which results in some eggs being carried by adults, and some being laid on plants where they develop unattended. Eggs derive a great benefit when carried by an adult because their survival rates improve considerably, given the lower rates of mortality caused by a parasitoid wasp (Reguera & Gomendio 2002). Only a small proportion of the female population carries eggs, and females carry a small number of eggs. The scenario is different for males, since all males in natural populations end up carrying eggs, and males carry a greater number of eggs than females do. Since females never carry their own offspring, the eggs they carry are likely to be the result of intraspecific parasitism by other females. The evolutionary significance of male carrying is the subject of an ongoing controversy. Some authors believe that egg carrying by males is also likely to be a result of intraspecific parasitism (Kaitala *et al.* 2001), while other authors have argued that, while intraspecific parasitism may account for a small proportion of egg-carrying cases equivalent to the small proportion of females who carry eggs, it is unlikely to explain the much higher level of egg carrying observed among males (Gomendio & Reguera 2001). These authors have suggested that, given the high costs of egg carrying for adults in terms of increased predation rates (Reguera & Gomendio 1999; Kaitala *et al.* 2000), males are unlikely to accept eggs unless there are chances that at least some of the eggs will be their true genetic offspring. In addition, egg carrying maximizes survival rates, and therefore could be

considered as a rudimentary form of parental investment, since the term refers to the effects on offspring survival and not to the degree of elaboration involved (see Clutton-Brock & Godfray 1991; Clutton-Brock 1991). This controversy will only be resolved with the use of molecular techniques to determine paternity.

Restriction Fragment Length Polymorphism (RFLP) and PCR amplified microsatellite loci are the most frequent molecular markers applied to address the study of relatedness and parentage (Quinn *et al.* 1987; Burke 1989; Westneat 1990; Queller *et al.* 1993; Avise 1994; Dixon *et al.* 1994; Schierwater *et al.* 1994; Pena & Chakraborty 1994; Topping & Millar 1998; DeWoody *et al.* 1998; Simmons & Achmann 2000). However, these techniques require knowledge of the genome under investigation and/or relatively large amounts of DNA. These requirements have prevented, to some extent, their use for addressing questions in taxa such as insects, where detailed genetic information about one taxon is rarely applicable to another (Fagerberg *et al.* 2001), and where only small quantities of DNA are usually available for analyses (for instance from embryos or larvae). A recent technique, Random Amplified Fragment Polymorphism (RAPD) fingerprinting (Welsh & McClelland 1990; Williams *et al.* 1990), has been able to solve these drawbacks, and it has been largely used as a method for relatedness and paternity analyses (Lewis & Snow 1992; Lynch & Milligan 1994), especially in insects (Hadrys *et al.* 1992, 1993; Hadrys & Siva-Jothy 1994; Hooper & Siva-Jothy 1996; Schierwater *et al.* 1997). However, the occurrence of non-parental bands has raised doubts concerning its use in paternity analyses (Riedy *et al.* 1992; but see Scott *et al.* 1992 and Hadrys *et al.* 1993).

Recently, another technique, Amplified Fragment Length Polymorphisms (AFLPs), based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA, has been developed (Vos *et al.* 1995). AFLP markers have been found to be suitable for genetic analyses such as relatedness and parentage, genetic structure and diversity, distinction of genotypes, populations, cultivars, species, etc. (Maughan *et al.* 1996; Arens *et al.* 1998; Escaravage *et al.* 1998; Winfield *et al.* 1998; Krauss 1999; Questiau *et al.* 1999; Loh *et al.* 1999, 2000a, 2000b; Creswell *et al.* 2001; Giannasi *et al.* 2001). This is possible because AFLP markers are virtually free of artifacts and because comigration of non-allelic fragments occurs at extremely low levels (Mueller & Wolfenbarger 1999). So far, the utility of the AFLP technique for paternity analysis has been assessed in a few studies (*Persoonia mollis*: Krauss & Peakall 1998; Krauss 1999; *Luscinia svecica*: Questiau *et al.* 1999). These pioneering studies have shown that dominant AFLP markers can be useful for an estimation of paternity in taxa for which no microsatellite primers are available.

There are no microsatellites' markers available for *P. laciniata*, and, in addition, only small quantities of DNA are available for analyses (golden egg bug eggs and nymphs are extremely small, and nymphs in captivity do not survive beyond the first instar). The aim of this study was to use AFLP markers for paternity analyses in the golden egg bug, as a tool to understand the evolutionary significance of egg carrying in this system. Because in this system we lack information on who the mother and the father are, a new statistical methodology is developed to assign paternity estimating the putative mother.

Materials and Methods

Samples and experimental conditions

We collected 45 individuals of *P. laciniata* (29 males and 16 females) in El Espinar (Segovia, Central Spain) at the end of May 2000. Individuals were transported in individual plastic vials to the laboratory at the Museo Nacional de Ciencias Naturales and placed in small Petri dishes (5.5 cm diameter). Prior to the experiment, eggs were removed from carrying males as well as from carrying females. Individuals were kept in constant conditions during the experimental period (25° C, lights on from 8:00 AM to 9:00 PM). One day after

Group	Sex-ratio (males:females)	Density
A	4:4	High (8 individuals/0.0272 m^2; 293.8 individuals/m^2)
B	6:2	High (8 individuals/0.0272 m^2; 293.8 individuals/m^2)
C	4:4	Low (8 individuals/ 0.5 m^2; 16 individuals/m^2)
D	6:2	Low (8 individuals/ 0.5 m^2; 16 individuals/m^2)

Table I. Sex ratio and density of the experimental groups established with 32 individuals of *Phyllomorpha laciniata*.

their capture, four groups of different sex ratios and densities were established with individuals taken at random (Table I).

Individuals in the high-density groups were kept in small plastic containers (16.5 cm x 16.5 cm x 10.5 cm) whereas individuals in the low-density groups were kept in large glass containers (1 m x 0.5 m x 0.5 m). Throughout the experimental period, individuals were provided daily with fresh branches of the host plant *Paronychia argentea* (Caryophyllaceae). They were provided with greater surface area of the plant on which they could lay eggs and feed than that represented by individuals to make sure that plant availability was not a limiting factor.

Individuals were allowed to mate and to oviposit freely and after 8 days they were isolated and eggs removed carefully from their backs. The thorax of adults was extracted, discarding the digestive tract, and frozen to -80° C. Each egg was placed in an Eppendorf tube and was checked daily until hatching. Recently emerged nymphs were also frozen to -80° C.

Eggs on plants were collected on the 4th and 8th day of the experiment. Under our experimental conditions, nymphs hatched after around 11 days (Reguera 1999). Therefore no egg, either laid on plant or laid on a conspecific could have hatched without being noticed. Only eggs laid on males were analysed since the aim of the study was to elucidate paternity of male-carried eggs.

To keep the conditions of sex ratio and densities in each group constant, if an individual died before the 6th day of the experiment it was replaced by another individual of the same sex. Dead individuals were frozen after removing the eggs that they were carrying, and the procedure described above was followed to obtain the nymphs. In group C, one male died on the 4th day and one female died on the 1st day of the experiment and they had to be replaced (male C201 replaced by male C224, female C200 replaced by female C237). In group D, 2 males died on the 3rd and 4th days (D300 and D301 replaced, respectively, by D320 and D324). Only one individual died after the 6th day (D303).

The sequence of copulations and oviposition were not monitored because in order to do this individuals should have been marked, and this could have affected the preference of females when copulating and when ovipositing on individuals. This implies that it was not possible to determine by observational data which female had produced each of the eggs.

Non-virgin females were employed in the experiment. So far, all attempts to rear this insect in captivity have been unsuccessful. Although we have managed to obtain virgin females from the field at the very beginning of the reproductive season (March-April), these females do not lay eggs when placed in captivity although they do engage in copulations. Thus, it was not possible to carry out this study using virgin females, which adds further complexity to the study of paternity in this insect.

DNA isolation

Genomic DNA was isolated from the thorax of adults previously cleaned from chitinous extensions (n=36 adults) and from the whole body of the nymphs emerged from eggs laid on

males (n=44), using a CTAB procedure modified from Weising *et al.* (1995, page 51), Möller *et al.* (1992) and Reineke *et al.* (1998). This method was chosen after evaluation of six different protocols (for details see García-González *et al.* 2002). Briefly, the tissue was frozen in liquid nitrogen and ground, followed by the addition of 200 mL, for adults, or 100 mL for nymphs, of CTAB buffer (100 mM Tris-HCl, pH 8.0, 20 mM ethylene diamine tetra acetic acid (EDTA), 1.4 M NaCl, 2% CTAB). Proteinase K (to a final concentration of 0.2 mg/mL) was added and samples were incubated at 65°C for 60 min followed by centrifugation at 13000 rpm for 6 min and transfer of supernatant to a new Eppendorf tube. Samples were then washed with an equal volume of chloroform:isoamyl alcohol (24:1) and centrifuged at 13000 rpm for 3 min followed by addition of 0.5 vol of 5M NH_4Ac, incubation on ice for 30 min and centrifugation at 14000 rpm for 20 min. After transferring the supernatant to a new Eppendorf tube, 10 mL of RNAse (10 mg/mL) was added and samples were incubated at 37°C for 30 min followed by addition of 2 vol of 100% ethanol and 0.1 vol of NaAc 3M pH 5.2 and subsequent precipitation for 20 min at -80°C. The samples were then centrifuged at 13000 rpm for 10 min and the supernatant was discarded. The precipitate was washed with 200 mL of 70% ethanol and centrifuged at 13000 rpm for 10 min. Finally, after extraction of the supernatant, isolated DNA was diluted in 20 mL of distilled water.

Integrity of DNA was examined by running a 0.8% agarose gel and staining with ethidium bromide.

AFLP analysis

Briefly, AFLPs were resolved using the AFLP™ Plant Mapping Protocol (Perkin-Elmer Applied Biosystems, Madrid, Spain), but the reactions were performed with half the volume described in the protocol, with the exception of the quantity of DNA and adapters used (for further details see García-González *et al.* 2002). In a series of preliminary tests we verified that the profile of AFLP fragments obtained was identical to that seen using the total reaction volume. All products were purchased from Perkin-Elmer, except enzymes MseI, EcoRI and T4 DNA ligase, which were obtained from New England Biolabs, Inc. (Beverly, USA).

Thirty different selective primer combinations were tested and two primer pairs were identified as the most polymorphic, and therefore informative, and the ones which offered the most clean and reproducible patterns: MseI-CAT and EcoRI-AAG (JOE-Green), and MseI-CAG and EcoRI-AAC (NED-Yellow) (García-González *et al.* 2002). For each of the two primer pairs selective PCRs were performed. The final product was run on an ABI PRISM 310 Genetic Analyser (Perkin-Elmer). Digitally converted raw data were saved on a computer as samples migrated past the fluorescence detector. Multilocus profiles were visualized using ABI Genescan software.

Data analysis

Fragment scoring

We considered each fragment as a dominant locus with two states: presence or absence. AFLP profiles were scored for the presence/absence of fragments in the 60-300 bp range. Only unambiguous AFLP markers that were easily scored were used. The size in base pairs was given by the comigration of a size standard. Two peaks were considered of the same size if they differed by less than 0.5 bp. Scoring was carried out without knowing the identity of the individuals or the potential relatedness between them. One individual did not amplify with any primer combination and it was excluded from the analyses.

The use of an index of correlation in migration for all peaks revealed that loci obtained migrated independently, and in a series of sample replications, repeatability of the method reached 96.6% for the combination of the two primer pairs used (García-González *et al.* 2002).

Exclusion probability

We considered only the adult individuals of our population for the allele frequency calculations. Assumption of Hardy-Weinberg equilibrium is necessary to calculate the allele frequencies since AFLP markers are considered dominant (Yan *et al.* 1999). We used the proportion of individuals with no peak for a given locus as the genotypic frequency of the recessive homozygotes (q^2), with q being the estimation of the frequency of the allele absence in the population for that locus. We defined *p* as the frequency of the allele presence with $p=1-q$. We focused only on polymorphic loci with $q^2>3/N$, with N being the number of adult individuals, as recommended by Lynch & Milligan (1994).

Exclusion probabilities were calculated using the equation of Chakraborty *et al.* (1974) to compute the probability of exclusion based upon at least two diagnostic markers. The exclusion probability (the probability that any one randomly chosen male can be excluded as the father of a chosen individual) is the most common measure of the potential of a given genetic system for use in paternity analysis (Lewis & Snow 1992). An exclusion diagnostic marker (also termed diagnostic marker, diagnostic peak or diagnostic fragment) is defined by the situation in which, for a given locus, both a potential father and the true mother lack the allele (fragment absence in the AFLP profile) whereas the particular offspring has it (fragment presence in the AFLP profile). Following Pena & Chakraborty (1994), the situation in which a male can be unambiguously excluded as the father is when, two or more than two exclusion diagnostic markers are revealed when analysing the AFLP profiles of the potential father, the true mother and the offspring, thus allowing for one mutation in the AFLP profile of the true sire without exclusion.

We calculated exclusion probabilities: (i) at the population level for loci with $q^2>3/N$, with N being the number of individuals; (ii) at the population level for all loci; and (iii) at the adult population level for loci with $q^2>3/N$, with N being the number of adults. For *k* markers, the cumulative probability *Q* of exclusion for at least one diagnostic marker, is:

$$Q = 1 - \prod_{i=1}^{k} (1 - p_i)$$

The exclusion probability *P* on at least two diagnostic markers is:

$$P = Q - \sum_{i=1}^{k} p_i \prod_{\substack{j=1 \\ j \neq i}}^{k} (1 - p_i)$$

being $_jp_i$

the probability of exclusion based upon the *ith* marker; that is, the probability of having no peak in both parents ($q^2_i \times q^2_i$) and one allele present *pi* in a nymph:

$$p_i = q_i^2 \times q_i^2 \times p_i$$

Paternity of eggs carried by males

Paternity assignment in *P. laciniata* is difficult because it is not possible to carry out studies with virgin females, which implies that the eggs laid may have been fertilised by sperm from males who copulated with the females in the field before the experiment started. Furthermore, under our conditions each experimental group has several possible mothers for any given nymph (from 2 to 5, depending on the number of females in each group), which adds complexity to the determination of the true genetic parents. The procedure we used to determine paternity was based on the determination of exclusion diagnostic markers (diagnostic peaks in the AFLP profile) where a peak (fragment) was absent in both parents (i.e., when both are recessive homozygotes), and present in the nymph. The existence of two or more than two diagnostic peaks is used to exclude a nonsire. We have applied this procedure with some modifications (see below) to allow paternity exclusions when multiple females are potential mothers. The steps to exclude paternity were as follows:

Step 1. We looked for AFLP fragments that were present in the nymph but absent in the carrier male as well as in all the potential mothers of the nymph (all the females in the group), comparing together the AFLP profiles of all females in each group. A carrier male was excluded as the father of the nymph when at least two diagnostic peaks were found in the profile comparison, i.e. when at least two peaks were present in the nymph and absent in the male as well as in the set of potential mothers.

Step 2. For the study of relatedness among individuals we did not use the similarity index (Lynch 1990; Lynch & Milligan 1994) but we calculated a pairwise genetic dissimilarity matrix for all individuals from the AFLP data by Euclidean distance (Krauss 1999) using NTSYSpc 2.02i software (Exeter Software, New York). Euclidean distance between individuals *i* and *j* for all loci *x* (where x_{ki} and x_{kj} are equal to either 1 or 0) is:

$$E_{ij} = \sqrt{\sum_{k=1}^{n} \left(x_{ki} - x_{kj}\right)^2}$$

These measures were then used to construct a UPGMA dendrogram for all individuals (Sneath & Sokal 1973) and it was used as a first approach to estimate the levels of relatedness between individuals.

Step 3. Values of genetic relatedness estimated from Euclidean distance were used to identify a putative mother from all the potential mothers of a given nymph (all the females in each group). For the 15 nymphs that were not excluded as sired by the carrier male in Step 1, the female with the highest degree of relatedness among the females within the nymph's group was selected as the putative mother.

Selection of the putative mother relies on a higher relatedness between nymph-putative mother than expected by chance. At the end of the paternity exclusion process, we verified this assumption exploring differences between (i) genetic similarity of nymph x_i -putative mother and genetic similarity of nymph x_i -females of the same group of the nymph, excluding the putative mother (individuals with probability $p>0$ of being the mother of the nymph), and (ii) genetic similarity of nymph x_i -putative mother and genetic similarity of nymph x_i -females of the other groups (individuals with probability $p=0$ of being the mother of the nymph), for x from $i=1$ to $i=n$, with n being the number of nymphs that were not excluded on step 1 as sired by the male who carried them during egg stage.

In these comparisons the data were not independent since for a given nymph there was only one value of relatedness with the putative mother and a series of values for the relatedness with other females (Danforth & Freeman-Gallant 1996). Thus, it was necessary to control by nymph when analysing differences in genetic relatedness. We carried out a mixed model of variance analysis (Statsoft 1996), in which nymph was entered as a random factor and parentage as fixed factor with the following levels: A. Nymph-putative mother, B. Nymph-females with probability $p>0$ of being the nymph's mother, C. Nymph- females with probability $p=0$ of being the nymph's mother. This analysis was preferred over a t-test for dependent samples since the latter uses the mean genetic value for each group of values for a nymph and does not consider variance of data in levels B and C.

The dependent variable (genetic dissimilarity) was transformed using logarithmic transformation (Sokal & Rohlf 1981).

Step 4. Once a putative mother for each nymph was selected, exclusion diagnostic peaks were sought between nymph-carrier male-putative mother for the 15 nymphs for which the carrier male was not excluded as the father in Step 1. A carrier male was excluded as the nymph's father when two or more than two diagnostic peaks were found in the profile comparison of these three individuals. A nymph possessing none or only one peak that was not present in both putative mother and carrier male, i.e. a nymph with none or only one diagnostic peak, was assumed to be sired by the carrier male. Profiles

of nymph-putative mother-non carrier males within the nymph's group were also compared to verify assignment of paternity. To do this, we examined if there were any nymphs possessing none or only one diagnostic marker with another male within the nymph's group. In this case, the male having a higher genetic relatedness with the nymph was assumed to be the father. We found this to be true on three occasions, with nymphs for which a non carrier male, instead of the carrier male, being found to have higher genetic relatedness and therefore assumed to be the father.

Step 5. As an alternative to Steps 2-4 we calculated the minimum rate of intraspecific parasitism experienced by carrier males irrespectively of the determination of the putative mother. One way to calculate this is to determine, for each nymph, the exclusion of the carrier male as his father comparing the profiles of nymph-carrier male-female x_i, for x from $i=1$ to $i=n$, with n being the number of females within the nymph's group. Two, or more than two, diagnostic peaks in all the profiles between nymph-carrier male with each one of the females in the group were indicative of non paternity by the carrier male. We thus established the minimum rate of intraspecific parasitism without carrying out identification of putative mothers.

Step 6. Finally, for nymphs that were not sired by the males who carried them, we determined whether the father was a male whitin the nymph's group or a male which whom the mother mated in the field. We looked for exclusion diagnostic peaks among nymph-putative mother-male x_i, for x from $i=1$ to $i=n$, with n being the number of males in the nymph's group. A male from the nymph's group was regarded as the nymph's father when none or only one diagnostic peak was found in this profile. When, for a given nymph, two or more than two diagnostic peaks were found for all the profiles concerning the males in the nymph's group, the nymph was assumed to be fathered by a male who mated with the female before the experiment started (i.e., in the field). In the case of a nymph having none or only one diagnostic peak with more than one male within the nymph's group, the male with the greater genetic relatedness with the nymph was assumed to be the father.

Finally, we verified the procedure of paternity assignment for those nymphs for which we concluded that they were sired by the carrier male or by another male in the nymph's group. We carried out a mixed model of analysis of variance (see Step 3) to check the existence of differences between genetic similarity of nymph x_i - putative father and genetic similarity of nymph x_i -other males (in this case all males in the experiment are individuals with probability $p>0$ of being the nymph's father since the nymph's mother could have mated with any of them in the field), for x from $i=1$ to $i=n$, with n being the number of nymphs fathered by males from the nymph's group. Each individual nymph was entered as a random factor and parentage as fixed factor with two levels: A. Nymph-putative father, B. Nymph-other males. The dependent variable (genetic dissimilarity) was transformed using logarithmic transformation.

Expected Probability of a male carrying eggs fertilized by him by chance

We used Monte Carlo methods (Manly 1991) to simulate the random distribution of eggs within groups. Probabilities for the allocation of eggs among males were calculated from the simulated distribution frequencies for the number of eggs fertilized divided by the number of eggs carried (taking into account the number of eggs fertilized by each male, the number of eggs carried by each male, the number of males, and the total number of eggs within the treated group). Random distribution of eggs assumes no differential female selection of males to oviposit and no differences among males in accepting or rejecting eggs, this being the null hypothesis. Our alternative hypothesis is a bias on the male acceptance of fertilized eggs following paternity certainty and paternal investment predictions (see for example Westneat & Sherman 1993; Wright 1998).

Results

The mean number of fragments generated per individual was 84.7 (standard error (SE)=0.86; n=79; range=59-98). Within the study population (79 individuals) two AFLP primer pairs generated a total of 116 fragments, 107 (92.2%) of them being polymorphic.

We used a total of 76 polymorphic loci with $q^2>3/N$, with N being the number of adult individuals (N=35), in order to calculate allele frequencies. Dominant allele frequencies (p), calculated from the frequency of the recessive phenotype q^2 (band absence) for each one of these polymorphic loci, varied from 0.014 (band absence in 34 of 35 individuals) to 0.66 (band absence in 4 of 35 individuals). The mean dominant allele frequency (p) over 76 polymorphic loci generated from 35 adults was 0.386 (SE=0.019; mean p for MseI-CAT/EcoRI-AAG=0.392, SE=0.029; mean p for MseI-CAG/EcoRI-AAC=0.381, SE= 0.026).

The global exclusion probability based on at least two diagnostic peaks (P), calculated for the combination of the two primer pairs, MseI-CAT/EcoRI-AAG ("Green") and MseI-CAG/EcoRI-AAC ("Yellow"), was 0.88 for: (i) all the population for loci with $q^2>3/N$, with N being the number of individuals; (ii) all the population for all loci; and (iii) for the adult population for loci with $q^2>3/N$, with N being the number of adults (Figure 1). The global exclusion probability considering a single diagnostic peak in order to exclude paternity by a carrier male (Q) was 0.98 for (i), (ii) and (iii).

From a total of 72 eggs carried by males, 44 nymphs (61.1%) could be analysed, with 75%, 58%, 50%, and 60% being analysed in groups A through D, respectively (Table II). In total, 79 individuals (35 adults and 44 nymphs developed from eggs carried by males) were analysed in 18 subgroups of carrying males-nymphs-potential mothers distributed among 4 different sex ratio and density groups (A through D, Table I).

We could resolve AFLP profiles for a total of 42 nymphs and for the males who carried them as eggs (see Table II). These 42 nymphs possessed between 0 and 10 fragments present in the nymph and not present in both the carrier male and the set of potential mothers, i.e. diagnostic peaks (Figure 2). Twenty-seven nymphs possessed two or more than two diagnostic peaks when the profiles of the nymph, the carrier male, and the set of potential nymph's mothers (all the females in the group) were compared. Thus, on this first step of exclusion of paternity by the carrier male, 64.3% of nymphs were regarded as not fathered by the carrier male (Table II, Step 1). In 15 nymphs out of

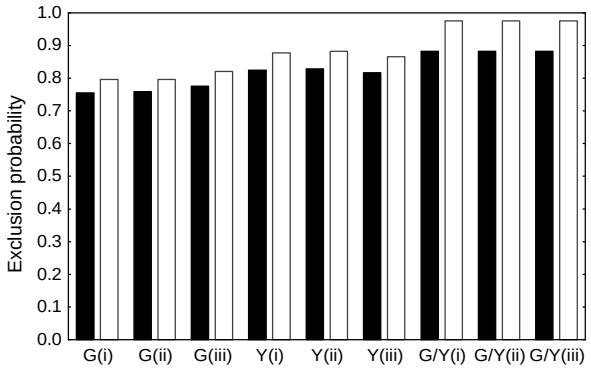

Figure 1. Exclusion probability for the Green (G) primer pair (MseI-CAT/EcoRI-AAG), Yellow (Y) primer pair (MseI-CAG/EcoRI-AAC), and Green/Yellow (G/Y) primer pair combination, considering a single diagnostic marker as relevant for excluding the carrying male as the father of the nymph (probability Q, open bar) or considering at least two diagnostic peaks (probability P, black bar). Exclusion probabilities (i) at the population level for *loci* with $q^2>3/N$, with N being the number of individuals; (ii) at the population level for all *loci*; and (iii) at the adult population level for *loci* with $q^2>3/N$, with N being the number of adults, are shown.

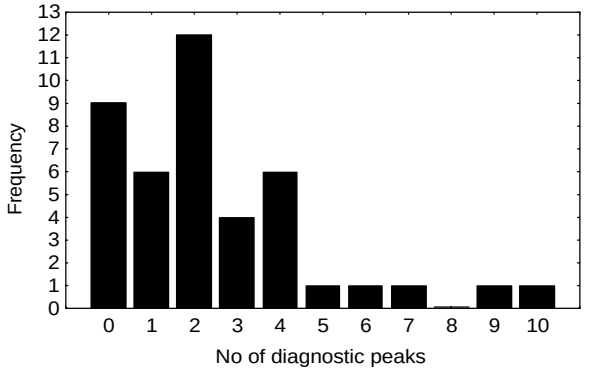

Figure 2. Distribution of the diagnostic markers between the profiles for sets of nymph-carrier male-potential mothers of the nymph.

Group (Potential mothers)	Male	No. of eggs carried	No. of eggs analysed (%)	No. of eggs (%) excluded as sired by the carrier male after different steps: Step 1	Step 4	Steps 1 & 4	Step 5
A	A9	7	5 (71.4%)	0 (0%)	3 (60%)	3 (60%)	1 (20%)
(A28, A33,	A12	2	2 (100%)	0 (0%)	1 (50%)	1 (50%)	1 (50%)
A36, A39)	A19	6	5 (83.3%)	1 (20%)	4 (80%)	5 (100%)	5 (100%)
	A25	5	3 (60%)	1 (33.3%)	1 (33%)	2 (66.7%)	2 (66.7%)
Subtotal A		**20**	**15 (75%)**	**2 (13.3%)**	**9 (60%)**	**11 (73.3%)**	**9(60%)**
B	B100	4	4 (100%)	4 (100%)	-	4 (100%)	4 (100%)
(B123, B125)	B110	5	1 (20%)	0 (0%)	1 (100%)	1 (100%)	1 (100%)
	B113	2	1 (50%)	1 (100%)	-	1 (100%)	1 (100%)
	B115	3	1 (33.3%)	1 (100%)	-	1 (100%)	1 (100%)
	B117	1	1 (100%)	1 (100%)	-	1 (100%)	1 (100%)
	B122	4	3 (75%)	2 (66.7%)	1 (33.3%)	3 (100%)	2 (66.7%)
Subtotal B		**19**	**11(57.9%)**	**9 (81.8%)**	**2 (18.2%)**	**11 (100%)**	**10 (91%)**
C	C201	1	0 (0%)	-	-	-	-
C200, C231, C237,	C206	1	1 (100%)	1 (100%)	-	1 (100%)	1 (100%)
C239, C242)	C211	4	3 (75%)	3 (100%)	-	3 (100%)	3 (100%)
	C224	12	5 (41.7%)	5 (100%)	-	5 (100%)	5 (100%)
	C225	0	0	-	-	-	-
Subtotal C		**18**	**9 (50%)**	**9 (100%)**	**0 (0%)**	**9 (100%)**	**9 (100%)**
D	D300	0	0	-	-	-	-
(D326, D328)	D301	1	1 (100%)	1 (100%)	-	1 (100%)	1 (100%)
	D303	0	0	-	-	-	-
	D307	1	1 (100%)	1 (100%)	-	1 (100%)	1 (100%)
	D309	2	2 (100%)	2 (100%)	-	2 (100%)	2 (100%)
	D312	7	2 (28.6%)	2 (100%)	-	2 (100%)	2 (100%)
	D320*	3	2 (66.7)*	-	-	-	-
	D324	1	1 (100%)	1 (100%)	-	1 (100%)	1 (100%)
Subtotal D		**15**	**7 (46.7%)**	**7 (100%)**	**0 (0%)**	**7 (100%)**	**7 (100%)**
TOTAL	**22**	**72**	**42(58.3%)***	**27 (64.3%)**	**11 (26.2%)**	**38 (90.5%)**	**35 (83.3%)**

Table II. Females and males in each group, potential mothers of the nymphs in each group, number of eggs carried by each male, number of eggs analysed, and number of nymphs excluded as sired by the carrier male after different steps of determination of paternity: Step 1, exclusion of paternity based on profiles nymph-carrier male- all potential mother for the nymph together ; Step 4, exclusion based on profiles nymph-carrier male-putative mother; Step 5, exclusion based on profiles nymph-carrier male- all potential mothers, one each time (see Materials and Methods for details). In some groups there are more individuals than the initial numbers established because some individuals died during the experimental period and they were replaced by another individuals (see Methods). * It was possible to determine paternity in only 42 nymphs out of 44 analysed because PCR did not amplify DNA from male D320.

42 (35.7%) there was none or a single mismatched peak. Thus, after Step 1 of paternity exclusion, and based on the level of acceptance defined in Materials and Methods, these nymphs were considered as possibly fathered by the male who carried them.

A putative mother for each nymph was determined among the females of the nymph's group. The female with the highest genetic similarity with the nymph was assumed to be the nymph's mother since (1) genetic distance for the relationship nymph-putative mother (A) was

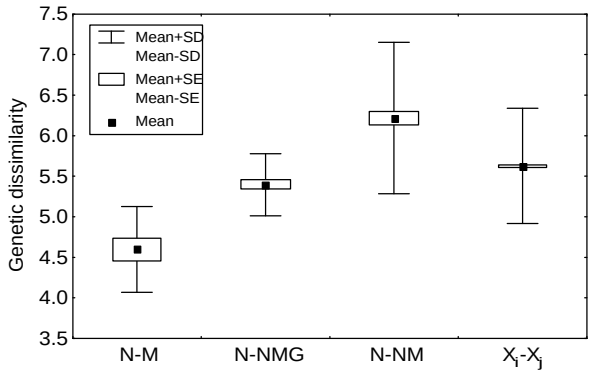

Figure 3. Genetic dissimilarity for different relationships between: N-M, nymph-mother (for 15 nymphs that were not excluded as sired by the carrier male in Step 1, 15 pairwise comparisons); N-NMG, nymph-non mother, with females, others than the putative mother, from the same group of the nymph (15 nymphs, 41 pairwise comparisons); N-NM, nymph-non mother, females from groups other than the nymph group (15 nymphs, 139 pairwise comparisons); Xi-Xj: all relationships between all individuals *i,j, i* different of *j,* from *i*=1 to *i*=79 (3081 pairwise comparisons).

significantly lower than that for nymph-females of the same group of the nymph (B), excluding the putative mother (Mixed Model Anova, $F_{1,14}$= 53.13; p<<0.001; n_A=15, n_B=41), and (2) genetic distance for the relationship nymph-putative mother (A) was significantly lower than that for nymph-females of the other groups (C) (Mixed Model Anova, $F_{1,14}$= 131.28; p<<0.001; n_A=15, n_C=139) (Figure 3).

Once the putative mother was assigned, exclusion diagnostic peaks among nymph-carrier male-putative mother were analysed. After this procedure, 7 nymphs exhibited none or only one diagnostic peak. However 3 of them exhibited none or only one diagnostic peak with another male from the nymph's group when comparing the profiles of nymph-putative mother-other males from the nymph's group. These 3 nymphs have a higher genetic relatedness with the non-carrier males, thus we excluded them as fathered by the carrier male. Therefore, after Step 4 of paternity exclusion, we considered 4 nymphs as sired by the males who carried them at the egg stage.

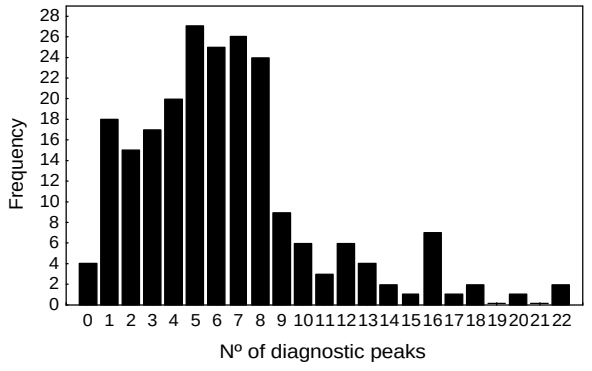

Figure 4. Distribution of the diagnostic markers between the profiles of the nymph, the putative mother, and the males in the group (for one male each time), for all nymphs in all groups.

The minimum rate of intraspecific parasitism experienced by carrier males calculated as described in Step 5 of Materials and Methods, without identification of putative mothers for each nymph, was 83.3% (35/42 eggs were carried by males which did not sire them) (Table II, Step 5). Therefore, the different steps based on the estimation of the putative mother to assign paternity raised the percentage of eggs not sired by the male who was carrying them from 83.3% to 90.5% in the overall study population (Table II).

For those nymphs not sired by the carrier male, we analysed, as described in Step 6 of Materials and Methods, whether their father was among the other males of the group or whether he was a previous partner of the mother with whom she mated in the field (i.e. before the female was brought to the laboratory). Figure 4 shows the distribution, for the 42 nymphs analysed, of the diagnostic peaks for all comparisons between profiles of the nymph-putative mother- male x_i, for *x* from *i=1* to *i=n*, with *n* being the number of males in the nymph's group (including the carrying male).

Genetic similarity between nymph-putative father (A) was significantly higher than that for nymph-other males (B) (Mixed Model Anova, $F_{1,12}$= 17.17; p=0.0014; n_A=13, n_B=280, see Step 6 of Materials and Methods) (Figure 5). This was calculated for 13 nymphs that were fathered by any male in the nymph's group (see below).

Out of 42 nymphs for which paternity could be assigned, 4 (9.5%) were sired by the male carrying them, 9 (21.4%) were sired by another male from

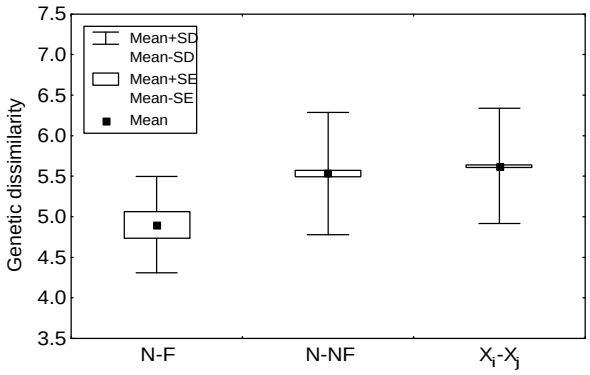

Figure 5. Genetic dissimilarity for the relationships between: N-F, nymph-putative father (for 13 nymphs sired by males from the nymph s group, 13 pairwise comparisons); N-NF, nymph-other males (13 nymphs, 280 pairwise comparisons); *Xi-Xj*: all relationships between all individuals *i,j, i* different of *j* from *i*=1 to *i*=79 (3081 pairwise comparisons).

the group, and 29 (69%) were sired by males with whom females mated before the start of our experiment (Figure 6 and Table III). Thus, the first term can be considered paternity by the carrier male, the second one can be considered intraspecific brood parasitism experienced by the carrier male, which is due to eggs fertilized by males from the same group, and the third term being eggs fertilized by sperm from males that mated with the female in the field.

Therefore, 13 nymphs out of 42 have been sired by males in the experimental groups, with 4 out of these 13 nymphs (30.8%) being sired by the carrier male, and 9 (69.2%) representing intraspecific brood parasitism due to eggs sired by other males from the same group. In groups C and D there were no eggs fathered by males from the group. Group A showed a moderate rate of paternity by carrier males in relation to eggs sired by others males from the group: 4 nymphs out of 11 sired by males in the group have been sired by the carrier male; this represents 36.4% paternity in carrier males (Figure 6 and Tables II and III).

Using Monte Carlo methods we simulated 2000 times the scenario of egg carrying in group A, the group where we detected paternity of carried eggs, to determine whether the probability of carrying fertilized eggs was due to random egg allocation. The real scenario was as follows: from 15 eggs laid by females in group A, male A9 carried 2 eggs fertilized by him and 3 others non-fathered by him, male A12 carried 1 egg fertilized by him and another egg non-fathered by him, male A19 carried 5 unrelated eggs, and male A25 one egg fertilized by him out of 3 carried eggs (see figure 6). Knowing from the paternity analyses that males A9, A12, A19, and A25, fertilized each a total of 5, 3, 1, and 2 eggs, respectively, we generated random distribution of these 11 eggs plus 4 other eggs fathered by males other than the males in the group. This process was repeated 2000 times and the frequencies of eggs carried by the father were recorded.

The probability of obtaining our real scenario for all males taken together was p=0.048. This

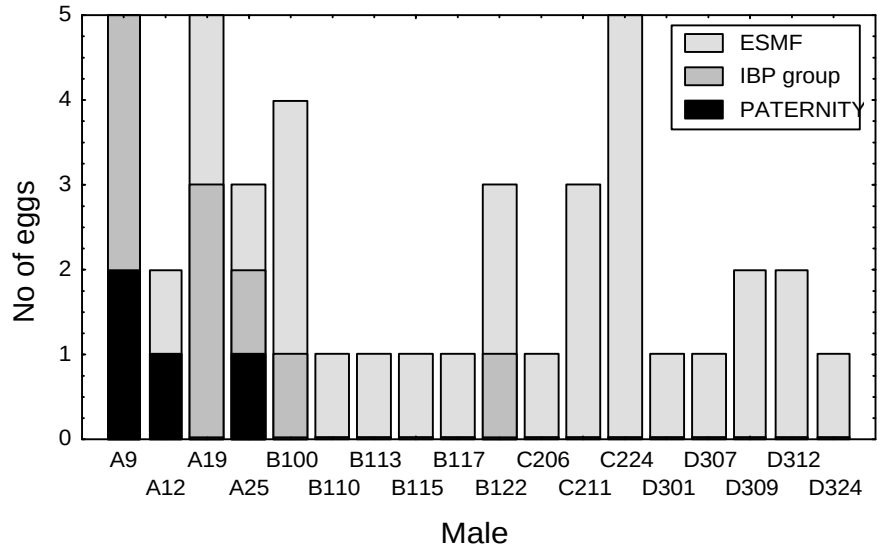

Figure 6. Number of eggs carried by each male discriminating between eggs sired by the male who carried the eggs (Paternity), sired by other male of the same group (IBPG), or sired by other male with whom the female mated before the experiment were set up (i.e. in the field) (ESMF). See text for details.

Carrier Male	%Paternity	% IBPG	% ESMF	% IBPG + % ESMF
A9	40	60	0	60
A12	50	0	50	50
A19	0	60	40	100
A25	33.3	33.3	33.3	66.7
Subtotal A	**26.7**	**46.7**	**26.7**	**73.3**
B100	0	25	75	100
B110	0	0	100	100
B113	0	0	100	100
B115	0	0	100	100
B117	0	0	100	100
B122	0	33.3	66.7	100
Subtotal B	**0**	**18.2**	**81.8**	**100**
C206	0	0	100	100
C211	0	0	100	100
C224	0	0	100	100
Subtotal C	**0**	**0**	**100**	**100**
D301	0	0	100	100
D307	0	0	100	100
D309	0	0	100	100
D312	0	0	100	100
D324	0	0	100	100
Subtotal D	**0**	**0**	**100**	**100**
TOTAL	**9.5**	**21.4**	**69**	**90.5**

Table III. Percentage of paternity (eggs sired by males who carried them), intraspecific brood parasitism due to eggs sired by other male of the same group (IBPG), or percentage of eggs sired by other males not present in the experimental group, i.e. males who mated with females before they were brought into the laboratory (eggs sired by other males in the field, ESMF).

probability is similar to that obtained using permutations with repetition (for n elements with n_1 elements of one type, eggs fertilized by the male x_i, and n_2 elements of another type, eggs non-fertilized by the male x_i) for each male separately, and then calculating the probability of the event for all males (p=0.046). For each particular male, the confidence of carrying eggs attending to predictions derived from paternity certainty and paternal investment (1-probability of the male carrying eggs fertilized by him at random) was as follows. For male A9 the confidence of that carrying 2 eggs fertilized by him is not the result of a random distribution (under the initial conditions established as the real conditions concerning total number of eggs fathered and carried in the real scenario) was between 43% and 84%. For male A12, the confidence of that carrying 1 egg fertilized by him is not the result of a random distribution (with identical conditions mentioned above) was between 63% and 97.5%. Finally, for male A25, the confidence of that carrying 1 egg fathered by him is not the result of a random distribution (with identical conditions mentioned above) was between 65.2% and 97.6%.

Discussion

This study is the first using AFLPs to understand the evolutionary significance of egg carrying in the golden egg bug. By using AFLP

markers, it has been possible to carry out paternity analysis in this species for which no codominant markers are available. In addition, the methodology developed may become useful to determine true genetic parents in other species in which neither the father, nor the mother, can be determined by observational methods.

AFLP markers revealed high levels of polymorphism in *P. laciniata*. A total of 107 polymorphic loci were obtained for a population of 79 individuals using two selective primer pairs. Previous work found no correlation in the migration of AFLP fragments in the two primer pair profiles, thus we considered each locus as independent (García-González *et al.* 2002). The mean dominant allele frequency, *p*, was 0.386, assuming Hardy-Weinberg equilibrium, for a total of 76 polymorphic loci with $q^2>3/N$, with N being the number of adult individuals (N=35). Given these estimated allele frequencies, the theoretical expected percentage of offspring for which all males except the true genetic father can be excluded (exclusion probability) was 88% for this population, which is higher than the confidence levels used in other studies (see for example Rossiter *et al.* 2000). This expected exclusion probability implies a number of assumptions including random mating and random extraction of individuals from the population (Lewis & Snow 1992). Another assumption is that closely related males do not compete for paternity, a fact that has been seen to overestimate the probability of excluding a randomly chosen nonfather (Double *et al.* 1997). In this study care was taken to ensure random collection of individuals from a natural population, and there is no evidence of non random mating in the field (Reguera 1999).

In the present study we had to face two main difficulties. First, we could not know by direct observation which female was the mother of a given nymph. There were, for each nymph, between 2 to 5 potential mothers, depending on the experimental group. This problem was solved by the statistical determination of the putative mother among all the potential mothers of a nymph. Differences were found for the genetic similarity between nymphs-putative mothers (the latter selected as the female with the highest degree of relatedness among the females with a probability $p>0$ of being the mother of the nymph) and nymphs-non putative mothers. By using a mixed model of ANOVA we avoided the problem of non-independence when analysing genetic relatedness between nymphs and females.

The second difficulty relates to the fact that the study could not be carried out with virgin females (see Materials and Methods). This implies that for each nymph there were multiple potential fathers, including both males from the nymph's experimental group and males from the natural population who could have copulated with the nymph's mother before the start of the experiment. To solve this problem we have developed a procedure to exclude non-fathers, based on the determination of exclusion diagnostic markers. More specifically, we could determine that a nymph was fathered by a male outside the nymph's experimental group when two or more than two diagnostic peaks were found for all profiles of nymph-putative mother-male x_i, for x from $i=1$ to $i=n$, with n being the number of males in the nymph's experimental group.

The exclusion of the egg-carrying male as the father of the nymph reached 83.3% of cases (without identification of putative mothers, as indicate in Step 5 of Materials and Methods), or 90.5% of nymphs (assessing first the putative mother as described in Materials and Methods). However, our results show that there was a high prevalence of eggs fertilized by sperm from males not present in the experimental groups (i.e., that mated with the female in the field before females were captured). In group B, 10 out of 11 eggs carried by experimental males were fathered by males from the field, and in groups C and D all nymphs were fathered by males not present in the experimental groups. Thus, in these experimental groups either females did not copulate with males during the 8 days that the experiments lasted, or if copulations took place, they did not involve sperm transfer (a common occurrence in this species,

pers. obs.). Taking all experimental groups into account, only 13 out of 42 (31%) of all eggs laid on males were fathered by a male within the group. Out of these 13 eggs sired by males in the experimental groups, 4 (30.8%) were sired by the carrier male, and 9 (69.2%) were carried by a male other than the father. In order to analyse whether females choose to lay eggs on fathers versus unrelated males, only eggs that are fathered by males from the experimental group should be considered. The reason is that when eggs are fertilized by males outside the experimental group, females are unable to lay eggs on fathers because they are absent (due to the obvious restrictions imposed by the experimental conditions). When we only take into account the eggs that have been fertilised by males present in the experimental group, around 30 % of the eggs are laid on their true genetic fathers.

This result could be explained by several factors. First, as already mentioned before, in some groups females may not have mated, or sperm transfer may have not taken place, which seems to be a common occurrence in this species. Secondly, in those experimental groups in which there are eggs fertilised by males present in the group, the relatively high proportion of eggs from males not present in the group suggests that last male sperm precedence may not be the norm in this species. An unexpected result, given that last male advantage occurs in a great number of heteropterans (Smith 1979; Sillén-Tullberg 1981; Arnqvist 1988; Rubenstein 1989).

Given the high proportion of eggs fathered by males not present in the experimental group, it is clear that the experimental females did not have the opportunity to lay those eggs on the true fathers and this may have biased the results, decreasing the rate of egg carrying by the true genetic fathers. In addition, the densities in experimental groups where much higher than those found in natural populations. Data gathered from adults found in an area of 400 m^2 in 44 different days throughout three reproductive periods (years 1998 to 2000, García-González & Gomendio, unpublished), showed densities ranging from 0.005 individuals per square meter to 0.265 individuals/m^2 (mean density=0.042 individuals/m^2, SE=0.007). On the other hand, densities in the experimental groups reached 16 individuals/m^2 in groups C and D, and 293.8 individuals/m^2 in groups A and B. Since we already know that high densities increase encounter rates and promote egg laying in conspecifics, it is likely that the experimental conditions also made it easier for females to lay eggs on conspecifics that were not the true genetic father. A recent study by Richardson & Burke (2001) has shown that, for an avian species, high breeding densities were associated with an increase in the frequency and extent of extra-pair paternity.

In conclusion, if we only take into account those cases in which females can choose to lay eggs on fathers versus unrelated males, 30.8% of those eggs were laid on fathers even under high densities that favour intraspecific parasitism. This, together with the probability of carrying fertilized eggs calculated from Monte Carlo simulations, strongly suggests that paternal care could play a role in this system.

The rates at which males carry eggs that are not their true genetic offspring still demand an explanation concerning the reasons why this occurs. According to theoretical models these should include: the impact of parental care on offspring survival which in this species is high, the ability to discriminate offspring which is unlikely to exist in this species, the costs of providing care which in this species seems high, and the mean parentage derived from different matings which is still unknown (Westneat & Sherman 1993).

Female golden egg bugs benefit from laying eggs on conspecifics because of the benefits derived for the offspring in terms of increased survival. However, carrying eggs is costly for adults because it increases predation rates (Reguera & Gomendio 1999; Kaitala *et al.* 2000). Thus, selection should favour individuals who modulate their investment of parental care in relation to genetic paternity to avoid investing in offspring who are not their own (Trivers 1972; Burke *et al.* 1989; Xia 1992; Dixon *et al.* 1994; Briskie *et al.* 1998; Sheldon & Ellegren

1998; Neff & Gross 2001). A low proportion of females carry eggs and these females carry few eggs. Thus, egg laying on females may be the result of low rates of intraspecific parasitism. However, all males in natural populations end up carrying eggs and they carry more eggs than females. It seems reasonable to ask then if males accept eggs because the probability that some of the eggs carried are his true genetic offspring, and the improved survival that they enjoy, are enough to compensate for the costs of carrying eggs. The results presented in this paper suggest that a proportion of eggs carried have been fertilised by the male, but at present we cannot judge whether the proportions observed in the study may reflect those found in natural populations because a high proportion of the eggs were fertilised before the experiment started, and because the densities present in the experimental groups were higher that those found in natural populations. Both factors may have led to an underestimation of the levels of paternity prevalent in natural populations.

The available evidence suggests that males may accept eggs despite some uncertainty about paternity because the benefits in terms of offspring survival are huge, because they cannot discriminate between own and unrelated offspring, and maybe because the level of parentage between successive egg laying events does not change substantially (Davies *et al.* 1992; Whittingham *et al.* 1992; Westneat & Sherman 1993; Whittingham *et al.* 1993; Houston 1995; MacDougall-Shackleton & Robertson 1998). In this sense it is interesting that last male precedence does not seem to occur, since this would make it difficult for males to assess when eggs are more likely to be fertilised by them. This point requires further attention since sperm competition mechanisms will determine levels of parentage between egg laying events.

Other studies have already suggested that "some" of the eggs carried by a golden egg bug male may have been fathered by him (Kaitala & Miettinen 1997; Kaitala *et al.* 2001). However, no evidence has been provided on the methodology, neither on the experimental conditions used, so it is difficult to judge how robust these results are and how they compare to ours. AFLP technique is robust and reliable because, among other reasons, stringent reaction conditions are used for primer annealing (Don *et al.* 1991; Vos & Kuiper 1997; Hecker & Roux 1996; Mueller & Wolfenbarger 1999). The use of AFLPs allows for a quick and accurate scoring of many genetic markers using an automated DNA sequencer.

The present study shows how AFLPs can be employed to determine paternity in the golden egg bug in particular, and more generally in groups in which neither the mother nor the father can be determined by other means, and suggests that paternal care could play an important role in explaining male egg carrying behaviour in the golden egg bug.

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CAPÍTULO 7

Mecanismos de competencia espermática, confianza de paternidad y evolución del cuidado paternal*

Resumen del Capítulo 7

Tradicionalmente se ha predicho que la inversión paternal debería ocurrir únicamente en especies en las cuales la confianza de paternidad (la probabilidad de un macho de ser el padre genético de la descendencia que se origina tras la realización del apareamiento) es alta. Sin embargo, el uso de técnicas moleculares ha puesto de manifiesto que, como resultado de la poliandria femenina, en algunas ocasiones los machos cuidan de descendencia de la cual no son los padres genéticos. Esta evidencia ha estimulado el desarrollo de estudios teóricos acerca de la relación entre la confianza de paternidad y el esfuerzo parental. Los modelos teóricos más recientes predicen que el esfuerzo parental de los machos depende de la confianza de paternidad pero también de los compromisos entre la reproducción presente y futura y del balance de costos/beneficios derivados del cuidado parental.

En el presente Capítulo se examinan los patrones de precedencia de esperma en *Phyllomorpha laciniata* para inferir el mecanismo de competencia espermática que opera en esta especie. El objetivo principal de este trabajo es obtener la confianza de paternidad característica de este insecto e integrar este conocimiento con los compromisos reproductivos y de inversión paternal que experimentan los machos. La paternidad de los huevos fecundados por los últimos machos que se aparean con hembras

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no-vírgenes se ha determinado por medio del análisis de los AFLPs. La probabilidad de exclusión que se ha obtenido en este estudio es del 98%, lo que demuestra que los marcadores AFLPs son una herramienta adecuada para afrontar la determinación de paternidad en esta especie. Hemos obtenido un valor promediado de P_n (la proporción de huevos que fecunda el último macho que se aparea con una hembra) de 0.43, que constituye el valor de confianza de paternidad para este insecto. Es decir, se espera que el último macho que se aparee con una hembra fecunde, en promedio, el 43% de los huevos puestos por la hembra tras la cópula. Los resultados obtenidos indican que el mecanismo de competencia espermática que opera en este insecto es la mezcla de esperma ("sperm mixing"), puesto que los machos disfrutan de una P_n intermedia y existe una alta variabilidad en torno al valor de P_n. Además, la confianza de paternidad se mantiene invariable con el tiempo. Toda esta evidencia sugiere que confianzas de paternidad intermedias pueden ser suficientes para que evolucione el cuidado paternal en esta especie puesto que (1) los beneficios del cuidado parental, en términos de supervivencia de la descendencia, son extremadamente altos, y (2) los machos no se benefician de una disminución del esfuerzo parental debido a que la confianza de paternidad se mantiene constante como consecuencia del mecanismo de competencia espermática. Por lo tanto, nuestros resultados no apoyan la idea tradicional que sostiene que la inversión paternal debería ocurrir únicamente en especies que disfrutan de una alta confianza de paternidad. Este estudio, además, sugiere que los conflictos de interés entre los sexos son una pieza clave en la comprensión de este sistema. Los datos apuntan a que las hembras podrían maximizar el número de machos que está dispuesto a aceptar huevos, y el número de huevos que está dispuesto a llevar cada macho, por medio de un mecanismo de mezcla de esperma. Este mecanismo asegura que todos los machos que se aparean con una hembra tengan probabilidades de producir descendencia, a la vez que dificulta que los machos puedan predecir el momento exacto en el que su descendencia genética será producida.

Sperm competition mechanisms, confidence of paternity, and the evolution of paternal care in the golden egg bug (*Phyllomorpha laciniata*)

Francisco Garcia-Gonzalez[1,2], Yolanda Núñez[3,4], Fernando Ponz[3,5], Eduardo R. S. Roldán[1,6] and Montserrat Gomendio[1,7]

[1]Departamento de Ecología Evolutiva, Museo Nacional de Ciencias Naturales (CSIC), José Gutiérrez Abascal 2, 28006 Madrid, Spain. [2]E-mail: mcng193@mncn.csic.es. [3]Departamento de Biotecnología, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Ctra. Coruña Km 7.5, 28040 Madrid, Spain.[4]E-mail: nmoreno@inia.es. [5]E-mail: fponz@inia.es [6]E-mail: roldane@mncn.csic.es. [7]E-mail: montseg@mncn.csic.es

Abstract

Theoretical models predict how parental effort should vary depending on confidence of paternity and on the trade offs between present and future reproduction. In this study we examine patterns of sperm precedence in *Phyllomorpha laciniata*, and how confidence of paternity influences the willingness of males to carry eggs. Females golden egg bugs show a flexible pattern of oviposition behaviour, which results in some eggs being carried by adults (mainly males), and some being laid on plants where mortality rates are near 100%. Adults are more vulnerable to predators when carrying eggs, thus it has been previously suggested that it should only pay males to accept eggs if there are chances that at least some of the eggs will be their true genetic offspring. We have determined the confidence of paternity for naturally-occurring individuals and its variation with the time. Paternity of eggs fertilized by the last males to mate with females previously mated in the field has been determined using AFLPs (Amplified Fragment Length Polymorphisms). The exclusion probability was 98%, showing that AFLP markers are suitable for paternity assignment. The mechanism of sperm competition that operates has been inferred from the mean proportion of eggs fathered by the last males and from the variance showed by this value. Sperm mixing seems the most likely mechanism of sperm competition, since the last male to copulate with field females fathers an average of 43% of the eggs laid during the next 5 days. More importantly, the proportion of eggs fathered does not change significantly during that period. We argue that intermediate levels of paternity can select for paternal care in this system because 1. benefits of care in terms of offspring survival are very high, and 2. males have nothing to gain from decreasing their parental effort in a given reproductive event since confidence of paternity remains constant with the time as a result of the mechanism of sperm competition. Thus, our findings do not support the traditional view that paternal investment is expected to arise only in species where confidence of paternity is high. The results suggest that females maximise the chances that several males will accept eggs by promoting a mechanism of sperm mixing which ensures that all males that have copulated with a female have some chance of fathering offspring, and that it is difficult for each male to predict when will his true genetic offspring be produced.

Key Words: Paternal care, confidence of paternity, paternity, sperm competition, sperm mixing, *Phyllomorpha laciniata*.

Introduction

Following Williams (1966) and Trivers (1972) the evolution of parental care will depend on the balance between the costs of care to the parent's residual reproductive value and the benefits to the fitness of offspring or other relatives. Because selection is expected to favour individuals that modulate their investment in relation to genetic paternity, in order to avoid investing in offspring that are not their own, paternal investment was predicted to arise only in species where confidence of paternity is high (Trivers 1972; Maynard Smith 1977; Gwynne 1984). However, the use of molecular techniques to determine paternity has revealed that, as a result of female polyandry, males often care for unrelated young (see for example Birkhead and Møller 1992; Møller and Birkhead 1993; Scott and Williams 1993; Westneat and Sargent 1996; Hughes 1998). Such unexpected results prompted a number of theoretical and empirical studies in an effort to understand under which conditions should parents invest in offspring which are not their own. Whereas some of these studies suggested that decreases on paternity levels should have no effect on paternal behaviour (Davies *et al.* 1992; Wagner *et al.* 1996; MacDougall-Shackleton and Robertson 1998, and see also Maynard Smith 1978; Grafen 1980; Whittingham *et al.* 1992; Whittingham *et al.* 1993; Houston 1995), others have found support for the association between confidence of paternity and paternal investment (Burke *et al.* 1989; Davies *et al.* 1992; Whittingham *et al.* 1992; Xia 1992; Westneat and Sherman 1993; Dixon *et al.* 1994; Briskie *et al.* 1998; Sheldon and Ellegren 1998; Neff and Gross 2001, and see for insects Smith 1979b; Müller and Eggert 1989; Scott and Williams 1993; Simmons *et al.* 1993; Wright 1998; Simmons and Achmann 2000).

Theoretical models have shown that the relationship between paternity and parental care is complex (Whittingham *et al.* 1992; Westneat and Sherman 1993). Current models predict that paternal care depends on confidence of paternity as well as on trade-offs between present and future reproduction (Whittingham *et al.* 1992; Westneat and Sherman 1993; Mauck *et al.* 1999). In a model developed by Westneat and Sherman (1993), which reconciles previous modelling attempts (see for example Maynard Smith 1978; Grafen 1980; Werren *et al.* 1980; Winkler 1987; Whittingham *et al.* 1992; Xia 1992), the following predictions were formulated: (1) When males do not know their own paternity and mean paternity is the same for all matings, a probability of siring offspring below 1 should have no effect on the optimal level of parental effort. (2) When parents can discriminate their own young, overall parental effort should be reduced, but nepotism should increase. (3) When parental behaviour is costly to care givers, paternity should have more effect than when caring is not costly. (4) Paternity should have less effect when care greatly increases offspring survival than when care is not crucial to offspring survival. Few studies have examined these predictions (see review of Wright 1998) and more empirical work is necessary, especially to test the prediction regarding variations in the confidence of paternity with time and its effect on parental effort.

Studies on sperm competition in insects have provided important insights into the relationship between paternity and paternal care. Sperm competition has important consequences for the evolution of paternal care since it is a form of male/male competition for paternity. Sperm competition mechanisms determine the link between male ejaculate expenditure and fertilisation success, thus defining which strategies can males adopt to adjust their investment levels to their confidence of paternity. In other words, sperm competition mechanisms determine how males can evaluate confidence of paternity. Since sperm competition mechanisms are largely the result of female reproductive morphology and physiology (Birkhead and Møller 1998), it is females who define the arena in which ejaculates compete, and thus the rules of the game (Gomendio *et al.* 1998). It was at first predicted that species with male parental investment should

be characterised by last male sperm precedence (Gwynne 1984), but high confidence of paternity may be also associated with first male sperm precedence if there is little risk that females will utilize sperm from future males, thus resulting in the first males investing parentally (Gwynne and Snedden 1995; Simmons 1995; Simmons and Siva-Jothy 1998). However, to our knowledge, there are no studies examining the effects of intermediate levels of confidence of paternity on parental investment.

P. laciniata is a good system to test the predictions of Westneat and Sherman (1993) model since in this species males carry eggs that they have fathered as well as unrelated eggs. Females mate with multiple males within a reproductive cycle and store the sperm in a spermatheca, conditions that promote the occurrence of sperm competition. Females lay one egg at a time continuously over the reproductive season (from March until August), and daily egg production ranges between 0 and 10 eggs. Some eggs are laid on conspecifics, while others are laid on plants where they develop unattended (Mineo 1984; Kaitala, 1996). Eggs derive a great benefit when carried by an adult because their survival rates improve considerably, given the lower rates of mortality caused by a parasitoid wasp (Reguera and Gomendio 2002). However, egg carrying conveys important costs for adults in terms of increased predation rates (Reguera and Gomendio 1999; Kaitala *et al.* 2000). Females are unable to oviposit on themselves, thus they are forced to search for conspecifics to lay eggs on them in order to increase their fitness. The evolutionary significance of male carrying has been the subject of an ongoing controversy (Gomendio and Reguera 2001; Kaitala *et al.* 2001), however, a recent study has shown that male egg carrying is a rudimentary form of parental investment since males carry a variable proportion of genetic offspring and egg carrying maximizes egg survival rates (Garcia-Gonzalez et al., unpublished). Given the high costs of egg carrying for adults, males are expected to accept eggs only if there are chances that at least some of the eggs will be their true genetic offspring. The confidence of paternity levels at which males become willing to accept eggs are likely to depend on the variation of this confidence through the time, the costs of no care for the offspring, and the trade off between parental effort, mating effort and somatic effort for caring males.

The assignment of paternity is a fundamental piece of information in studying patterns of sperm use and the relationship between paternal care and confidence of paternity. AFLP fingerprinting (Vos *et al.* 1995) is a very reliable technique that generates hundreds of genetic markers (Vos and Kuiper 1997; Mueller and Wolfenbarger 1999). However, the utility of the AFLP technique for paternity analysis has been assessed in a few studies (Krauss and Peakall 1998; Krauss 1999; Questiau *et al.* 1999 and see Gerber *et al.* 2000). So far, there are no studies using AFLPs to investigate patterns of sperm use. Here we use for the first time AFLP markers to determine sperm precedence patterns in a species. The aim of this study is double folded. On the one hand, we wish to understand the mechanisms of sperm use in the golden egg bug to determine how sperm competition translates into male reproductive success. Our final aim is to understand the connection between sperm precedence patterns observed in natural populations, and the extent of egg carrying by male golden egg bugs under the light of current theory on the relationship between paternal care and confidence of paternity.

Materials and Methods

Definitions

In the present study Paternity is used as synonymous of Westneat and Sherman's (1993) "parentage" and of the term "actual fatherhood" of Wright (1998) in the sense that it is the proportion of juveniles that are genetic offspring of a parent. Confidence of paternity is a male's average probability of siring offspring following copulation with a given female (Alexander 1974;

Simmons 2001), with no implications about an individual's ability to assess his own paternity. This term was used by Wright (1998) as the "probability of paternity" and by Westneat and Sherman (1993) as "reduced parentage". We follow the definitions of Westneat and Sherman (1993) of Parental behaviour: any action by a parent that increases the survival of young (see Clutton-Brock 1991) and of Reproductive effort: the sum of Parental effort (effort expended on parental behaviour), Mating effort (effort expended in acquiring fertilizations), and Somatic effort (effort that increases and individual's chances of survival to another breeding attempt). P_n is the proportion of offspring sired by the last male (the nth) to mate, usually calculated for the second male to mate with a female in a double mating trial, i. e., P_2 (Boorman and Parker 1976; Birkhead and Møller 1998).

Samples and Experimental Conditions

The aim of the study was to allow mating females collected in the field to lay eggs during the five days following the end of the copulation, and then to determine the number of eggs fathered by the last male to mate with the female.

Eighteen mating pairs were collected in three close localities of Central Spain (mean distance among them: 26.1 km): El Espinar (eight pairs collected on the 7th June 2000), Robledo de Chavela (eight pairs collected on the 12th June 2000), and Navas del Rey (2 pairs collected on the 14th June 2000). The position and the stage of development of eggs (assessed by their colour) carried by mating males, if they were carrying any, were recorded each time a mating pair was collected. At these dates, individuals were at the peak of their reproductive activity in the area of collection. Monitoring of the population in Central Spain from the beginning of their reproductive cycles allowed us to determine this particular stage. Data collected throughout 3 years and a series of preliminary studies examining sperm contained in the females' spermatheca showed that at this moment of the reproductive period 100% of all females in the population have mated at least once. Females of this species are polyandrous, and data from laboratory experiments have shown that females mate multiple times with the same and with different males. Observational data from capture-recapture studies carried out in natural populations supports this fact. Thus, it is very likely that females in this study had mated multiple times in the field before the copulation in which they were captured.

Copulation in this insect lasts on average more than 15 hours (23 hours on average as Kaitala 1998; 32.5 hours on average as Reguera 1999; 17 hours on average as Garcia-Gonzalez, unpublished) and may last for more than 48 hours. In order to avoid interrupting sperm transfer by the mating male, mating pairs were carefully carried in individual plastic vials to the laboratory. Pairs were placed in small Petri dishes (5.5 cm diameter) and kept at constant conditions (25° C, lights on from 8:00 AM to 9:00 PM). For each mating female the start of the five days laying period was marked as the end of the current copulation. We checked for the end of copulations at least four times during the day at 9:00, 13:00, 17:00 and 21:00 hours. To determine the end of each copulation in those instances in which the exact time was not observed, we used the middle point between two intervals.

Once copulation was finished the male was removed and frozen to -80°C, and the female was allowed to lay eggs freely during the subsequent five days. Our aim was to examine sperm precedence patterns in naturally occurring golden egg bugs by determining paternity of eggs laid by females on the plant without confounding effects by male presence (for instance, repeated matings). However, in some cases, some females laid eggs on the mating male before the end of copulation was detected by us. These eggs could be distinguished from those already carried by males at the time they were captured because recently laid eggs are white. In addition, the position of eggs carried by males when they were captured was registered. Eggs laid on mating males were processed and used to make and estimate of the paternity of eggs

that were laid on males soon after copulation has ended.

Throughout all the experimental period, individuals were provided ad libitum daily with fresh branches of the host plant *Paronychia argentea*. Extreme care was taken to make sure that there were no eggs glued to the plant each time it was added to the animals, including the first time when the mating pairs were collected. This prevented that any alien egg, previously laid on plants by females in the field, could have entered the container. Eggs laid by females were collected with intervals of 24 hours from the end of the copulation, for each particular female, and at the same time host plant replacement was carried out. All eggs were laid on plants except those laid on males immediately after the end of the copulation. Each egg was labelled with the date of laying, and was placed in an Eppendorf tube and checked daily until hatching. Recently emerged nymphs were frozen as well as adults to -80°C.

Paternity of eggs was analysed for seven of the 18 families in which eggs were laid along the five experimental days (families 1, 3, 7, 11, 12, 16 and 18; see Table 1). In addition, the parents and the eggs laid on males of another two families (families 2 and 4) were included to increase sample size when analysing paternity of eggs laid on males immediately after copulation (93.33% of the eggs laid on males in families 1, 2, 4, 7, 11, 12, 16 and 18 were analysed; see Table 1). Moreover, another two adults were included to increase sampling in order to calculate allele frequencies (see samples sizes below and in Table 1).

DNA Isolation

Genomic DNA was isolated from the thorax of adults previously cleaned from chitinous extensions (n = 20 adults) and from the whole body of the nymphs emerged from eggs laid by females from nine families (n = 89, see Table 1), using a CTAB procedure modified from Weising *et al*. (1995, page 51), Möller *et al*. (1992) and Reineke *et al.* (1998).

AFLP Analysis

A total of 109 individuals were processed. AFLPs were resolved using the AFLP™ Plant Mapping Protocol (Perkin-Elmer Applied Biosystems, Madrid, Spain), but the reactions were performed with half the volume described in the protocol, with the exception of the quantity of DNA and adapters used. In a series of preliminary tests, we verified that the profile of AFLP fragments obtained was identical to that seen using the total reaction volume. All products were purchased from Perkin-Elmer, except enzymes MseI, EcoRI and T4 DNA ligase, which were obtained from New England Biolabs, Inc. (Beverly, USA).

In a previous study two selective primer pairs were identified as highly polymorphic, offering clean and reproducible patterns: MseI-CAT and EcoRI-AAG (JOE-Green), and MseI-CAG and EcoRI-AAC (NED-Yellow). For each of these two primer pairs selective PCRs were performed. The final product was run on an ABI PRISM 310 Genetic Analyser (Perkin-Elmer) (further details will be published elsewhere). Digitally converted raw data were saved on a computer as samples migrated past the fluorescence detector. Multilocus profiles were visualized using ABI Genescan software.

Data Analysis

Only unambiguous AFLP markers that were easily scored were used. We considered each fragment as a dominant locus with two states: presence or absence. AFLP profiles were scored for the presence/absence of fragments in the 60-300 bp range. The size in base pairs was given by the comigration of a size standard. Two peaks were considered of the same size if they differed by less than 0.5 bp.

In a previous study, a series of sample replications were conducted to check the repeatability of the method. This repeatability reached 96.6% for the combination of the two primer pairs used (Garcia-Gonzalez et al.,

unpublished).

We have used an index of correlation in migration (IC) for all *loci i* and *j*, with *i* different of *j*, to check for correlation between *loci* (Questiau *et al.* 1999). For this purpose, we calculated all pairwise comparisons between *loci* for all individuals (N), using two states for each locus: 1 for presence of a peak, 0 for the absence. The index of correlation in migration is:

$$IC = \sum_{n=1}^{N} \left| state_{ith} locus - state_{jth} locus \right| / N$$

A value of one between two fragment positions means that when a peak appears at the *ith* position, another peak does not appear at the *jth* position, or vice versa. A value of zero means identical appearance or absence in both *ith* and *jth* position, which could indicate comigration of the two fragments. No correlation in the migration of bands was detected in the two primer pair profiles. A total of 167,860 pairwise comparisons were calculated for Green and 192,494 for Yellow. No value of 0 or 1 were detected for the sum of all individuals from 1 to N within each comparison for *loci i* and *j*. The average index for Green was IC_{Green} = 0.49 ± 0.13 (mean±standard deviation, n = 1540) and for Yellow was IC_{Yellow} = 0.34 ± 0.12 (n = 1776). This indicates the independence of the *loci*.

Exclusion Probability

We considered only the adult individuals of our population for the allele frequency calculations. We used the proportion of individuals with no peak for a given locus as the genotypic frequency of the recessive homozygotes (q^2), with *q* being the estimation of the frequency of the allele absence in the population for that locus. We defined *p* as the frequency of the allele presence with *p = 1 - q*. We focused only on polymorphic *loci* with $q^2 > 3 / N$, with N being the number of adult individuals, as recommended by Lynch and Milligan (1994).

Exclusion probabilities were calculated using the equation of Chakraborty *et al.* (1974) to compute the probability of exclusion based upon at least two diagnostic markers. The exclusion probability (the probability that any one randomly chosen male can be excluded as the father of a chosen individual) is the most common measure of the potential of a given genetic system for use in paternity analysis (Lewis and Snow 1992). An exclusion diagnostic marker (also termed diagnostic marker, diagnostic peak or diagnostic fragment) is defined by the situation in which, for a given locus, both a potential father and the true mother lack the allele (fragment absence in the AFLP profile) whereas the particular offspring has it (fragment presence in the AFLP profile). Following Pena and Chakraborty (1994), the situation in which a male can be unambiguously excluded as the father is when, two or more than two exclusion diagnostic markers are revealed when analysing the AFLP profiles of the potential father, the true mother and the offspring, thus allowing for one mutation in the AFLP profile of the true sire without exclusion.

We calculated exclusion probabilities at the adult population level for *loci* with $q^2 > 3 / N$, with N being the number of adults. For *k* markers, the cumulative probability *Q* of exclusion for at least one diagnostic marker, is:

$$Q = 1 - \prod_{i=1}^{k} (1 - p_i)$$

The exclusion probability *P* on at least two diagnostic markers is:

$$P = Q - \sum_{i=1}^{k} p_i \prod_{\substack{j=1 \\ j \neq i}}^{k} (1 - p_i)$$

being $_{i}p_{i}$

the probability of exclusion based upon the *ith* marker; that is, the probability of having no peak in

both parents (q^2_i *x* q^2_i) and one allele present p_i in a nymph:

$$p_i = q_i^2 \times q_i^2 \times p_i$$

Determination of paternity

The procedure we used to determine paternity was based on the determination of exclusion diagnostic markers (diagnostic peaks in the AFLP profile), where a peak (fragment) was absent in both parents (i.e., when both are recessive homozygotes), and present in the nymph. Following Pena and Chakraborty (1994), the male (in this case the last male to copulate with a female) was excluded as the genetic father of the nymph when at least two diagnostic peaks were found in the profile comparison, i.e. when at least two peaks were present in the nymph and absent in the male as well as in the mother.

The number of eggs laid, and the proportion of eggs fertilized by the last male (P_n), were used as dependent variables in repeated measures ANOVAs. These variables were log-transformed and arcsin-transformed, respectively, to fulfil parametric assumptions (Sokal and Rohlf 1981). Other assumptions of the repeated measures ANOVA were checked by specific tests; the compound symmetry assumption was checked by the tests of Greenhouse-Geisser and Huynh-Feldt, and the Sphericity assumption was checked by Mauchley's Sphericity Test (Statsoft 1996).

Results

Oviposition after Copulation

The females from the 18 pairs laid a total of 236 eggs throughout the 5 days period (see Table 1). The mean number of eggs laid by females during this period was 13.1 eggs (standard error (SE) = 0.7, Min. = 9, Max. = 21). The first day after copulation females laid 36.02% of the eggs laid throughout the laying period monitored, in the following days they laid 18.64%, 19.92%, 16.10% and a 9.32% for the second, third, fourth and fifth days, respectively. The number of eggs laid decreases as the time from the end of the last copulation increases (repeated measures ANOVA,

Family	No. of eggs laid each day					No. of eggs hatched	No. of eggs included in paternity analyses	No. of eggs laid on males
	d1	d2	d3	d4	d5			
1	8	3	2	2	2	17	16	3
2	8	0	3	0	0	11		4
3	2	3	2	2	2	10	10	0
4	4	2	3	1	0	10		3
5	8	5	2	1	0	16		0
6	1	2	4	5	0	12		0
7	3	2	3	5	2	15	13	3
8	1	4	2	2	0	9		0
9	4	3	3	1	0	11		1
10	5	2	0	5	1	11		1
11	7	1	3	2	2	15	14	7
12	5	1	3	1	1	11	9	3
13	5	5	2	1	2	14		3
14	2	3	2	2	3	11		0
15	5	4	1	0	1	11		0
16	5	0	3	3	2	13	12	5
17	10	1	6	2	2	21		0
18	2	3	3	3	2	9	9	2
TOTAL	85	44	47	38	22	227	89[1]	35

Table 1. Number of eggs laid every day, from the first day after the copulation to the fifth day (d1 to d5, respectively), number of eggs hatched, number of eggs included in paternity analyses, and number of eggs laid on males immediately after copulation. [1]This number includes 4 and 2 eggs analysed in pairs 2 and 4, respectively, in which only eggs laid on males were analysed.

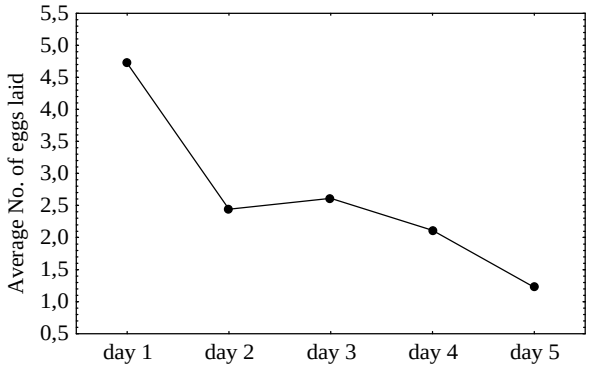

Figure 1. Mean oviposition rate of females (n = 18) along the experimental period.

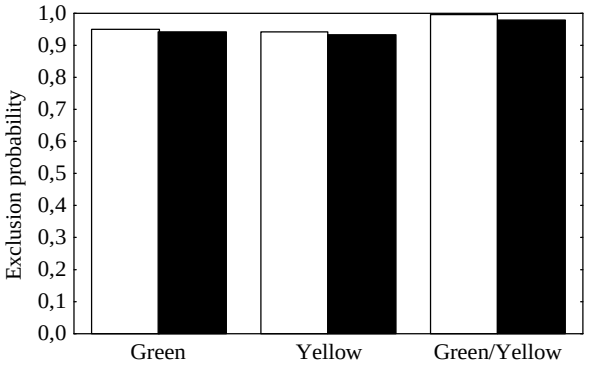

Figure 2. Exclusion probability for the Green primer pair (MseI-CAT/EcoRI-AAG), Yellow primer pair (MseI-CAG/EcoRI-AAC), and Green/Yellow primer pair combination, considering a single diagnostic marker as relevant for excluding the carrying male as the father of the nymph (probability Q, open bar) or considering at least two diagnostic peaks (probability P, black bar).

$F_{4,68}$ = 7.82, p << 0.001) (see Fig. 1). The first day after copulation females (n = 18) laid a mean (± SE) of 4.72 ± 2.65 eggs, followed by 2.44 ± 1.50, 2.61 ± 1.24, 2.11 ± 1.57, and 1.22 ± 1.00 eggs, for the second, third, fourth and fifth days, respectively.

Despite the short time interval between the end of copulation and experimental male removal, 41.2% (35 out of 85) of the eggs laid by females in the first day after copulation were laid on mating males.

A total of 227 out of the 236 eggs laid hatched. The mean percentage of hatchability was 96.1% (S.E. = 1.9, Min. = 69.2, Max. = 100, n = 18) (see Table 1).

Paternity of Eggs and Sperm Precedence Patterns

Within the study population (109 individuals) two AFLP primer pairs generated a total of 116 scorable fragments (see Table 2). The mean number of fragments generated per individual was 52 (SE = 0.9; n = 109; range = 25-72). All scored fragments were polymorphic, as assessed by band absence in at least one individual. We used a total of 91 polymorphic *loci* with $q^2 > 3 / N$, with N being the number of adult individuals (N = 20), in order to calculate allele frequencies. Dominant allele frequencies (p), calculated from the frequency of the recessive phenotype q^2 (band absence) for each one of these *loci*, varied from 0.0 (band absence in all adults) to 0.55 (band absence in 4 of the 20 adults). The mean dominant allele frequency (p) over 91 polymorphic *loci* generated from 20 adults was 0.239 (see Table 2). The global exclusion probability based on at least two diagnostic peaks (P), calculated, as described in Material and Methods, for the combination of the two primer pairs, MseI-CAT/EcoRI-AAG ("Green") and MseI-CAG/EcoRI-AAC ("Yellow"), was 0.98. The global exclusion probability considering a single diagnostic peak in order to exclude paternity by the last male to mate with a

Table 2. Polymorphism for two amplified fragment length polymorphism primer pairs in a sample of 109 individuals (10 males, 10 females, 89 nymphs) of *Phyllomorpha laciniata* from Central Spain, with mean dominant allele frequencies (p) and standard error (SE) calculated for adults (20 individuals) for polymorphic *loci* with $q^2 > 3 / N$ (for details see text).

Primer pair	No. of fragments	Polymorphic fragments	Mean (p)	SE
Green (MseI-CAT/EcoRI-AAG)	56	56	0.275	0.02
Yellow (MseI-CAG/EcoRI-AAC)	60	60	0.204	0.02
Total	116	116	0.239	0.02

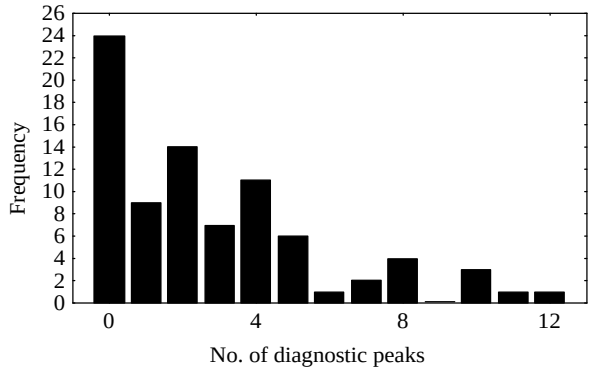

Figure 3. Distribution of the diagnostic markers between the profiles of the nymph-mother-last mating male, for the families analysed to determine P_n.

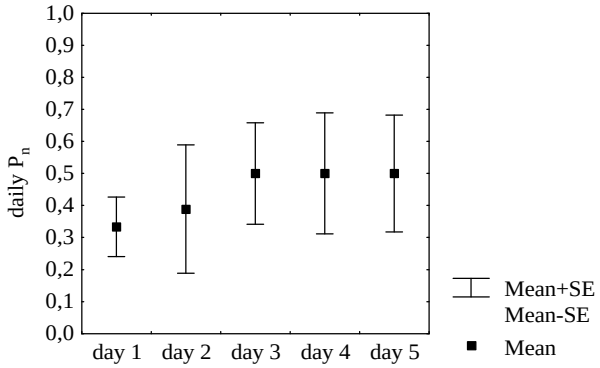

Figure 4. Mean proportion of eggs sired by the last mating male (P_n) calculated from the daily P_n of the families analysed for the determination of the sperm precedence patterns.

female (*Q*) was 0.997 (see Fig. 2).

In order to determine the proportion of eggs fertilized by the last male (P_n), 83 nymphs, distributed among seven families (families 1, 3, 7, 11, 12, 16 and 18), and obtained from the eggs laid along the five days period, were analysed. The proportion of eggs analysed out of the total number of eggs laid by the females of these families was 87.4% (see Table 1). We resolved AFLP profiles for these nymphs and for the females and males of these families. The 83 nymphs analysed possessed between 0 and 12 fragments present in the nymph and not present in both the mother and the last mating male, i.e. diagnostic peaks (see Fig. 3). In 33 nymphs out of 83 there was none or a single mismatched peak. Thus, based on the level of acceptance defined in Materials and Methods, and on the probability of exclusion obtained, these nymphs, being a 39.8% of all nymphs emerged from eggs laid, were considered as fathered by the last mating male.

The average proportion of eggs fertilized by the last mating male for each family over the whole laying period varied from 0.1 to 0.78% (see Table 3). The mean P_n obtained from the values for each family was 0.43 (SE = 0.11); thus, this value represents the confidence of paternity for the whole population. The proportion of eggs sired by the last male calculated for each one of the five days varied from 0.0 to 1.0 (see Table 3). An analysis of the variation of P_n shows that there are no differences in the proportion of eggs sired by the last male (arcsin-transformed) between days (Repeated measures ANOVA, $F_{4,16} = 0.13$, $p = 0.97$). The proportion of variance in the dependent variable that is explained by differences among groups is 0.031 (as assessed by the Eta squared coefficient), i.e. a 3% of P_n variance would be explained by the effect "time after the last copulation" in case of statistical significance. The repeated measures analysis did not included families 12 and 16 because in family 12 the only

Family	day 1	day 2	day 3	day 4	day 5	days 1-5
	E/F	E/F	E/F	E/F	E/F	P_n
1	8/1	3/1	2/0	1/0	2/1	0.19
3	2/0	3/0	2/1	2/0	1/0	0.1
7	3/2	2/0	3/1	3/0	2/0	0.23
11	7/1	1/0	2/0	2/2	2/1	0.29
12	4/2	1/1	3/3	1/1	0/0	0.78
16	5/2	0/0	3/2	2/2	2/2	0.67
18	2/1	1/1	3/3	2/1	1/1	0.78

Table 3. Number of eggs included in paternity analyses (E) and number of eggs fertilized by the last mating male (F), and the proportion of eggs sired by the last mating male (P_n) for the whole five days laying period, for each particular family.

egg laid on the 5th day could not be analysed, and in family 16 no eggs were laid in the 2nd day (see Tables 1 and 3). This implies empty cells, thus repeated measures cannot be conducted for these families. Calculating the mean P_n of all families (including families 12 and 16) for each day clearly supports that P_n remains nearly constant as the time after the last copulation increases, for the laying period considered (see Fig. 4).

Paternity of Eggs carried by Males

Eggs laid on males during the short interval between the end of copulation and their experimental removal were processed. In addition to the eggs carried by males in those families included in the study of sperm precedence patterns, eggs on males from families 2 and 4 were included (see Table 1). The same procedure of determination of paternity based on the number of diagnostic peaks that has been described above was conducted for this set of data. A percentage of 93.33% of the eggs carried by males in these families were analysed (see Table 1). The proportion of eggs carried by a male that were fertilized by him varied from 0.0 to 1.0 (see Fig. 5). There were no differences among males in the proportion of carried eggs that were sired by themselves ($c27 = 10.16$, $p = 0.18$). Males carried a mean percentage of 38% of their own offspring (SE = 12.16).

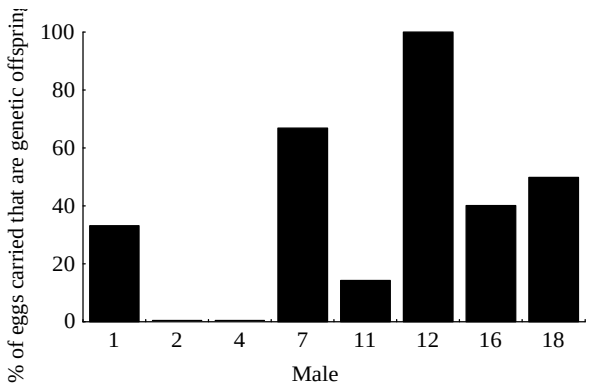

Figure 5. Proportion of eggs carried by the male that were fertilized by him, for cases in which females laid eggs on males immediately after the copulation.

Discussion

Mechanisms of sperm utilization

The results of this study show that in naturally occurring *P. laciniata* the last male to mate with a female experiences an intermediate level of fertilization success (mean P_n value is 0.43), characterised by a moderate variance (standard deviation of 0.30, range 0.1 to 0.78). In addition, our results show that averaged P_n value remains constant throughout the 5 days laying period.

Our results strongly suggest that a mechanism of sperm mixing is responsible for the pattern of sperm precedence in the golden egg bug. The patterns of sperm use in studies on sperm competition are typically inferred from the mean specific P_2 value (Birkhead and Møller 1998). Intermediate values are usually taken as indicative of sperm mixing while extreme values are assumed to be the result of mechanisms such as sperm stratification, sperm removal or sperm displacement by sperm flushing (Birkhead and Møller 1992; Simmons and Siva-Jothy 1998; Simmons 2001). Recently, it has been acknowledged that intraspecific variation in the proportion of offspring fathered by the last male to copulate with a female provides better information about the mechanisms involved in sperm use, than just the species mean value (Lewis and Austad 1990; Cook *et al.* 1997; Simmons and Siva-Jothy 1998; Harvey and Parker 2000; Simmons 2001). Low variance is expected where there are mechanisms to pre-empt previously stored sperm through for instance sperm displacement, or if there is a depletion of the sperm-storage organ prior to the second mating. On the contrary moderate variance is expected when sperm mixing occurs, being the magnitude of variation associated with the degree of sperm mixing. Finally, high levels of variation in P_2 are expected if, for example, first mating males use mating plugs to prevent future inseminations (values can be very low if the plug remains intact or very high when the plug's protection is breached) (Simmons and

Siva-Jothy 1998).

There are a number of mechanisms of sperm competition that, a priori, could have been thought to be applicable to *P. laciniata*. However, only sperm mixing predicts the results obtained in this study and is compatible with the rest of the evidence gathered so far in this species. The alternative mechanisms that are not supported by our results are the following:

1. Sperm removal. To our knowledge sperm removal in Heteroptera has been never been documented. Apart from the fact that male genitalia doesn't seem to be adapted to remove sperm from previous rivals, the sperm removal hypothesis seems unlikely in this insect since this mechanism does not predict longer copulations in male biased sex ratios when males are mated with virgin females, as occurs in *P. laciniata* (Garcia-Gonzalez and Gomendio, unpublished).

2. Sperm displacement by sperm flushing. This mechanism predicts high values of P_n that remain constant with the time, as long as complete displacement is achieved. If complete displacement is not achieved and sperm mixing follows sperm displacement high values of P_n that decrease with time are predicted. None of these predictions is supported by our data. The fact that the rate of sperm transfer is very slow in this species (Garcia-Gonzalez and Gomendio, unpublished) is also against this hypothesis.

3. Sperm precedence by sperm stratification ("last in-first out" principle). This mechanism also predicts very high values of P_n for, at least, the first eggs laid by females after copulation. In general the predictions are the same than for sperm displacement. In this case, high P_n values can decrease with time depending of the relative sperm representation of each male in the spermatheca. Thus, the sperm stratification hypothesis is clearly not supported by our results.

4. Another mechanism that has been proposed is the passive loss of sperm (sperm depletion), resulting in the sperm from the first male or males being lost from the female storage organs before the sperm from the last male enters the sperm-storage organ (Lessells and Birkhead 1990; Birkhead and Biggins 1998). Depending on the time interval between matings and the rate of sperm depletion this mechanism predicts either high values of P_2 that remain constant with time if the sperm from the first male is completely lost before the next male copulates, or intermediate values if the sperm from the first male is not completely lost and sperm mixing occurs to some degree. The fact that none of the pairs sampled show high P_n values that remain invariable through time does not support a mechanism of sperm loss. The existence of a pump in the spermatheca of females could be also against this mechanism in the golden egg bug. The hypothesis of Retnakaran (1974) to explain bimodal distributions of P_2 in Lepidoptera should be noted here. This hypothesis would predict the contrary of the sperm depletion hypothesis. Retnakaran (1974) suggested that the filling of the sperm-storage organ by the sperm from the first male can eliminate room for the sperm of subsequent competitors. P_2 would then reach 0, 1, or intermediate values depending on the filling by the first male (Gwynne and Snedden 1995, and see also Simmons and Achmann 2000). We have found a low number of sperm stored in multiple mated females in the field (averaging around 500 sperm, unpublished data) that could indicate a fixed storage capacity for the spermatheca of golden egg bug females, however this is in agreement with the very low rate of sperm transfer seen in this species. The hypothesis of Retnakaran (1974) predicts low values of P_2 that remain constant with the time, however none of the pairs analysed by us show this pattern.

In short, none of the predictions derived from these sperm competition mechanisms are supported by our data. P_n values obtained in this study evidences that sperm from at least two males are present within the female sperm-storage organ, and the mean P_n and the magnitude of its variation indicates that homogeneous sperm mixing is the norm. When sperm mixing takes place, the best strategy for males to improve fertilisation success is to increase sperm numbers in the female's sperm storage organ. In a previous

study (Garcia-Gonzalez and Gomendio, unpublished) we have shown that males respond to the presence of rivals by increasing copulation duration as well as by increasing the rate of sperm transfer. The combination of both mechanisms results in greater numbers of sperm being transferred to the female. Thus, copula duration could also account for variation in the output of sperm competition, as occurs in other heteropterans (McLain 1989; Rubenstein 1989) and other insect species (Dickinson 1986; Nuyts and Michiels 1993; Parker and Simmons 2000, and see for review Simmons 2001)

From a comparative perspective, the order heteroptera summarises well the general picture concerning P_2 values in insects: out of 11 heteropteran species reviewed by Simmons (2001) four species show median P_2 values higher than 0.75 (2 species show near complete second male precedence with values higher than 0.9), one species show variable mean P_2 values between 0.4 and 1, and the majority (6 species) show intermediate values (ranging from 0.5 to 0.68) (Economopoulus and Gordon 1972; Harwalkar and Rahalkar 1973; Smith 1979b; Sillén-Tullberg 1981; McLain 1985; Arnqvist 1988; McLain 1989; Rubenstein 1989; Carroll 1991; Ueno and Ito 1992; Arnqvist and Danielsson 1999a, 1999b). In this order, high variance in P_2 values is characteristic of intermediate values (standard deviations of P_2 between 0.23 and 0.49, range from 0 to 1), as it has been proposed to be a general pattern in insects (Simmons and Siva-Jothy, 1998). The mean P_n value of *P. laciniata* and its variance associated are close to those found for other heteropterans such as *Oncopeltus fasciatus* whose mean P_2 value is 0.50 (range 0.03-0.63, Economopoulus and Gordon 1972), *Jadera haematoloma* (mean P_2 = 0.62, range 0.05-0.95, Carroll, 1991), and *Nezara viridula* (mean P_2 = 0.51, range 0-1, McLain, 1985).

Paternal care and confidence of paternity in natural populations of the golden egg bug

Female golden egg bugs do not lay clutches of eggs, but rather they lay eggs continuously over several months. This means that females can lay between 0 and 10 eggs on any given day. Just after copulation has ended females tend to lay a greater number of eggs. The first day after copulation females laid a mean of 4.72 ± 2.65 eggs followed by lower numbers for the next four days (ranging from 1.22 to 2.61). This fact favours the occurrence of paternal care because it means that males are likely to be present when females lay eggs after copulation. In our study 41.18% of eggs laid the first day were laid on males, despite the short interval between the end of copulation and the experimental removal of males.

Our results indicate that in a natural population a male who copulates with a female will enjoy an intermediate confidence of paternity that remains constant with the time, i. e. it is expected that he would sire on average around 43% of the offspring produced by the female, independently of the time since the last copulation The prediction of an expected paternity around intermediate values is supported by the fact that the mean paternity for a subsample of males on which eggs were laid immediately after copulation is 0.38. This value also suggests that males do not accept preferentially their true genetic offspring.

According to the model by Westneat and Sherman (1993) males are predicted to decrease their parental effort as a response to a reduction in paternity when:

1. Males can discriminate their own young, and may chose to care only for their true genetic offspring. In general, data provided by the study of avian systems suggest that males are not able to discriminate their own from unrelated chicks (see Westneat and Sherman 1993, and references therein for a discussion about this topic on birds and other taxa). Males tend to rely upon indirect cues to estimate probability of paternity in the brood as a whole, such as the proportion of exclusive mating access with the female (Burke *et al.* 1989; Davies *et al.* 1992). No cases are known in which males can discriminate eggs according to their genetic relationship, and it is very unlikely that golden egg bug males can discriminate their

genetic offspring since it would imply an ability to discriminate eggs fathered by the male from other eggs (males only carry eggs until the nymphs hatch). Unlike avian systems in which males have to decide whether to care for a whole brood, golden egg bug males make individual decisions every time an egg is laid; thus, they are likely to assess the probability that each egg has of being their true genetic offspring, and not the probability in a brood as a whole.

2. Survival costs to offspring as a result of male desertion are not high. Whittingham *et al.* (1992) predicted that when male care is critical to offspring survival, males might provide care in an all-or-nothing way, caring for young unless confidence of paternity falls below a very low threshold, and empirical tests have found support for this prediction (Whittingham *et al.* 1993; MacDougall-Shackleton and Robertson 1998). It is clear now that in *P. laciniata* eggs laid on plants suffer very high mortality rates: only around 3% of the eggs laid on plants survive until hatching because of parasitation and predation, while 25% of eggs laid on adults survive (Reguera and Gomendio 2002). Thus, male caring is crucial to offspring survival and males could invest parentally in the eggs laid after mating if there are chances of that those eggs have been fertilized by him (specifically, a male's probability of siring an egg after mating is, on average, 0.43, which provides, on average, the evolutionary equivalent of 4 genetic descendants for each 10 eggs accepted).

3. Confidence of paternity is expected to improve in future breeding attempts. Westneat and Sherman's (1993) model predicts that, if individuals' levels of paternity in future reproductive episodes are, on average, the same as in the present, there will be nothing to be gained by males reducing their levels of care in any one breeding attempt. In a similar way, Maynard Smith (1978) and Grafen (1980) pointed out that if paternity is the same in all breeding attempts, then it should have no effect on the optimal form of parental behaviour. In the golden egg bug system each egg represents a "breeding attempt" and confidence of paternity remains, on average, constant through time. Last mating males have, on average, a probability of siring an offspring around 0.43, irrespective of the time since the end of the copulation. This intermediate level of paternity by the last male to mate is likely to be the result of sperm mixing, and will therefore remain at such intermediate levels in future matings either with the same female or with other females. The lack of better paternity prospects in the future thus explains in part why males accept eggs at intermediate levels of confidence of paternity.

In most cases the main cost for males of investing in offspring is a reduction in the possibilities of mating with other females (Zeh and Smith 1985; Clutton-Brock 1991; Smith 1997). However, in *P. laciniata* this does not seem to be the case since egg-carrying males continue to search for other females and mate with them. This is because eggs are glued on the backs and males do not have to remain in a particular place looking after the eggs, neither does egg carrying imply any additional activities that could reduce the time assigned to mate searching and copulation. The reduction in mobility that egg carrying entails is unlikely to affect the search for mates, since it only limits the ability of males to fly, a rare behaviour which only occurs when adults have to escape from a predator. Thus, egg carrying by males does not entail a cost in terms of mating effort, but it does imply a survival cost to males who become more vulnerable to predators (Reguera and Gomendio 1999; Kaitala *et al.* 2000). In other species, such as giant water bugs from the subfamily Belostomatinae, male water bugs that are completely egg-covered are rejected as mates by females because they have no room for any additional eggs (Smith 1976; Smith 1979a). This does not happen in *P. laciniata* because males accept eggs from different females sequentially over the reproductive season, and as they hatch after several days they are lost. Lack of space in males' backs does not seem to be a limiting factor for females.

In summary, female golden egg bugs are polyandrous and store sperm in the spermatheca. When a male copulates with a non-virgin female

his probability of siring offspring is around 0.43 for at least the following 5 days if no other copulations take place. This strongly suggests that sperm mixing is the most likely sperm competition mechanism. Males are unlikely to distinguish eggs sired by them from unrelated eggs, and are therefore unable to accept their own eggs exclusively. Despite the intermediate levels of confidence of paternity males accept eggs because sperm mixing implies that there are no chances of improving confidence of paternity levels in future breeding attempts, and because eggs suffer very high mortality rates when laid on plants. For males the risk of mistakenly leaving one's own offspring on plants, seems higher than the risk of accepting unrelated eggs. In addition, males do not suffer a loss of opportunities to mate with other females while they carry eggs, although they do become more vulnerable to predators.

From the point of view of the female it is worth asking why is sperm mixing the mechanism favoured by female genital morphology. It has been widespread accepted that when females benefit from male investment in offspring, mechanisms which ensure high levels of confidence of paternity should be favoured. Such mechanisms tend to be those resulting in high levels of last male precedence. Thus, the fact that sperm mixing occurs in *P. laciniata* and that it results in intermediate levels of paternity seems paradoxical. Closer scrutiny of this model suggests other interpretations. Golden egg bug females are unable to lay eggs on themselves, but they derive major fitness benefits if they lay eggs on other individuals because of the high mortality rates that eggs laid on plants suffer. Thus, female are forced to "parasitise" the care provided by other adults. Data from natural populations show that, as expected, other females do not accept eggs frequently since they are unlikely to be related to the offspring. This means that males are the best option for females, but males will be unlikely to accept eggs unless their confidence of paternity is high enough. In this sense female have two main choices: either they ensure high levels of paternity to the last male to copulate, in which case they may maximise the chances that males will accept eggs only after copulation, but minimise the chances that males will accept eggs under other circumstances, or they may provide intermediate levels of paternity to all males who have copulated with the female at some point. When females lay clutches of eggs it may be a good option to ensure high levels of paternity to the last male and in this way maximise the chances that he will accept the whole clutch. On the contrary, *P. laciniata* females lay eggs almost continuously over several months and it is in their interest to maximise the chances that each time an egg is laid (an event which may take place several times each day) there will be a male willing to accept it. In this context it may be a better strategy for females to develop a mechanism that results in sharing of paternity between several males, than a mechanism that gives high paternity to just one male. In addition, sperm mixing implies that, as long as other copulations do not take place, paternity remains constant through time, which means that males will tend to accept eggs not just after copulation but also for longer periods of time. Finally, when offspring have low survival prospects when left unattended, as in this case, males will not be too strict about the levels of paternity needed to accept eggs. Under this perspective it seems reasonable to suggest that in a system in which offspring depend strongly on the care from other conspecifics, and females need to maximise the number of males willing to accept eggs, the best strategy for females may be to give a share of paternity to all males with whom they have copulated. Male willingness to care for offspring in this system has evolved because offspring survival prospects are more important than the risk of accepting unrelated eggs, and because males suffer no costs in terms of mating effort.

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CAPÍTULO 8

Discusión General

1. Avances en la comprensión de *Phyllomorpha laciniata* a partir del estudio de poblaciones naturales

El estudio de poblaciones naturales de *Phyllomorpha laciniata* ha permitido que se avance en el conocimiento del ciclo reproductor, comportamiento, y ecología de este insecto. Esta información ha sido particularmente útil para comprender un modelo del que hasta hace poco no se conocía prácticamente nada de su ciclo y de sus patrones de comportamiento en el medio natural. La evidencia recopilada ha servido, pues, para poder interpretar en un contexto ecológico el significado adaptativo de los caracteres bajo estudio, y para poder diseñar en el laboratorio experimentos cuyos resultados sean generalizables al medio natural. Además de los datos presentados en los Capítulos que componen esta Tesis, durante su realización se ha recopilado información de estudios de captura-recaptura que han sido de gran utilidad para entender de una forma más global el ciclo biológico de *P. laciniata*. Estos datos se han obtenido en estudios realizados en el campo en tomas de datos semanales o quincenales durante la estación reproductiva de este insecto a lo largo de tres años (1998: 18 muestreos con periodicidad semanal, desde el 20 de abril al 17 de agosto; 1999: 18 muestreos con periodicidad semanal desde el 13 de abril hasta el 9 de agosto; 2000: 9 muestreos con periodicidad quincenal desde el 7 de abril hasta el 28 de julio) en la misma población estudiada por Reguera (1999), situada en Villaviciosa de Odón (Madrid). En resumen, algunos de los aspectos más destacables que los datos de poblaciones naturales han aportado son los siguientes:

1. Una diferencia entre los sexos en los patrones naturales de transporte de huevos: una mayor proporción de machos que de hembras porta huevos y los machos portan significativamente más huevos que las hembras.

2. La razón de sexos se mantiene invariable a lo largo de la estación reproductiva en 1:1.

3. Las hembras ponen huevos a lo largo de la estación reproductiva de manera continua. Se puede considerar que cada evento de ovoposición de las hembras da lugar a un único huevo y más raramente a una secuencia en la que se ponen dos o tres huevos en serie; las hembras no realizan puestas aisladas de muchos huevos separadas por largos intervalos de tiempo.

4. Los huevos sufren altas tasas de parasitismo debido al himenóptero parasitoide *Gryon bolivari* y parece haber un ajuste entre los ciclos del parásito y los de *P. laciniata* (ver más adelante la sección "El parasitismo de los huevos como posible presión selectiva que conduce al cuidado paternal").

5. La información obtenida del seguimiento de las poblaciones naturales también ha mostrado una pieza clave en el conocimiento del ciclo: los adultos que surgen de los huevos puestos el año en curso no se aparean ese año, ya que su actividad reproductiva no comienza hasta la próxima estación reproductiva tras el invierno. No es de extrañar, por lo tanto, que a partir de la fecha en la que empiezan a surgir estos nuevos adultos, los apareamientos, y la intensidad del transporte de huevos en la población, vaya declinando. Los individuos de segunda generación no se aparearán hasta después de la diapausa, en la primavera del siguiente año, por lo que podemos considerar el periodo invernal como una diapausa reproductiva, cuyo final (en la primavera del año siguiente) marca el inicio de un nuevo ciclo. Estudios realizados en el laboratorio han corroborado este aspecto: de un total de 46 parejas establecidas con hembras que emergieron de huevos producidos el año en curso sólo tres realizaron cópulas y ninguna de ellas puso huevos.

6. El ciclo biológico de esta especie, a su vez, nos informa sobre un hecho importante a tener en cuenta en diseños experimentales en los que es deseable el empleo de hembras vírgenes (por ejemplo, estudios que investiguen aspectos de la competencia espermática como el que se muestra en el Capítulo 4): únicamente hembras colectadas muy al principio de la estación reproductora y que cumplan una serie de requisitos pueden ser consideradas vírgenes. El uso de hembras adultas

llegadas al estado de imago por la cría de estadios ninfales avanzados (al menos superiores al primer estadio ninfal) también da como resultado la obtención de hembras vírgenes, sin embargo la gran mayoría de estas hembras no se apareará hasta el año siguiente. Se pueden obtener hembras vírgenes receptivas dejando que las hembras hibernen en instalaciones naturales aisladas de machos, y recogiéndolas en la estación reproductiva siguiente. A pesar de la obtención de hembras vírgenes y de que éstas hembras se apareen con normalidad todavía queda sin explicación la razón por la cual las hembras vírgenes apareadas en cautividad no ovipositan con normalidad. Esto limita en cierta medida su uso en, por ejemplo, estudios en los que sea necesario conocer el éxito reproductivo de las hembras vírgenes, y complica la experimentación en estudios de paternidad bajo condiciones experimentales concretas (por ejemplo, como las del Capítulo 6).

El análisis detallado de los patrones naturales en cuanto al transporte de huevos realizado en el Capítulo 2 ha puesto de manifiesto que los machos y no las hembras muestran diferencias en cuanto al número de huevos recién puestos que portan y en cuanto a la proporción de individuos que portan estos huevos, según se considere individuos que se están apareando o individuos aislados. En conjunto, los resultados obtenidos en dicho Capítulo apuntan a que los huevos puestos recientemente que portan los machos en cópula han sido puestos por la hembra con la cual se están apareando. La situación más probable que conduce a la aceptación de esos huevos es la realización de cópulas repetidas por parte de los machos. Los machos realizarían una estrategia de aseguración de la paternidad por medio del establecimiento de cópulas repetidas, lo que se alternaría con la aceptación de huevos durante los intervalos entre las cópulas. Esto es lo que ocurre en las chinches acuáticas gigantes: Smith (1979) mostró que los machos de *Abedus herberti* sólo aceptan huevos tras copular con una hembra, y alternan la aceptación con la realización de cópulas repetidas con la misma hembra. De manera similar, el macho del escarabajo enterrador con cuidado paternal *Necrophorus vespilloides* realiza cópulas repetidas para incrementar la probabilidad de que los huevos puestos por la hembra hayan sido fecundados por él (Müller y Eggert, 1989). En *P. laciniata* se ha observado que, a menudo, las hembras en cautividad se aparean de manera múltiple con el mismo macho (Kaitala, 1998; García-González, no publicado). Por otro lado, se ha visto también que los machos permanecen junto a la hembra con la que previamente se han apareado con mayor frecuencia que junto a otras hembras (Kaitala, 1998). Por último, las hembras depositan huevos sobre los machos despúes de la cópula (Kaitala y Miettinen, 1997, y presente Tesis). En esta Memoria de Tesis se muestra que los huevos son puestos en un corto periodo después de la cópula y que una proporción de estos huevos son fecundados por el último macho que ha copulado con la hembra. Por lo tanto, a la luz de todos estos resultados, la hipótesis de la aseguración de la paternidad por cópulas repetidas debe ser considerada como una interpretación plausible del origen de la aceptación de huevos por parte de los machos.

Los datos obtenidos en el Capítulo 2 han refutado la hipótesis de puesta de huevos sobre individuos en cópula por hembras ajenas a las que se encuentran apareándose, propuesta por Kaitala (1996) para explicar el comportamiento de transporte de huevos en *P. laciniata*. A pesar de que esta hipótesis pudiera explicar en parte la carga de huevos que portan los machos en cautividad (cuando los individuos están en condiciones de alta densidad de hembras), no tiene un valor predictivo aceptable para explicar el transporte de huevos en la naturaleza. Es importante en este punto matizar el término de "parasitismo intraespecífico de puesta" que se ha aplicado a *P. laciniata*. El parasitismo de puesta intraespecífico se define como la puesta de huevos por parte de una hembra en el nido de otra, y, consecuentemente, las hembras que practican esta estrategia lo que parasitan es el cuidado parental de otros individuos (Petrie y Møller, 1991). El parasitismo intraespecífico de puesta es, entonces,

necesariamente y por definición, posterior a la aparición del cuidado parental, puesto que no se puede parasitar el cuidado parental si éste previamente no existe. Por ello, es incorrrecto proponer que las hembras de *P. laciniata* desarrollan parasitismo intraespecífico en ausencia de cuidado paternal tal y como sugiere la hipótesis del "mating pair intraspecific brood parasitism" (Kaitala, 1996; Kaitala, 1998; Härdling y Kaitala, 2001; Katvala y Kaitala, 2001; Tallamy, 2001).

De los estudios realizados en esta Tesis se desprende que el análisis del transporte de huevos en *P. laciniata* no es simple y que requiere la formulación y contraste de varias hipótesis. Queremos hacer hincapié en el alto valor que tiene el conocimiento de lo que ocurre en la naturaleza para sacar conclusiones a partir de estudios realizados en condiciones controladas. Con respecto a esto podemos hacer notar aquí que se ha citado que, en cautividad, los individuos de *P. laciniata* que portan huevos se descargan de algunos de estos huevos al frotarse activamente con las plantas (Kaitala, 1999). Esta autora ha sugerido que este comportamiento puede ser una contra-adaptación a la puesta de huevos. Sin embargo, ni durante el estudio de Reguera (1999) ni durante el presente estudio se ha observado tal situación. En el momento de la ovoposición el huevo queda firmemente pegado al substrato (ya sea planta o coespecífico) debido a una sustancia pegajosa que recubre la zona basal del huevo. La eliminación de los huevos es, por tanto, extremadamente difícil, y puede producir graves daños al individuo portador, aunque puede producirse casualmente, especialmente en individuos que portan un gran número de huevos y alguno de ellos ha sido situado en una posición precaria. En *Abedus herberti*, especie en la que, como se ha citado anteriormente, los machos muestran cuidado paternal por transporte y cuidado de los huevos en el dorso, Smith (1976) describe que los individuos que se encuentran en cautividad a altas densidades y en condiciones de stress muestran un comportamiento de eliminación de huevos, pero que sin embargo este comportamiento nunca es observado en la naturaleza. Las observaciones de Kaitala (1999) podrían tal vez responder a esta situación, puesto que en condiciones de limitación de espacio y de alta densidad los individuos acaban portando un número de huevos muy superior al rango encontrado en poblaciones naturales, por lo que los huevos que en lugar de estar pegados al cuerpo del adulto acaban pegados a otros huevos situados en capas inferiores si que podrían ser eliminados voluntariamente. Sin embargo, esta situación de sobrecarga de huevos nunca se da en poblaciones naturales, por lo que el valor de dicho comportamiento como estrategía de eliminación de huevos por parte de los individuos no parece relevante.

En definitiva, los resultados de los estudios sobre estrategias reproductivas son difícilmente generalizables, o las conclusiones sobre su valor adaptativo poco acertadas, si existen carencias notables en el conocimiento de la ecología de la especie que no permitan identificar las fuerzas selectivas que están operando sobre poblaciones naturales. Durante la realización de esta Tesis, los estudios longitudinales en el tiempo de poblaciones naturales, los patrones de transporte de huevos inferidos de recolecciones de individuos a lo largo de toda la estación reproductora durante varios años, y la realización de experimentos básicos han aportado una importante base biológica en la que se han enmarcado los estudios llevados a cabo.

2. Los intereses de la hembra

Este estudio ha demostrado que las hembras de *P. laciniata* muestran una preferencia por realizar la ovoposición sobre coespecíficos, ya sean del sexo masculino o femenino. La razón de esta preferencia reside en los beneficios en términos de supervivencia que obtiene la descendencia como resultado del transporte por parte de los adultos (Reguera y Gomendio, 2002). Las preferencias y elección de los lugares de ovoposición han sido puestas de manifiesto en otras especies, principalmente de insectos y anuros (Resetarits y Wilbur, 1989; Thompson y

Pellmyr, 1991; Godfray, 1994; Resetarits, 1996), sin embargo, un caso como el que se presenta en el sistema de *P. laciniata* en el que el substrato óptimo de ovoposición son los individuos de la misma especie no ha sido descrito hasta la fecha en ninguna otra especie de insecto terrestre.

Es importante considerar que, debido a que en esta especie las hembras ponen un huevo a la vez, y que estos eventos de ovoposición se repiten de forma continuada (al menos cada 2 ó 3 días) a lo largo de toda la estación reproductora, una hembra se enfrenta un gran número de veces a la decisión de ovopositar un huevo determinado en la planta hospedadora (con los consiguientes riesgos en la supervivencia de los huevos) o buscar un sitio de ovoposición óptimo (esto es, un coespecífico), lo que a su vez podría afectar a la fecundidad total femenina si retrasa de forma significativa la puesta de cada huevo. Este tipo de compromisos reproductivos son comunes en insectos, especialmente en parasitoides (Rosenheim, 1999; Rosenheim et al., 2000), y en el caso de *P. laciniata* la solución parece ser la ovoposición, cuando no hay individuos coespecíficos disponibles, sobre la planta hospedadora. Esta estrategia, podría considerarse como "lo mejor dentro de lo malo" ("the best of a bad job"), bajo la cual una hembra en ausencia de coespecíficos sobre los que intentar la ovoposición se beneficiaría de la puesta en planta y de la continuación de la producción de huevos teniendo en cuenta que, en este substrato, existe una probabilidad, aunque muy baja, de que el huevo sobreviva.

Sin embargo, sería lógico esperar que, dados los enormes beneficios de depositar huevos sobre coespecíficos, la ovoposición se viera estimulada ante la presencia de adultos, pues las bajas densidades encontradas en poblaciones naturales sugieren que las hembras no se encuentran a menudo cerca de coespecíficos sobre los que depositar huevos. Los resultados obtenidos apoyan esta hipótesis pues demuestran que la ovoposición de las hembras se ve duplicada ante la presencia de coespecíficos. La presencia de coespecíficos no sólo estimula la ovoposición, sino que el efecto de los coespecíficos actúa sobre los niveles de producción y maduración de los huevos. Los resultados, por lo tanto, apoyan la idea de que el comportamiento en la ovoposición, y en general, la fisiología reproductiva está determinada de una forma adaptativa por la variabilidad en la calidad de los diferentes substratos de ovoposición y por su disponibilidad. Esta relación ha sido propuesta con anterioridad para otros insectos (Engelman, 1970; Papaj y Messing, 1996; Papaj, 2000), sin embargo en ninguno de estos casos el substrato óptimo de ovoposición lo constituyen los coespecíficos.

La estimulación por simple presencia de coespecíficos de los eventos reproductivos femeninos ha sido demostrada en muy pocas especies de insectos. Por el contrario, en este grupo si se han documentado abundantes casos de estimulación ligada al apareamiento. Por otra parte, se ha visto que la estimulación de la ovoposición en insectos generalmente incrementa el éxito reproductivo masculino. Un ejemplo claro de esto es *Drosophila melanogaster*, especie en la que el macho transfiere junto con el eyaculado sustancias que además de provocar un aumento de la ovoposición, retrasan la receptividad de las hembras e imponen un coste en la supervivencia femenina (Partridge et al., 1987; Chapman et al., 1995; Rice, 1996; Rice y Holland, 1997; Holland y Rice, 1999; Johnstone y Keller, 2000; Wolfner, 2002). Sin embargo, los posibles beneficios para las hembras resultantes de la estimulación no han sido suficientemente explorados en ésta y otras especies de insectos en las que ocurre estimulación de la ovoposición durante el apareamiento, o en algunas especies de mamíferos en las que ocurre estimulación por la presencia de coespecíficos o por el apareamiento (ver por ejemplo McComb, 1987).

La asociación, en *P. laciniata*, entre la respuesta femenina a la presencia de substratos de ovoposición óptimos y el aumento de las probabilidades de supervivencia de los huevos depositados sobre coespecíficos apunta claramente a que las hembras se benefician de la estimulación de la ovoposición. Es especialmente

interesante para la comprensión del sistema el hecho de que la estimulación ocurra igualmente si el individuo coespecífico receptor de los huevos es macho o hembra. Como la supervivencia de los huevos se ve incrementada por el transporte de los adultos, independientemente del sexo del individuo transportador, el sentido adaptativo de este comportamiento es claro: la estimulación de la ovoposición es, muy probablemente, una estrategia femenina que se ha seleccionado debido a que incrementa el éxito reproductivo de las hembras, por medio de la mejora en las esperanzas de supervivencia de la progenie.

3. Una herramienta molecular para la comprensión del sistema

La comprensión del comportamiento de este insecto estaba en gran medida limitada por la necesidad de una herramienta molecular que permitiera determinar las relaciones genéticas entre los individuos que portan huevos y los huevos portados (ver Gomendio y Reguera, 2001). En el Capítulo 5 se ha mostrado el desarrollo de los marcadores AFLPs para su aplicación sobre *P. laciniata*. Primero, se han mostrado y evaluado varios métodos para realizar la extracción de ADN en este insecto. El método basado en la extracción en un tampón de CTAB es el que ofrece los mejores resultados y es el que se propone para realizar futuros estudios que necesiten aislar el material genético de este organismo. Segundo, se han evaluado combinaciones de cebadores AFLPs y se han obtenido dos parejas que ofrecen resultados satisfactorios. Tercero, mediante la aplicación de los cebadores elegidos se ha valorado (1) el grado de polimorfismo en poblaciones naturales de *P. laciniata*, que alcanza valores superiores al 92%, (2) el grado de repetibilidad que alcanza la técnica (96.6%), y (3) la correlación en la migración de los fragmentos, que ha demostrado la existencia de independencia en la migración (independencia de los *loci*).

Recientemente, y trás la conclusión de los estudios expuestos en esta Tesis, se ha tenido acceso a un estudio no publicado (Miettinen, 2001), en el que se utilizan microsatélites para analizar paternidad en esta especie. Es importante subrayar que las dos técnicas utilizadas para abordar el problema de paternidad en este insecto (microsatélites y AFLPs) se han desarrollado de manera independiente y que son consideradas herramientas de trabajo complementarias. Esto es, no realizan una descripción única o universal del material genético de la especie, sino que describen características genéticas de poblaciones o relaciones genéticas entre individuos, y son utilizadas, en este caso en concreto, para inferir el valor adaptativo de patrones biológicos o comportamientos. Las técnicas moleculares pueden ser empleadas de diferentes maneras para dar respuesta a múltiples preguntas relacionadas con un mismo aspecto, para contrastar hipótesis diferentes, para analizar poblaciones diferentes, o poblaciones sujetas a diferentes condiciones ambientales o experimentales, por poner unos ejemplos. Por otra parte, el uso de herramientas diferentes en la resolución de un problema científico puede ser ventajoso al reforzar las hipótesis por el uso de diferentes metodologías, o bien al ofrecer controversias o resultados opuestos que incitan y aceleran la resolución del problema. Por todo ello, se considera positivo el desarrollo y uso de estas dos técnicas (microsatélites o AFLPs) en la intención de responder a preguntas de índole ecológica o evolutiva en *P. laciniata*. En esta Tesis se ha comprobado que la aplicación de los marcadores AFLPs tiene un gran potencial para responder, en *P. laciniata*, a preguntas en las que sea preciso conocer las relaciones genéticas entre los individuos. Mediante esta técnica se pueden obtener multitud de fragmentos de restricción que determinen la identidad genética de los individuos, poblaciones, etc. El uso de esta técnica conlleva una serie de ventajas en comparación con otros marcadores genéticos como son (1) que no es necesario tener un conocimiento previo sobre la secuencia de nucleótidos del ADN, (2) que cantidades ínfimas de tejido pueden ser procesadas, (3) que muestras de ADN

parcialmente degradadas pueden ser utilizadas, (4) que es altamente fiable debido a su alta repetibilidad, y (5) que la obtención de un gran número de marcadores es económicamente viable en cualquier especie (Vos et al., 1995; Vos y Kuiper, 1997; Mueller y Wolfenbarger, 1999; Gerber et al., 2000).

4. Una primera aproximación a la existencia de cuidado paternal en *Phyllomorpha laciniata*

En el presente estudio se han aplicado los AFLPs para determinar paternidad en *P. laciniata*. Por otra parte, se ha desarrollado una metodología para determinar el padre genético, cuando no se conoce éste ni tampoco la madre genética, de entre una serie de madres potenciales. Esta metodología puede ser de utilidad en estudios futuros sobre otros sistemas con la misma problemática experimental mostrada en el Capítulo 6.

Los resultados obtenidos muestran que bajo las condiciones experimentales en las que se ha llevado a cabo el estudio, los machos portan una gran proporción de huevos que no han sido fertilizados por ellos. Sin embargo, la mayoría de huevos que componen la fracción de los que no han sido fecundados por el macho portador fueron fecundados por machos con los que la hembra se apareó previamente a su recolección en el campo, y no por los machos utilizados en los grupos experimentales. Esto se podría explicar por la ausencia de inseminación de las hembras en algunos grupos experimentales, ya sea por ausencia de cópulas o por ausencia de transferencia de esperma (que como se ha visto en el Capítulo 4 puede ocurrir con relativa frecuencia). Sin embargo hay otra explicación, que tras la conclusión de los otros estudios de esta Tesis, parece, en gran medida, ser responsable de este patrón. Ésta sería la existencia de un mecanismo de competencia espermática de mezcla de esperma ("sperm mixing") que, como se ha visto en el Capítulo 7, es el mecanismo que determina los patrones del uso de los espermatozoides de machos rivales en este insecto. Con un mecanismo de mezcla de esperma la representación gamética de los machos utilizados en los experimentos probablemente estuvo limitada, y dió lugar a bajas tasas de paternidad por parte de estos machos, puesto que las hembras presentaban espermatozoides de otros machos con los que se aparearon con anterioridad a su recolección en el campo.

Considerando los huevos que fueron fertilizados por machos de los grupos experimentales, un 30.8% era transportado por el padre genético. La magnitud de la tasa de paternidad se valora de forma adecuada teniendo en cuenta que las condiciones experimentales promovían en gran medida el parasitismo por parte de las hembras. Las condiciones de densidad en las que se mantuvieron los individuos fueron varias veces superiores a las que se encuentran en la naturaleza. Esta alta densidad no sólo puede haber operado con interferencias en el establecimiento de parejas, o acoso a parejas en cópula. Las altas condiciones de densidad también deben haber operado en los grupos en los que si ha habido fertilización de huevos por los machos utilizados en el experimento. En estos casos las condiciones de alta densidad dificultan que la hembra deposite los huevos sobre el padre genético, ya que en estos grupos se espera que los machos tengan una baja paternidad debida a la alta densidad del sexo masculino. Además, la alta densidad favorece que un número elevado de huevos que no han fecundado los machos experimentales sean depositados sobre ellos puesto que los verdaderos padres genéticos no se hallan presentes, y los machos se ven forzados a una proximidad espacial con las hembras ,independientemente de que hayan copulado con ellas o no. El diseño del experimento, realizado bajo la presunción de que el mecanismo de competencia espermatica más probable era el de precedencia del último macho que copula con la hembra (por ser el más común en insectos), y con una elevada densidad para facilitar las cópulas, parece haber forzado la puesta sobre machos no relacionados con los huevos. A la vista de los

resultados ello se debe a que las hembras ya tenían espermatozoides almacenados de machos con los que habían copulado en el campo. Al ser el mecanismo de competencia espermática el de mezcla de esperma, los niveles de paternidad de los machos experimentales fueron muy bajos, pero al estar espacialmente limitados es muy posible que tuvieran más dificultad para evitar las puestas que los machos en poblaciones naturales.

Miettinen (2001) utilizando microsatélites arroja una tasa de paternidad para los huevos portados por machos colectados en el campo de alrededor del 20%. Esta autora considera que una tasa baja de paternidad refleja la ausencia de cuidado paternal. Sin embargo, hay que tener presente que, primero, el cuidado paternal se define como cualquier cuidado realizado por los progenitores que incremente con alguna probabilidad la supervivencia de la progenie (Clutton-Brock, 1991). Por lo tanto, una baja tasa de paternidad en absoluto indica que no exista cuidado parental. Segundo, en un estudio experimental como el que se ha realizado en el Capítulo 6 el papel del cuidado paternal en la evolución del comportamiento de transporte de huevos por parte de machos debe ser examinado contrastando la tasa de paternidad real y la que se esperaría por simple ovoposición y aceptación de huevos al azar. Los resultados obtenidos en dicho Capítulo, utilizando métodos de Monte Carlo, apuntan a que, en conjunto, los machos portan más huevos fecundados por ellos mismos que los que se esperaría si hubiera una distribución al azar, lo que indica un papel activo del macho receptor de huevos en la aseguración de paternidad y posterior aceptación de huevos. Finalmente, la tasa de paternidad hay que ponerla en relación con el balance de costos/beneficios para la descendencia y para los progenitores que efectúan el cuidado parental (ver más adelante la sección "Cuidado parental y confianza de paternidad en poblaciones naturales de *P. laciniata*"). Es importante mencionar que, en el caso de muchas especies de aves en las que los machos participan del cuidado de las crías a pesar de que una elevada proporción no son sus descendientes genéticos, el problema no se ha resuelto negando que los machos lleven a cabo cuidado paternal, sino desarrollando modelos que ayuden a explicar bajo qué condiciones les compensa a los machos cuidar de crías aunque algunas de ellas no sean suyas.

Al margen de los resultados obtenidos en este estudio hay que recalcar que esta ha sido una primera aproximación experimental al problema de la paternidad de los huevos portados por los machos. Hay que enfatizar que la importancia del trabajo expuesto en el Capítulo 6 reside en el desarrollo de una técnica y una metodología asociada que permite determinar paternidad en este insecto, y en la demostración de que el cuidado paternal puede jugar un papel importante en este sistema. La puesta a punto de estas técnicas ha supuesto un ingente esfuerzo puesto que no se disponía de una herramienta molecular adecuada. Además, en éste modelo no sólo se desconoce el padre, sino también la madre, por lo que ha sido necesario determinar estadísticamente, a partir de los resultados moleculares, tanto la probabilidad de que una hembra sea la madre, como la probabilidad de que un macho sea el padre. En base a los resultados obtenidos es difícil saber si la tasa de paternidad encontrada en este estudio refleja la que ocurre en poblaciones naturales debido a que (1) una alta proporción de huevos fueron fecundados antes de que empezara el experimento, (2) el mecanismo de competencia espermática ha resultado ser diferente al esperado, y (3) las densidades experimentales que se han utilizado sobrepasan en gran medida las densidades de individuos en la naturaleza. Estos aspectos apuntan, como se ha mencionado, a que posiblemente la tasa de paternidad natural esté infravalorada en el estudio realizado en el Capítulo 6. No obstante, con el trabajo mostrado en dicho Capítulo se establece el punto de partida a partir del cual se pueden realizar estudios futuros que manipulen condiciones experimentales cercanas a las encontradas en la naturaleza en busca de su efecto sobre las tasas de paternidad, y así comprobar si se cumplen las predicciones teóricas acerca de la relación entre la confianza de paternidad y el cuidado paternal.

5. Los intereses del macho en condiciones de competencia espermática

Los machos de *P. laciniata* ajustan el tamaño del eyaculado de acuerdo con las predicciones de los modelos de riesgo de competencia espermática (ver por ejemplo Parker, 1990, 1998; Parker et al., 1997; Ball y Parker, 1998). Los resultados obtenidos en el Capítulo 4 han puesto de manifiesto que la presencia de machos rivales tiene un efecto doble sobre la inversión en eyaculado transferido por parte del macho que se aparea. Por un lado, la cópula se prolonga, lo que se traduce en más tiempo dedicado a la transferencia de espermatozoides, y por otro lado, el macho incrementa la tasa de transferencia de espermatozoides por unidad de tiempo. Todo ello probablemente incrementa las probabilidades de fecundación de los huevos, debido a una mayor representación de espermatozoides en la espermateca femenina, en un contexto en el que la competencia espermática es altamente probable. Esto explica la razón de la existencia de cópulas largas y prolongadas en esta especie.

Las hipótesis de la guarda de la pareja (Alcock, 1994) y de "sperm loading" (Dickinson, 1986) predicen que las cópulas se prolongarán en situaciones en las que exista riesgo de competencia espermática. Para discriminar ambas hipótesis se requiere, o bien un análisis de la transferencia de espermatozoides a lo largo de la duración de la cópula, o bien un análisis del éxito de fertilización de los machos implicados (Simmons, 2001). En el presente estudio se ha llevado a cabo el primer análisis, y con él se ha podido rechazar la hipótesis de la guarda de la pareja, mientras que la del "sperm loading" se ha visto apoyada: el presente estudio ha demostrado que la transferencia de espermatozoides no ocurre solamente en los primeros instantes de la cópula, sino a lo largo de ella. Dentro de un marco comparativo con otros heterópteros, orden en el que se ha documentado con anterioridad la existencia de cópulas prolongadas (McLain, 1980; Sillén-Tullberg, 1981; Clark, 1988; McLain, 1989; Rubenstein, 1989; Carroll, 1991; Carroll, 1993), *P. laciniata* se diferencia de especies con cópulas prolongadas en las cuales la transferencia de esperma ocurre exclusivamente en los primeros instantes de la cópula. En estas otras especies, por lo tanto, parece ocurrir una estrategia de guarda de la pareja (Sillén-Tullberg, 1981; McLain, 1989; Carroll, 1991). Lo que ocurre en *P. laciniata* está en consonancia con especies en las que la transferencia ocurre también a lo largo de la cópula (por ejemplo ver Arnqvist y Danielsson, 1999). Sin embargo, la existencia de sperm loading llevado a cabo por un alargamiento de la cópula, junto con un incremento en la tasa de transferencia de espermatozoides, no ha sido documentada en ninguna especie, ni en estudios que han analizado el valor adaptativo de las cópulas prolongadas, ni en estudios que han analizado la inversión en eyaculado en el contexto de la competencia espermática.

La presencia de machos rivales durante el apareamiento es usada como señal por los machos de *P. laciniata* para percibir el riesgo de competencia espermática y eyacular de manera estratégica de acuerdo a este riesgo (recordemos que la producción de espermatozoides no está exenta de costos, por lo que a los machos les interesa optimizar la inversión en eyaculado). Desde una perspectiva general, las señales usadas por los machos para eyacular de manera estratégica en este contexto son variadas: los machos pueden usar la presencia de rivales como en *P. laciniata*, pueden usar el tiempo empleado en guardar a la hembra antes de la cópula como un indicador del riesgo pasado, o pueden usar el propio estatus de apareamiento que tiene en ese momento el macho (revisión en Parker et al., 1997; Ball y Parker, 1998). Otra señal puede ser el estado de apareamiento de la hembra; los machos de determinadas especies son capaces de discriminar si la hembra es virgen o no, proveyendo a las hembras no vírgenes con más espermatozoides (Cook y Gage, 1995; Wedell, 1998; Wedell y Cook, 1999; pero ver Wedell, 1992). Un caso interesante, por ejemplo, es el de la polilla *Plodia interpunctella*. En esta especie, la

presencia de machos rivales no tiene ningún efecto sobre el número de espermatozoides que insemina el macho que se está apareando, sin embargo, la presencia de espermatozoides de machos rivales previamente almacenados en la espermateca incrementa el número de espermatozoides transferidos (Cook y Gage, 1995). También los machos del heteróptero *Lygaeus equestris* permanecen más tiempo en cópula con hembras previamente apareadas que con hembras vírgenes (Sillén-Tullberg, 1981), y lo mismo ocurre en *Jadera haematoloma* (Carroll, 1991), a pesar de que la cópulas prolongadas no incrementan el tamaño del eyaculado en estas especies. En *P. laciniata*, sin embargo, la duración de la cópula no depende del estatus de la hembra (virgen vs. no virgen), como se ha visto en este estudio, sino que el gasto en eyaculado está determinado por la presencia de machos rivales.

En conjunto, los resultados no sólo corroboran las predicciones de los modelos teóricos de competencia espermática, sino que demuestran la estrategia que pueden seguir los machos de *P. laciniata* para maximizar el éxito reproductivo. Por otra parte, los resultados son importantes en la consideración de la existencia de cuidado paternal: la estrategía de los machos conduce a una maximización de las probabilidades de que el huevo o los huevos puestos trás la cópula sean fecundados por ellos. Esto constituye una pieza clave para la comprensión del sistema, puesto que es probable la conexión entre la maximización de las probabilidades de fecundación y la aceptación de huevos tras la cópula por parte de los machos.

6. Precedencia de esperma y mecanismos de competencia espermática en *P. laciniata*

Los resultados obtenidos en el Capítulo 7 han mostrado que el éxito en la fecundación del último macho que se aparea con una hembra es, en promedio, intermedio, y con una alta variabilidad. Por otra parte, este valor se mantiene constante con el tiempo a nivel poblacional, al menos durante un periodo de 5 días tras la cópula. Estos resultados indican que el mecanismo de competencia espermática que con mayor probabilidad opera en esta especie es la mezcla de esperma. Una serie de mecanismos alternativos han sido contemplados, pero las predicciones derivadas de ellos no se ven apoyadas por los resultados obtenidos en este Capítulo ni tampoco por la evidencia obtenida en los otros Capítulos. Entre los datos que apoyan la mezcla de esperma se encuentra la relación positiva entre la duración de la cópula y la transferencia de esperma que hemos observado en el Capítulo 4. La inversión en el número de espermatozoides transferidos bajo condiciones de competencia espermática es ventajosa en dos situaciones: cuando el esperma de los diferentes machos en competición se mezcla de manera más o menos homogénea en la espermateca, o bien cuando existe desplazamiento de los espermatozoides de machos rivales almacenado en la espermateca debido al flujo de esperma del último macho que copula con la hembra (Dickinson, 1986; Parker y Simmons, 1991; Eady, 1995; Parker, 1998; Wedell y Cook, 1998; Arnqvist y Danielsson, 1999). En esta especie, por lo tanto, la duración de la cópula sería un factor fundamental en la variación en el resultado de la competencia espermática, como ocurre en otras especies (Dickinson, 1986; McLain, 1989; Rubenstein, 1989; Nuyts y Michiels, 1993; Parker y Simmons, 2000; y ver, para una revisión, Simmons, 2001). Por ejemplo, en el heteróptero Ligeido *Neacoryphus bicrucis* la duración de la cópula del segundo macho se correlaciona positivamente con P_2 (McLain, 1989), mientras que en el Gérrido *Gerris remigis* únicamente hay precedencia del segundo macho (P_2>0.5) si su copula es más larga que la duración de la cópula del primer macho (Rubenstein, 1989).

En relación con los factores que pueden afectar a la proporción de descendencia producida por el último macho, aparte de la duración de la cópula se ha visto que en algunas especies el tamaño del macho, el intervalo entre las cópulas o la longevidad de los espermatozoides pueden influir en el valor de P_2 (Simmons et al., 1996; Bisoondath y Wiklund, 1997; Simmons y Siva-Jothy, 1998; Parker y Simmons, 2000). Por ejemplo, en la

chinche pentatómida *Nezara viridula*, se obtienen valores de P_2 bajos sólo si el tamaño del primer macho es mayor que el tamaño del segundo macho (McLain, 1985). En *P. laciniata* la influencia del tamaño parece negligible puesto que esta variable no afecta ni a la duración de la cópula ni al número de espermatozoides transferido, como se vió en el Capítulo 4. El intervalo entre las cópulas es un factor sobre el que no existe información hasta la fecha y que sería interesante examinar, aunque la mezcla de esperma posiblemente amortigua en gran medida el efecto que este factor pueda tener sobre la paternidad en *P. laciniata*.

En resumen, el Capítulo 7 muestra que la varianza y el patrón de variación con el tiempo de P_n, a un nivel individual y poblacional, apoyan la existencia de que exista una mezcla de esperma. El hecho de que P_n se mantenga constante a lo largo de los 5 días posteriores a la cópula sugiere de manera clara que el esperma de varios machos se mezcla tan pronto como llega a la espermateca femenina y así se mantiene a lo largo del tiempo. En cualquier caso, y a efectos prácticos, una media poblacional de 0.43 evidencia que, en general y a nivel poblacional, el éxito de fecundación por parte del último macho que copula con la hembra es intermedio.

El presente estudio constituye además la primera aproximación que se realiza a la determinación de los patrones de uso del esperma, y en definitiva, de los mecanismos de competencia espermática, por medio de la determinación de la paternidad con el uso de AFLPs. Como es evidente, la determinación de la paternidad es una pieza fundamental en el estudio de los patrones de precedencia de esperma y de la relación entre la confianza de paternidad y el esfuerzo parental. Tradicionalmente se han usado varias técnicas para inferir los patrones de uso del esperma. La técnica de esterilización de los machos ha sido la más usada para obtener información acerca de la proporción de descendencia producida por el último macho que se aparea con una hembra (ver por ejemplo Boorman y Parker, 1976; Sillén-Tullberg, 1981; Carroll, 1991; Siva-Jothy y Tsubaki, 1994; Cook et al., 1997; Wedell y Cook, 1998; Arnqvist y Danielsson, 1999). Esta técnica, sin embargo, presenta el inconveniente de que los individuos sufren una exposición a sustancias que pueden afectar a la producción de espermatozoides, a la viabilidad de la progenie, o a la capacidad de fecundación. El uso de marcadores morfológicos, es decir, mutaciones fenotípicas con herencia Mendeliana, se ha usado también para determinar la paternidad cuando más de un macho se aparea con una hembra (ver por ejemplo Smith, 1979; McLain, 1985; Lewis y Austad, 1990). Sin embargo, un problema inherente a este método es la posibilidad de un sesgo en la competitividad del esperma de los diferentes tipos de machos utilizados. Por último, los polimorfismos enzimáticos (alozimas), los cuales han sido utilizados, aunque menos que las técnicas anteriores, para determinar paternidad (Dickinson, 1986; Dickinson, 1988; LaMunyon, 1994; Conner, 1995; Sakaluk y Eggert, 1996; Calos y Sakaluk, 1998), presentan el inconveniente de que es necesario el conocimiento del genotipo de los padres previamente a la realización de los experimentos. El uso de los AFLPs, como ha quedado patente en este estudio, puede ser una alternativa seria a todas estas técnicas.

Por último, hay que hacer notar que la determinación de la paternidad en insectos ha sido mayoritariamente realizada en el laboratorio, donde las condiciones son controladas y los potenciales padres genéticos son conocidos y, en la mayoría de los casos, se limitan a dos machos que copulan en el orden y en el momento que determina el diseño experimental. Los estudios de paternidad llevados a cabo en poblaciones naturales han sido escasos (ver Dickinson, 1988; LaMunyon, 1994), debido, principalmente, a la dificultad de registrar todos los compañeros de apareamiento de las hembras. Hasta 1994, año en el cual LaMunyon determinó paternidad en una población natural de la polilla *Utetheisa ornatrix* por medio del empleo de alozimas, ningún trabajo estimó paternidad más allá de calcular umbrales mínimos y máximos de paternidad (Dickinson, 1988).

Por otra parte, los patrones de precedencia de esperma inferidos a partir de los valores de P_2 son generalmente extrapolados a los patrones que ocurren en la naturaleza. Sin embargo, se ha mostrado que en algunas especies los valores de P_2 obtenidos bajo condiciones controladas no reflejan lo que ocurre cuando la hembra se aparea con más de dos machos. Por ejemplo, Zeh y Zeh (1994) demostraron que, en el pseudoescorpión *Cordylochernes scorpioides*, la precedencia del segundo macho calculada en experimentos de doble apareamiento femenino arrojaba valores de P_2 cercanos a 1 (es decir, ventaja del último macho), mientras que en experimentos de triple apareamiento (calculo de P_3), el último macho no tenía predominancia en la fecundación de los huevos. El desarrollo reciente de métodos moleculares facilita el estudio de los sistemas de insectos en condiciones naturales, pero todavía hay pocos estudios que los hayan empleado con este propósito. El presente trabajo ha analizado los patrones de precedencia de esperma de hembras que se han apareado de manera natural en condiciones de libertad. Este tipo de estudios son necesarios para validar los resultados que se han obtenido para un gran número de especies en el laboratorio acerca de los mecanismos que explican la paternidad en especies poliándricas.

7. Cuidado parental y confianza de paternidad en poblaciones naturales de *P. laciniata*

En el Capítulo 3 y en el Capítulo 7 hemos visto que las hembras ponen un número elevado de huevos poco tiempo después de la cópula. Por ejemplo, las hembras de las parejas utilizadas en el experimento del Capítulo 7 depositaron el primer día después de la cópula una media (± error estandar) de 4.72 ± 2.65 huevos. Este hecho favorece claramente la posilibilidad de que ocurra cuidado paternal en *P. laciniata*. Una evidencia de esto es que el 41.18% de los huevos puestos por las hembras el primer día fue depositado sobre los machos inmediatamente después de que tuviera lugar la cópula. En términos medios, un 38% de los huevos que fueron depositados sobre los machos instantes después de acabar la cópula habían sido fecundados por ellos mismos. Es decir, la tasa de paternidad de los huevos portados por esos machos es del 38%. No existen datos claros acerca de la duración de la asociación entre el macho y la hembra una vez que ha acabado la cópula en condiciones naturales, pero datos experimentales sugieren que esta asociación puede ser lo suficientemente prolongada como para que se favorezca el cuidado paternal. Por un lado, se ha sugerido que los machos exhiben una forma de guarda de la pareja postcopulatoria (Kaitala y Miettinen, 1997). Por otro lado, las cópulas múltiples por parte del mismo macho son frecuentes en el laboratorio, y hemos visto en el Capítulo 2 que este comportamiento puede ser el causante de gran parte de la carga de huevos que portan los machos. En definitiva, los resultados obtenidos en el Capítulo 6, en el que se muestran que los machos portan descendencia genética con una probabilidad superior a la que se esperaría por azar, junto con los datos del Capítulo 7, y los patrones de comportamiento y de transporte de huevos, muestran que el cuidado paternal juega un papel importante en esta especie.

En términos generales hemos obtenido que un macho de *P. laciniata* de las poblaciones de España Central disfruta de una confianza de paternidad de 0.43 después de aparearse con una hembra. En otras palabras, se espera que un macho que se ha apareado cogido al azar sea el responsable del 43% de los huevos depositados por la hembra (hasta el momento que otro macho rival se aparee con la hembra).

El modelo de Westneat y Sherman (1993) contempla que la inversión creciente en cualquiera de los componentes del esfuerzo reproductivo dentro de un periodo reproductivo: esfuerzo parental (esfuerzo empleado en el comportamiento parental), esfuerzo en el apareamiento (esfuerzo empleado en adquirir adquirir parejas y fecundarlas), y esfuerzo somático (esfuerzo que incrementa la probabilidad de un individuo de sobrevivir hasta un próximo intento reproductivo), conlleva una menor

capacidad para invertir en los otros dos componentes restantes. La decisión de los machos, desde una perspectiva de estrategias vitales ("life history") es, entonces, optimizar la inversión en cada uno de los tres componentes del éxito reproductivo a lo largo de varios intentos reproductivos para maximizar la eficacia biológica a lo largo de toda la vida (Wright, 1998). Un análisis de las predicciones del modelo de Westneat y Sherman (1993), el cual resume los principales modelos realizados hasta ese momento en cuanto a la relación entre la paternidad y el cuidado paternal (Maynard Smith, 1978; Grafen, 1980; Werren et al., 1980; Winkler, 1987; Whittingham et al., 1992; Xia, 1992), nos ha indicado que los machos de *P. laciniata* podrían aceptar huevos a pesar de una confianza de paternidad intermedia debido a las siguientes razones:

1. Un macho de esta especie no puede discriminar si el huevo que va a depositar la hembra ha sido fecundado por él o no.

2. El costo de supervivencia para los huevos que no son portados por individuos es extremadamente alto. El cuidado paternal es, por lo tanto, crucial para la supervivencia de las crías y los machos deberían cuidar siempre que exista alguna probabilidad de que alguno de los huevos aceptados sean descendencia genética. Whittingham y colaboradores (1992) predijeron que cuando el cuidado paternal es crucial para la supervivencia de la progenie, los machos cuidarían a no ser que la confianza de paternidad cayera por debajo de un umbral mínimo, momento en el cual desertarían. Otros estudios teóricos igualmente han sugerido que únicamente niveles muy bajos de paternidad tendrían un efecto negativo sobre el cuidado paternal (Westneat y Sherman, 1993; Houston, 1995). La realización de estudios empíricos contrastando estas predicciones han encontrado que los machos pueden tolerar una cierta reducción en la confianza de paternidad sin que por ello se vea afectado el cuidado paternal efectuado, siempre y cuando el cuidado paternal sea imprescindible para la supervivencia de las crías (Whittingham et al., 1993; MacDougall-Shackleton y Robertson, 1998). Un ejemplo ilustrativo de esto es lo que pasa en el calamón (*Porphyrio porphyrio*). En este ave los machos no proveen cuidado paternal en relación con la paternidad. Los machos, a veces, se constituyen en tríos y cada uno de los machos de dicho trío copula con la hembra. A pesar de que los machos no dominantes (machos beta) acceden a menos cópulas y fecundan menos huevos que el macho dominante del trío (macho alfa), todos los machos realizan el mismo cuidado paternal puesto que cada uno de ellos disfruta de alguna probabilidad de haber engendrado, al menos, un pollo, y el cuidado es fundamental para la supervivencia de las crías (Jamieson et al., 1994; Westneat y Sargent, 1996).

3. La confianza de paternidad se mantiene constante con el tiempo como resultado del mecanismo de mezcla de esperma. Es decir, el nivel de paternidad que espera obtener un macho, en promedio, en un episodio de reproducción futuro, no es significativamente mejor que el que obtiene en el presente y, por ello, no se beneficiará de una deserción del cuidado paternal (es decir, no se beneficiará de anular el esfuerzo parental en el episodio de reproducción presente y ampliar el esfuerzo somático y en el apareamiento).

Otro hecho importante que hay que tener en cuenta es que en *P. laciniata* la inversión creciente en esfuerzo parental no influye en la inversión en el apareamiento puesto que, en esta especie, el cuidado paternal no supone un costo en la adquisición de pareja, ya que los machos que portan huevos continúan copulando con otras hembras.

En resumen, la constancia en la confianza de paternidad con el tiempo, derivada del mecanismo de competencia espermática, unido al inmenso costo para la supervivencia de los huevos que son puestos sobre la planta hospedadora, probablemente ha impuesto una fuerte selección para que evolucione el cuidado paternal en esta especie.

8. Los intereses del macho y de la hembra en conflicto

En *P. laciniata* el conflicto sexual sobre el cuidado parental es inevitable puesto que las hembras no pueden depositarse los huevos sobre ellas mismas. Como las hembras no pueden cuidar ellas mismas de las crías, su única oportunidad para mejorar la supervivencia de las crías es depositar los huevos sobre los padres o, alternativamente, sobre otros coespecíficos que no mantienen relación genética con la descendencia. Los machos, a su vez, sufren costos de predación cuando portan huevos, por lo que es improbable que los acepten a no ser que una proporción de ellos sean sus descendientes genéticos y se beneficien de aumentar la supervivencia de su propia desdendencia. Bajo este marco, los intereses femeninos radican en el mantenimiento de unos mecanismos de competencia espermática que ofrezcan una confianza de paternidad intermedia y estable a lo largo del tiempo. Un macho debe de sopesar los costos de portar huevos que no son suyos, contra los costos que supone que sus propias crías acaben en plantas. Una confianza de paternidad intermedia es, probablemente, suficiente para compensar a los machos, dadas las bajísimas posibilidades de supervivencia de los huevos sobre la planta hospedadora. Por otra parte, la poca variación de la confianza de paternidad con el tiempo hace que a un macho no le sea ventajoso aceptar huevos únicamente en momentos muy específicos (por ejemplo, justo después de la cópula), lo que maximiza las posibilidades de poner varios huevos en un macho después de realizar la cópula con él. Esta es la estrategia más apropiada en una especie en la que no se ponen todos los huevos simultáneamente tras las cópula, sino que se ponen en una secuencia que es muy variable a lo largo del tiempo. Finalmente, este mecanismo de competencia espermática también maximiza el número de machos dispuestos a aceptar huevos, puesto que todos los machos que hayan copulado con la hembra mantienen un cierto nivel de paternidad mucho tiempo después de la cópula.

Por lo tanto, el sistema parece estar dominado por un conflicto entre los intereses de los sexos. Mientras que las hembras salen beneficiadas del mantenimiento de unos mecanismos de competencia espermática que les revierten los mayores beneficios, los machos intentarían incrementar su éxito en la fecundación por medio de, por ejemplo, la realización de cópulas repetidas o de cópulas largas, que conllevan un mayor número de espermatozoides transferidos.

9. El parasitismo de los huevos como posible presión selectiva que conduce al cuidado paternal

La presión selectiva debida al parasitismo de los huevos por parte de himenópteros parasitoides puede haber influido en gran medida en la evolución de la ovoposición sobre individuos y en la evolución del cuidado paternal en este sistema.

Una serie de factores se han propuesto como decisivos en la evolución del cuidado parental en insectos. Estos factores, que pueden actuar de manera independiente o conjunta se pueden resumir principalmente en cuatro grupos: a) ambientes estables que favorecen la filopatría, grandes tamaños estructurales, iteroparidad y tamaños de puesta reducidos, b) ambientes con condiciones físicas extremas que inducen altas tasas de mortandad, c) una especialización trófica que conduce a la protección de recursos frente a competidores, y d) altas tasas de predación y parasitismo (ver Tallamy y Denno, 1981). Están documentadas las altas tasas de parasitismo que sufren los huevos de *P. laciniata* por parte del himenóptero Scelionido *Gryon bolivari*, especialmente los huevos que son dejados sobre la planta hospedadora (Reguera, 1999; Reguera y Gomendio, 2002). Por poner un ejemplo, el porcentaje de individuos que porta algún huevo parasitado en las poblaciones estudiadas llega hasta el 40% (García González y Gomendio, datos inéditos). El parasitismo es, en la actualidad, una presión selectiva que incide fuertemente sobre los patrones de transporte de huevos en *P. laciniata*.

Reguera (1999) obtuvo que cuando el riesgo de parasitismo de los huevos disminuye también lo hace la tendencia de los machos a portar huevos, pero no así la de las hembras. Esta autora también obtuvo que a medida que aumenta la presión por parasitismo dentro de una población, aumenta la proporción de machos que porta huevos, mientras que la proporción de hembras que porta huevos se mantiene invariable. Por otro lado también comprobó el efecto del parásito en la intensidad de cuidado a nivel interpoblacional: en poblaciones con menores tasas de parasitismo una menor proporción de machos transportan huevos en relación a poblaciones en las que la tasa de parasitismo es mayor (la proporción de hembras que porta huevos se mantiene igualmente invariable a nivel interpoblacional). A la luz de estos resultados esta autora concluyó que los machos, y no las hembras, muestran una flexibilidad en su capacidad de adaptarse a situaciones donde los beneficios de portar huevos varían. Por lo tanto, el parasitismo podría influir sobre el grado de cuidado paternal, haciendo que los machos aceptaran huevos con mayor frecuencia debido a que un parasitismo intenso significaría que los huevos tienen muy pocas probabilidades de eclosionar si no son portados por un individuo adulto. Es importante hacer notar que el grado diferencial de parasitismo sufrido por las poblaciones podría explicar diferencias entre poblaciones en el grado de cuidado paternal efectuado. Por otra parte, datos no publicados resultantes de los estudios de captura-recaptura llevados a cabo durante la realización de este trabajo a lo largo de los años 1998 a 2000, muestran un acople entre el ciclo de la chinche y la presión de parasitismo. El acople de los ciclos muestra una típica dinámica parásito-huésped y refleja la importancia del parasitoide en la modulación del ciclo de *P. laciniata*.

Todo ello, por lo tanto, muestra una dinámica poblacional de *P. laciniata* ajustada a la intensidad de parasitación de los huevos y una respuesta comportamental condicionada por la intensidad de parasitismo. Creemos por lo tanto probable que el transporte de huevos se haya desarrollado como una estrategia para incrementar la eficacia biológica femenina debida a presiones selectivas como el parasitismo. Una vez aparecido este comportamiento, los machos intentarían maximizar su inversión parental de acuerdo a los patrones de precedencia de esperma, mientras que las hembras serían objeto de parasitismo intraespecífico (sólo una pequeña proporción de hembras portan huevos, en escaso número).

10. Otras consideraciones sobre el cuidado paternal en *P. laciniata*

El comportamiento de transporte de huevos por los individuos adultos de *P. laciniata* recuerda al comportamiento de las chinches acuáticas gigantes (Belostomatidae). Como se ha citado con anterioridad, los machos de algunas especies de belostomátidos aceptan huevos sobre el dorso tras haber copulado con una hembra y luego realizan un comportamiento de aireación en la superficie de la carga de huevos (Smith, 1976; Smith, 1980). El comportamiento de *P. laciniata* y el de los belostomátidos tiene varias diferencias. Por ejemplo, los machos de *P. laciniata* no parecen modificar su comportamiento de acuerdo a si portan huevos o no. Sin embargo, ambos grupos comparten un hecho importante: los huevos, en general, tienen muy pocas probabilidades de sobrevivir si no son portados por los adultos. Posiblemente esta es una de las claves para la evolución, por medio de la selección natural, del cuidado paternal en *P. laciniata*.

Tallamy (2000; 2001) ha sugerido recientemente que ha sido la selección sexual y no la selección natural la que ha dirigido el curso de la evolución en la mayoría de casos en los que el cuidado paternal ha evolucionado en artrópodos. Según este autor, en estos casos las hembras prefieren como pareja a los machos que están dispuestos a hacerse cargo de la prole, puesto que de esta manera las hembras incrementan su éxito reproductivo por dos posibles vías: (1) las hembras se liberan de las restricciones que impone el cuidado maternal sobre la fecundidad (hipótesis de la fecundidad incrementada), o (2) las

hembras identifican a los machos que poseen buenos genes (principio del handicap, bajo el cual los machos que realizan cuidado parental indicarían que son capaces de sobrevivir a pesar de los costos del cuidado y, por ello, que son de una calidad genética superior a los machos que no realizan cuidado). La evolución del cuidado paternal en *P. laciniata* no parece haber sido favorecida por la selección sexual puesto que está claro que las hembras no realizan una elección de pareja preferente hacia a los machos que portan huevos (Kaitala, 1998; Reguera, 1999). Tallamy (2001) ha sugerido que el cuidado paternal que surge a través de la selección natural no debería incluir los casos en los que se cuida de progenie de paternidad cuestionable. Nosotros estamos en desacuerdo con esta proposición, puesto que se debe tener en cuenta la existencia de un conflicto sexual en cuanto al cuidado paternal, se debe tener en cuenta la existencia de compromisos entre los componentes de éxito reproductivo, y se debe tener en cuenta las variaciones o la ausencia de éstas en cuanto a la confianza de paternidad que pueden experimentar los machos en episodios de reproducción futuros (aspectos que han sido expuestos con anterioridad). De lo contrario se debería revisar toda la literatura científica sobre cuidado parental en aves y mamíferos, puesto que en muchas especies de estos grupos las cópulas extra-pareja y las fecundaciones extra-pareja son frecuentes (ver por ejemplo Kempenaers et al., 1992; Dixon et al., 1994; Birkhead, 1995; Petrie y Kempenaers, 1998). En estas especies, por lo tanto, los machos cuidan, a menudo, de crías que no son suyas, y sin embargo no hay duda de que el comportamiento efectuado es cuidado parental que ha surgido por los beneficios en términos de supervivencia que adquieren las crías, y por lo tanto por selección natural.

11. Consideraciones finales

En conjunto los diferentes aspectos tratados en esta Tesis suponen un avance en la comprensión del sistema y en el significado adaptativo del transporte de huevos. El punto de inflexión en el cual se originara la ovoposición femenina sobre individuos probablemente haya sido causado por presiones debidas al parasitismo de los huevos o por algún otro factor biótico o abiótico que haya supuesto una mortalidad alta de la descendencia. A partir de ese momento, el sistema se puede considerar dominado por intereses en conflicto entre el sexo femenino y masculino. Teniendo en cuenta el interés femenino en cuanto al destino de los huevos, la estrategia de las hembras sería explotar el cuidado de los individuos coespecíficos. La estrategia de los machos, sin embargo, radica en maximizar el número de huevos fecundado por ellos mismos y ajustar el cuidado parental de acuerdo a la confianza de paternidad, puesto que de esa manera incrementan su éxito reproductivo al mejorar la probabilidad de supervivencia de sus crías. Un mecanismo de competencia espermática que se traduce en una confianza de paternidad intermedia puede haber sido suficiente para favorecer el cuidado paternal, puesto que la confianza de paternidad se mantiene constante con el tiempo y la descendencia experimenta unos costos extremadamente altos si no es portada por un individuo adulto. Una selección apuntando a maximizar la certeza de paternidad podría haber ocurrido desde la aparición del comportamiento de puesta sobre individuos, lo que explicaría el comportamiento de cópulas repetidas, las cópulas prolongadas, y el "sperm loading" para incrementar el éxito del último macho que cópula con una hembra en la fecundación de los huevos puestos tras la cópula. En conjunto, las evidencias recogidas hasta la fecha tanto en poblaciones naturales como en estudios experimentales apuntan a que el cuidado paternal juega un papel importante en la evolución del transporte de huevos en esta especie.

El presente estudio no sólo mejora la comprensión del comportamiento de *P. laciniata*, sino que provee una metodología específica para abordar cuestiones de índole ecológica y evolutiva en éste y otros organismos, y lo que es más importante, provee avances en el campo de

disciplinas como la reproducción, la selección sexual, la competencia espermática, el cuidado parental y el conflicto sexual, que se pueden sintetizar brevemente en los siguientes puntos: (1) Los ciclos reproductivos, incluyendo la dinámica ovárica y la ovoposición, se ajustan de manera adaptativa a la disponibilidad y calidad de los substratos de ovoposición, (2) Las respuestas de los machos enfrentados a competencia espermática pueden ser múltiples y estar mediadas tanto por procesos fisiológicos como comportamentales. Los modelos de riesgo de competencia espermática parecen ser adecuados para explicar la inversión estratégica en el eyaculado, (3) Confianzas de paternidad intermedias y no sólo altas pueden hacer que la selección natural favorezca el cuidado paternal dependiendo de los compromisos de reproducción presentes y futuros y dependiendo de los costos y beneficios de dicho cuidado, (4) Los mecanismos de competencia espermática pueden reflejar la existencia de conflictos de intereses entre los sexos con respecto a la confianza de paternidad y a la inversión parental.

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Conclusiones

1. Los estudios de poblaciones naturales y la realización de experimentos han permitido construir una imagen bastante completa del ciclo biológico y patrones de comportamiento de *Phyllomorpha laciniata* en un contexto ecológico. Las diferencias entre los sexos en cuanto a los patrones del transporte de huevos en poblaciones naturales refutan la hipótesis del parasitismo intraespecífico sobre parejas en cópula y apoyan las predicciones de la hipótesis de aseguración de la paternidad por cópulas repetidas.

2. Las hembras de este insecto realizan una selección de los lugares de ovoposición: realizan la puesta preferentemente sobre coespecíficos, lo que constituye un comportamiento adaptativo puesto que de esta manera incrementan su eficacia biológica por medio de una mejora de las probabilidades de supervivencia de las crías.

3. La ovoposición de las hembras se ve estimulada por la presencia de adultos coespecíficos, lo que igualmente se interpreta como un proceso adaptativo al conferir un incremento de la eficacia biológica femenina. La selección de los coespecíficos para realizar la ovoposición y la estimulación de la ovoposición ocurre frente a la presencia de machos y hembras, lo que pone de manifiesto que el interés de las hembras radica en realizar la ovoposición sobre individuos adultos independientemente del sexo del individuo receptor. Los resultados indican que las hembras se benefician de la estimulación de la ovoposición por coespecíficos, lo que no ha sido documentado con claridad hasta la fecha.

4. La existencia de ambos procesos (selección de ovoposición y estimulación de la ovoposición) muestra que la presencia de coespecíficos modula la reproducción de este insecto y que la dinámica ovárica responde de una manera adaptativa a la disponibilidad y calidad de los substratos de ovoposición.

5. Los machos de *P. laciniata* ajustan la inversión en el eyaculado dependiendo del riesgo de competencia espermática, de acuerdo con las predicciones de los modelos teóricos. Los machos en presencia de machos rivales incrementan la duración de la cópula (lo que se traduce en una transferencia mayor de espermatozoides) y la tasa de transferencia de espermatozoides. Los datos muestran que la respuesta de los machos enfrentados a competencia espermática puede ser múltiple, y que el significado adaptativo de las cópulas largas y prolongadas en este insecto radica en el incremento en la transferencia de espermatozoides, y por extensión en la maximización de las probabilidades de fecundación, lo que incrementaría el éxito reproductivo masculino.

6. Se ha desarrollado la aplicación de una herramienta molecular para responder, en este insecto, a preguntas ecológicas y evolutivas que impliquen el conocimiento de las relaciones genéticas entre los individuos. Los marcadores moleculares AFLP ofrecen unos niveles de polimorfismo adecuados para la realización de esta tarea. Los resultados obtenidos en este estudio y las ventajas que presentan estos marcadores moleculares frente a otros métodos de ADN "fingerprinting" sugieren que es una técnica apropiada para su aplicación en este sistema.

7. La aplicación de los AFLPs para la asignación de paternidad en este insecto ha mostrado que el principal problema que mostraba este sistema hasta la fecha, a saber, la determinación de la relación entre las crías y los individuos que realizan el cuidado parental, puede ser solucionado. Los AFLPs ofrecen unas probabilidades de exclusión (la probabilidad de que un individuo elegido al azar pueda ser excluido como el padre de una cría dada) adecuadas para el análisis de la paternidad, y este estudio es uno de los primeros que muestran que

la técnica de los AFLPs puede ser usada con fiabilidad en la determinación de la paternidad. El análisis de la paternidad de los huevos portados por los machos indica que algunos de los huevos que portan los machos han sido fecundados por ellos. La probabilidad con la que los machos portan huevos de los que son los padres genéticos es superior a la que se esperaría por azar, incluso bajo condiciones que claramente no favorecen el cuidado paternal.

8. Por vez primera se ha realizado un análisis de los patrones de precedencia de esperma en esta especie, que, además, se ha llevado a cabo sobre hembras apareadas de forma múltiple en condiciones naturales. Los resultados indican que el mecanismo que rige la competencia espermática es una mezcla de esperma. Este mecanismo, junto con las estrategias masculinas para incrementar el éxito en la fecundación (copulas largas, aumento de la tasa de transferencia de espermatozoides, copulas repetidas) determina la paternidad que obtienen los machos tras copular con las hembras.

9. El mecanismo de competencia espermática se traduce en una confianza de paternidad poblacional intermedia que no varía con el tiempo transcurrido desde la realización de la cópula. Este hecho junto con los beneficios derivados del cuidado y los altos costos para las crías de la ausencia de inversión parental parece suficiente para que evolucione el cuidado paternal en *P. laciniata*.

Por vez primera se aportan datos empíricos que sugieren que confianzas de paternidad intermedia pueden estar asociadas con la inversión paternal, dependiendo de los compromisos reproductivos presentes y futuros y de los beneficios y costos del cuidado. De esta manera se apoya la idea de que no sólo los machos de especies con confianzas de paternidad altas serían proclives a realizar un esfuerzo parental.

10. En conjunto, el sistema está dominado por un conflicto entre los sexos. El conflicto sexual y el mecanismo de competencia espermática están íntimamente ligados. El interés femenino radica en la ovoposición sobre individuos, independientemente de la relación genética entre el receptor y las crías. Por ello, el interés femenino se traduce en el mantenimiento de un mecanismo de competencia espermática que determine una confianza de paternidad no tan alta como para que los machos únicamente acepten huevos tras la cópula y no tan baja como para que los machos no acepten huevos en ninguna ocasión. Un mecanismo de mezcla de esperma, que se traduce en unas probabilidades de fecundación de huevos intermedias que se mantienen constantes con el tiempo, en un sistema en el que el cuidado es fundamental para la supervivencia de las crías, incrementa tanto el número de machos que están dispuestos a portar los huevos como el número de huevos que está dispuesto a portar cada macho. Por el contrario, los machos intentarían incrementar su éxito en la fecundación por medio de diferentes estrategias y de esta manera maximizarían su tasa de paternidad para evitar realizar inversión parental en descendencia no genética, puesto que el cuidado parental supone un costo de predación para quien lo efectúa.

11. Se propone que el comportamiento de ovoposición sobre individuos puede haberse originado por presiones de parasitismo o predación de los huevos. Sin embargo, lo que en esta Tesis queda probado es que una vez que este comportamiento está establecido en el sistema los machos intentan maximizar la tasa de paternidad de los huevos que portan y que, de hecho, una proporción de los huevos que portan la constituyen huevos fecundados por ellos mismos. Por lo tanto, el cuidado paternal juega un importante papel en la evolución del comportamiento de *P. laciniata*.